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Sulindac-derived reactive oxygen species induce apoptosis of human multiple myeloma cells via p38 mitogen activated protein kinase-induced mitochondrial dysfunction

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Abstract Non-steroidal anti-inflammatory drugs are well known to induce apoptosis of cancer cells independent of their ability to inhibit cyclooxygenase-2, but the molecular mechanism for this effect has not yet been fully elucidated. The purpose of this study was to elucidate the potential signaling components underlying sulindac-induced apoptosis in human multiple myeloma (MM) cells. We found that sulindac induces apoptosis by promoting ROS generation, accompanied by opening of mitochondrial permeability transition pores, release of cytochrome c and apoptosis inducing factor from mitochondria, followed by caspase activation. Bcl-2 cleavage and down-regulation of the inhibitor of apoptosis proteins (IAPs) family including cIAP-1/2, XIAP, and survivin, occurred downstream of ROS production during sulindac-induced apoptosis. Forced expression of survivin and Bcl-2 blocked sulindac-induced apoptosis. Most importantly, sulindac-derived ROS activated p38 mitogen-activated protein kinase and p53. SB203580, a p38 mitogen-activated protein kinase inhibitor, and RNA inhibition of p53 inhibited the sulindac-induced apoptosis. Furthermore, p53, Bax, and Bak accumulated in mitochondria during sulindac-induced apoptosis. All of these events were significantly suppressed by SB203580. Our results demonstrate a novel mechanism of sulindac-induced apoptosis in human MM cells, namely, accumulation of p53, Bax, and Bak in mitochondria mediated by p38 MAPK activation downstream of ROS production.

Keywords Apoptosis · Multiple myeloma · NSAIDs · Reactive oxygen species

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Introduction

Apoptosis is the physiological process whereby most cells, including B lymphocytes, are eliminated, thus leading to homeostasis. Recent evidence indicates that many cancer chemotherapy agents induce apoptosis of tumor cells. Dexamethasone and other chemotherapeutic agents, such as melphalan, vincristine, and doxorubicin [1], induce apoptosis in MM, suggesting that apoptosis is one of the mechanisms mediating their therapeutic effect.

Mitogen-activated protein kinases (MAPKs) transduce signals from the cell membrane to the nucleus and thus contribute to a wide spectrum of cellular processes including cell growth, differentiation, and apoptosis. Activation of extracellular signal-regulated kinases (ERKs) contributes to cell differentiation, proliferation, and survival, whereas c-Jun NH_2-terminal kinase (JNK) and p38 MAPK are activated by proinflammatory cytokines and environmental stresses and promote apoptosis [2]. There are a number of reports indicating an association of MAPKs with p53 induction and stabilization. Specifically, upon activation, JNK phosphorylates

several transcriptional factors such as c-Jun, thereby modifying the expression of genes including p53. It is also well known that p53 is post-translationally stabilized through phosphorylation at multiple sites by protein kinases including JNK and p38. p53 escapes from ubiquitin-dependent degradation [3, 4].

Sulindac is the most extensively investigated clinically relevant chemopreventive non-steroidal anti-inflammatory drug (NSAID), and it has been shown to reduce the number and size of the colorectal tumors in genetically susceptible humans and animals [5–7]. Sulindac sulfide and sulindac sulfone are the two major metabolites of sulindac. Sulindac sulfide is a selective inhibitor of cyclooxygenase, whereas sulindac sulfone is believed to lack the ability to inhibit cyclooxygenase [8]. Both metabolites induce apoptosis in a variety of cell types [8–11]. Although progress has been made in understanding the mechanisms by which NSAIDs such as sulindac trigger apoptosis in neoplastic cells, very little is known about the hierarchy of events, particularly those related to MAPK signaling, mitochondrial injury, and generation of reactive oxygen species (ROS). In this context, many studies have shown a connection between oxidative stress and perturbation of MAPK pathways for other noxious stimuli [12].

The purpose of the current study was to define the signaling components underlying sulindac-induced apoptosis in human MM and the relationship between mitochondrial injury, ROS generation, p38 MAPK activation, and apoptosis. We report that ROS generation plays a central role in these events and is responsible for activation of p38 MAPK, which subsequently promotes mitochondrial accumulation of p53, Bak, and Bax, release of proapoptogenic proteins from mitochondria, caspase activation, and apoptosis. We also report that the members of the IAP family, including cIAP-1/2, XIAP, and survivin, are down-regulated and that Bcl-2 is cleaved during sulindac-induced apoptosis. Furthermore, enforced expressions of survivin and Bcl-2 attenuated sulindac-induced apoptosis. These results suggest the presence of an amplification loop in which sulindac-induced mitochondrial dysfunction promotes the further activation of the apoptotic cascade.

Materials and methods

Cell culture and reagents

The MM cell line IM9 (p53 wild type) was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO_2. Sulindac, Z-VAD-fmk, SB203580, and valinomycin were purchased from Calbiochem (San Diego, CA). *N*-acetyl-L-cysteine (NAC) was from Sigma-Aldrich (St. Louis, MO), and antibodies against apoptosis inducing factor (AIF), caspase 8, caspase 9, survivin, cIAP-1, cIAP-2, p38, Bax, β-actin, and Hsp60 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated p38 and phosphorylated p53 (p-p53; Ser 15) from Cell signaling Technologies (Cambridge, MA); antibodies against caspase 3, caspase 7, Bak, cytochrome c, p21, and XIAP were from BD Biosciences Pharmingen (San Diego, CA); antibodies against p53 was from Novastatin, and antibodies against Bcl-2 and proliferating cell nuclear antigen (PCNA) were from DakoCytomation (Denmark).

Analysis of apoptosis

Apoptosis was determined using an annexin V-fluorescein isothiocyanate/propidium iodide (PI) kit (BD Pharmingen, San Jose, CA) as described previously [13]. Briefly, cells were washed with cold PBS and then resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. The cells were incubated with 5 μl each of annexin V-fluorescein isothiocyanate and PI and then analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Measurement of the mitochondrial transmembrane potential (MMP)

Loss of the MMP was measured using an ApoAlert mitochondrial membrane sensor kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Briefly, after drug treatment, cells were incubated with Mitosensor for 15 min at 37°C. After centrifugation, they were resuspended in washing buffer and analyzed by fluorescence channel using flow cytometry. The results were obtained as mean value of stained cell histogram.

Detection of ROS

ROS in cells were measured using dichlorofluorescein diacetate (DCFH-DA, Calbiochem), an oxidation-sensitive fluorescent probe. After incubation with drugs, cells were stained with 20 μM DCFH-DA for 30 min at 37°C in the dark. The cells were then analyzed using a Becton-Dickinson FACScan (Becton Dickinson) to determine the level of ROS [13].

Measurement of caspase activity

The activities of caspases 3/7, 8, and 9 were assessed using the CaspaTag^{TM} Caspase Activity Kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. This kit employs carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitors of caspases 3/7 (FAM-DEVD-FMK), 8 (FAM-LETD-FMK), and 9 (FAM-LEHD-FMK), which are

cell-permeable and non-cytotoxic fluorochrome inhibitors of that covalently bind to a reactive cysteine residue on the large submit of the active caspase heterodimer, inhibiting enzymatic activity and producing green fluorescence. Thus the green fluorescent signal directly corresponds to the amount of active caspases present in the cell at the time the reagent was added. Fluorescein-conjugated caspase substrate was added directly to the cell suspension and left for 1 hour at 37°C under 5% CO_2 and protected from the light. After washing, labeled live cells were detected by flow cytometry.

Subcellular fractionation

Cytosolic and mitochondrial fractions were prepared using a mitochondria isolation kit (Pierce, Rockford, IL). Briefly, cells treated under various conditions were washed in ice-cold PBS and resuspended in ice-cold cytosol extraction buffer. After incubation on ice, the preparation was centrifuged at $3000 \times g$ for 5 min, and supernatant was kept as the cytosolic fraction. The pellets were resuspended in Tris-buffered saline containing 2% CHAPS, incubated for 1 min on ice, and centrifuged at $12{,}000 \times g$ for 2 min. The supernatant was collected as the mitochondrial fraction. All samples were stored at -20°C until use.

Western blotting

Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Concentrations of each lysates were determined by Bradford assay. Equal amounts of protein (20 μg–50 μg) were separated by SDS-PAGE and then transferred to a nitrocellulose membrane. The membranes were then incubated with relevant primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody, and immunoreactive proteins were visualized with enhanced chemiluminescence reagents (Amersham Bioscience, Little Chalfont, United Kingdom).

Establishment of IM9 cells stably overexpressing Bcl-2 and survivin

IM9 cells were stably transfected with plasmids encoding Bcl-2 (pcDNA3-Bcl-2, 14) or survivin or with a control vector (pcDNA3-survivin-Myc and empty pcDNA vector were kindly provided by Dr. Jin Q. Cheng, University of South Florida College of Medicine). Log-phase IM9 cells were mixed and incubated with 5 μg of plasmid DNAs at 37°C for 15 min, and then electroporated in a 0.4cm using the Gene Pulser electrophoration apparatus (Bio-Rad Laboratories Inc., CA, USA) and using a single-pulse protocol (voltage 300 V and capacitance 960 μF). Cells were incubated at 37°C for 15 min and then resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and 200 μg/ml G418 [15].

Transfection with small interfering RNA (siRNA)

Prior to electroporation, IM9 cells were mixed either with 0.05 nmol of siRNA against p53 (#106141) or nonsilencing control siRNA (#4611, scrambled sequence) obtained from Ambion (Austin, TX) and incubated at 37°C for 15 min. Following one pulse of electroporation at 280 V and 960 μF, cells were incubated at 37°C for 15 min and then resuspended in RPMI 1640 supplemented with 10% fetal bovine serum [15]. Finally, cells were treated with sulindac for 48 h.

Immunoprecipitation assays

For measurement of p38 MAPK activity, cell lysates were immunoprecipitated using the protein A/G-agarose (Santacruz, CA) and anti-p38 MAPK antibody (Santacruz, CA). After washing the immunocomplex, kinase reactions were carried out by the addition of kinase buffer (25 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 2 mM EGTA) in presence of ATF2 protein (Santacruz, CA) and [γ-^{32}P]ATP at 37°C for 30 min to precipitated immunocomplex. Phosphorylated ATF2 protein was resolved in 10% SDS-polyacrylamide gels and visualized by autoradiography [16].

Statistical analysis

Data are presentated as mean $\pm$ SD. Comparisons between groups were used to the paired Student's *t*-test. Asterisk ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$) was considered to be statistically significant.

Results

Sulindac induces caspase dependent apoptosis in MM cells

To examine whether sulindac can induce apoptosis, we treated IM9 human MM cells with sulindac (50–150 μM) over a 48-h period. Upon exposure to sulindac, we detected significant dose- and time-dependent apoptosis as assessed by annexin V/PI staining, followed by flow cytometry (Fig. 1(A, B)). In general, the extent of cytotoxicity increased significantly at higher concentrations of sulindac and longer exposure times. Specifically, approximately 21% and 55% of the cells died following a 48-h exposure to 100 and 150 μM sulindac, respectively (Fig. 1(A)), and 21% and 34% of the

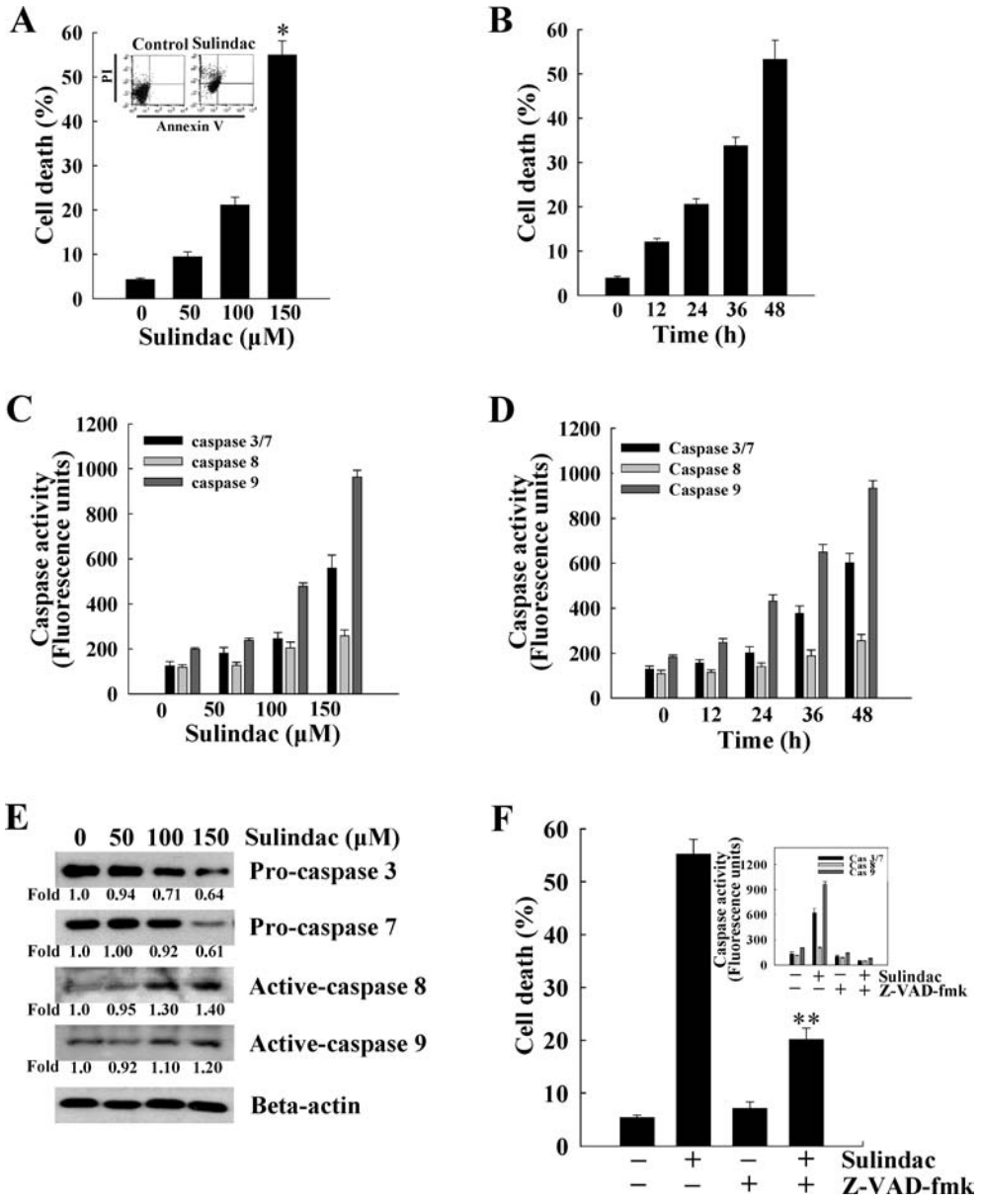

Fig. 1 Sulindac induces caspase dependent apoptosis in IM 9 cells. (A–B), Sulindac induces cell death in a dose- and time-dependent manner. Cells were treated with 0 to 150 μM sulindac for 48 h (A) or for the indicated times with 150 μM sulindac (B). Cell death was than measured as the percentage of cells with PI(+) or annexin V(+) using flow cytometry (A inlet, Sulindac; 150 μM treatment). $^{*}P < 0.05$ vs. the control groups. (C–D), Sulindac induces caspase activation in a does (C)- and time (D)-dependent manner. Cells were treated as described in A and B. Caspase activity was detected by staining with fluorescein isothiocyanate-labeled caspase inhibitors, followed by flow cytometry. (E), Caspases are activated in sulindac-treated cells. Cells were treated with 0 to 150 μM sulindac for 48 h, lysed, and subjected to Western blot analysis. Equal protein loading was confirmed by Western blotting for β-actin. Bands were subjected to densitometric scanning using the Sicon image software. Immunoblots are representative of at least two independent experiments. (F), Z-VAD-fmk blocks sulindac-induced cell death. Cells were treated with 150 μM sulindac with or without 20 μM Z-VAD-fmk for 48 h. Cell death was determined by annexin V/PI staining, followed by flow cytometry. 20 μM Z-VAD-fmk almost completely inhibit the caspase activity (inlet).$^{**}P < 0.01$ vs. the sulindac-treated groups. Each values represent the mean $\pm$ s.d. of three independent experiments

cells died after exposure to 150 μM sulindac for 24 and 36 h, respectively (Fig. 1(B)).

We next examined whether sulindac-induced apoptosis was caspase dependent. Treatment of the cells with sulindac caused a dose- and time-dependent increase in the activities of caspase 3/7, 8, and 9 (Fig. 1(C, D)). Western blotting revealed a dose-dependent decrease in the levels of pro-caspase 3, and 7 and increase in active caspase 8 and 9 (Fig. 1(E)). Also, Z-VAD-fmk, a caspase inhibitor, significantly blocked sulindac-induced apoptosis (Fig. 1(F)). These results suggest

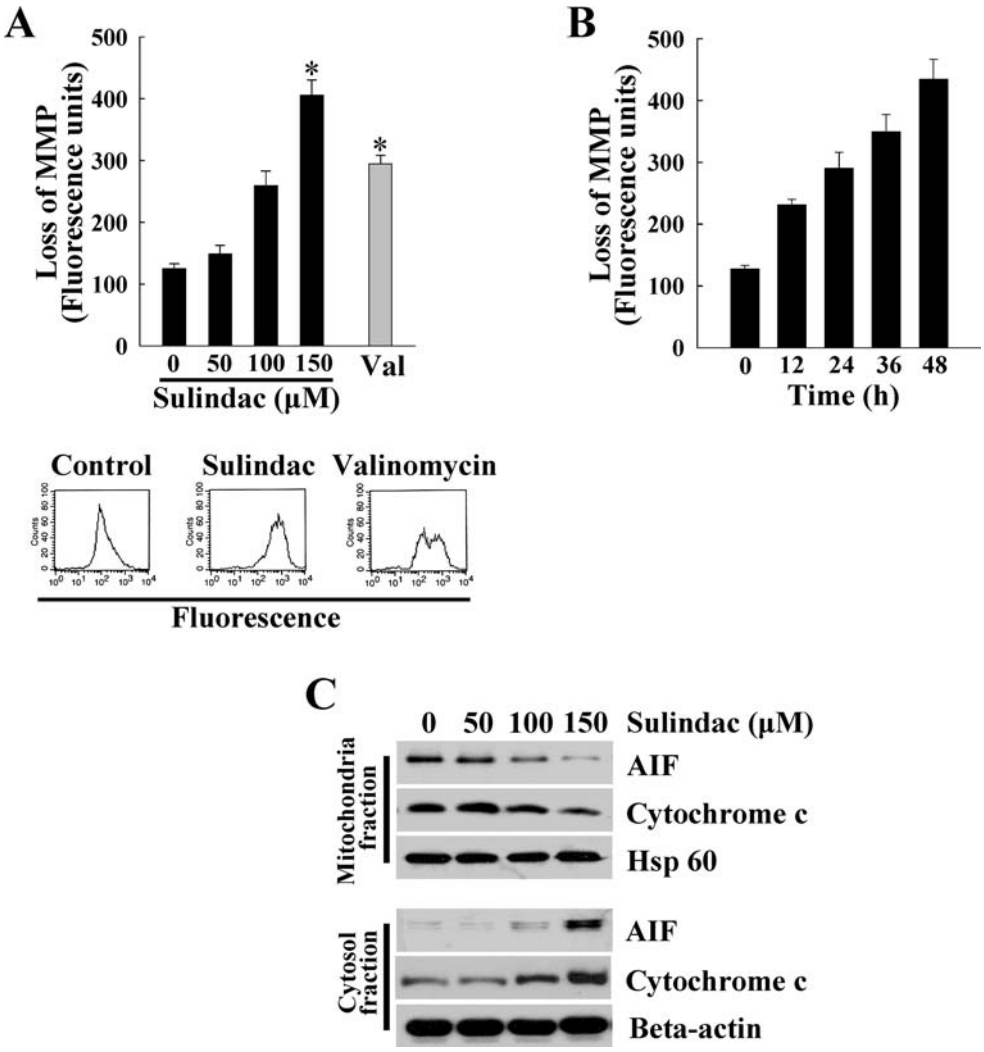

Fig. 2 Involvement of mitochondrial dysfunction in sulindac-induced apoptosis. (A–B), Sulindac induces a loss of MMP in a dose- and time-dependent manner. Cells were treated with 0 to 150 μM sulindac or 5 nM valinomycin (Val, as the positive control) for 48 h (A) or with 150 μM sulindac for indicated times (B). The MMP was analyzed by staining with Mitosensor, followed by flow cytometry (A below, Sulindac; 150 μM treatment). $^*P < 0.05$ vs. the vehicle-treated groups (control groups). (C), Sulindac causes the release of AIF and cytochrome c from mitochondria to the cytosol. Cells were treated with 0 to 150 μM sulindac. After 48 h, cells were separated into cytosolic and mitochondrial fractions and analyzed by Western blotting. Equal protein loading in the cytosolic and mitochondrial fractions was confirmed by Western blotting for β-actin and Hsp60, respectively. Immunoblots are representative of at least two independent experiments. Each values represent the mean $\pm$ s.d. of three independent experiments

that sulindac mainly induces caspase dependent apoptosis in IM9 multiple myeloma cells.

Mitochondria dysfunction is involved in sulindac-induced apoptosis

We next assessed the change in the MMP and the release of cytochrome c and AIF from mitochondria following exposure of IM9 cells to sulindac. As shown in Fig. 2(A) and (B), sulindac caused a time- and dose-dependent reduction of the MMP. Western blotting of subcellular fractions showed that a 48-h treatment with sulindac increased the level of cytochrome c and AIF in the cytosol and decreased their levels in the mitochondria (Fig. 2(C)). The purity of these fractions was confirmed by Western blotting for actin, a cytosolic protein, and Hsp60, a mitochondrial protein. These data suggest that the mitochondrial-mediated apoptotic pathway plays an important role in sulindac-induced death of MM cells.

The antioxidant NAC blocks sulindac-mediated ROS generation, mitochondrial injury, and apoptosis in IM9 cells

We next examined the role of ROS generation in the action of sulindac in IM9 cells. As shown in Fig. 3(A), 2 mM NAC markedly reduced the induction of ROS by 150 μM sulindac. This treatment also blocked sulindac-induced apoptosis and activation of caspase 3/7, 8, and 9 (Fig. 3(B, C)) and suppressed the loss of MMP (Fig. 3(D)) and the release of cytochrome c and AIF from mitochondria (Fig. 3(E)). Together, these findings indicate that ROS generation plays a primary role in sulindac-mediated mitochondrial injury and apoptosis in IM9 MM cells.

IAP family members are down-regulated during sulindac-induced apoptosis downstream of ROS generation

A recent report showed that sulindac attenuates the expression of survivin, a member of the IAP family, in colorectal

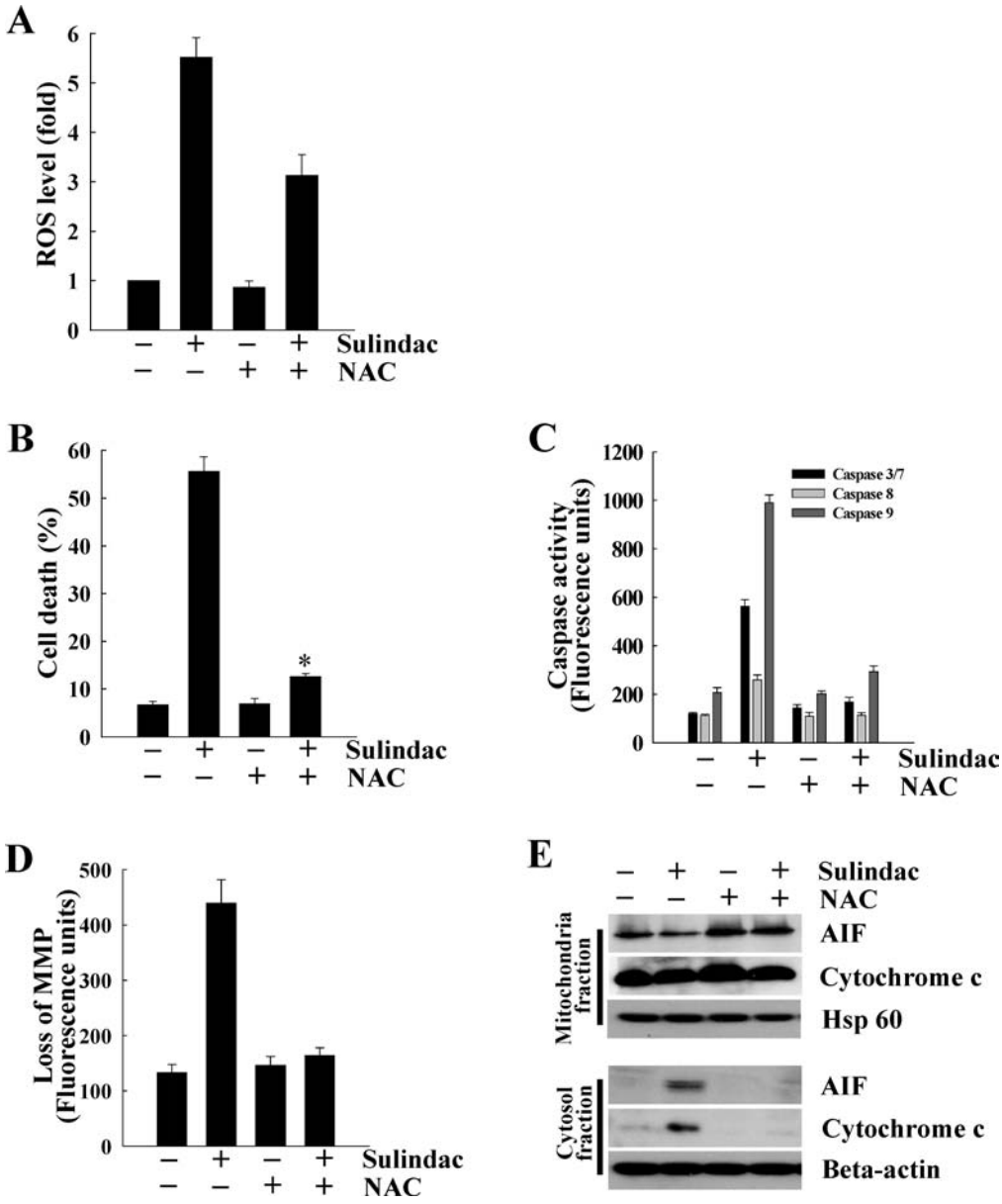

Fig. 3 Inhibition of sulindac-mediated ROS generation, mitochondrial injury, and apoptosis by NAC in IM9 cells. (A), Sulindac induces ROS production, which is inhibited by NAC. Cells were treated with 150 μM sulindac with or without 2 mM NAC for 3 h. ROS levels were measured using DCFH-DA, followed by flow cytometry. (B–E), NAC prevents sulindac-induced cell death (B), caspase activation (C), loss of MMP (D), and release of AIF and cytochrome c from mitochondria (E). Cells were treated with 150 μM sulindac together with or without 2 mM NAC for 48 h. Cell death, caspase activity, and MMP were assessed by flow cytometry, and mitochondrial release of AIF and cytochrome c were determined by Western blotting of subcellular fractions. Immunoblots are representative of at least two independent experiments. Each values represent the mean ± s.d. of three independent experiments. $^*P < 0.05$ vs. the sulindac-treated groups

carcinoma cells [17]. To determine whether sulindac has similar effects in human MM cells, we examined the expression of the IAP family, including survivin, cIAP-1/2, and XIAP, after treatment with or without sulindac. As shown in Fig. 4(A) and (B), sulindac caused a dose- and time-dependent decrease in the levels of survivin, cIAP-1, and XIAP, whereas there was only a slight decrease in the level of cIAP-2.

To gain further insight into the relationship between ROS generation and the down-regulation of IAP family proteins, we examined the effect of NAC on the reduction of their expression by sulindac. As shown in Fig. 4(C), NAC markedly attenuated the ability of sulindac to reduce the levels of IAP proteins, indicating that the product of ROS mediates sulindac-induced down-regulation of IAP family proteins.

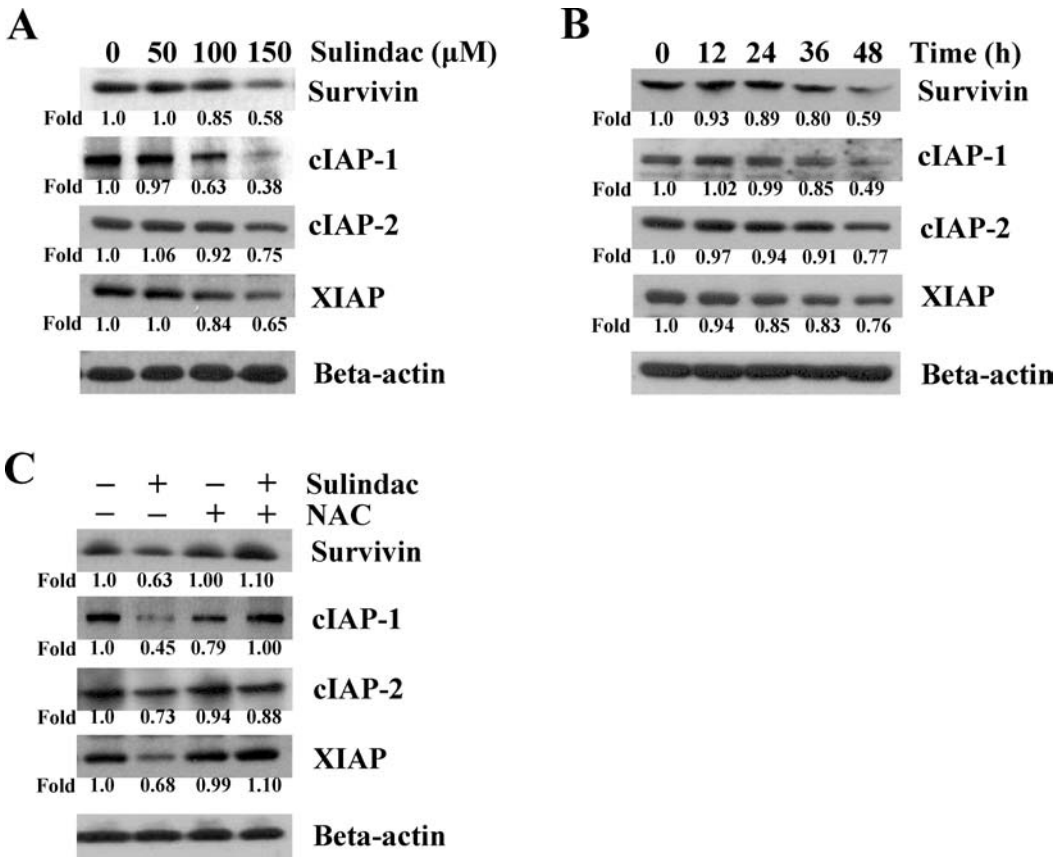

Fig. 4 Down-regulation of IAP family during sulindac-induced apoptosis downstream of ROS. Total cell lysates were analyzed by Western blotting for IAP family members. (A–B), Sulindac reduces the level of IAP family proteins. Cells were treated with 0 to 150 μM sulindac for 48 h (A) or with 150 μM sulindac for the indicated times (B). (C), NAC prevents the reduction of IAP family proteins by sulindac. Cells were treated with 150 μM sulindac with or without 2 mM NAC for 48 h. Immunoblots are representative of at least two independent experiments

Sulindac induces Bcl-2 cleavage and mitochondrial accumulation of Bax/Bak

The Bcl-2 family pro- and anti-apoptotic proteins comprise an important control point for mitochondria-mediated apoptosis [18, 19]. We therefore examined whether the levels of Bcl-2-related proteins, including the anti-apoptotic protein Bcl-2 and the proapoptotic proteins Bak and Bax, change during sulindac-induced apoptosis in MM cells. As shown in Fig. 5(A), sulindac significantly increased the level of Bak, whereas it only slightly increased the level of Bax. Interestingly, sulindac did not change the level of Bcl-2, but Western blotting did detect production of the cleaved form of Bcl-2 during sulindac-induced apoptosis.

Bak is an integral mitochondrial membrane protein, and Bax can be translocated from the cytosol to mitochondria when activated [20]. We therefore examined whether the increased levels of these proteins accumulate in the mitochondria. The levels of Bak and Bax were measured by Western blotting in subcellular fractions following treatment of IM9 cells with 150 μM sulindac for 48 h. As shown in Fig. 5(B), sulindac markedly increased levels of Bak and Bax in the mitochondrial fraction, whereas does not change the levels of Bak and Bax in vehicle-treated (DMSO) cells.

Next, to elucidate the role of ROS in Bcl-2 cleavage and mitochondrial accumulation of Bak and Bax, we examined the effects of NAC on sulindac-induced changes in the levels of Bcl-2, Bak, and Bax. As shown in Fig. 5(C), sulindac-induced Bcl-2 cleavage was inhibited by NAC. Similarly, the mitochondrial accumulation of Bak and Bax was markedly inhibited by NAC. These results indicate that Bcl-2 cleavage and mitochondrial accumulation of Bak and Bax are mediated by sulindac-induced ROS generation.

Enforced expression of survivin and Bcl-2 inhibits sulindac-induced apoptosis

To further investigate the pathways underlying sulindac-induced apoptosis, we stably transfected IM9 cells with survivin and Bcl-2. As shown in Fig. 6, much less apoptosis was induced in survivin- and Bcl-2-overexpressing IM9 cells than in control cells in response to 150 μM sulindac.

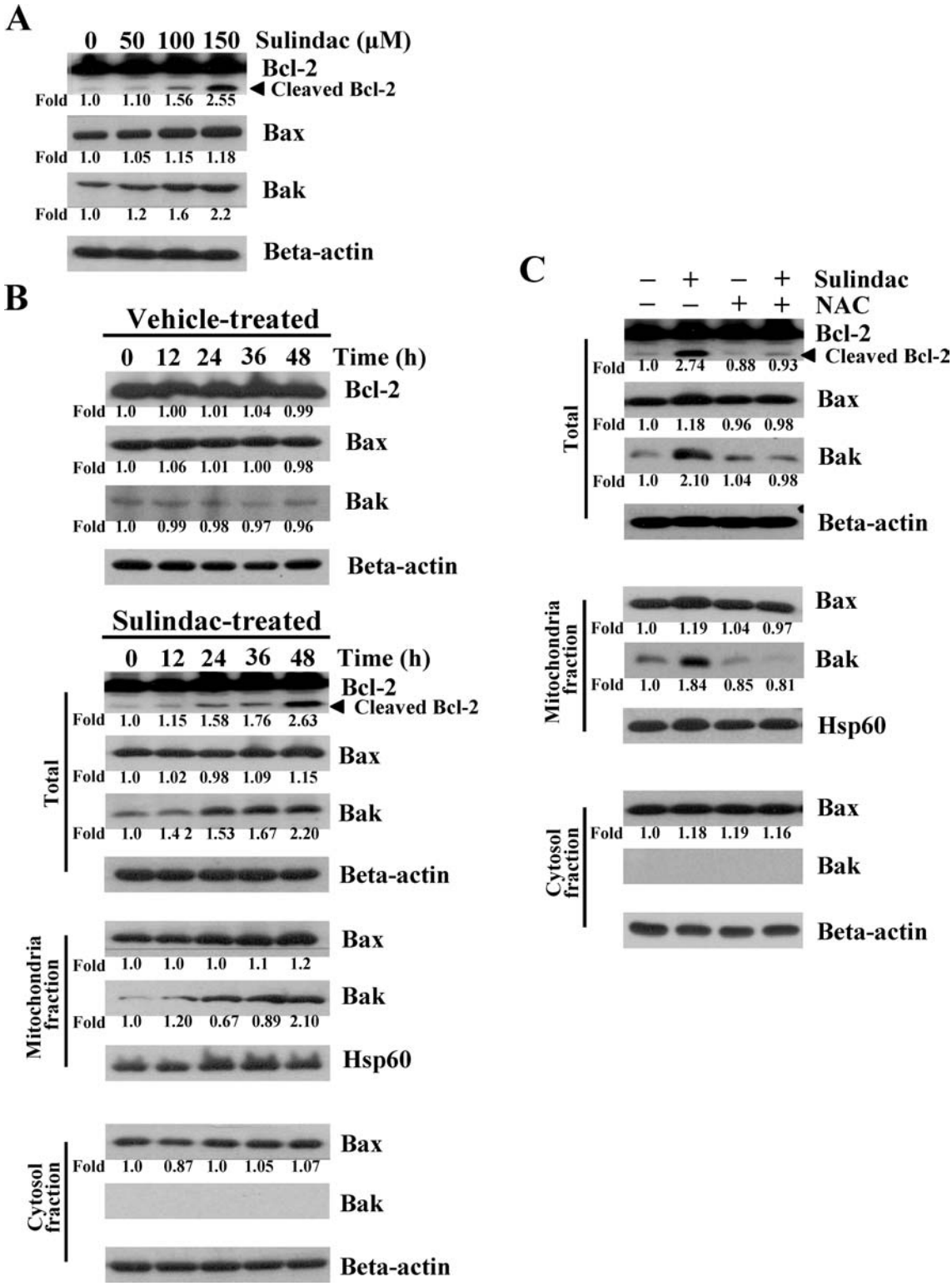

Fig. 5 Sulindac induces Bcl-2 cleavage and mitochondrial accumulation of Bax and Bak. Sulindac treated-cells were fractionated into cytosolic and mitochondrial fractions and analyzed by Western blotting. (A–B), Sulindac causes the dose- and time-dependent cleavage of Bcl-2 and increase in the levels of Bax and Bak. Cells were treated with 0 to 150 μM sulindac for 48 h (A) or for the indicated times with 150 μM sulindac (B). Vehicle-treated (DMSO) as the positive control does not change levels of Bcl-2, Bax, and Bak. (C), NAC prevents sulindac-induced Bcl-2 cleavage and mitochondrial accumulation of Bax and Bak accumulation. Cells were treated with 150 μM sulindac with or without 2 mM NAC for 48 h. Immunoblots are representative of at least two independent experiments

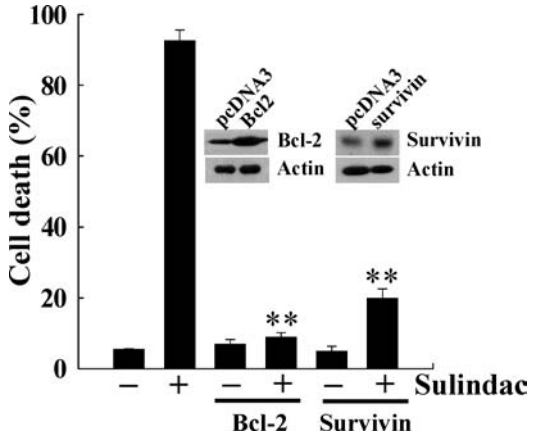

Fig. 6 Inhibition of sulindac-induced apoptosis in Bcl-2- and survivin-overexpressing cells. Overexpression of Bcl-2 and survivin prevent sulindac-induced cell death. IM9 cells were stably transfected with vectors encoding Bcl-2 and survivin. The extent of overexpression was determined by Western blot analysis. Wild-type cells, Bcl-2-overexpressing cells, and survivin-overexpressing cells were treated without (−) or with (+) 200 μM sulindac for 48 h. Cell death was determined by annexin V/PI staining, followed by flow cytometry. The data represent the mean ± s.d. of three independent experiments.$^{**}P < 0.01$ vs. the sulindac-treated groups

p53 activation is involved in sulindac-induced apoptosis

To determine whether p53 is activated by sulindac treatment, we examined the levels of total p53 and its target gene p21^{Waf1} by Western blotting. As shown in Fig. 7(A) and (B), sulindac caused a dose- and time-dependent elevation in the total levels of p53 and p21^{Waf1} in IM9 cellsWe further examined the role of p53 in sulindac-induced apoptosis using siRNAs. As shown in Fig. 7(C), the cells transfected with p53 siRNA had decreased sensitivity to sulindac, whereas sulindac sensitivity was unchanged in cells transfected with a control siRNA. Western blot analysis showed that the level of total p53 but not actin was suppressed by p53 siRNA transfection, indicating that the effect of p53 siRNA was specific. These results suggest that p53 activation plays a role in sulindac-induced apoptosis in MM cells.

Sulindac induces the accumulation of p53 to mitochondria downstream of ROS generation

Although sulindac markedly activates p53, we found that Bax, a transcriptional target gene of p53, was only slightly increased by sulindac. In addition, evidence from several

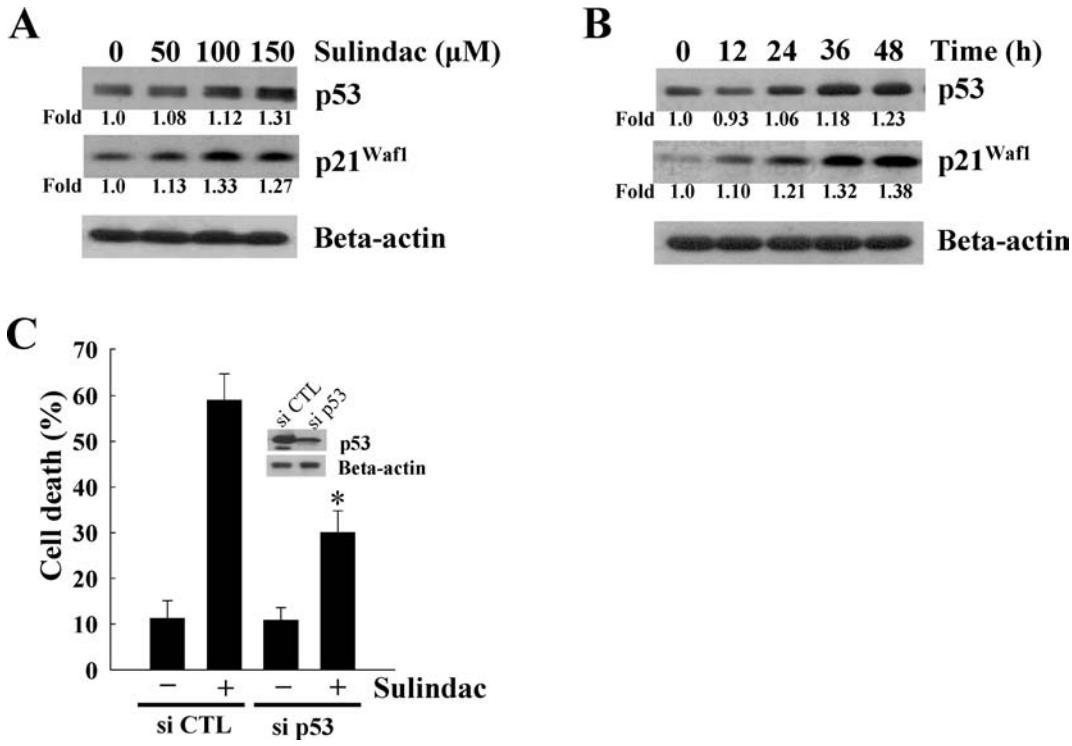

Fig. 7 Activation of p53 during sulindac-induced apoptosis. (A–B), p53 and p21 protein expression levels were increased by sulindac. Cells were treated with 0 to 150 μM sulindac for 48 h (A) or with 150 μM sulindac for the indicated times (B). Total cell lysates were analyzed by Western blotting. Immunoblots are representative of at least two independent experiments. (C), Reduction of p53 levels by p53 siRNA confers resistance to sulindac-induced cell death. IM9 cells were transfected with p53 siRNA by electroporation. The extent of RNA interference was determined by Western blot analysis. Control siRNA-transfected cells (si CTL) and p53 siRNA-transfected cells (si p53) were treated without (−) or with (+) 150 μM sulindac for 48 h. Cell death was determined by annexin V/PI staining, followed by flow cytometry. The data represent the mean ± s.d. of three independent experiments. $^{*}P < 0.05$ vs. the sulindac-treated groups

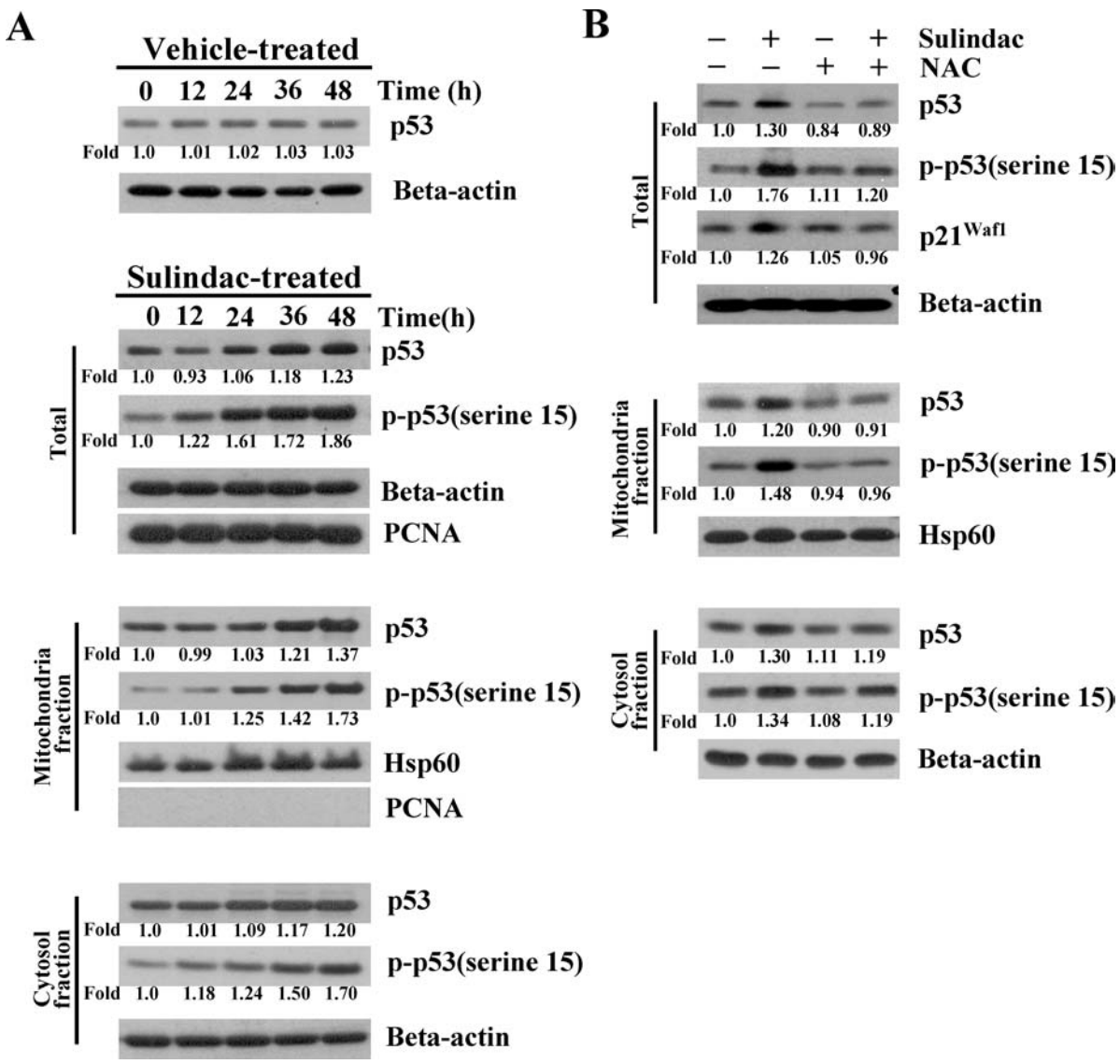

Fig. 8 Sulindac-induced ROS generation causes accumulation of p53 to mitochondria. Sulindac treated-cells were separated into cytosolic and mitochondrial fractions and analyzed by Western blotting as described. (A), Sulindac induces the activation of p53 and causes accumulation of p53 and p-p53 (Ser15) to mitochondria. Cells were treated with 150 μM sulindac for the indicated times. PCNA was used as a marker for nuclear contamination. Vehicle-treated (DMSO) as the positive control does not change level of p53. (B), NAC prevents sulindac-induced accumulation of p53 and p-p53 (Ser15) to mitochondria. Cells were treated with 150 μM sulindac with or without 2 mM NAC for 48 h. Immunoblots are representative of at least two independent experiments

experimental systems and laboratories suggest that p53 induces apoptosis independent of its effects on transcription by acting directly on mitochondria [21–24]. We therefore examined whether sulindac causes p53 to accumulate in mitochondria. A cell fractionation experiment (Fig. 8(A)) revealed that sulindac causes a time-dependent accumulation of p53 and p-p53 (Ser15) in mitochondria, suggesting that p53 accumulates in mitochondria during sulindac-induced apoptosis.

We next examined the role of ROS in sulindac-induced p53 activation and accumulation in mitochondria using the ROS scavenger NAC. As shown in Fig. 8(B), sulindac-induced p53 activation and accumulation in mitochondria was inhibited by NAC. Also, sulindac-induced accumulation of p-p53 (Ser15) was markedly inhibited by NAC. These results indicate that p53 activation and accumulation in mitochondria are mediated by sulindac-induced ROS generation.

p38 MAPK activation is involved in sulindac-induced apoptosis downstream of ROS production

To investigate the role of MAPK signal transduction pathways in sulindac-induced apoptosis of IM9 MM cells, we measured the level of MAPK phosphorylation by Western

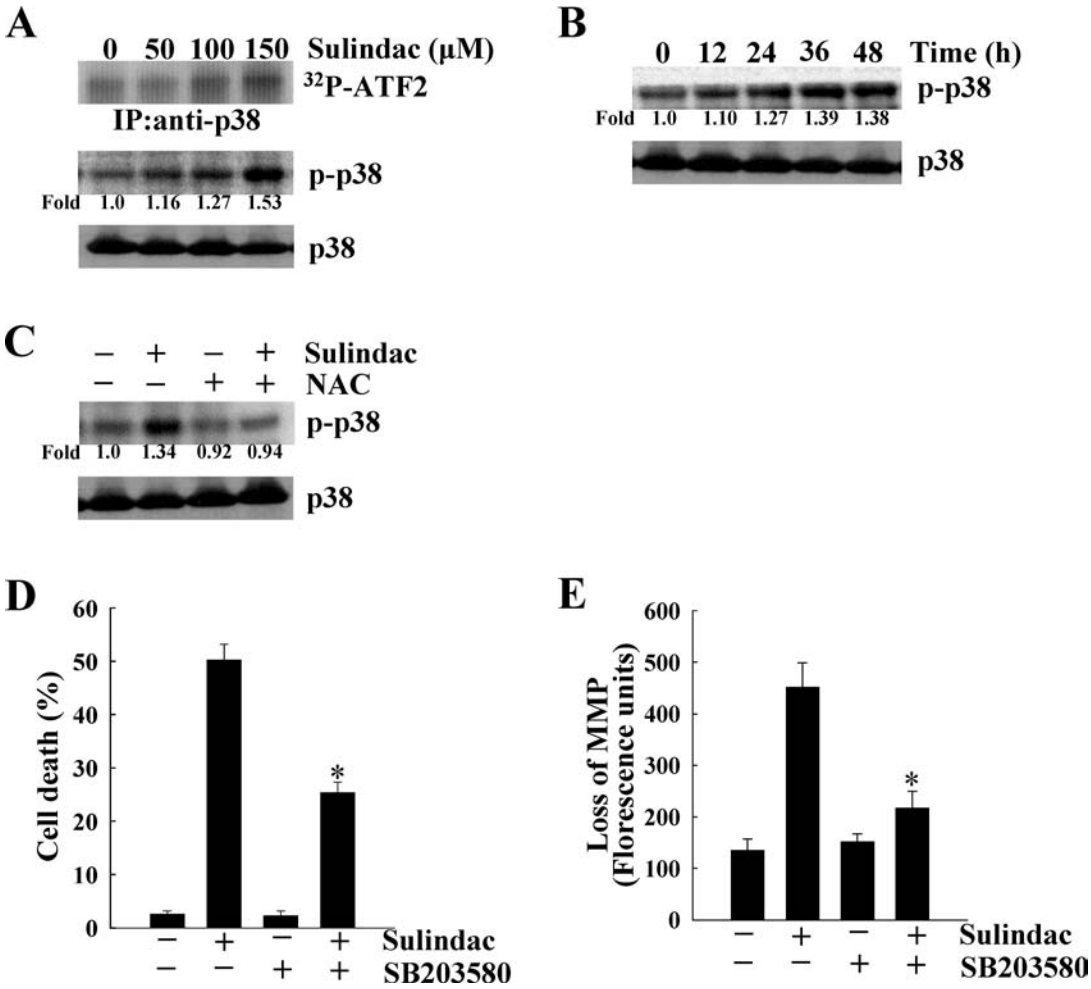

Fig. 9 Sulindac induces p38 MAPK activation downstream of ROS generation. (A–B), Sulindac induces p38 MAPK phosphorylation. Cells were treated with 0 to 150 μM sulindac for 48 h (A) or with 150 μM sulindac for the indicated times (B). Total cell lysates were analyzed by Western blotting. (C), NAC prevents sulindac-induced p38 MAPK activation. Cells were treated with 150 μM sulindac with or without 2 mM NAC for 48 h. Immunoblots are representative of at least two independent experiments. (D–E), SB203580 partially prevents sulindac-induced cell death (D) and loss of MMP (E). Cells were treated with 150 μM sulindac with or without 15 μM SB203580 for 48 h. Each values represent the mean $\pm$ s.d. of three independent experiments. $^{*}P < 0.05$ vs. the sulindac-treated groups

blot analysis. We found that sulindac induces the dose- and time-dependent activation of p38 (Fig. 9(A, B)), but it did not induce the phosphorylation of JNK or ERK (data not shown). We next examined the role of ROS in p38 MAPK activation by measuring sulindac-induced phosphorylation in the presence and absence of NAC. As shown in Fig. 9(C), NAC eliminated prevented sulindac from activating p38 MAPK.

We further examined whether p38 MAPK is important in sulindac-induced apoptosis of IM9 cells by pretreating them with or without SB203580, a specific inhibitor of p38 MAPK. As seen in Fig 9(D), sulindac-induced apoptotic cell death was markedly decreased by SB203580. We also found that SB203580 blocks sulindac-induced mitochondrial membrane permeability (Fig. 9(E)). These results indicate that sulindac can induce prolonged activation of p38 MAPK in IM9 MM cells downstream of ROS generation and that p38 MAPK may play a partial but significant role in sulindac-induced apoptosis.

p38 MAPK activation mediates the accumulation of Bak, Bax, and p53 in mitochondria

Having obtained clear evidence for the involvement of p38 MAPK in sulindac-induced apoptosis, we next asked how p38 MAPK mediates mitochondrial dysfunction. Because sulindac causes the mitochondrial accumulation of proapoptotic proteins such as p53, Bak, and Bax (Fig. 5(A, B)), we evaluated the possibility that p38 MAPK mediates sulindac-induced apoptosis by causing p53, Bak, and Bax to translocate to mitochondria. To test this possibility, we pretreated IM9 cells with SB203580 and then analyzed the subcellular distribution of p53, Bak, and Bax by Western blot analysis.

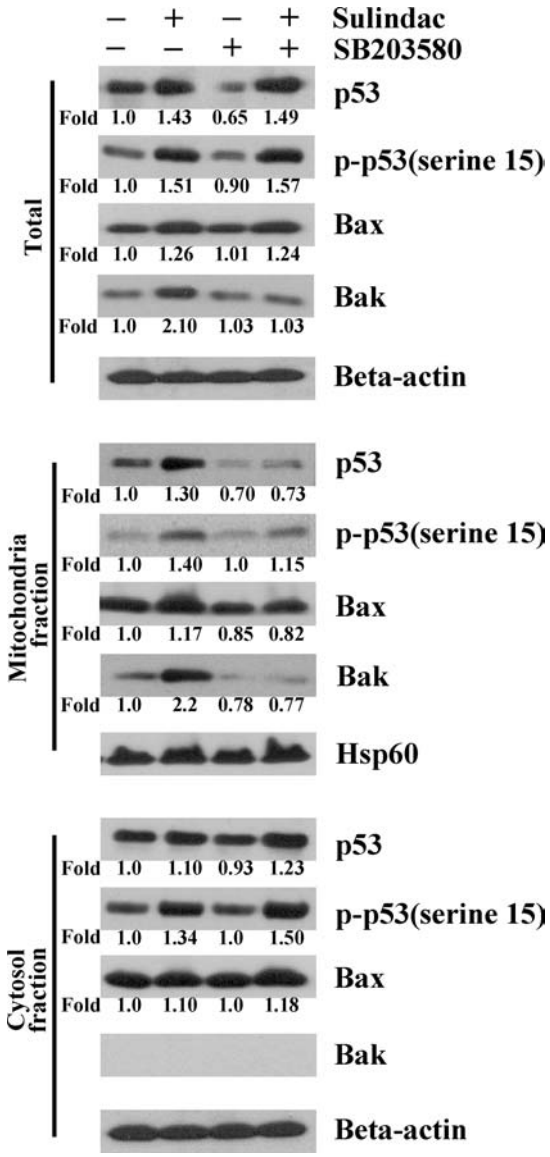

Fig. 10 Accumulation of Bak, Bax, and p53 in mitochondria by p38 MAPK activation during sulindac-induced apoptosis. SB203580 prevents sulindac-induced accumulation of Bak, Bax, and p53 in mitochondria but does not block increases in the total levels of Bax, p53, and p-p53 (Ser15) protein. Cells were treated with 150 μM sulindac with or without 15 μM SB203580 for 48 h. Sulindac treated-cells were separated into cytosolic and mitochondrial fractions and analyzed by Western blotting. Immunoblots are representative of at least two independent experiments

SB203580 markedly decreased the mitochondrial accumulation of p53, Bak, and Bax in response to sulindac treatment (Fig. 10). Furthermore, the accumulation of p-p53 (Ser15) in mitochondria was also markedly inhibited by the p38 MAPK inhibitor. These results indicate that p38 MAPK is activated upstream of mitochondrial dysfunction during sulindac-induced apoptosis and that p38 mediates the mitochondrial accumulation of p53, Bak, and Bax.

Discussion

Many chemopreventive agents have antitumorigenic activities due to their ability to induce apoptosis, suggesting that the induction of apoptosis is an effective strategy for inhibiting tumorigenesis. Sulindac is the most extensively investigated clinically relevant chemopreventive NSAID. Previous reports have shown that sulindac-induced apoptosis in human epithelial cancers requires cyclic GMP-dependent protein kinase-mediated activation of JNK1 [25], down-regulation of β-catenin [26], up-regulation of death receptor 5 [27], down-regulation of ERK1/2 [28], and the presence of Bax [26]; however, the mechanism of apoptosis and the possible relationship between MAPK pathways and oxidative stress in response to sulindac have not been defined in MM cells. The results presented here indicate that sulindac-induced ROS generation plays a critical and primary role in sulindac-induced cell death response in IM9 human MM cells and that this process involves the activation of p38 MAPK and p53. Our results also indicate that p38 MAPK activation-mediated accumulation of p53, Bak, and Bax in mitochondria is accompanied by mitochondrial injury, caspase activation, and apoptosis.

ROS are one class of agents known to damage the mitochondria, and many apoptosis-inducing drugs have been shown to promote the generation of ROS [29, 30]. Thus, we investigated the possibility that ROS plays a role in sulindac-induced apoptosis. We found that sulindac induced the generation of ROS in MM cells. This response was dependent on a cellular event because the addition of sulindac to medium alone did not result in ROS production (data not shown). If these ROS are truly signaling molecules involved in apoptosis, quenchers of ROS such as antioxidants should prevent apoptosis. Indeed, we found that sulindac-induced apoptosis, mitochondrial dysfunction, and caspase activation were greatly reduced by addition of NAC. These results suggest that, in this model system, the generation of ROS has a primary role in the induction of apoptosis by sulindac.

We found that sulindac treatment of IM9 cells caused a marked decrease in the level of antiapoptotic IAP family proteins, including survivin, cIAP-1/2, and XIAP. The sulindac-mediated decrease in the protein level of IAP family proteins was not observed until 24 h, whereas activation of caspases was evident as early as 12 h after sulindac treatment. Because IAP family proteins inhibit caspases [31], these results suggest that the decrease in the level of IAP family proteins amplifies rather than initiates sulindac-induced apoptosis of IM9 cells. Also, sulindac markedly increased the cleavage of Bcl-2 in IM9 cells. This cleavage of Bcl-2 was associated with caspase activation and production of a 23-kDa Bcl-2 fragment, both of which were inhibited by zVAD-fmk (data not shown), indicating that the cleavage is caspase dependent. Because the primary role of Bcl-2 is thought to be the

regulation of cytochrome c release from mitochondria [32, 33], it is possible that this cleavage of Bcl-2 is required for promoting mitochondrial dysfunction such that it serves as part of a positive feedback loop in apoptotic signaling. Furthermore, overexpression of Bcl-2 and survivin potently inhibited sulindac-induced apoptosis, supporting the idea that the mitochondrial pathway and caspase activation play an important role in sulindac-induced apoptosis.

Cell death is as important as cell proliferation in regulating development and homeostasis in multicellular organisms. Physiological cell death is usually mediated through apoptosis, which is positively or negatively regulated by various extracellular factors. A wide range of anticancer and chemopreventive agents have been shown to induce apoptosis in malignant cells in vitro [34, 35]. Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, and the activation of caspases. In many apoptotic responses, mitochondria play a major role in coordinating caspase activation through the release of apoptogenic factors, such as cytochrome *c*, AIF, and endonuclease G [36, 37]. Members of the Bcl-2 family control the release of these proteins, and several other proteins with sequence homologies to Bcl-2 are also implicated in regulation of apoptosis in response to various stimuli [38–41]. Bak is one such protein that promotes cell death by neutralizing the antiapoptotic effect of Bcl-2 [42, 43]. Bax is another key protein that heterodimerizes with Bcl-2, counteracting its antiapoptotic function [44]. Mutations in *Bak* and *Bax* genes have been shown to cause resistance to the induction of apoptosis by certain stimuli [45–47]. The results of the present study revealed that sulindac treatment causes a marked change in the level of Bak protein in IM9 cells, whereas the level of Bax was only slightly increased, although its accumulation to mitochondria was significantly increased. Sulindac treatment of IM9 cells disrupted the MMP, which triggered the release of cytochrome *c* and the activation of caspases. Thus, it seems reasonable to postulate that Bak and Bax proteins play an important role in sulindac-induced apoptosis.

The activation of the p38 MAPK pathway by cellular stress is generally associated with activation of the apoptotic program [48]. Our present results revealed that exposure of cells to sulindac stimulate the phosphorylation of p38 MAPK downstream of ROS generation. Furthermore, a p38 MAPK inhibitor suppressed the sulindac-induced accumulation of Bax to mitochondria, up-regulation of Bak, mitochondrial dysfunction, and apoptosis. These findings indicate that the p38 MAPK-Bak/Bax pathway plays an important role in sulindac-induced apoptosis in MM cells.

In the present study, we also found that induction of p53 participates in sulindac-induced apoptosis, although transfection of siRNA did not completely abolish sulindac-induced cell death. The levels of p53, p-p53 (Ser15), and the p53 target gene, $p21^{Waf1}$, were enhanced by sulindac, effects that were suppressed by NAC. Thus it appears that sulindac initially promotes ROS generation, which subsequently activates p53, suggesting that ROS-dependent activation of p53 activation and phosphorylation mediates the anticancer effect of sulindac. Surprisingly, p53 protein accumulated in mitochondria during the sulindac-induced apoptosis of IM9 cells. Recent work by Moll and coworkers provided strong evidence that p53 contributes to the induction of apoptosis not only through target gene stimulation in the nucleus but also through initiation of proapoptotic processes in the mitochondria due to physical interaction of the core DNA-binding domain of p53 with apoptosis regulators such as Bcl-2 and Bcl-X_L [21–23]. Although we cannot rule out a possible role of p53 transactivation in sulindac-induced apoptosis, our results suggest that sulindac induces apoptosis in MM cells through accumulation of p53 to mitochondria.

Although the mechanisms of p53-mediated apoptosis in response to cellular stress remain poorly understood, a recent study suggests that the phosphorylation of p53 at Ser15 plays a pivotal role in the activation of p53 and the induction of apoptosis [49]. Furthermore, this phosphorylation of p53 has been shown to play a critical role in its stabilization, up-regulation, and functional activation [50]. Interestingly, p38 MAPK phosphorylates p53 at Ser15 in response to UV irradiation and resveratrol treatment [51, 52]. In the present study, we found that the level of p53 phosphorylation at Ser15 was increased during sulindac-induced apoptosis, suggesting that sulindac activates p53 to promote apoptosis of IM9 MM cells. The phosphorylation of p53 is known to be mediated by many protein kinases, including DNA-dependent protein kinase, ATM, ATR, JNKs, ERKs, and p38 MAPK [53–56]. In the present study, sulindac was shown to activate p38 MAPK, which played an important role in the induction of apoptosis. Pretreatment of IM9 MM cells with SB203580, a p38 MAPK-specific inhibitor, did not inhibit the phosphorylation of p53 at Ser15, but it substantially inhibited the accumulation of the phosphorylated protein to mitochondria. Therefore, activation of p38 MAPK does not appear to be required for the activation of p53, but it plays an important role in the accumulation of p-p53 (Ser 15) to mitochondria. Finally, further studies are needed to determine the role of other protein kinases that phosphorylate p53 on Ser15 in sulindac-induced apoptosis.

In conclusion, the present results indicate that sulindac-induced ROS generation in human MM cells increases the levels of p53, Bak, and Bax proteins and causes their accumulation in mitochondria, triggering the release of apoptogenic molecules from the mitochondria to the cytosol, which leads to caspase activation and cell death. The accumulation of p53, Bak, and Bax in the mitochondria was suppressed by SB203580, a selective p38 MAPK inhibitor. The sulindac-induced mitochondrial dysfunction and caspase activation were probably amplified by stimulation of Bcl-2 cleavage

and the down-regulation of IAP family proteins including survivin, XIAP, and cIAP-1/2. Furthermore, our results suggest that p53 and p38 MAPK are essential for sulindac-induced apoptosis at a point downstream of ROS generation. Taken together, these results indicate that sulindac may be clinically promising alone or in combination with other therapies for the treatment of MM.

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