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Attenuating Smac Mimetic Compound 3-Induced NF-κB Activation by Luteolin Leads to Synergistic Cytotoxicity in Cancer Cells

Lang Bai,[1] Wenjie Chen,[1] Xia Wang,[2] Wei Ju,[1] Shanling Xu,[1] and Yong Lin[1*]

[1]*Molecular Biology and Lung Cancer Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest DR. SE, Albuquerque, New Mexico 87108*

[2]*Laboratory of Molecular and Translational Medicine, West China Second University Hospital, Sichuan University, 3-17 Renminnanlu, Chengdu 610041, China*

ABSTRACT

Smac mimetics are potential anticancer therapeutics selectively killing cancer cells through autocrine tumor necrosis factor (TNF)-mediated apoptosis pathway. Our recent study reveal that the Smac mimetic compound 3 (SMC3)-activated NF-κB protects cancer cells against apoptosis, thus blunting SMC3's anticancer activity. Based on our previous observations that the nutrient flavonoid luteolin potently blocks TNF-induced NF-κB activation in cancer cells, we investigated if the combination of SMC3 and luteolin would achieve a synergistic anticancer activity. The results show that luteolin had no effect on autocrine TNF but it effectively blocked SMC3-induced nuclear factor kappa B (NF-κB) activation and expression of anti-apoptotic NF-κB targets. When SMC3 and luteolin were combined in treating cancer cells derived from lung and liver tumors, the activation of TNF-dependent apoptosis was markedly sensitized and a synergistic cytotoxic effect was achieved. In addition, the SMC3 and luteolin co-treatment had marginal effect on immortalized normal human bronchial epithelial cells. The results suggest that combination of SMC3 and luteolin is an effective approach for improving the anticancer value of SMC3, which has implications in cancer prevention and therapy. J. Cell. Biochem. 108: 1125–1131, 2009.

KEY WORDS: NF-κB; SMAC MIMETIC; LUTEOLIN; CYTOTOXICITY; APOPTOSIS

Second mitochondria-derived activator of caspase (Smac, also called direct IAP [inhibitor of apoptosis] binding protein with low p*I*, DIABLO) was identified as an important signal amplifier for the intrinsic (mitochondrial) apoptosis pathway [Chai et al., 2000; Du et al., 2000; Verhagen et al., 2000]. When the intrinsic apoptosis pathway is initiated, the mitochondrial protein Smac is released to the cytosol where it suppresses the inhibitor of apoptosis protein (IAP) family members c-IAP1, c-IAP2, and XIAP so as to release the brake for apoptosis [Chai et al., 2000; Du et al., 2000]. Many cancer cells have acquired resistance to apoptosis. Thus, to lower the apoptosis threshold by modulation of apoptosis-regulating molecules such as Smac is a potential approach for improving anticancer chemo- or radiotherapy [Hanahan and Weinberg, 2000; Wu et al., 2007]. Several Smac mimetics have been developed and shown to have an anticancer property. Interestingly, the anticancer activity of Smac mimetics appears to be executed mainly through induction of autocrine tumor necrosis factor (TNF)-mediated activation of the extrinsic apoptosis pathway [Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007; Bertrand et al., 2008], although other cancer cell-killing mechanisms may also be utilized [Bank et al., 2008; Lu et al., 2008].

Besides triggering apoptosis, Smac mimetics also activate nuclear factor kappa B (NF-κB), a cell survival signal that protects cells against death [Aggarwal, 2003; Varfolomeev et al., 2007; Vince et al., 2007]. Our recent studies unveil that Smac mimetic compound 3 (SMC3), a Smac mimetic with a potent anticancer activity in a variety of tumor cells, activates NF-κB through autocrine TNF; and subsequently NF-κB upregulates expression of anti-apoptotic genes

Abbreviations used: EB, ethidium bromide; IAP, inhibitor of apoptosis protein; LDH, lactate dehydrogenase; NF-κB, nuclear factor B; Smac, second mitochondria-derived activator of caspase; SMC3, Smac mimetic compound 3; ROS, reactive oxygen species; RT-PCR, reverse transcriptase polymerase chain reaction; TNF, tumor necrosis factor.

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*Correspondence to: Dr. Yong Lin, MD, PhD, Molecular Biology and Lung Cancer Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr., SE, Albuquerque, NM 87108. E-mail: ylin@lrri.org

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such as Bcl-XL and MnSOD to attenuate the anticancer activity of the Smac mimetic. Thus, blocking the SMC3-induced NF-κB activation would be an effective approach to improve the anticancer activity of SMC3 [Bai et al., 2009], akin to sensitizing cancer cells to TNF-induced apoptosis using NF-κB blocking agents [Wang et al., 2006; Karin, 2008; Wang and Lin, 2008].

Luteolin (3′,4′,5,7-tetrahydroxyflavone), a common flavonoid found in many plant types such as fruits, vegetables, and medicinal herbs, has been shown to have various anti-inflammation, anti-allergy, and anticancer biological effects [Lin et al., 2008; Seelinger et al., 2008; Lopez-Lazaro, 2009]. Recent studies have attributed the anticancer property of luteolin at least partly to its NF-κB blocking activity [Lin et al., 2008]. In addition, luteolin functions as an anticancer adjunct. For example, luteolin potently blocked TNF-induced NF-κB activation, but it had little effect on the apoptosis signaling pathway activated by TNF, thereby shifting the cellular signaling balance to the side of cell death [Shi et al., 2004; Ju et al., 2007]. Thus, luteolin could be used to sensitize TNF-induced apoptosis in cancer cells.

Because NF-κB blunts the anticancer activity of the SMC3 and luteolin functions as an effective NF-κB blocker, we examined whether a combination of luteolin and SMC3 could achieve increased cancer cell killing activity. The results showed that although luteolin did not interfere with autocrine TNF, it potently blocked SMC3-induced NF-κB activation, resulting in a synergistic cytotoxicity in cancer cells. This observation implies that the combination of luteolin and SMC3 is an effective approach to anticancer chemotherapy.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

SMC3 was a generous gift from Dr. Xiaodong Wang, University of Texas Southwest Medical Center. Luteolin was from Cayman Chemical (Ann Arbor, MI). The following antibodies were used for Western blot: anti-caspase-8 and -caspase-3 (Pharmingen, San Diego, CA), anti-PARP (BioSource, Camarillo, CA), anti-Bcl-XL (Cell Signaling, Beverly, MA), anti-MnSOD (BD Biosciences, San Diego, CA), anti-β-tubulin (Sigma, St. Louis, MO). The human TNF detection ELISA kit was purchased from eBioscience, Inc. (San Diego, CA).

CELL CULTURE

The human lung cancer cell line H23, and human hepatoma cell lines HepG2 and Huh7 were obtained from American Type Culture Collection (Manassas, VA). H23 cells were grown in RPMI 1640 with 10% fetal bovine serum, 1 mmol/L glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin. HepG2 and Huh7 cells were cultured in DMEM with 4.5 g/L glucose, 10% fetal bovine serum, 1 mmol/L glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin. HCCBE-2 and -3 are human bronchial epithelial cells immortalized by insertion of cyclin-dependent kinase 4 and human telomerase reverse transcriptase [Ramirez et al., 2004], a gift from Dr. Jerry Shay and Dr. John Minna (University of Texas Southwestern Medical Center) and cultured in keratinocyte serum-free medium in collagen-coated plates.

CYTOTOXICITY ASSAY

Cytotoxicity was determined using a lactate dehydrogenase (LDH) release-base cytotoxicity detection kit (Promega, Madison, WI). Cells were seeded in 48-well plates at 70–80% confluence. After culture overnight, cells were treated as indicated in each figure legend. LDH release was determined and cell death was calculated as described previously [Chen et al., 2007; Ju et al., 2007]. In order to morphologically study cell death, H23 cells were cultured on cover slides and pretreated with luteolin (20 μM) for 30 min followed by SMC3 (10 nM) treatment for 16 h or remained untreated. Cells were stained with 50 μg/ml of acridine orange and 50 μg/ml ethidium bromide (EB), and immediately visualized and photographed under a fluorescent microscope [Lin et al., 2004].

MEASUREMENT OF AUTOCRINE TNF SECRETION BY ELISA

Cells were plated onto 12-well plates at 70–80% confluence. After culture overnight, cells were treated as described in the figure legends. The culture media were collected and the concentration of TNF was detected by ELISA analysis with the human TNF-α ELISA kit following the instruction of the manufacturer (eBioscience, Inc.) [Bai et al., 2009].

WESTERN BLOT

Cells were harvested and lysed in M2 buffer (20 mM Tris–HCl, pH 7.6; 0.5% NP-40; 250 mM NaCl; 3 mM EGTA; 3 mM EDTA; 2 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride; 20 mM β-glycerophosphate; 1 mM sodium vanadate; and 1 μg/ml leupeptin). Equal amounts of protein extracts were resolved in 12% SDS–PAGE and the proteins of interest were probed by Western blot and visualized by enhanced chemiluminescence according manufacturer's instructions (Amersham, Piscataway, NJ) [Lin et al., 2006; Wang et al., 2007].

TRANSFECTION AND LUCIFERASE REPORT ASSAY

Cells grown in 24-well plates were transfected with p5 × κB-Luc and pRSV-LacZ with FuGENE 6 according to manufacturer's instruction (Roche, Indianapolis, IN). Twenty-four hours after transfection, cells were treated as indicated in each figure legend. Luciferase activity was measured using a luciferase assay kit (Promega) and normalized to β-galactosidase activity [Lin et al., 1999].

REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted with the RNAeasy kit (Qiagen, Valencia, CA). One microgram of RNA from each sample was used as a template for cDNA synthesis with a reverse transcription kit (Promega). An equal volume of cDNA product was used in the PCR. The primers used were: MnSOD, AGTTGCTGGAAGCCATCAAACGTG and TAAGGCCTGTTGTTCCTTGCAGTG; Bcl-XL, TGGGCTCACTCTTCAGTCGGAAAT and ATGTAGTGGTTCTCCTGGTGGCAA; and β-actin, CCAGCCTTCCTTCCTGGGCAT and AGGAGCAATGATCTTGATCTTCATT. The reaction condition was 94°C, 45 s; 55°C, 40 s; and 72°C, 45 s. For MnSOD, Bcl-XL, and β-actin, the cycles for PCR were 25, 27, and 21, respectively. PCR products were run on 2% agarose gel with 0.5 μg/ml EB, visualized, and photographed.

STATISTICAL ANALYSIS

Data are expressed as means ± standard deviation (SD). Statistical significance was examined by one-way analysis of variance (ANOVA) pair-wise comparison. To assess the potential for interactions between factors, we included interaction terms in the models. When tests indicated an interaction, 95% confidence intervals for the differences in means were obtained to assess the magnitude of the interaction. In all analyses, $P < 0.05$ was considered statistically significant.

RESULTS

LUTEOLIN DOES NOT INTERFERE WITH SMC3-INDUCED AUTOCRINE TNF

Our recent study found that SMC3-induced autocrine TNF is independent of NF-κB, and NF-κB attenuates SMC3-induced cell death. Thus, blockage of NF-κB could enhance the cancer cell killing efficacy of SMC3 [Bai et al., 2009]. Considering that luteolin is a potent NF-κB blocker, we hypothesized that this flavonoid could be an adjunct agent in sensitizing SMC3's anticancer activity. In order to augment SMC3-induced cytotoxicity, luteolin should not suppress autocrine TNF triggered by SMC3. Therefore, we first investigated if luteolin affects the SMC3-triggered TNF secretion from H23 and HepG2 cells, two SM-responsive cell lines derived from distinct human tumors. The results show that regardless of the presence of luteolin, SMC3 equally increased the TNF in culture media at all the tested time points (Fig. 1A,B). Notably, luteolin by itself did not induce autocrine TNF (Fig. 1A,B). These results indicate that luteolin has no inhibitory effect on SMC3-induced TNF secretion, an essential process for tumor cell apoptosis triggered by this Smac mimetic compound.

LUTEOLIN EFFICIENTLY BLOCKS NF-κB ACTIVATION INDUCED BY SMC3

Next, the effect of luteolin on SMC3-induced NF-κB activation in H23 and HepG2 cells was examined. SMC3 potently activated NF-κB in both cell lines, which was detected by a reporter assay with an NF-κB-responsive luciferase reporter (Fig. 2A). Inclusion of luteolin markedly blocked the NF-κB activity induced by SMC3 (Fig. 2A). Similarly, the SMC3-stimulated expression of the two representative NF-κB target anti-apoptotic genes Bcl-XL and MnSOD was also completely suppressed by luteolin (Fig. 2B,C). Remarkably, expression of both the mRNA and protein levels of these genes was effectively suppressed by luteolin, suggesting that the down-regulation of these anti-apoptotic factors is through blocking SMC3-induced NF-κB transcriptional activity.

LUTEOLIN POTENTIATES SMC3-INDUCED APOPTOSIS PATHWAY

The TNF-induced NF-κB pathway in cancer cells is dominant over the apoptosis pathway, resulting in cancer cell survival and proliferation in response to TNF. Thus, blocking the NF-κB pathway would release the apoptosis brake to ensure the initiation of TNF-induced apoptosis [Wang and Lin, 2008; Balkwill, 2009]. We then examined the activation of caspase-8, the initiator caspase that is recruited and activated at the TNF receptor 1 signaling complex by SMC3 and luteolin. In H23 cells, SMC3 alone induced a weak and slow (at 24 h posttreatment) activation of caspase-8, which was detected by Western blot as the appearance of the cleaved active form of caspase-8 (p43/36 and p23). When luteolin was included, the activation of caspase-8 was significantly stronger and faster (began at 8 h posttreatment). The subsequent activation of the effector caspase-3 and cleavage of the caspase-3 substrate PARP was also dramatically enhanced (Fig. 3A). Remarkably, SMC3 selectively eliminated c-IAP1 while it had a marginal effect on c-IAP2 expression, whereas luteolin had no detectable effect on these two IAP proteins (Fig. 3A). Although under the experimental conditions SMC3-induced caspase-8 activation was too weak to be detected in HepG2 cells, the combined treatment with SMC3 and luteolin dramatically activated caspase-8 and downstream caspase-3 (Fig. 3B). Furthermore, sub-G1 distribution, which reflects apoptosis, was significantly increased when cells were treated with SMC3 plus luteolin (Fig. 3C). In addition, apoptotic cell death was

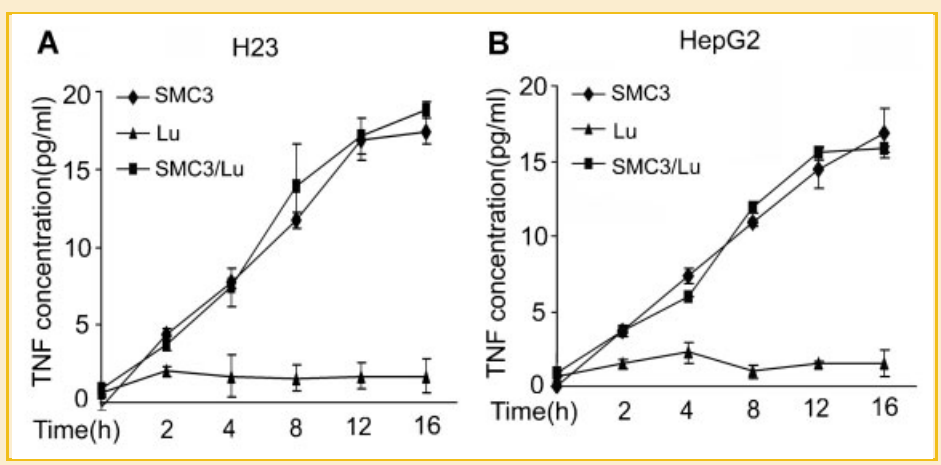

Fig. 1. Luteolin has no effect on SMC3-induced TNF secretion. H23 (A) or HepG2 (B) cells were pretreated with luteolin (20 μM) for 30 min follow by SMC3 (50 nM) for the indicated times or left untreated (0 min). The concentrations of TNF in cell culture media were measured by ELISA.

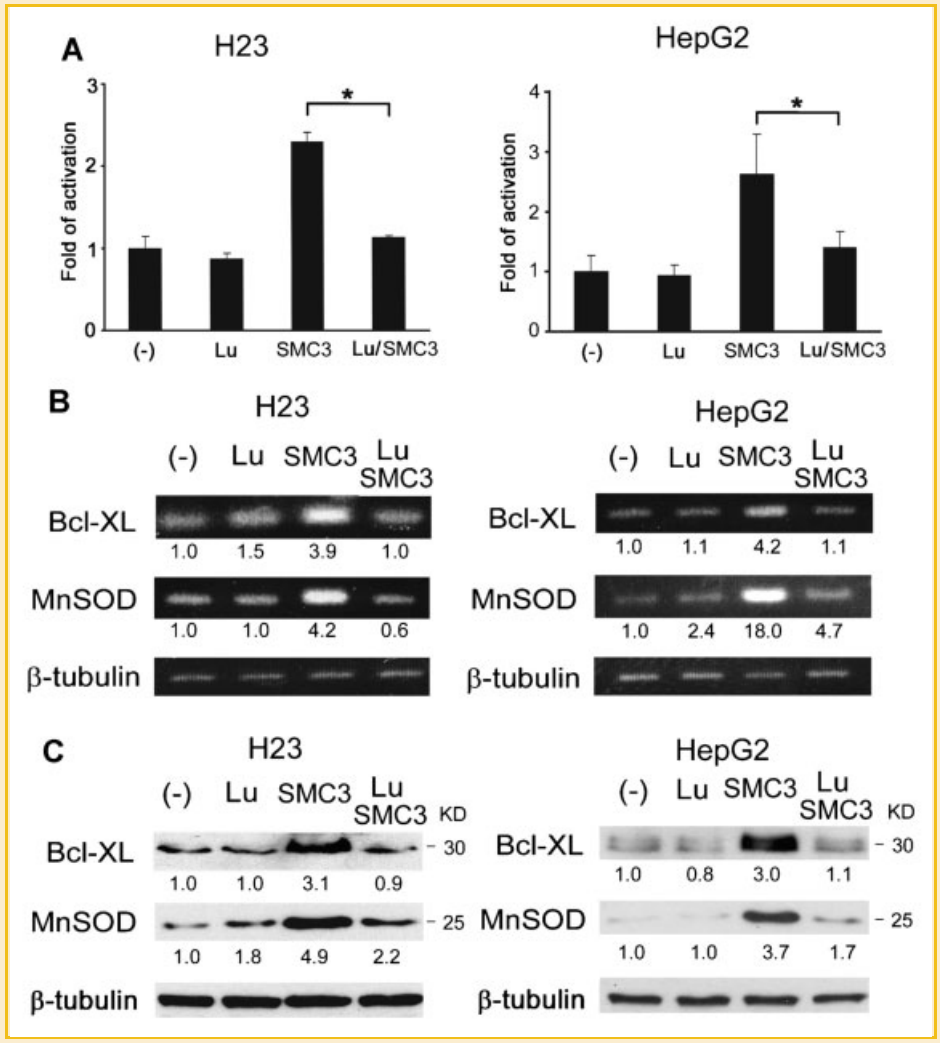

Fig. 2. Luteolin blocks SMC3-induced NF-κB activation. A: H23 and HepG2 cells were co-transfected with p5 × κB-Luc and pRSV-LacZ. Twenty-four hours after transfection the cells were pretreated with luteolin (20 μM) for 30 min followed by SMC3 treatment (10 nM for H23 and 25 nM for HepG2 cells) for 24 h or left untreated. Luciferase activity was detected and normalized to β-galactosidase activity. Data shown are the mean ± SD, $^{*}P < 0.01$. B: H23 and HepG2 cells were pretreated with luteolin (20 μM) for 30 min followed by SMC3 treatment (10 nM for H23 and 25 nM for HepG2 cells) for 6 h or left untreated. TNF mRNA was detected by RT-PCR. β-Actin was detected as an input control. C: H23 and HepG2 cells were treated as in subpart (B). Bcl-XL and MnSOD proteins were detected by Western blot. β-Tubulin was detected as an input control.

detected morphologically by acridine orange and EB staining. The combination of SMC3 and luteolin dramatically increased cell death and the dead cells exerted typical apoptotic features (data not shown). These results suggest that luteolin is able to sensitize SMC3-induced apoptosis in both H23 and HepG2 cells.

SYNERGIC CYTOTOXICITY IS INDUCED BY COMBINED TREATMENT WITH SMC3 AND LUTEOLIN

Next we examined whether co-treatment of luteolin and SMC3 result in enhanced cytotoxicity in cancer cells by detecting LDH release. We first treated H23 cells with an increasing concentration of luteolin and a fixed SMC3 dose (10 nM). SMC3 alone caused little cell death (<10%). However, a dramatic increase in cell death was detected when increasing concentrations of luteolin were included. Consistently, a potentiated cytotoxicity was seen with a fixed luteolin concentration and increased concentrations of SMC3 (Fig. 4A,B). A similar observation was also made in HepG2 and Huh7 cells (Fig. 4C). The substantial increase in cytotoxicity in luteolin/SMC3 compared to SMC3 or luteolin alone was significantly higher than would be expected under an additive model, indicating a significant synergy ($P < 0.001$ for all cell lines). In addition, an MTT assay was employed to detect cell viability, which showed results consistent with that of the LDH release assay (data not shown). These results suggest that combined treatment with luteolin and SMC3 could achieve a synergistic anticancer activity. Interestingly, in the two immortalized human bronchial epithelial cell lines (HCCBE-2 and -3), SMC3 and luteolin combination did not cause detectable cytotoxicity (Fig. 4D), suggesting that co-treatment of SMC3 and luteolin selectively kill malignant cells.

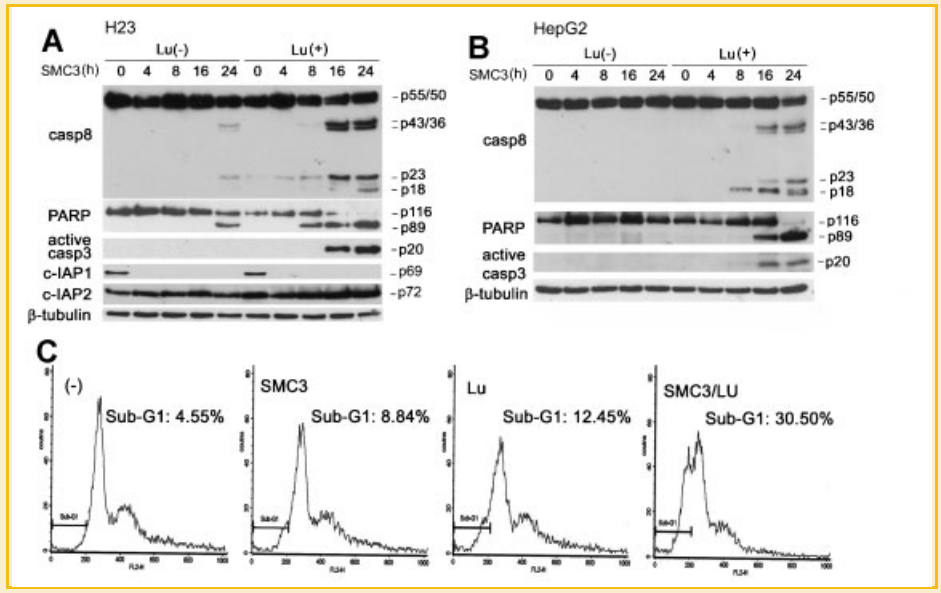

Fig. 3. Luteolin potentiates SMC3-induced apoptosis. A: H23 cells were pretreated with 20 μM of luteolin for 30 min or left untreated and followed by SMC3 treatment (10 nM) for the indicated time periods. Caspase-8, PARP, activated caspase-3, c-IAP1, and c-IAP2 were detected by Western blot. β-Tubulin was detected as an input control. B: HepG2 cells were treated with 20 μM of luteolin for 30 min or left untreated and followed by SMC3 treatment (25 nM) for the indicated time periods. Caspase-8, PARP, and activated caspase-3 were detected by Western blot. β-Tubulin was detected as an input control. C: H23 cells were pretreated with 20 μM of luteolin for 30 min or left untreated and followed by SMC3 treatment (10 nM) for 16 h. The cells were stained with propidium iodide (100 μg/ml) for 30 min and detected by flow cytometry.

THE POTENTIATED CYTOTOXICITY BY SMC3 AND LUTEOLIN CO-TREATMENT IS TNF-DEPENDENT

Finally, we examined whether the synergistic cytotoxicity caused by SMC3 and luteolin co-treatment is through potentiating autocrine TNF-induced apoptosis. A TNF neutralizing antibody was used to block the function of secreted TNF from H23 cells. Consistent with previous reports that cell death induced by SMC3 alone was dramatically inhibited by pretreatment with the TNF neutralizing antibody [Petersen et al., 2007; Bai et al., 2009], the synergism in cell death by SMC3 and luteolin was also abolished by the TNF neutralizing antibody. As a negative control, the control antibody had no effect on SMC3 and luteolin-induced cytotoxicity (Fig. 5). Together with the results shown in Figure 3, this observation further supports the finding that the synergistic cancer cell killing by the SMC3 and luteolin combination is mainly through sensitization of autocrine TNF-induced apoptosis.

DISCUSSION

In this report, we provide evidence validating the hypothesis that luteolin can sensitize SMC3-induced cancer cell apoptosis through blocking the NF-κB pathway. We found that luteolin does not interfere with SMC3-induced autocrine TNF that is essential for apoptosis caused by this Smac mimetic. However, luteolin potently blocked SMC3-induced NF-κB activation, and the co-treatment of luteolin and SMC3 resulted in potentiated apoptosis and a synergistic cytotoxicity in cancer cells. The results suggest that combination of luteolin and SMC3 could be an effective approach for anticancer chemotherapy. Similar to other anticancer drugs, combining SMC3 with other drugs such as luteolin would increase their therapeutic effect while reducing their doses in order to minimize adverse effects [Mabuchi et al., 2004; Maschek et al., 2004].

Our recent work found that the process of SMC3-induced autocrine TNF is independent of NF-κB activation and NF-κB plays a negative role in attenuating SMC3-induced cytotoxicity [Bai et al., 2009]. The fact that NF-κB is distinctively required for cell survival but not death during cancer cells' response to SMC3 provides an opportunity to sensitize the anticancer activity of SMC3 by shutting off the NF-κB pathway. Indeed, directly targeting key mediators of the TNF-induced NF-κB activation pathway, such as IKKβ or the NF-κB RelA subunit, dramatically potentiated SMC3-induced cytotoxicity in a variety of cancer cells [Bai et al., 2009]. In addition, in the lung cancer cell line A549, which does not secret TNF and is resistant to SMC3-induced death, the combination of SMC3, TNF, and luteolin caused substantial cytotoxicity (data not shown). Taken together with our previous observation that luteolin sensitizes TNF-induced apoptosis in A549 cells through blocking the NF-κB pathway, these results fully support our hypothesis that blocking NF-κB with luteolin sensitizes SMC3-induced cell death. The observations suggest that the combination of other anticancer drugs that have NF-κB inhibition properties will achieve potentiated anticancer activity. In this study, we show clearly that the combination of luteolin and SMC3 is able to reach this goal. The blockage of SMC3-induced NF-κB activation by luteolin may underlie the main mechanism of the synergistic cytotoxicity caused by these two agents, although other mechanisms are not excluded [Leung et al., 2005; Lin et al., 2008].

Luteolin blocks TNF-induced NF-κB activation through a mechanism that required accumulation of the reactive oxygen species (ROS) superoxide [Ju et al., 2007]. This process does not

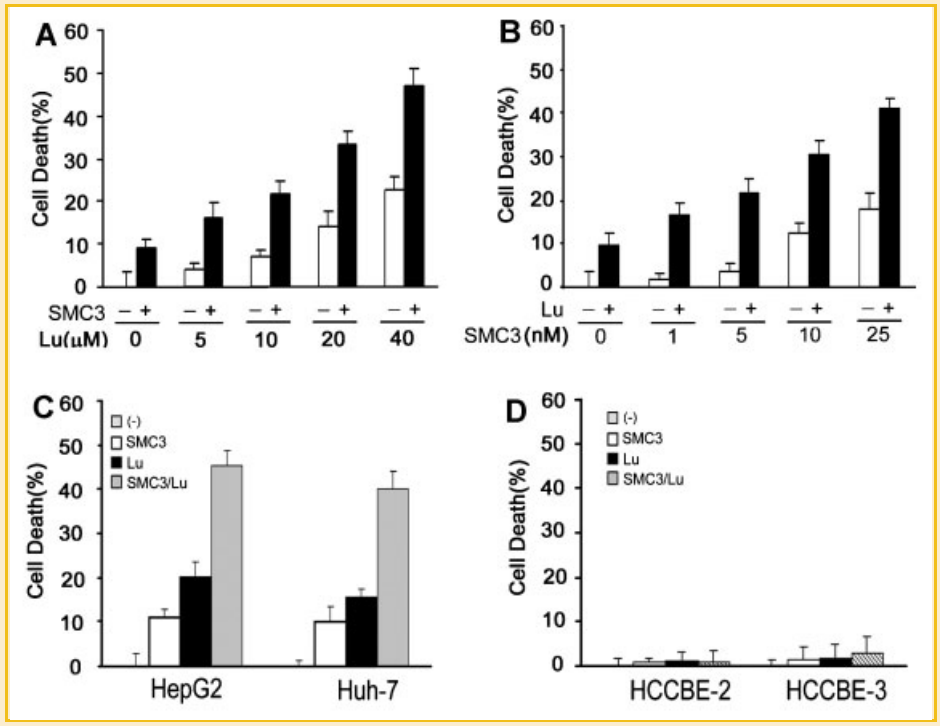

Fig. 4. Synergistic cytotoxicity is induced by SMC3 plus luteolin co-treatment in cancer but not untransformed cells. A: H23 cells were pretreated with luteolin (5–40 μM) for 30 min or remained untreated and followed by exposure to SMC3 (10 nM) for 36 h. Cell death was measured with an LDH release assay kit. B: H23 cells were pretreated with luteolin (20 μM) for 30 min or remained untreated and followed by exposure to SMC3 (1–25 nM) for 36 h. Cell death was measured as described in subpart (A). C: HepG2 and Huh-7 cells were pretreated with 20 μM of luteolin for 30 min followed by 25 nM of SMC3 treatment for 36 h. Cell death was measured as described in subpart (A). D: HCCBE-2 and -3 cells were pretreated with luteolin (40 μM) for 30 min followed by 36 h of treatment with 25 nM of SMC3. Cell death was measured as described in subpart (A).

affect the early steps of NF-κB activation, such as activation of IKK and degradation of IκB, but suppresses NF-κB-triggered expression of anti-apoptotic genes [Shi et al., 2004; Ju et al., 2007]. Although our previous studies demonstrated that induction of autocrine TNF is independent of either transcription or translation, the signal leading to this process has not been revealed [Wang et al., 2008; Bai et al., 2009]. The results in this report show that the SMC3-induced autocrine TNF is not affected by luteolin, a strong ROS inducer in cancer cells [Ju et al., 2007], suggesting that the change of the cellular redox status is unlikely involved in SMC3-stimulated TNF secretion.

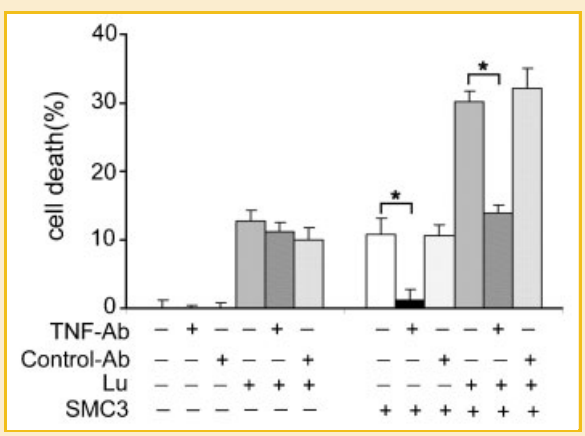

Fig. 5. Blocking SMC3-induced TNF secretion suppresses SMC3 plus luteolin co-treatment-induced cytotoxicity. H23 cells were pretreated with 20 μM luteolin for 30 min, TNF neutralizing antibodies (1 μg/ml), or control antibody (1 μg/ml) for 1 h, followed by SMC3 (10 nM) treatment for 36 h. Cell death was measured by LDH leakage assay.

We further found that SMC3 and luteolin, alone or in combination, caused no detectable cytotoxicity in immortalized human bronchial epithelial cells, suggesting the approach of combining these two drugs has relatively low toxicity in normal or untransformed cells. Because luteolin is rich in diets and is implicated to be preventive against cancer [Lin et al., 2008; Seelinger et al., 2008], combining SMC3 with luteolin may also be used in cancer prevention. The results shown here warrant further animal experiments to validate the cancer therapy and prevention values of combining these potential anticancer agents in vivo.

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