Sulindac enhances arsenic trioxide-mediated apoptosis by inhibition of NF-κB in HCT116 colon cancer cells

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Abstract. To study whether the apoptotic effect of arsenic trioxide (As2O3) on colon cancer cells could be enhanced by the addition of sulindac, HCT116 cells were treated with As2O3 (1, 5, 10 μM) and sulindac (0.5 mM), either alone or in combination. As2O3 alone slightly inhibited the growth of HCT116 cells, whereas the combination of As2O3 and sulindac reduced cell growth by 30-40%. Annexin V staining indicated that the synergistic effect of the combination was mediated through increased apoptosis. We examined whether the combination of As2O3 and sulindac on apoptosis is mediated by inhibition of the NF-κB pathway in HCT116 colon cancer cells. Western blot analysis showed that the level of nuclear NF-κB (p65) was not changed significantly by As2O3 or sulindac treatment alone, while the level of nuclear NF-κB (p65) was drastically decreased in the combination treatment by inhibiting the phosphorylation and the degradation of IκB-α. These results suggest that sulindac enhances apoptosis when combined with As2O3 by inhibiting NF-κB activation mediated through the blocking of phosphorylation and degradation of IκB-α.

Introduction

During the last decade, arsenic trioxide (As2O3) has been proven to be a promising alternative treatment in relapsed patients with acute promyelocytic leukemia (APL) as well as newly diagnosed APL patients. Even in relapsed APL patients, complete remission rates have reached 80-100% (1). Moreover, toxicities do not seem to be serious with only minimal myelosuppression. The most common side effects of As2O3 were gastrointestinal symptoms. The mechanisms of action were shown to exert dose-dependent dual effects in APL cells, i.e., by triggering apoptosis and inducing partial differentiation (2,3). This promotes differentiation of APL cells at low concentrations, however at high concentrations, it triggers apoptosis and inhibits the growth of cells. These effects were associated with the modulation and degradation of the t(15;17)-specific fusion protein PML-RARα (4,5).

Recent studies show that the apoptotic effect of As2O3 is not specific to APL cells, and that it can be observed in non-APL leukemia and lymphoma cells, as well as in other solid tumor cell lines (6-9). Antitumor effects may be enhanced when As2O3 is combined with other agents such as all-trans-retinoic acid (ATRA) and interferon-α (IFN-α). These drugs have been reported to synergistically promote apoptosis when combined with As2O3 (10,11). The mechanism of As2O3 for inducing apoptosis is explained by the activation of caspases, the downregulation of Bcl-2 and the modulation of p53, as well as the uncoupling of the mitochondrial potential (12-14).

A transcriptional factor, NF-κB is known to induce genes that promote cell survival and block apoptosis. In addition, NF-κB can upregulate genes which are significant in tumor growth and metastasis (15). Sulindac (nonsteroidal anti-inflammatory drug, NSAID) may cause regression of colonic polyps and block their progression to cancer. Sulindac and its metabolites are known to inhibit the NF-κB pathway (16,17). According to Berman et al, sulindac enhances tumor necrotic factor (TNF)-mediated apoptosis in lung carcinoma by suppressing NF-κB (15). This suggests that sulindac and other NSAIDs may serve as useful agents in cancer chemotherapy.

To find out whether the apoptotic effect of As2O3 on colon cancer cells could be enhanced by the addition of sulindac, HCT116 cells were treated with As2O3 and sulindac, either alone or in combination. In addition, our study revealed that this synergistic effect was mediated by inhibition of the NF-κB pathway in HCT116 colon cancer cells.

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Abbreviations: APL, acute promyelocytic leukemia; NSAID, nonsteroidal anti-inflammatory drug; NF-κB, nuclear factor-κB

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Materials and methods

Cell cultures and reagents. HCT116 human colon cancer cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in MacCoy's 5A medium (Gibco, Rockville, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified 5% carbon dioxide and 95% air incubator at 37°C. Sulindac and As2O3 were obtained from Sigma (St. Louis, MO, USA). Sulindac was dissolved in methanol at a concentration of 100 mM stock solution and As2O3 was dissolved in NaOH. All chemicals were obtained from Sigma, unless otherwise stated.

Antibodies. The rabbit anti-active caspase-3, rabbit anti-caspase-8, mouse anti-IκB-α phospho-specific, mouse anti-IκB-α, and mouse anti-NF-κB p65 antibodies were obtained from BD Phar-mingen (Mountain View, CA, USA).

Cell viability assays. Cell viability was assessed by MTT assay kit (Roche, Mannheim, Germany). MTT assays were used to measure the number of viable cells. In this assay, the membrane permeant dye is reduced by mitochondrial dehydrogenases in living cells, and spectrophotometric measurement allows quantification of cell viability. Equal numbers of cells were cultured in 96-well plates. HCT116 colon cancer cells were pretreated for 2 h with or without 0.5 mM sulindac, and then treated for 24 h with As2O3 (1, 5, 10 μM). The slides were washed three times with TTBS for 20 min at room temperature. The slides were incubated in blocking solution (10% normal goat serum, 0.1% (v/v) Tween-20) for 1 h at room temperature for 2 h with FITC-conjugated goat anti-mouse IgG which had been diluted at 1:100 in 0.1% BSA/PBS overnight at 4°C. After washing with PBS, the slides were incubated at room temperature for 2 h with FITC-conjugated goat anti-mouse IgG which had been diluted at 1:200 in the 0.1% BSA/PBS (Jackson ImmunoResearch, West Grove, PA, USA). After washing with PBS and H2O, the slides were mounted. The slides were examined under a fluorescent microscope.

Results

Sulindac synergistically enhances As2O3 induced cell growth inhibition. As2O3 is known to inhibit cell proliferation, and sulindac also causes regression of cell growth in colon cancer. The goal of this study was to investigate whether the combination of As2O3 and sulindac is able to work synergistically. To examine whether sulindac can enhance the effect of As2O3 on cell proliferation and cell death, an MTT assay was performed. As2O3 alone had a minimal effect in inhibiting cell growth, while the combination of As2O3 and sulindac resulted in about a 30-40% reduction of cell growth in a dose-dependent manner over the same time period (Fig. 1). This indicates that sulindac synergistically enhances As2O3 induced cytotoxicity.

Sulindac and As2O3 synergistically induce cell death. To clarify whether the reduction of viable cells after the combined treatment with As2O3 and sulindac is mediated through the induction of apoptosis, cells were stained with Wright stain solution and annexin V-FITC, and analyzed by flow cytometry. Wright staining showed that treatment with As2O3 and sulindac induced more apoptotic cells than treatment with As2O3 alone (Fig. 2). Fig. 3 shows the apoptotic effect of the combined treatment on HCT116 colon
cancer cells using flow cytometric analysis after annexin V-FITC staining. The combination of As$_2$O$_3$ 5 μM and sulindac 0.5 mM significantly increased the percentage of apoptotic cells to 42%, while As$_2$O$_3$ 5 μM only slightly increased the percentage of apoptotic cells to 13.2%. Notably, a concentration of As$_2$O$_3$ 10 μM alone resulted in a 13.8% increase, while the combined treatment resulted in a 45% increase in apoptotic cells, which is only slightly higher when compared to those of a concentration of As$_2$O$_3$ 5 μM (Fig. 3). These results suggest that sulindac enhances As$_2$O$_3$-induced apoptosis synergistically, and the concentration of As$_2$O$_3$ should be at least 5 μM in order to induce the synergistic effect of the combination with sulindac.

Sulindac promotes As$_2$O$_3$-induced apoptosis through a caspase-8- and -3-mediated apoptotic pathway. As$_2$O$_3$ has been shown to cause cell death through activating caspases that trigger a death pathway in several types of cancer cells, including leukemic, myeloma and lung cancer cells. In order to confirm that increased apoptosis caused by the combination of As$_2$O$_3$ and sulindac was mediated by activation of caspases in the HCT116 colon cancer cells, we assessed different induction markers, such as caspase-8, and caspase-3 activation by Western blot analysis. Treatment of cells with As$_2$O$_3$ alone did not activate the initiator, caspase-8, whereas treatment with As$_2$O$_3$ and sulindac resulted in activation of caspase-8 in a dose-dependent manner (Fig. 4). This became noticeable at a concentration of As$_2$O$_3$ 5 μM and sulindac 0.5 mM. The precursor form of caspase-3 was cleaved to 17 kDa after treatment with the combination of the two agents, indicating that caspase-3 was also activated by treatment with As$_2$O$_3$ and sulindac. This result suggests that the combination of As$_2$O$_3$ and sulindac promotes apoptosis by a caspase-8- and -3-mediated pathway in HCT116 colon cancer cells.
Combination of As$_2$O$_3$ and sulindac inhibits phosphorylation and degradation of IκB-α. It can be hypothesized that the synergistic effect of apoptosis caused by the combination of sulindac and As$_2$O$_3$ is attributable to the inhibition of NF-κB. The NF-κB transcription factor resides in cytosol in an inactive state, complexed with the inhibitory protein IκB. Activation occurs by phosphorylation of IκB-α at Ser32 and Ser36. Phosphorylation of IκB-α causes proteosome-mediated degradation of IκB-α, resulting in the release of active NF-κB and nuclear translocation of activated NF-κB. As phosphorylation of IκB-α at Ser32 is essential for the release of active NF-κB, phosphorylation at this site is an excellent marker of NF-κB activation. To ascertain whether the addition of sulindac to As$_2$O$_3$ inhibits phosphorylation of IκB-α and ultimately inhibits NF-κB activation, we measured the level of phosphorylated IκB-α, cytosolic IκB-α and nuclear NF-κB by Western blot analysis. Fig. 5 shows that As$_2$O$_3$ alone failed to suppress the phosphorylation of IκB-α, whereas As$_2$O$_3$ plus sulindac suppressed the phosphorylation (42 kDa) and degradation (38 kDa) of IκB-α in a dose-dependent manner. Treatment with As$_2$O$_3$ alone activates NF-κB (65 kDa) in a dose-dependent manner. However, the combination of the two agents can inhibit NF-κB activation. The decreased level of nuclear NF-κB was paralleled to the increased level of cytoplasmic IκB-α in the combination treatment. The result was further supported by a decrease in phosphorylated IκB-α in the combination treatment. It showed that the inhibition of NF-κB activation began at a concentration of As$_2$O$_3$ 1 μM.
and sulindac 0.5 mM, with a concentration of As$_2$O$_3$ 10 μM and sulindac 0.5 mM being able to almost completely block NF-κB activation. Surprisingly, treatment with sulindac alone slightly activates NF-κB in HCT116 colon cancer cells (Fig. 5).

Combination of As$_2$O$_3$ and sulindac inhibits translocation of activated NF-κB into the nucleus. To confirm that the combination of As$_2$O$_3$ and sulindac inhibits the translocation of activated NF-κB into the nucleus, immunofluorescence staining was performed (Fig. 6). As we expected, the combination of the two agents blocked nuclear translocation of NF-κB, while As$_2$O$_3$ alone allowed some nuclear translocation. It can also be compared to treatment with sulindac alone in that it did not suppress the nuclear translocation of NF-κB. This indicates that a combination of As$_2$O$_3$ and sulindac ultimately blocks the translocation of activated NF-κB through the inhibition of phosphorylation and degradation of IκB-α.

Discussion

As$_2$O$_3$ has been reported to induce complete remission in patients with relapsed APL as well as newly developed APL. The use of As$_2$O$_3$ is increasing, since this agent has diverse pathways in malignant cells and multiple potential molecular targets which indicate that it could be used as a targeted cancer therapy. Via numerous pathways, As$_2$O$_3$ participates in the induction of apoptosis and differentiation, and the inhibition of angiogenesis. There are several possible mechanisms that As$_2$O$_3$ involves in apoptosis and differentiation in cancer cells. These are mediated by elevated intracellular H$_2$O$_2$ leading to cytochrome c release and by the activation of the caspase cascade, by increased bax expression and inhibition of NF-κB (1,6,18-20). Currently, many scientists are attempting to use As$_2$O$_3$ as a chemotherapeutic agent in treating solid tumors. In fact, in vitro studies with As$_2$O$_3$ of several solid tumors including lung, prostate, renal cell and colon cancer have been carried out (21-25). Researchers have studied ways of enhancing As$_2$O$_3$-induced cytotoxicity, and have tried the combination with several agents such as ATRA, imatinib, IFN-α, vitamin C, and doxahexanoic acid (10,11,23).

Sulindac may be a good candidate for enhancing the As$_2$O$_3$ induced effect as sulindac has been demonstrated to play a role in the prevention of colon cancer from precancerous lesions (26,27). Sulindac is a nonsteroidal anti-inflammatory agent that inhibits cyclooxygenase activity to block prostaglandin synthesis. Sulindac sulfide is the most active metabolite of sulindac and blocks prostaglandin synthesis by non-selective inhibition of cyclooxygenase 1 and 2 (27). However, alternative mechanisms of sulindac action other than the inhibition of prostaglandin synthesis have demonstrated that sulindac is also capable of inhibiting activation of the NF-κB pathway, PPARδ and phosphodiesterase (17,28,29). Collectively, these findings suggest that it is feasible to combine As$_2$O$_3$ and sulindac to enhance cytotoxicity that can lead to cancer cell death. There have been several studies which support the hypothesis that a combination of the two agents can work synergistically on cancer cell lines (30,31). As we expected in our study, sulindac was able to enhance the As$_2$O$_3$-induced cytotoxic effect in colon cancer cells. In fact, in this study the combination of As$_2$O$_3$ and sulindac resulted in a dramatic

Figure 6. Sulindac inhibits nuclear translocation of NF-κB in HCT116 colon cancer cells. HCT116 colon cancer cells were cultured in an 8-well Lab-Tek chamber slide (Nalge Nunc International, Naperville, IL, USA) and pretreated for 2 h with or without sulindac 0.5 mM and then treated with arsenic trioxide (1, 5, 10 μM). The slides were stained with the NF-κB p65 antibody and FITC-conjugated goat anti-mouse IgG. The slides were examined under a fluorescent microscope. The control was untreated HCT116 colon cancer cells. As$_2$O$_3$, arsenic trioxide; C, control.
reduction in cell growth as compared to treatment with As$_2$O$_3$ alone.

The question is how the combination of As$_2$O$_3$ and sulindac enhances cytotoxicity. To address this question, we assessed an apoptosis pathway that is known to be associated with As$_2$O$_3$-induced cytotoxicity. Our study showed that treatment of colon cancer cells with As$_2$O$_3$ induced apoptosis, and was markedly enhanced when used in combination with sulindac. This indicates that sulindac synergistically enhances As$_2$O$_3$-induced apoptosis. As$_2$O$_3$ exposure has been shown to activate caspases both in vitro, and in vivo (1,22,33). Whether the activation of caspases by As$_2$O$_3$ is a direct or indirect effect of the agent is not clear. In clinical trials with relapsed APL patients, responses to As$_2$O$_3$ were associated with enhanced expression of proenzymes for caspase-2 and -3, and activation of caspase-1 and -2 (34). In another study using myeloma cells, As$_2$O$_3$ induced apoptosis via caspase-9 activation at a clinically relevant level (2-5 μM) (35). As$_2$O$_3$ also promotes apoptosis by activating caspase-3 in neuroblastoma cell lines and in myeloid leukemia cells (36,37). Ishitsuka et al reported that exposure of an adult T-cell leukemia cell line to As$_2$O$_3$ in vitro activates caspase-8 and -3 (38). Our study showed that enhanced apoptosis by the combination of the two agents is a result of activation of caspases. In this study, the combination of As$_2$O$_3$ and sulindac activated caspase-8 and caspase-3, whereas As$_2$O$_3$ alone did not seem to activate caspase-8. However, the cleaved form of caspase-3 slightly increased at a concentration of As$_2$O$_3$ 10 μM alone, indicating that a high dose of As$_2$O$_3$ (10 μM) activates caspase-3. Therefore, our study suggests that sulindac promotes As$_2$O$_3$-induced apoptosis through caspase-8 and -3 activation. Recently, Jin et al reported that synergistic induction of apoptosis by As$_2$O$_3$ and sulindac in lung cancer cells is a result of the down-regulation of survivin, a member of the inhibitor of apoptosis protein (IAP) family (21).

NF-kB is a ubiquitously expressed transcription factor that plays a pivotal role in the expression of various inducible target genes that regulate apoptosis, among several other vital functions. It also controls cell proliferation, differentiation, and immune and inflammatory responses (39). NF-kB can be activated by many stress stimuli and in turn stimulate the transcription genes that allow resistance to apoptosis. When NF-kB is activated, it promotes the expression of several antiapoptotic genes, such as the cellular inhibitors of apoptosis c-IAP1 and c-IAP2 (40,41). These genes induced by NF-kB can inhibit the activation of the caspase cascade that causes cell death. Thus, being able to block the activation of NF-kB may be a measure to enhance the cytotoxic effect of As$_2$O$_3$. In this study, we showed that sulindac enhanced As$_2$O$_3$ induced apoptosis, however it was not able to block the activation of NF-kB at concentrations of 1 through 10 μM of As$_2$O$_3$ in HCT116 colon cancer cells. As$_2$O$_3$ blocks NF-kB activation, activates the caspase-8 and -3 pathways and eventually induces apoptosis in colon cancer cells. As$_2$O$_3$ at a dose of 1-2 mM has been shown to have a therapeutic effect on APL cells, even in patients resistant to ATRA or conventional chemotherapy, with minimal toxicity. However, the dose and dosing regimen required for a clinical response to As$_2$O$_3$ in solid cancers has not been established yet. Clinical trials in treating hematologic and solid cancers with As$_2$O$_3$ are currently being conducted (43). These should clarify the prospects of the clinical potential for managing solid tumors with As$_2$O$_3$. According to our study, either treatment with As$_2$O$_3$ alone or treatment with sulindac alone in the therapeutic dose range was not able to induce effective apoptosis in HCT116 colon cancer cells. However, the combination of As$_2$O$_3$ and sulindac induced apoptosis mediated by the activation of the caspase-8 and -3 pathway through blocking NF-kB activation. A concentration of As$_2$O$_3$ 5 μM and sulindac 0.5 mM seems to be the optimal synergistic combination to induce apoptosis in colon cancer cells. Therefore, a combination of As$_2$O$_3$ and sulindac can result in a synergistic effect on colon cancer cells and may be useful in treating colon cancer patients.

In conclusion, we demonstrated that the combination of As$_2$O$_3$ and sulindac enhances cytotoxicity and apoptosis mediated by activation of caspase-8 and -3. This results from the blocking of NF-kB activation through the inhibition of phosphorylation and degradation of IkB-α. A concentration of As$_2$O$_3$ 5 μM and sulindac 0.5 mM can work in an effective synergistic manner on colon cancer cells. Therefore, the combination of As$_2$O$_3$ and sulindac may be useful in treating patients with colon cancer.

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References


