Artesunate enhances TRAIL-induced apoptosis in human cervical carcinoma cells through inhibition of the NF-κB and PI3K/Akt signaling pathways

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis and kills cancer cells with little or no adverse effects on normal cells. TRAIL is relatively safe for clinical applications. However, TRAIL resistance is widely found in cancer cells leading to limitations in utilizing TRAIL as a therapeutic agent for cancer treatment. Recently, artesunate, an effective and safe anti-malarial drug, was also described as a promising candidate for cancer therapy. It would be of importance to determine whether combination treatment of TRAIL together with artesunate could overcome drug-resistance of tumors. Here, we demonstrate the first evidence that artesunate effectively enhances TRAIL-mediated cytotoxicity by suppressing pro-survival proteins, such as survivin, XIAP and Bcl-XL. Upon treatment with artesunate, the levels of survival proteins were strongly suppressed in HeLa cells. The down-regulation of these survival proteins could be regulated by repressing activation of NF-κB and Akt. Artesunate also inhibited TRAIL-induced transcriptional activity of NF-κB. In addition, this substance significantly enhanced both extrinsic and intrinsic apoptosis, which were induced by TRAIL. Taken together, the results of the present study suggest that artesunate exhibits an ability to overcome TRAIL resistance and combination treatment of TRAIL together with artesunate may be an effective strategy for cancer therapy.

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Abbreviations: TNF, tumor necrosis factor; TRAIL, related apoptosis inducing ligand; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, phosphoinositide 3-kinase; Akt, serine/threonine protein kinase; MAPK, mitogen-activated protein kinase; DISC, death-inducing signaling complex; cFLIP, cellular FLICE-like inhibitory protein; Bid, BH3 interacting domain death agonist; Bcl-xL, B-cell lymphoma-extra large; XIAP, X-linked inhibitor of apoptosis protein

Introduction

The tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), a member of the tumor necrosis factor super-family, has attracted great interest due to its specific anti-tumor potential without toxic side effects. It selectively induces proteins that initiates apoptosis in a variety of neoplastic cells while displaying minimal or absent cytotoxicity to most normal cells (1,2). Studies in non-human primates such as chimpanzees and cynomolgus monkeys show no toxicity upon intravenous infusion, even at high doses (3). TRAIL has become a recognized target for cancer therapy. TRAIL binds to TRAIL-R1 or TRAIL-R2, two death domain-containing receptors, also called DR4 and DR5, to trigger apoptosis (4,5). It has been also described that TRAIL can induce survival signals in some cell types especially by inducing the pro-survival and proinflammatory transcription factor NF-κB, phosphoinositide 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways via JNK activation (6). Unfortunately, a considerable range of cancer cells, especially some highly malignant tumor, is resistant to induction of apoptosis by TRAIL (5). Resistance to TRAIL seems to be mediated through multiple mechanisms in the signaling pathways of TRAIL-induced apoptosis, including mutations in the death receptors, defects in the molecules involved in formation of the death-inducing signaling complex (DISC), dysregulation of DISC activation by TRAIL receptor antagonists, overexpression of cellular FLICE-like inhibitory protein (cFLIP) and enhancement in expression of caspase inhibitors and other cell survival proteins (4-7). The emergence of resistance to TRAIL is a major problem limiting its utility as a therapeutic agent. Combination of TRAIL with chemotherapy or radiotherapy significantly enhances cytotoxicity to tumors and overcome this problem, and unfortunately, they come with severe toxic side effects inducing cell death processes in both malignant and non-malignant cells (7-9). Therefore, there is a necessity for agents that are safe and efficacious in potentiating the effects of TRAIL leading to overcome such resistance and successful use of TRAIL for cancer therapy. The

search for novel antitumor agents that circumvent several limitations has increased its attention toward natural sources, in particular plant compounds. Artesunate is one of semi-synthetic derivatives of artemisinin isolated from decoctions of traditional Chinese medicine Artemisia annua L (qinghao, sweet wormwood) (10). During recent years artemisinin and its derivatives have emerged as the most effective and safe drugs for the treatment of severe and chloroquine-resistant malaria (11). There is increasing evidence to support the notion that artesunate might be a highly interesting compound to be used in cancer. First encouraging investigation in clinical treatment of uveal melanoma suggested further clinical trials with artesunate for cancer treatment in the near future (12). Artesunate triggers apoptotic cell death in various tumor cells in both a p53-dependent and -independent manner (13,14). Artesunate has been shown to inhibit the growth of Kaposi's sarcoma cells, a highly angiogenic multifocal tumor, and that activity of cell growth inhibition correlated with the induction of apoptosis (15). So far, the effects of artesunate in enhancing TRAIL-induced apoptosis in tumor cells have not been elucidated. It would be interesting to know, whether combination of artesunate and TRAIL could overcome drug-resistance of tumors. This combination treatment might be an alternative promising strategy for cancer therapy. In the present study, we investigated the efficacy of artesunate to sensitize human cervical cancer cells to TRAIL, and further investigated its intracellular signaling mechanism in sensitizing TRAIL-induced apoptosis by artesunate.

Materials and methods

Chemicals and reagents. Artesunate (Guilin Pharmaceutical Corp. Ltd., China) was dissolved in dimethyl sulfoxide (DMSO) and kept as a stock solution at -20˚C. The final concentration of DMSO was kept below 0.1% throughout the study. Recombinant human TRAIL and recombinant human tumor necrosis factor (TNF-α) were purchased from Peprotech (London, UK). Primary antibodies specific to caspase-3, caspase-7, caspase-8, poly (ADP-ribose) polymerase (PARP), BH3 interacting domain death agonist (Bid), B-cell lymphoma-extra large (Bcl-xL), X-linked inhibitor of apoptosis protein (XIAP), survivin and phosphorylated form of Akt (Ser473), extracellular-signal-regulated kinase (ERK) (Thr202/Tyr204), p38 (Thr180/Tyr182), c-Jun NH2-terminal kinase (JNK) (Thr183/Tyr185), p65 (Ser536) were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies specific to actin, caspase-9, Akt, ERK, p38, JNK, and p65 were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

Cell culture and treatment. HeLa human cervical cancer cells (ATCC, Rockville, MD, USA) and stably transfected with NF-κB-luciferase reporter plasmid were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2/95% air.

Cell viability assay. Viability of cells after treatment was determined by WST-1 Cell Counting kit (Wako Pure Chemical

Industries, Osaka, Japan) as previously described (16). Cells were seeded into a 96-well plate (6x10³/80 μl/well). After 24 h of incubation, medium containing artesunate (10 μl) was added to the wells and cells were incubated for 30 min. TRAIL in medium (10 μl) was added to each well, and further incubated for 24 h. WST-1 solution (10 μl) was added to each well and incubated at 37˚C for 1 h. The absorbance at 450 nm was measured using a microplate reader. Cell viability was determined from the absorbance of soluble formazan dye generated by living cells.

Immunoblot analysis. Immunoblot analysis was performed as previously described (17). Cell was seeded and incubated overnight in a 60 mm culture dish (1x10⁶/4 ml/well). After treatment, whole cell lysates from HeLa cells were prepared with lysis buffer (25 mM HEPES pH 7.7, 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM b-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenyl-methylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 mg/ml aprotinin and 10 mg/ml leupeptin). Cell lysates were subjected to electrophoresis in SDS-PAGE (10%) and electrophoretically transferred to Immobilon-P nylon membrane (Millipore, Bedford, MA, USA). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co., Ltd., Suita, Osaka, Japan) for at least 2 h, and probed with the indicated primary antibodies overnight, followed by horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark). The results were visualized using ECL reagents (Amersham Bioscience, Piscataway, NJ, USA).

Luciferase reporter assay. NF-κB transcriptional activity in the HeLa cells stably transfected with NF-κB-luciferase reporter was determined by luciferase assay. Cells were seeded in 24-well plate (1x10⁵/500 μl/well) and left overnight. The cells were pretreated with artesunate (60 μM) for 30 min, and further incubated with TRAIL (200 ng/ml) or TNF-α (20 ng/ml) for 12 h. Cells were then lysed with 200 μl passive lysis buffer (Promega, Madison, WI, USA) and 5 μl of cell lysate were mixed with 20 μl of luciferase substrate solution (Picagene, Toyo Inc, Tokyo, Japan). Luminescence was determined using a luminometer (Atto, Tokyo, Japan). The luciferase activity was normalized by total protein and then calculated as relative luciferase activity.

Statistical analysis. Data are expressed as the mean ± SD and analyzed by Student's t-test using SPSS software to determine the significance of differences between groups. A $p<0.05$ was considered to be significant.

Results

Artesunate enhances TRAIL-induced cell cytotoxicity. Initially, cytotoxicity of artesunate in HeLa cells was determined after 24 h of treatment with 0-300 μM artesunate. The concentration range of 10-60 μM was observed to be non-toxic for HeLa cancer cells, with cell viability being greater than 80%. The cytotoxicity of combination treatment of TRAIL and artesunate was also examined and the results clearly and surprisingly showed the synergist effect of artesunate in TRAIL-induced cell cytotoxicity (Fig. 1A). In addition, the enhancement of
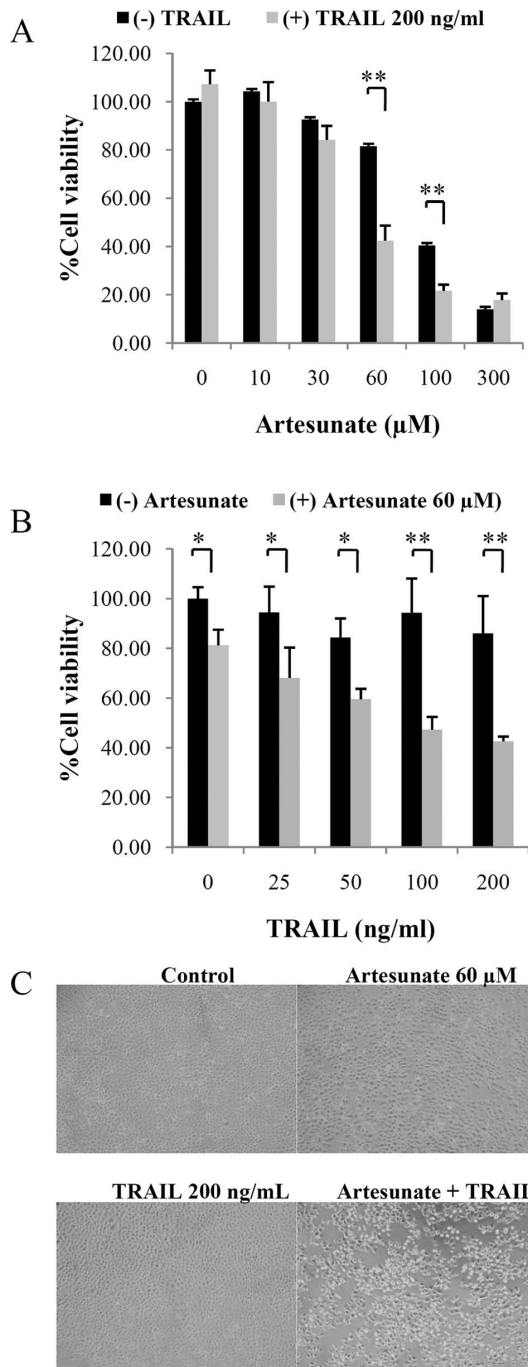
Figure 1. Effect of artesunate on TRAIL-induced cell cytotoxicity. Viability of HeLa cells after treatment was determined by WST-1 assay. (A) Cells were pretreated with artesunate (60 μM) or vehicle (0.1% DMSO) for 30 min, and further incubated with different concentrations of TRAIL for 24 h. (B) Cells were pretreated with different concentration of artesunate for 30 min, and further incubated in the presence or absence of TRAIL (200 ng/ml) for 24 h. Data are expressed as the mean ± SD from at least three independent experiments, and significant differences between groups are shown by *p<0.05, **p<0.01. (C) Photographs were taken at original magnification of x50. Cells were pretreated with artesunate (60 μM) for 30 min, followed by incubation in the presence or absence of TRAIL (200 ng/ml) for 24 h.

Figure 2. Artesunate enhances TRAIL-induced apoptosis. HeLa cancer cells were pretreated with artesunate (60 μM) or vehicle (0.1% DMSO) for 30 min, and further incubated with TRAIL (200 ng/ml) for 12 h. Whole cell lysates were analyzed by immunoblot for apoptotic proteins, caspase-9, caspase-8, caspase-7, caspase-3, Bid and PARP, using actin as loading control.

TRAIL-induced cell death by artesunate presented in dose-dependent manner with the correlation coefficients of 0.972. The artesunate concentration at 60 μM was selected for further studies owing to non-toxic and significantly increased 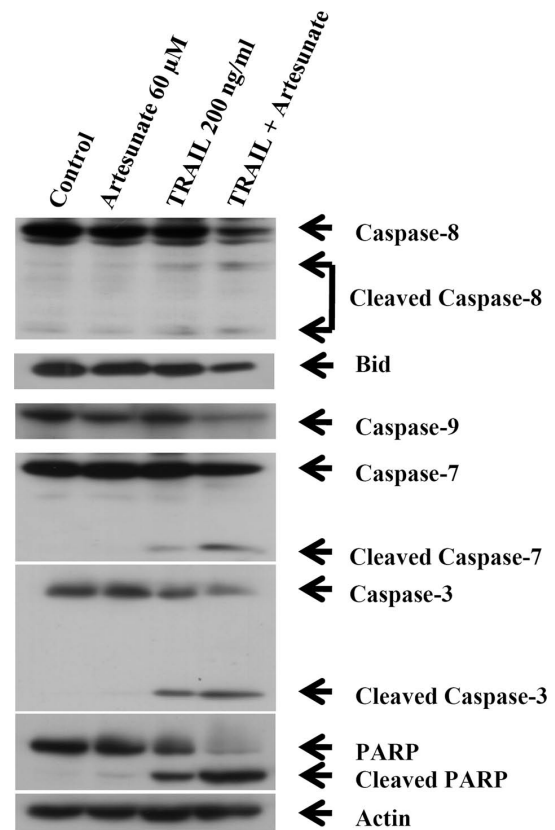cell death in the combination treatment with TRAIL. The 24-h treatment of recombinant human TRAIL in the range of concentrations of 25-200 ng/ml on HeLa cancer cells showed only less than 16% cell death. These results implied that HeLa cancer cells were resistant to TRAIL-mediated cytotoxicity. However, pretreatment of artesunate in the concentration of 60 μM for 30 min before 24-h TRAIL treatment could increase the percentage of cell death significantly up to 30-60% depending on the concentration of TRAIL (Fig. 1B). Typical morphological changes of apoptotic cell death were observed under microscopy. Massive cell death was observed in HeLa cells at 24 h after treatment with a combination of 60 μM artesunate and TRAIL 200 ng/ml as shown in Fig. 1C.

Sensitization of artesunate to TRAIL-induced apoptosis through the activation of caspase-mediated death signal. To confirm the activation of apoptotic signals by combined treatment with artesunate and TRAIL, we performed Western blot analysis of the cleavage of PARP, Bid and caspase-8, -9, -3, -7, which are hallmarks of cells undergoing apoptosis, in HeLa cells treated with artesunate and/or TRAIL (Fig. 2). The apoptosis -inducing effect of TRAIL was enhanced remarkably by artesunate pretreatment as shown in Fig. 2 by the increased cleavage of pro-caspase -8, -9, -3, -7, Bid and PARP. These results clearly showed that artesunate could sensitize HeLa cells in TRAIL-induced apoptosis both the extrinsic apoptosis pathway (mitochondria independent pathway) and the intrinsic apoptosis
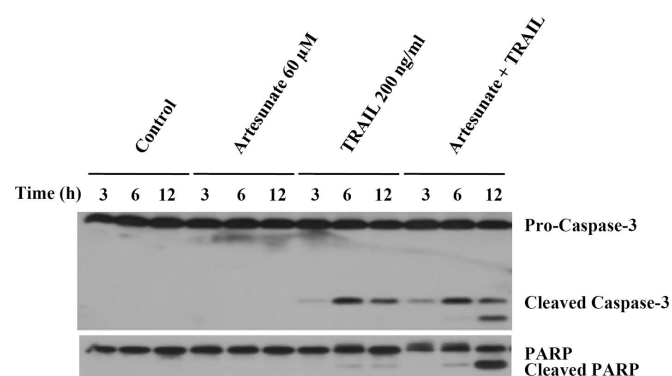
Figure 3. Kinetics of caspase-3 activation and PARP cleavage after single and combination treatment of TRAIL and artesunate. HeLa cancer cells were pretreated with artesunate (60 μM) or vehicle (0.1% DMSO) for 30 min, and further incubated with TRAIL (200 ng/ml) for 3, 6 and 12 h. Whole cell lysates were immunoblotted as described above with anti-caspase-3 and anti-PARP antibodies.
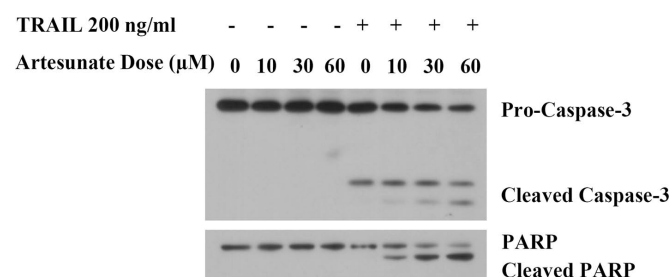

Figure 4. TRAIL-induced apoptosis enhanced by artesunate in a dose-dependent manner. HeLa cancer cells were pretreated with artesunate (10, 30 and 60 μM) or vehicle (0.1% DMSO) for 30 min, and further incubated with TRAIL (200 ng/ml) for 12 h. Whole cell lysates were immunoblotted as described above with anti-caspase-3 and anti-PARP antibodies.

Figure 5. Effect of artesunate on signaling pathway of TRAIL. HeLa cells were pretreated with artesunate (60 μM) for 30 min, followed by incubation in the presence or absence of TRAIL (200 ng/ml) for 12 h. Whole cell lysates were then analyzed by immunoblot for levels of total and phosphorylated forms of Akt, Erk, JNK, p38, NF-κB p65 and the total forms of survival protein; Bcl-xL, XIAP and survivin.

pathway (mitochondria dependent pathway). In addition, kinetics of caspase-3 activation and PARP cleavage after treatment with TRAIL, artesunate and their combination in the period time of 3-12 h were also investigated and the results were shown in Fig. 3. TRAIL-induced apoptosis occurred within 3 h, showed the highest apoptosis at 6 h and then declined after 12 h of treatment. The combination treatment of TRAIL and artesunate could accelerate TRAIL-induced apoptosis continuously until 12 h of treatment by increasing the level of the activated caspase-3 and the cleaved PARP remarkably. In addition, artesunate exhibited the synergist effect in inducing apoptosis selectively with TRAIL but not with TNF-α (data not shown). This synergist effect of artesunate in TRAIL-induced apoptosis was dose-dependent as shown in Fig. 4.

Effect of artesunate on other signaling pathway of TRAIL. Both apoptotic and non-apoptotic cell signaling could be influenced by NF-κB, PI3K/Akt and MAPK. Therefore, we further investigated effect of artesunate on other TRAIL-induced signal pathway in HeLa cancer cells i.e., the NF-κB, PI3K/Akt and the MAPK and pro-survival protein signaling pathways. After 12 h of treatment, TRAIL (200 ng/ml) triggered survival signaling pathway, especially MAPKs, as revealed by increased phosphorylation of ERK, JNK and p38, whereas
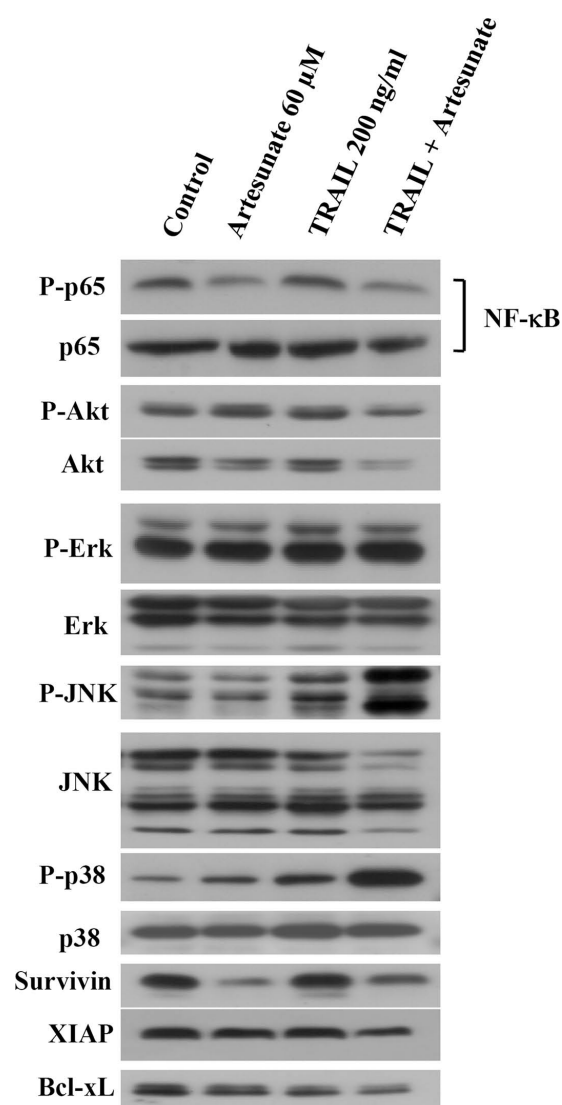
only a little effect or no effect by TRAIL on NF-κB p65 and Akt pathway, which was already constitutively activated in common HeLa cancer cells (Fig. 5). After the combined treatment of TRAIL and artesunate, the phosphorylation of MAPKs, i.e., ERK, JNK and p38, was induced significantly and TRAIL-induced NF-κB and Akt activation was significantly suppressed by artesunate. The signal of pro-survival proteins, survivin, XIAP, Bcl-xL, were also determined and the results clearly showed down-regulation of these pro-survival proteins signals depending on the concentration of artesuante as shown in the Fig. 6. In addition, the decrease of these pro-survival proteins after the combined treatment of TRAIL and artesunate was observed markedly at 12 h of treatment.

Artesunate inhibits TRAIL-induced NF-κB activation. As the NF-κB is a major regulator of proinflammatory cytokine expression in TNF-α and TRAIL stimulation. We focused the
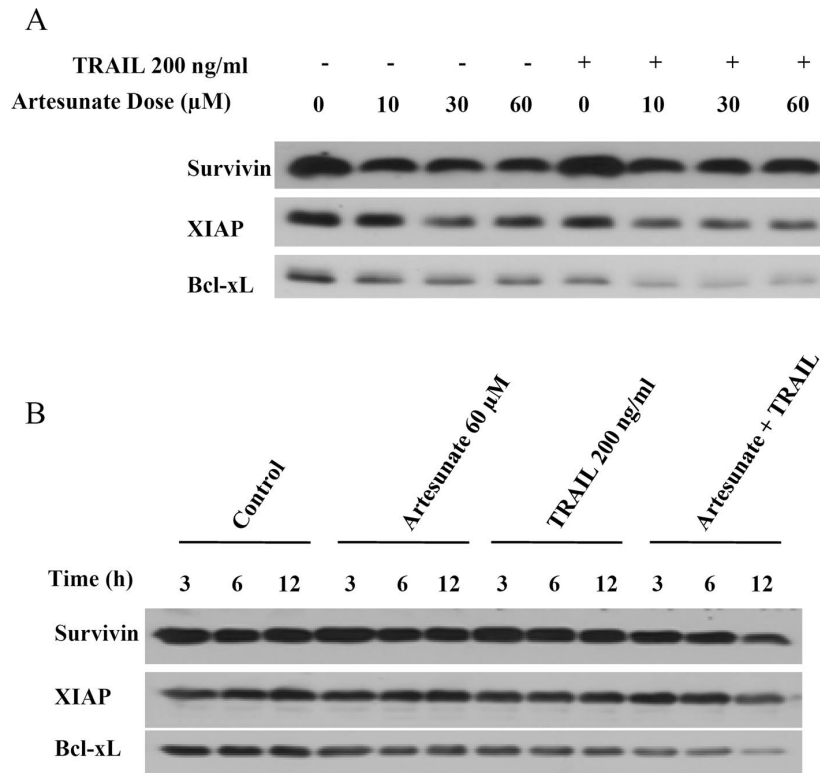
Figure 6. The inhibitory effect of artesunate on pro-survival proteins in a dose- (A) and (B) time-dependent manner. HeLa cancer cells were pretreated with artesunate (10, 30 and 60 µM) or vehicle (0.1% DMSO) for 30 min, and further incubated with TRAIL (200 ng/ml) for 3-12 h. Whole cell lysates were immunoblotted as described above with anti-survivin, anti-XIAP and anti-Bcl-xL antibodies.

effect of artesunate on the NF-κB-dependent pathway. To investigate this effect, the inhibitory effect of artesunate on TRAIL-induced NF-κB activation in luciferase expressing HeLa cancer cells, which containing NF-κB transcription factor, was confirmed by using luciferase reporter assay. TNF-α was used as a potent inducer of NF-κB activation. Treatment with 20 ng/ml TNF-α or 200 ng/ml TRAIL for 12 h induced activation and increased transcriptional activity of NF-κB by 7.1- and 2.05-fold, respectively, compared to control (Fig. 7A and B). Artesunate pretreatment suppressed the TNF-α-induced NF-κB transcriptional activity by approximately 33%, and completely abolished the TRAIL-induced NF-κB transcriptional activity. These results clearly confirmed the inhibitory effect of artesunate on TRAIL-induced NF-κB activation.

Discussion

Resistance to TRAIL can occur at different points in the signaling pathways of TRAIL-induced apoptosis. Over-expression of anti-apoptotic proteins and activation of different subunits of NF-κB is an important reason to affect on development of TRAIL resistance in certain types of cancer cells (18). In addition, there are numberous reports to suggest that activation of Akt could inhibit TRAIL-induced apoptosis in a wide variety of tumor cells (19-21). Activated Akt phosphory-lates multiple proteins implicated in the control of cell survival (22). Our data clearly demonstrated that human cervical carci-noma cells exhibited resistance to TRAIL-induced apoptosis as shown by low cytotoxicity after TRAIL treatment for 24 h in Fig. 1B, possibly owing to high expression of activated NF-κB

and Akt. Similarly, Bernard et al (23) reported high expression levels of c-Rel or activation of endogenous Rel/NF-κB factors in HeLa cells. TRAIL was demonstrated to promote the survival and proliferation of primary human vascular endo-thelial cells by activating the Akt pathways (24), perhaps leading to developing TRAIL resistance of cancer cell by itself. The NF-κB and Akt activations clearly played important roles in failure to undergo apoptosis in response to TRAIL treatment. More recently, there is increasing evidence to support that artesunate may have a potential anti-cancer effect (25). In search for strategies to overcome TRAIL resistance of tumor cells, we tested the antitumor activity of artesunate on HeLa cancer cells. We provided evidence for the first time that artesunate was a potent sensitizer for TRAIL-induced apoptosis in HeLa cells. The mechanism of artesunate for TRAIL sensitization in HeLa cells was, at least in part, due to suppression of the NF-κB and PI3K/Akt signal pathway by inhibition of phosphorylation of p65 and Akt. Correspondingly, artesunate significantly inhibited transcriptional activity of NF-κB induced by TRAIL and TNF-α as shown in Fig. 7. Some reports have shown that artesunate inhibited NF-κB activation induced by lipopolysaccharide (LPS), an inflam-matory stimuli (26). Anti-apoptotic function of NF-κB activation in TRAIL signaling is mediated by the up-regulation of several anti-apoptotic genes such as cFLIP, Bcl-xL and XIAP (27). In addition, blocking PI3K/Akt has been reported to induce significant down-regulation of survivin and cytotoxicity in human multiple myeloma and liver carcinoma cells (28,29). We further investigated the signal of pro-survival proteins, survivin, XIAP, Bcl-xL and the results clearly showed
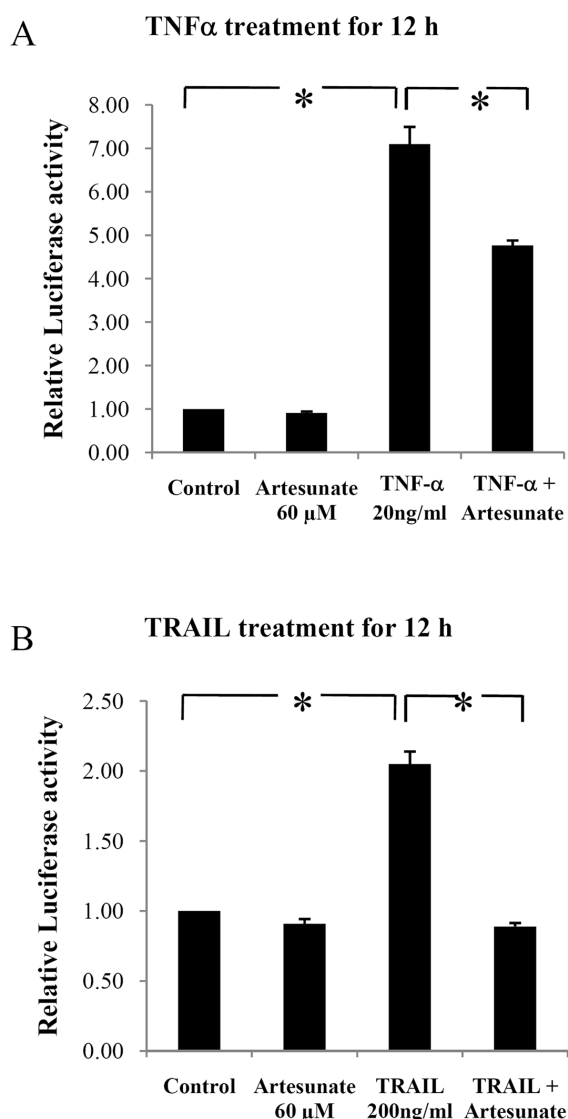
A

TNFα treatment for 12 h

B

TRAIL treatment for 12 h

Figure 7. Effect of artesunate on TRAIL-induced NF-κB transcriptional activity. HeLa cells stably transfected with NF-κB luciferase reporter plasmid were pretreated with artesunate (60 μM) for 30 min, followed by incubation in the presence of TRAIL (200 ng/ml) (A) or TNF-α (20 ng/ml) (B) for 12 h. NF-κB-mediated luciferase gene expression was then determined, normalized by total protein and then calculated as relative luciferase activity. Data are expressed as the mean ± SD from three independent experiments.

down-regulation of these pro-survival proteins signals. These obviously decreased pro-survival proteins exhibited an important influence to augment and facilitate TRAIL-induced apoptosis in HeLa cancer cells leading to cancer cell death. These studies provided additional evidence regarding the role of survival protein mediated by the NF-κB and PI3K/Akt pathway, in the regulation of apoptosis. It has been reported that antiapoptotic factors, especially survivin, play a potential role in the resistance to activation-induced cell death and prognosis of neuroblastoma (22). Consistent with the report by Azuhata *et al* (30) in which higher levels of survivin correlated with greater resistance to TRAIL. Similar enhancement of TRAIL-induced apoptosis by down- regulation of survivin has been reported for resveratrol (31). Not only NF-κB or PI3K/Akt inhibition, but also down-regulation of pro-survival proteins by

artesunate resulted in enhancement of TRAIL-induced apoptosis. We also found that this combined treatment of TRAIL and artesunate could induce phos-phorylation of p38, JNK, MAPK/ERK, which are implicated in propagating the apoptotic signal. The sensitization of artesunate in TRAIL-induced apoptosis clearly involved both the extrinsic (mitochondria-independent pathway) and the intrinsic apoptosis pathway (mitochondria-dependent pathway). Artesunate enhanced the cleavage of caspase-8 induced by TRAIL. Activated caspase-8 triggers apoptosis, either directly activating downstream executioner caspase such as caspase-3, and caspase-7, or by cleaving Bid that activates the mitochondrial pathway to cell death. Bid is cleaved by caspase-8 to a truncated version of the molecule tBid, which translocates from cytosol to mitochondria and induces the release of cytochrome C and finally activates caspase-9. Activated caspase-9 then activates downstream executioner caspases such as caspase-3, which subsequently cleave many important intracellular proteins such as PARP and DNA fragmentation factor, resulting in morphological changes in apoptosis and cell death. It was possible that artesunate induced the cleavage of Bid and the activation of caspase-9 in Bcl-xL-sensitive manner as shown by the increase of the degradation of caspase-9 and Bid together with the decrease of Bcl-xL, anti-apoptotic protein. Artesunate also inhibited the XIAP protein, which was up-regulated by NF-κB facilitating the process of activation of caspase-3 and-7 by the activated caspase-9. Among the IAP family proteins, XIAP exhibits the strongest anti-apoptotic properties and inhibits apoptosis signaling by binding to active caspase-3 and -7 and by preventing caspase-9 activation (32). It has been reported that inhibition of the apoptosis pathway involves up-regulation of apoptotic protein inhibitors, IAP, which leads to attenuation of the death receptor-mediated signal pathway (33). The obvious enhancement of the caspase-3 activation and the cleavage of PARP by synergist effect of artesunate on TRAIL-induced apoptosis were dose- and time-dependent.

In conclusion, we demonstrated the first evidence that artesunate effectively enhances TRAIL-mediated cytotoxicity by suppressing the activation of NF-κB and Akt, resulting in the decrease of pro-survival proteins, leading to trigger both extrinsic and intrinsic apoptosis. This suggests that the combination treatment of TRAIL and artesunate may be an effective strategy for cancer therapy in the near future.

Acknowledgements

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