Andrographolide induces apoptotic and non-apoptotic death and enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in gastric cancer cells

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Abstract. Andrographolide, a natural compound isolated from Andrographis paniculata, has been reported to possess antitumor activity. In the present study, the effect of andrographolide in human gastric cancer (GC) cells was investigated. Andrographolide induced cell death with apoptotic and non-apoptotic features. At a low concentration, andrographolide potentiated apoptosis and reduction of clonogenicity triggered by recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL). Exposure of GC cells to andrographolide altered the expression level of several growth-inhibiting and apoptosis-regulating proteins, including death receptors. It was demonstrated that activity of the TRAIL-R2 (DR5) pathway was critical in the development of andrographolide-mediated rhTRAIL sensitization, since its inhibition significantly reduced the extent of apoptosis induced by the combination of rhTRAIL and andrographolide. In addition, andrographolide increased reactive oxygen species (ROS) generation in a dose-dependent manner. N-acetyl cysteine prevented andrographolide-mediated DR5 induction and the apoptotic effect induced by the combination of rhTRAIL and andrographolide. Collectively, the present study demonstrated that andrographolide enhances TRAIL-induced apoptosis through induction of DR5 expression. This effect appears to involve ROS generation in GCs.

Introduction

Gastric cancer (GC) is one of the most common types of malignancy and is the leading cause of cancer-associated mortality worldwide (1). A large number of chemotherapy drugs have been tested for patients with advanced gastric cancer, and considerable progress has been achieved with platinum drug-based regimens (2). However, the overall prognosis of GC remains poor (3). Therefore, additional efforts are ongoing to develop safer and more effective therapeutic strategies.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has emerged as an attractive anticancer agent, since it selectively induces cell death in various human cancer cells with little effect on normal cells (4). Thus far, five TRAIL receptors have been identified in humans. Among them, TRAIL-R1 (DR4) and TRAIL-R2 (DR5) mediate apoptosis through characteristic interactions of their cytoplasmic death domains (5). Engaged TRAIL receptors recruit adaptor proteins and form the death-inducing signaling complex, which subsequently activates caspase cascades with or without mitochondrial amplification (6). However, it has also been shown that numerous TRAIL-resistant cancer cells exist (7). Therefore, a number of approaches, such as combined administration of TRAIL with various sensitizers, including synthetic small molecules, natural compounds and enzyme inhibitors, are currently being tested in attempt to overcome TRAIL resistance (8-10).

The diterpenoid lactone andrographolide is one of the biologically active constituents of Andrographis paniculata, a medicinal plant traditionally used for prevention and treatment of various diseases (11-13). A number of studies have demonstrated that andrographolide and its analogues possess potential anti-inflammatory and antitumor effects mediated by attenuation of nuclear factor-κB activation in various systems (11-14). Andrographolide also induces cell cycle arrest and apoptosis of cancer cells by inhibiting phosphoinositide 3-kinase/protein kinase B, mitogen-activated protein kinase and other tumor growth pathways, depending on the type of treated cells (15-18). Andrographolide triggers intrinsic and extrinsic apoptotic pathways in different cancer cells via mechanisms involving activation of p53, reactive oxygen species (ROS) and topoisomerase II (17,19,20). Furthermore, andrographolide demonstrated a potent anticancer effect when it was applied in combination with other anticancer agents, including cisplatin and doxorubicin (21,22). In the present study, the effect of andrographolide was determined in GCs, and it was reported to perform as a GC sensitizer to the action of TRAIL.
Materials and methods

Cell culture and dosing. The human GC SNU601, SNU638 and AGS cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured for 2-10 weeks in the Roswell Park Memorial Institute-1640 medium (In vitrogren; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (PAA Laboratories; GE Healthcare, Chalfont, UK) and 1% Penicillin-Streptomycin (Welgene, Inc., Gyeongsan, Korea) at 37°C in a 5% CO₂ atmosphere. Drug treatment of the cells was performed by adding 0-50 µM andrographolide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) alone or with 5-20 ng/ml recombinant human TRAIL (rhTRAIL; a gift from T.H. Kim, Department of Biochemistry and Molecular Biology, Chosun University, Korea) (23) to the culture medium at 37°C for 24-48 h. Anti-oxidants N-acetyl cysteine (NAC), butylated hydroxyanisole (BHA), Trolox and catalase, and caspase inhibitors z-DEV, z-IETD, z-LEHD, z-LEVD and z-VAD were purchased from EMD Millipore (Billerica, MA, USA).

MTT viability assays. For the MTT assay, cells were plated in the wells of a 96-well plate at a density of 1x10⁴ cells/well, incubated at 37°C for 24 h, and then treated with 0.2% dimethyl sulfoxide as a vehicle or 10-50 µM andrographolide at 37°C for 48 h. The MTT solution (0.5 mg/ml) was added to the cells and incubated at 37°C in a CO₂ incubator for the last 4 h. The plates were centrifuged at 600 x g for 10 min at room temperature and the culture medium was removed. The cells were solubilized using 100 µl of 100% dimethyl sulfoxide and the solubilized formazan product was quantified using an enzyme-linked immunosorbent assay plate reader at 595 nm. The absorbance of the untreated cells was set as 100% and cell survival was expressed as a percentage of this value.

Hoechst 33342 (HO)/propidium iodide (PI) double staining. Treated cells were stained with 1 µg/ml of HO and 5 µg/ml of PI for 15 min at room temperature in Shandon Cytospin II (Thermo Fisher Scientific, Inc.). Slides were prepared, air dried, mounted with aqueous mounting medium (Gel Mount; Biomedica, Foster City, CA, USA) and observed under a fluorescent microscope (magnification, x200; DM5000; Leica Microsystems, GmbH, Wetzlar, Germany) at respective excitation/emission wavelengths of 430/425 nm (HO) and 580/630 nm (PI). For each slide, five fields were randomly chosen. Morphological assessments of apoptotic and non-apoptotic death were performed. Intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei and intact or crushed pink nuclei were considered viable, early apoptotic, late apoptotic or non-apoptotic dead cells, respectively. A total of 500 cells distributed across random microscope viewing fields were counted and the number of apoptotic or non-apoptotic cells was expressed as a percentage of the total number of cells scored.

Immunoblotting. Protein extracts (50 µg) were electrophoretically separated using 10-12% SDS-PAGE and transferred to a nitrocellulose membrane using a standard technique (24). Antibodies specific to B-cell lymphoma-2 (Bcl-2; dilution, 1:200; catalog no., 2876S) and B-cell lymphoma-extra-large (Bcl-xL; dilution, 1:500; catalog no., 2762S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-p53 (dilution, 1:1,000; catalog no., sc-126), anti-p21 (dilution, 1:1,000; catalog no., sc-6246) and anti-α-tubulin (dilution, 1:500; catalog no., sc-32293) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-Bcl-2 associated X protein (Bax; dilution, 1:200; catalog no., BD 610983) was purchased from BD Biosciences (San Jose, CA, USA) and anti-Bcl-2 homologous antagonist/killer (Bak; dilution, 1:200; catalog no., 06-536) was purchased from EMD Millipore. Antibody signals were detected using an Image Station 4000 MM image analyzer (Kodak, Rochester, NY, USA).

RNA interference (RNAi). For the RNAi experiment, the sequences of small interfering (si)RNA were as follows: DR4 forward, 5’-CUGGAAAGUCAUCUAUCCU(dtdt)-3’ and reverse, 5’-AGUGAGUGACUUUCCAG(dtdt)-3’; DR5 forward, 5’-CAGACUGUGGCCCUUUG(dtdt)-3’ and reverse, 5’-UCAAAGGGCACCAGUCUG(dtdt)-3’; and control siRNA forward, 5’-CCUACGCACCAAUUCGUU(dtdt)-3 and reverse, 5’-ACGAAAAUGUGGCAG(dtdt)-3’. Bcl-2 (Bcl-xL; dilution, 1:500; catalog no., 2762S) and B-cell lymphoma-extra-large (Bcl-xL; dilution, 1:500; catalog no., 2876S) were individually transfected with siRNA oligonucleotides using an Amaxa Transfection System™ (Basel, Switzerland) and grown at 37°C for 24-36 h prior to the drug treatment.

Clonogenic assay. Clonogenic activity was measured according to established procedures with certain modifications in cell numbers and incubation period (25). For the clonogenic assay, 2.5x10² cells/35-mm dishes were re-plated with andrographolide or vehicle at 37°C for 18 h, followed by incubation with rhTRAIL 37°C for another 6 h. Cells were then trypsinized, and counted under light microscope at x100 magnification. The mean value of the cell number from 5 counts per sample was calculated and 2,000 cells were re-plated in 60-mm dishes in duplicate, and maintained at 37°C/5% CO₂ for 14 days in a humidified atmosphere. The grown cells were fixed with 3.7% formaldehyde, stained with 0.5% crystal violet, and colonies (>0.7 mm diameter) were scored to determine cell proliferating ability (26).

Detection of ROS generation. Cells were treated with andrographolide for 8, 18 and 24 h and loaded with 50 µM 2’,7’-dichlorofluorescin diacetate (DCFDA; Molecular Probes; Thermo Fisher Scientific, Inc.) to measure ROS generation and 0.5 µg/ml HO to quantify cell number for 30 min. Subsequent to rinsing twice with PBS, fluorescent images were captured with an inverted fluorescence microscope (magnification, x200) or fluorescence intensities were obtained with a Fluorocount (PerkinElmer, Inc., Port Richey, NJ, USA) at excitation/emission wavelengths of 490/530 nm (DCFDA) and 525/530 nm (H2DCFDA) and 490/580 nm (H2DCFDA) and 490/580 nm (H2DCFDA).
340/425 (HO), and values of ROS production were obtained by determining the ratio of DCFDA/HO signals per well.

Statistical analysis. All numerical data are reported as the mean ± standard error. P<0.05 was considered to indicate a statistically significant difference. All data represent the results of ≥3 independent experiments. Student’s t-test was used to evaluate the differences between control and treated group values, and one-way analysis of variance was applied to analyze the significance of differences caused by the effects of gene silencing or caspase inhibition.

Results and Discussion

Andrographolide inhibits cell growth and triggers apoptotic and non-apoptotic cell death in human GC cells. To investigate the antitumor efficacy of andrographolide in GC, AGS, SNU601 and SNU638 human GC cells were treated with andrographolide at several concentrations and the effect of the compound on cell growth and cell viability was assessed by counting the number of live cells and by performing the MTT assay, respectively. Andrographolide evidently decreased the cell growth rate and viability of GC cells (Fig. 1A and B). To examine whether a decrease of cell viability was accompanied by andrographolide-induced cell death, occurrence of apoptotic and non-apoptotic cell death was detected using the HO/PI double staining method. Apoptotic and non-apoptotic cell death was assessed by the percentage of condensed or cleaved apoptotic nuclei following HO staining (apoptotic death) and PI-stained red cells without apoptotic features (non-apoptotic death). As shown in Fig. 1C, andrographolide induced apoptotic and non-apoptotic cell death in GC cells. While the total number of dead cells was linearly associated with the concentration of andrographolide, the number of apoptotic dead cells with typical cleaved or condensed nuclei peaked in presence of 20 µM andrographolide. At concentrations of andrographolide exceeding 20 µM, PI-stained cells with rigid shapes of nuclei became predominant, indicating an increase of andrographolide-induced non-apoptotic cell death.

Andrographolide regulates expression of intrinsic and extrinsic apoptotic proteins. To investigate the mechanisms involved in andrographolide-induced cytotoxicity, expression levels of cell cycle inhibitory proteins and apoptosis-inducing proteins were analyzed by immunoblotting assay. As shown in Fig. 2, andrographolide increased expression of cyclin inhibitor p21 in all tested GC cells and levels of the p27 protein in SNU638 cells. Andrographolide also induced expression of membrane death receptors DR4 and DR5 in GC cells. DR5 expression was induced by relatively low concentrations of andrographolide (10-20 µM), whereas induction of DR4 expression was observed following exposure to increased concentrations of andrographolide (30-40 µM). Andrographolide also increased p53 levels in AGS and SNU638 cells, but not in SNU601 cells. Andrographolide likely induces p53 via p16 induction.
cells. The transcription factor p53 has been reported to regulate expression of p21, DR4 and DR5 in response to various cytotoxic stimuli (27-29). However, the role of p53 as a transcription regulator may not be essential in this system, since SNU638 and SNU601 cells carry transcriptionally mutant p53 protein (30). The effects of andrographolide on the expression level of Bcl-2 family members (intrinsic apoptotic regulators) was also detected. The level of anti-apoptotic Bcl-2 was inversely proportional to the concentration of andrographolide in all three GC cells, while expression of another anti-apoptotic protein, Bcl-xL, was unaffected. At high concentrations, andrographolide also affected the levels of pro-apoptotic Bcl-2 proteins: Expression of Bak was elevated in all three GC cells tested, while the level of Bax was increased only in SNU638 cells. At high concentrations, andrographolide also affected the levels of pro-apoptotic Bcl-2 proteins: Expression of Bak was elevated in all three GC cells tested, while the level of Bax was increased only in SNU638 cells. Based on these results, it was hypothesized that at high concentrations, andrographolide triggers various stress signaling events, including activation of the extrinsic and intrinsic apoptotic pathways in GC cells. However, although expression of pro-apoptotic proteins was directly proportional to the concentration of andrographolide, apoptotic death or total cell death was not considerably increased in the presence of high concentrations of andrographolide (Fig. 1C). This potential discrepancy may be explained by overload of apoptotic signaling under severe stress conditions: Extremely harsh stress or high doses of chemotherapeutic drugs may cause abrupt and multiple stimulation of destructive pathways as opposed to the sequential apoptotic cascades.

Andrographolide enhances rhTRAIL-induced apoptotic cell death. Although andrographolide triggered cell death of GCs and induced expression of several death-inducing proteins in the present study, the use of this drug at high doses may be unsafe due to the toxic side effects. Recently, the utility of combinations of drugs with different mechanisms of action for cancer treatment has been gaining increasing attention (31-34). Effective combinations of anticancer drugs enhance therapeutic efficacy, as well as reduce toxicity, since each constituent may be used at a lower, non-toxic dose. Thus, it was investigated whether a combination of a low concentration of andrographolide with rhTRAIL may enhance rhTRAIL-induced apoptosis of GC cells. Combined treatment was performed by a pretreatment of cell cultures with 10 or 15 µM andrographolide for 24 h, and a subsequent incubation with rhTRAIL for another 24 h. As demonstrated in Fig. 3A, the number of apoptotic bodies was significantly increased following the combined treatment with andrographolide and rhTRAIL compared with following incubation with rhTRAIL alone. The effect of the combined treatment with andrographolide was particularly evident in AGS cells, which demonstrated resistance to rhTRAIL action. The andrographolide-mediated enhancement of the apoptotic rate was confirmed again by flow cytometric analysis, in which the increased proportion of cells in the sub-G1 phase. Subsequently, to determine whether the combined treatment with andrographolide reduced colony-forming ability of GC cells, the clonogenic assay was performed, which is based on the capacity of a single cell to proliferate into a clone. It was revealed that the combined treatment with rhTRAIL and andrographolide caused a more substantial decrease in clonogenic activity compared with the effect of rhTRAIL alone (Fig. 3C). Together, these results indicated that andrographolide promotes tumor-suppressing activity of rhTRAIL in GC cells.
Figure 3. Andrographolide enhanced rhTRAIL-induced apoptosis and clonogenicity. (A) Cells were pretreated with 10 or 15 µM andrographolide for 24 h and exposed to rhTRAIL at the indicated concentration for 24 h. Apoptosis was detected by staining cells with Hoechst 33342 and assessing the ratio of apoptotic nuclei to normal nuclei under a fluorescence microscope. (B) Cells pretreated with 15 µM andrographolide for 24 h and exposed to rhTRAIL for 24 h were stained with propidium iodide and then quantified for sub-G1 DNA content by flow cytometry. *P<0.05. (C) Cells were incubated in the absence or presence of 15 µM andrographolide for 24 h and exposed to 0, 5, 10 and 20 µM rhTRAIL for the last 1 h. Cells (2,000) were then re-plated on 60-mm dishes. Colonies were stained and counted at 2 weeks post-incubation. rhTRAIL, recombinant human tumor necrosis factor-related apoptosis-inducing ligand; Andro, andrographolide.

Figure 4. Apoptosis triggered by the combined action of andrographolide and rhTRAIL involves activation of caspase-3, caspase-8 and caspase-9, but not caspase-4. (A) AGS and SNU638 cells were treated with a combination of 15 µM andrographolide and 10 ng/ml rhTRAIL in the absence or presence of caspase-3 inhibitor z-DEVD-fmk, caspase-8 inhibitor z-IETD-fmk, caspase-9 inhibitor z-LEHD-fmk, caspase-4 inhibitor z-LEVD-fmk or pan-caspase inhibitor z-VAD-fmk for 48 h. The treated cells were stained with Hoechst 33342 to allow detection of apoptotic cells. *P<0.05 vs. control; #P<0.05 vs. andrographolide and rhTRAIL treated cells. (B) AGS cells were treated with 0, 5 and 10 ng/ml rhTRAIL in the absence or presence of 15 µM andrographolide and subjected into caspase activity assays. *P<0.05. (C) Cells treated with the combination of 15 µM andrographolide and 10 ng/ml rhTRAIL in the absence or presence of z-DEVD, z-IETD and z-LEHD were collected for analysis of activity of different caspases. #P<0.05 vs. andrographolide and rhTRAIL-treated cells. rhTRAIL, recombinant human tumor necrosis factor-related apoptosis-inducing ligand; Andro, andrographolide.
Figure 5. Andrographolide-mediated sensitization to rhTRAIL pro-apoptotic action is partially regulated by DR5. AGS cells were transfected with scrambled small interfering RNA (CTL RNAi), DR4 RNAi or DR5 RNAi and exposed to the combination of andrographolide and rhTRAIL. The treated cells were then subjected to Hoechst 33342 staining to detect apoptotic body formation by assessing the ratio of apoptotic nuclei to normal nuclei under a fluorescence microscope. *P<0.05 vs. untreated CTL RNAi transfected cells; **P<0.05 vs. andrographolide and rhTRAIL-treated CTL RNAi-transfected cells. Silencing effect of DR4 and DR5 was confirmed by immunoblotting. rhTRAIL, recombinant human tumor necrosis factor-related apoptosis-inducing ligand; DR4, tumor necrosis factor-related apoptosis-inducing ligand-receptor 1; DR5, tumor necrosis factor-related apoptosis-inducing ligand-receptor 2; CTL RNAi, scrambled small interfering RNA.

The effect of various caspase inhibitors on apoptosis triggered by the combination of andrographolide and rhTRAIL was then examined to estimate the signal pathways involved. As shown in Fig. 4A, the pan-caspase inhibitor z-vad-fmk and the caspase-3 inhibitor z-DEVD-fmk almost completely prevented apoptosis. The caspase-8 inhibitor z-IETD-fmk also significantly inhibited apoptosis, while the caspase-9 inhibitor z-LEHD-fmk caused weaker but statistically significant inhibition. At the same time, the caspase-4 inhibitor z-LEVD-fmk had little effect on apoptosis. These results indicated that the combined treatment with andrographolide and rhTRAIL causes apoptosis primarily by stimulating the extrinsic apoptotic pathway (caspase-8/caspase-3) and, to a lesser extent, via activation of mitochondria-linked caspase-9. Pro-apoptotic effects of the combined treatment with andrographolide and rhTRAIL did not appear to involve the endoplasmic reticulum stress-associated apoptotic cascade in this system. The TRAIL-sensitizing effect of andrographolide was examined in TRAIL-resistant AGS cells by assessing actual activation of caspases during induction of apoptosis by the combined treatment. In response to the treatment with 5 and 10 ng/ml rhTRAIL, activity levels of caspase-3, caspase-8 and caspase-9 slightly increased. Treatment of AGS cells with 10 μM andrographolide alone did not induce any increase in activity of these enzymes. However, the combined treatment with andrographolide and rhTRAIL enhanced activity of these three caspases (Fig. 4B). To determine the identity of the caspase cascades involved, several selective inhibitors of corresponding enzymes in AGS cells were used. It was revealed that z-IETD-fmk significantly blocked activity levels of caspase-9 (P=0.035) and caspase-3 (P=0.012), whereas z-DEVD-fmk partially inhibited activation of caspase-8. At the same time, z-LEHD-fmk had little effect on activation of caspase-8 and only partially decreased caspase-3 activity (Fig. 4C). Collectively, these results indicated that the combined administration of andrographolide with rhTRAIL induces caspase-8 activation upstream of caspase-9 and caspase-3.

DR5 signaling is essential for andrographolide-mediated sensitization to the action of rhTRAIL. The extrinsic apoptotic pathway appeared to be important in mediating apoptosis caused by the combined treatment with andrographolide and rhTRAIL, as demonstrated by the major role of the caspase-8/caspase-3 axis in this process. It was observed that incubation with andrographolide led to increased expression levels of DR4 and DR5, the receptors mediating TRAIL-induced apoptosis in GC cells. Thus, the RNA interference approach was performed to confirm the role of DR4 and DR5 in sensitization to the effects of rhTRAIL caused by andrographolide. AGS cells transfected with scrambled control RNA, DR4 siRNA or DR5 siRNA were incubated with andrographolide and rhTRAIL, and apoptosis was then assessed by evaluating apoptotic body formation. Knockdown of DR5 significantly reduced the extent of apoptosis (P=0.028). By contrast, DR4 knockdown failed to prevent apoptosis induced by the combined treatment with andrographolide and rhTRAIL (Fig. 5). This may be explained by the evidence that the concentration of andrographolide used for the sensitizing activity was reduced compared with the concentration required for efficient DR4 induction. Thus, DR5 appears to perform a more important role compared with DR4 in mediating andrographolide-induced sensitization of AGS cells to the action of rhTRAIL. However, activation of multiple signaling apoptotic pathways cannot be ruled out, since apoptosis was not completely rescued by DR5 knockdown.

ROS is involved in andrographolide-induced sensitization to effects of rhTRAIL through potentiation of DR5 expression. Oxidative stress by chemopreventive agents, including curcumin and casticin, has been implicated in DR5 upregulation and apoptosis (35-37). Furthermore, numerous natural antitumor compounds have been reported to induce ROS production (35,37). The present study explored whether ROS is involved in apoptosis triggered by the combined action of andrographolide and rhTRAIL by examining the effects of various antioxidants. The general ROS scavenger N-acetyl cysteine (NAC) profoundly suppressed apoptosis and activation of caspases following the combination treatment, whereas application of catalase had a weaker, but
Figure 6. Andrographolide-induced ROS generation induces DR5 expression and is important for rhTRAIL-sensitizing effect. (A and B) AGS cells were treated with a combination of 15 µM andrographolide and 10 ng/ml rhTRAIL in the absence or presence of 5 mM NAC, 500 U catalase, 50 µM BHA and 50 µM trolox for 48 h. The treated cells were (A) stained with Hoechst 33342 to allow detection of apoptotic cells or (B) subjected to caspase-8 and -3 activity assay. *P<0.05 vs. control; †P<0.05 vs. andrographolide and rhTRAIL-treated cells. (C) Cells treated with 15 µM andrographolide in the presence or absence of 5 mM NAC were analyzed for ROS generation using DCFH-DA at 8, 18 and 24 h post-incubation. (D) Cells exposed to 15 µM andrographolide in the absence or presence of 5 mM NAC, 500 U catalase, 50 µM BHA and 50 µM trolox for 48 h were analyzed by immunoblotting. ROS, reactive oxygen species; DR5, tumor necrosis factor-related apoptosis-inducing ligand-receptor 2; rhTRAIL, recombinant human tumor necrosis factor-related apoptosis-inducing ligand; NAC, N-acetyl cysteine; BHA, butylated hydroxyanisole; Andro, andrographolide.

statistically significant, inhibitory effect (Fig. 6A and B). However, treatments with the superoxide anion scavenger butylated hydroxyanisole (BHA) or the lipid peroxidation inhibitor trolox had no effect (Fig. 6A and B). Based on these results, ROS, including hydrogen peroxide but not superoxide anion or superoxide radicals, appear to possess a critical role in apoptosis induced by the combined treatment with andrographolide and rhTRAIL. As determined by the dichloro-dihydro-fluorescein diacetate assay, exposure of AGS cells to andrographolide significantly increased ROS production, which may be reduced by application of NAC (Fig. 6C). The effect of antioxidants on the increase of DR5 expression induced by andrographolide was also examined. Similar to its inhibitory effect on apoptosis induction, NAC almost completely blocked induction of DR5 expression (Fig. 6D). BHA and trolox had no effect on DR5 induction, while catalase caused only a partial decrease in DR5 expression (Fig. 6D). Therefore, induction of oxidative stress by andrographolide may be the essential mechanism for DR5 induction and sensitization to the pro-apoptotic effects of rhTRAIL in GCs.

In conclusion, the present study revealed a possible role for andrographolide as a sensitizer of GC cells to the action of TRAIL. Based on the present results, co-application of these drugs may improve therapeutic efficacy of GC treatment, although additional clinical studies are required.

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