Abstract. Mesothelioma is an aggressive tumor associated with asbestos exposure. Auranofin as an inhibitor of thioredoxin reductase (TrxR) affects many biological processes such as inflammation and proliferation. In the present study, we investigated the cellular effects of auranofin on patient-derived mesothelioma cells in relation to reactive oxygen species (ROS) and glutathione (GSH) levels. Basal TrxR1 levels have no difference between mesothelial cells and certain mesothelioma cells. In particular, ADA, CON and Hmeso mesothelioma cells showed lower levels of TrxR1 expression. Auranofin inhibited the proliferation of mesothelioma cells in a dose-dependent manner. Among mesothelioma cells were ADA and CON cells sensitive to auranofin. This agent also induced caspase-independent apoptosis and necrosis in ADA cells. In addition, auranofin increased ROS levels including \( \text{O}_2 \cdot \) and induced GSH depletion in mesothelioma cells. While N-acetyl cysteine (NAC) prevented cell death and decreased ROS levels in auranofin-treated mesothelioma cells, L-buthionine sulfoximine (BSO) intensified apoptosis and GSH depletion in these cells. In conclusion, auranofin induced mesothelioma cell death through oxidative stress and the death was regulated by the status of GSH content.

Introduction

Thioredoxin (Trx) and glutathione (GSH) are major antioxidant systems in the cells to defend excess reactive oxygen species (ROS) production. Trx system consists of Trx and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent Trx reductase (TrxR) (1). Trx, having two active sites in cysteine residue, exists as a dithiol, reduced form. When Trx is oxidized, it is reduced by TrxR (1). GSH is a non-protein antioxidant and stabilizes the oxidized molecules by supplying electron. Trx and GSH systems control not only redox status but also affect many cellular events such as proliferation and apoptosis (2-4). Especially, TrxR1 is overexpressed in breast and oral cancer patients (5,6). It has been reported that the inhibition of TrxR increases the sensitivity of cancer cells to radiotherapy and anticancer drugs in melanoma, colon and breast cancers (7-9). Therefore, the regulation of Trx system can be a promising target for cancer therapy (10).

Auranofin, as a TrxR inhibitor, is used for the treatment of rheumatoid arthritis. Originally, this agent was considered as anti-inflammatory drug (11). However, recently many studies demonstrate that auranofin has an anticancer effect in leukemia and ovarian cancer cells (12,13). In addition, It has been suggested that auranofin induces FOXO3 activation, ROS accumulation, DNA damage and ERK inactivation in cancer cells (13,14). Mesothelioma is a rare tumor mainly derived from the pleura of lung and it has a poor prognosis (15). Although it is reported that TrxR1 is overexpressed in mesothelioma cells (16), little is known about the anti-growth effect of auranofin in mesothelioma cells.

In the present study, we investigated the effects of auranofin on cell proliferation and death in patient-derived human mesothelioma cells in relation to ROS and GSH levels.

Materials and methods

Cell culture. Human mesothelial cells (HM69 and HM72) and human mesothelioma cells (ADA, CON, Hmeso, Mill, Phi, REN and ROB) were obtained from Queen's Medical Center (Honolulu, HI, USA). These cells were cultured in Ham's F-12 media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (both from Gibco BRL, Grand Island, NY, USA). Mesothelial and mesothelioma cells were maintained in incubator containing 5% CO\(_2\) at 37°C. Cells were grown in
100 mm plastic cell culture dishes (BD Falcon, Franklin Lakes, NJ, USA) and harvested with a trypsin-EDTA (Gibco BRL).

**Reagents.** Auranofin purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) at 10 mM as a stock solution. The pan-caspase inhibitor (Z-VAD-FMK; benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) was obtained from R&D Systems, Inc. (Minneapolis, MN, USA) and were dissolved in DMSO at 10 mM. NecroX-2 and necrostatin-1 from Enzo Life Science (Plymouth Meeting, PA, USA) were dissolved in DMSO at 1 and 50 mM, respectively. NAC and BSO obtained from Sigma-Aldrich Chemical Co. were dissolved in 20 mM HEPES (pH 7.0) and water at 100 mM, respectively. Cells were pretreated with 15 µM Z-VAD, 1 µM NecroX-2, 50 µM necrostatin-1, 2 mM NAC or 10 µM BSO for 1 h prior to auranofin treatment.

**Western blot analysis.** The protein expression levels were evaluated by western blot analysis. In brief, 1x10⁶ cells in 60 mm culture dish (BD Falcon) were incubated with or without 3 µM auranofin for 24 h. Then cells were washed with phosphate-buffered saline (PBS) and added in 4 volumes of protein extract buffer (Life Technologies, Carlsbad, CA, USA). The concentrations of protein were determined using the Bradford method. A total of 30 µg total proteins were resolved by 4-20% SDS-PAGE gels, and then transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA, USA) by electroblotting. Then membranes were probed with anti-PARP and anti-c-PARP (Cell signaling Technology, Danvers, MA, USA) and anti-TrxR1, anti-GAPDH and anti-β-actin (Santa Cruz Biotechnology). Membrane was incubated with fluorescence-conjugated secondary antibodies. Bands were visualized by using a LI-COR Odyssey Imager (LI-COR Biosciences, Lincoln, NE, USA).

**Cell proliferation assay.** The effect of auranofin on proliferation in mesothelioma cells was determined by CellTiter 96® AQ®-One Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA). In brief, 5x10⁵ cells in 96-well microtiter plate (BD Falcon) were incubated with the indicated concentrations of auranofin with or without NAC or BSO for 24 h. Then, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) mixture was added to each plate. The plates were incubated for 3 h at 37°C. The optical density was measured at 490 nm using a microplate reader (VersaMax plate reader; Molecular Devices, Sunnyvale, CA, USA).

**Sub G-1 analysis.** Sub-G1 analysis was determined by propidium iodide (PI; Sigma-Aldrich Chemical Co.; Ex/Em=488/617 nm) staining. Briefly, 1x10⁶ cells in 60 mm culture dish (BD Falcon) were incubated with the indicated concentrations of auranofin with or without Z-VAD, NecroX-2 or necrostatin-1 for 24 h. Cells were washed with PBS and then incubated with 10 µg/ml PI with RNase at 37°C for 30 min. Sub-G1 DNA content cells were analyzed with an Accuri C6 flow cytometer (BD Sciences, Franklin Lakes, NJ, USA).

**Annexin V/PI staining.** Apoptosis was detected by staining cells with Annexin V-fluorescein isothiocyanate (FITC; Life Technologies; Ex/Em=488/519 nm) and PI (Sigma-Aldrich Chemical Co.). Briefly, 1x10⁶ cells in 60 mm culture dish (BD Falcon) were incubated with the indicated concentrations of auranofin with or without Z-VAD, NecroX-2, necrostatin-1, NAC or BSO for 24 h. Then cells were washed twice with cold PBS and added 300 µl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 1x10⁶ cells/ml. Five microliters of Annexin V-FITC and PI were added to these cells, which were analyzed with Accuri C6 flow cytometer (BD Sciences).

**Lactate dehydrogenase (LDH) release assay.** Necrosis in cells was evaluated by LDH kit (Sigma-Aldrich Chemical Co.) Briefly, 1x10⁵ cells in 60 mm culture dish (BD Falcon) were incubated with the indicated concentration of auranofin with or without NAC or BSO for 24 h. After treatment, the cell culture media were collected and centrifuged for 5 min at 1,500 rpm. A total of 50 µl of the media supernatant was added to a fresh 96-well plate (SPL Life Sciences, Pocheon, Gyeonggi-do, Korea) with LDH assay reagent and then incubated at room temperature for 30 min. The absorbance values were measured at 490 nm using a microplate reader (Synergy™ 2; BioTek® Instruments Inc., Winooski, VT, USA). LDH release was expressed as the percentage of extracellular LDH activity compared with the control cells.

**Detection of intracellular ROS levels.** Intracellular ROS such as H₂O₂, ‘OH and ONOO⁻ were detected by an oxidation-sensitive fluorescent probe dye, 2,7’-dichlorodihydrofluorescein diacetate (H₂DCFDA, Life Technologies; Ex/Em=495/529 nm). As H₂DCFDA is poorly selective for O₂⁻, dihydroethidium (DHE, Life Technologies; Ex/Em=518/605 nm), which is highly selective for O₂⁻, was used for its detection. Briefly, 1x10⁶ cells in 60 mm culture dish (BD Falcon) were incubated with the indicated concentrations of auranofin with or without NAC or BSO for 24 h. The cells were washed in PBS and incubated with 20 µM H₂DCFDA and DHE at 37°C for 30 min. DCF and DHE fluorescences were detected by using Accuri C6 flow cytometer (BD Sciences).

**Measurement of intracellular GSH level.** Cellular GSH levels were analyzed using a 5-chloromethylfluorescein diacetate dye (CMFDA, Ex/Em=522/595 nm; Life Technologies). In brief, 1x10⁶ cells were incubated in a 60 mm culture dishes (BD Falcon) with 3 µM auranofin with or without NAC or BSO for 24 h. Cells were then washed with PBS and incubated with 5 µM CMFDA at 37°C for 30 min. CMF fluorescence intensity was determined using Accuri C6 flow cytometer (BD Sciences). Negative CMF staining (GSH-depletion) of cells is expressed as the percentage of (+) CMF cells.

**Statistical analysis.** The results represent the mean of at least three independent experiments (mean ± SD). Data were analyzed using Instat software (GraphPad Prism4, San Diego, CA, USA). The Student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis using Tukey's multiple comparison test was used for parametric data. P<0.05 was considered to indicate a statistically significant difference.
Results

Auranofin inhibits proliferation and induces the death of mesothelioma cells. Firstly, we observed the protein expression levels of TrxR1 in human mesothelial and mesothelioma cells. As a result, there was no difference of TrxR1 expression in either mesothelial or mesothelioma cells (Fig. 1A). Instead, the levels of TrxR1 in ADA, CON and Hmeso were lower than those in other mesothelioma cells (Fig. 1A). Treatment with auranofin attenuated the proliferation of mesothelioma cells in a dose-dependent manner (Fig. 1B). ADA and CON cells, which showed lower TrxR1 levels, were more sensitive to auranofin than Mill and Phi cells (Fig. 1B). Auranofin completely reduced the level of TrxR1 in ADA cells and this agent also decreased that of Phi cells (Fig. 1B). In addition, auranofin increased LDH release, sub-G1 cells and Annexin V positive cells in ADA and Phi cells (Fig. 1C-E). LDH release was high in Phi cells whereas sub-G1 cells and Annexin V positive cells were high in ADA cells. It also induced a cleavage in PARP protein in ADA and Phi cells (Fig. 1F).

Auranofin leads to necrosis in ADA cells. It was investigated whether auranofin induces apoptosis and/or necrosis in ADA cells. When auranofin-treated ADA cells were co-incubated with Z-VAD, a pan-caspase inhibitor, Z-VAD did not change the percentages of sub-G1 and Annexin V positive cells in these cells (Fig. 2A and B). In contrast, NecroX-1, necrosis inhibitor, decreased the numbers of sub-G1 and Annexin V positive cells in auranofin-treated ADA cells and necrostatin-1, necroptosis inhibitor, reduced the numbers of decreased sub-G1 and Annexin V positive cells in these cells as well (Fig. 2C and D).

NAC prevents auranofin-induced cell death in ADA and Phi cells. Auranofin is an inhibitor of TrxR1. Therefore, it can induce cell death through an oxidative stress. We pre-treated ADA and Phi cells with 2 mM NAC for 1 h prior to the treatment of auranofin. NAC significantly recovered the reduced cell proliferation caused by auranofin in ADA and Phi cells (Fig. 3A). NAC also inhibited auranofin-induced LDH release in both cells (Fig. 3B). NAC significantly prevented cell death in auranofin-treated ADA and Phi cells, and the prevention was dramatic in ADA cells (Fig. 3C). As expected, auranofin increased ROS levels including O$_2^•−$ in ADA and Phi cells at 24 h, and NAC decreased the levels in these cells (Fig. 4A and B). Auranofin also induced GSH depletion in ADA and Phi cells (Fig. 4C and D). NAC completely blocked the GSH depletion caused by auranofin in ADA and Phi cells (Fig. 4C and D).

BSO enhances auranofin-induced cell death in ADA and Phi cells. There are two main antioxidant systems, Trx and GSH in cells. For this reason, inhibition of GSH synthesis might be a novel strategy to disturb the redox status and finally lead to cell death. As expected, BSO intensified the inhibition of cell proliferation in Phi cells, which were relatively resistant to auranofin compared with ADA cells (Fig. 3A). BSO did not
additionally increase the LDH release in auranofin-treated ADA and Phi cells (Fig. 3B). However, BSO significantly increased apoptotic cell death in auranofin-treated ADA and Phi cells (Fig. 3C). BSO also augmented the increased ROS levels including $O_2^{•-}$ in auranofin-treated cells and the augmentation was strong in Phi cells (Fig. 4A and B). Moreover, BSO significantly increased the numbers of GSH-depleted cells in auranofin-treated Phi cells (Fig. 4D).

Discussion

Auranofin as an inhibitor of TrxR has an anti-inflammatory effect (17) and can treat rheumatoid arthritis (18). In addition, auranofin shows anticancer effects in ovarian, prostate, breast and lung cancer cells (14,19-21). It is also reported that many cancer cells contain high level of TrxR expression (6,22). Thus, auranofin can be a strong candidate agent for treatment of cancer. Likewise, in the present study, auranofin inhibited the proliferation in mesothelioma cells and induced caspase-independent apoptosis and necrosis in these cells. Interestingly, the basal TrxR1 expression levels were not different between normal mesothelial cells and mesothelioma cells. This result is contrary to the report that Trx and TrxR were upregulated in mesothelioma (16). This discrepancy will be clarified in relation to expression and activity in Trx and TrxR proteins between normal and cancer cells. We observed that ADA and CON cells showed low level of TrxR1 expression and these cells were more sensitive to auranofin than other mesothelioma cells. These results support that the level of TrxR1 expression is involved in the cytotoxic effectiveness of drug among cancer cells (23).

Excess ROS production or an imbalance of antioxidant can lead to oxidative stress and finally damages the cells (24). Trx and GSH are the two main antioxidant systems in the cells (25). TrxR is a key component in the Trx system. Therefore, an inhibition of TrxR can induce cell death via causing oxidative stress (26). Correspondingly, auranofin increased the ROS levels including $O_2^{•-}$ in relatively auranofin-sensitive ADA cells and auranofin-resistant Phi cells. An increase in ROS levels was strong in Phi cells. This result suggests that auranofin-resistant Phi cells have a high threshold to oxidative stress to induce cell death. Furthermore, NAC, an antioxidant, attenuated the inhibition of proliferation in auranofin-treated ADA and Phi cells. This agent also prevented cell death in these cells. The prevention was accompanied by a decrease in ROS levels. These results suggest that auranofin induce cell growth inhibition and cell death in an oxidative stress-dependent manner.
GSH is a non-protein antioxidant and prevents cells from damage caused by oxidative stress (27). The thiol group of cysteine in GSH supplies an electron to unstable molecules and then GSH itself is oxidized. When GSH is converted to oxidized-form, it is reduced back by GSH reductase (28,29). GSH is also critical for cell proliferation and apoptosis (30,31). Therefore, an inhibition of GSH is a reasonable strategy to enhance cytotoxicity in anticancer drug resistant cancer cells (32). Likewise, auranofin increased the depletion of GSH in both ADA and Phi cells. Auranofin-sensitive ADA cells showed a strong depletion in GSH content. NAC significantly blocked GSH depletion in auranofin-treated ADA and Phi cells. Thus, NAC plays a role as a precursor of GSH as well as an antioxidant in mesothelioma cells. BSO, an inhibitor of GSH synthesis, intensified cell growth inhibition and cell death in auranofin-treated ADA and Phi cells. The enhancement of cell death by BSO was remarkable in auranofin-resistant Phi cells. BSO also accelerated the increase in ROS level and...
GSH depletion in auranofin-treated Phi cells. These results demonstrated that an inhibition of GSH is effective to enhance cell growth inhibition and cell death in these cells through an oxidative stress. In addition, mesothelioma cell death caused by auranofin was affected by the status of GSH content.

Acknowledgements

We thank Professor Peter R. Hoffmann and Dr Pietro Bertino for kindly providing the mesothelioma cells. The present study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (no. 2008-0062279) and supported by the Basic Science Research Program through the NRF funded by the Ministry of Education (2013006279).

References