Cigarette smoke contains many harmful chemicals, which contribute to the pathogenesis of smoking-related diseases such as chronic obstructive pulmonary disease, cancer and cardiovascular disease. The cytotoxicity of cigarette smoke is well documented, but the definitive mechanism behind its toxicity remains unknown. Ingredients in cigarette smoke are known to deplete intracellular glutathione (GSH), the most abundant cellular thiol antioxidant, and to cause oxidative stress. In the present study, we investigated the mechanism of cigarette smoke extract (CSE)-induced cytotoxicity in B16-BL6 mouse melanoma (B16-BL6) cells using liquid chromatography-tandem mass spectrometry. CSE and ingredients in cigarette smoke, methyl vinyl ketone (MVK) and crotonaldehyde (CA), reduced cell viability in a concentration-dependent manner. Also, CSE and the ingredients (m/z 70, each) irreversibly reacted with GSH (m/z 308) to form GSH adducts (m/z 378) in cells and considerably decreased cellular GSH levels at concentrations that do not cause cell death. Mass spectral data showed that the major product formed in cells exposed to CSE was the MVK-GSH adduct via Michael-addition and was not the GSH-CA adduct. These results indicate that MVK included in CSE reacts with GSH in cells to form the GSH-MVK adduct, and thus a possible reason for CSE-induced cytotoxicity is a decrease in intracellular GSH levels.

Key words: cigarette smoke extract (CSE); glutathione (GSH); methyl vinyl ketone (MVK); B16-BL6 mouse melanoma (B16-BL6) cell; LC/MS; LC/MS/MS

Cigarette smoking has been known as a major risk factor implicated in increased incidence of cardiovascular disease, cancer and chronic obstructive pulmonary disease. Cigarette smoke contains more than 4800 identified chemical compounds, many of which are toxic and harmful to the human body. Oxidants and aldehydes, major constituents in the gaseous phase of cigarette smoke, are thought to mediate oxidative stress which has been implicated in the pathogenesis of smoking-related diseases.

Reactive α,β-unsaturated carbonyl compounds such as acrolein and crotonaldehyde, are abundant in the gas phase of cigarette smoke and are major mediators of cigarette smoke-induced macrophage activation. These compounds are also thought to contribute to oxidative stress-induced inflammation and vascular dysfunction. Furthermore, acrolein and crotonaldehyde have been reported to directly react with the thiol groups, especially glutathione (GSH), via a Michael-type addition reaction, resulting in the formation of nonreducible GSH-aldehyde derivatives, thereby depleting the total available GSH pool. Because GSH plays a key role in cellular antioxidant defense against oxidant injury, GSH depletion leads to cigarette smoke-induced cytotoxicity. Similarly, methyl vinyl ketone (MVK), an α,β-unsaturated carbonyl compound, has been shown to react with free GSH in nucleophilic Michael-type addition. The MVK-induced cell apoptosis was associated with depletion of GSH, disruption of the mitochondrial transmembrane potential, and increased generation of reactive oxygen species.

We have previously demonstrated, using gas chromatography-mass spectrometry, that a lot of MVK is present in cigarette smoke extract (CSE) and readily reacts at 37°C with tyrosine, a chemically reactive amino acid, to form N-(3-oxobutyl)-Tyr via the Michael reaction.

The purpose of the present study was to investigate the molecular mechanism of the cytotoxicity induced by CSE in B16-BL6 mouse melanoma (B16-BL6) cells. We hypothesized that α,β-unsaturated carbonyl compounds present in CSE may irreversibly react with intracellular GSH to form GSH adducts, and that consequently lowered GSH levels may be associated with CSE-induced cytotoxicity. Since this melanoma cell line established by Poste et al. is highly metastatic, we tried to measure the influence of the formation of GSH adducts and lowered GSH levels on the function of this distinct cell line in the near future. As an initial step, the detection and identification of the GSH adducts formed in the cells exposed to CSE were confirmed using a highly sensitive LC/MS and LC/MS/MS system.

Experimental

Materials Caster Frontier One (cigarette brand name) cigarettes were purchased from Japan Tobacco Inc. (Tokyo, Japan), and Cambridge filters were purchased from Hiener Borgwaldt KC (Hamburg, Germany). Crotonaldehyde (CA) and methyl vinyl ketone (MVK) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N-Acetyl cysteine (NAC) was from Sigma-Aldrich (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was from BioWest Co. (Nuaille, France). The other chemicals were of reagent grade or higher and were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was from BioWest Co. (Nuaille, France). The other chemicals were of reagent grade or higher and were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

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France). Ethylenediaminetetraacetic acid (EDTA) trypsin solution (EDTA: 2.2 mg, trypsin: 0.25%) was from Mediatech, Inc. (Manassas, VA, U.S.A.). Penicillin/streptomycin solution (penicillin: 50,000 U/mL, streptomycin: 50 mg/mL) was from Cosmo Bio Co., Ltd. (Tokyo, Japan). Dulbecco’s modified Eagle’s medium (DMEM) with 1-glutamine was from Invitrogen Corp. (Carlsbad, CA, U.S.A.). Dulbecco’s phosphate-buffered saline without calcium and magnesium [DPBS(−)] was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). LC/MS grade H₂O and CH₃OH were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and LC/MS grade formic acid and an octa decyl silyl (ODS) column (Cosmosil 5C₁₈-AR-II 4.6 mm × 150 mm) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Preparation of CSE CSE was prepared by modification of a technique described in a previous report. Briefly, CSE was prepared by bubbling into phosphate-buffered saline [DPBS(−)] (1 mL per three cigarettes) the mainstream of smoke (gas phase) from which the particulate phase, including tars and nicotine, had been almost completely removed by passage through a Cambridge filter using an aspiration pump. The pump flow rate was kept constant (1 L/min). Smoke was bubbled for only 1 min after lighting the cigarette. The CSE solution was immediately filtered through a 0.22-μm filter. The resulting solution, designated 100% CSE, was stored at −80°C and diluted to various concentrations with DPBS(−) before use. The final concentrations of these solutions are expressed as percent values.

Cell Cultures, Treatment and Cell Proliferation The highly metastatic B16-BL6 cell line was kindly provided by Dr. Futoshi Okada of Tottori University (Yonago, Japan). The cells were cultured in DMEM containing 10% FBS and 0.1% penicillin/streptomycin solution at 37°C in humidified 5% CO₂−95% air. Cells were seeded onto 12-well culture plates at a density of 1×10⁵ cells per well in 2 mL of the growth medium. The cells were then treated with CSE (0.03, 0.1, 0.3, 1%, CA and MVK (3, 10, 30, 100 μM, each) for 24 h. As necessary, NAC was added just before the CSE, or MVK treatment. To assess cell proliferation, the attached viable cells were trypsinized and enumerated using a Coulter Counter (Coulter Electronics Inc., Hialeah, FL, U.S.A.). Cell viability was calculated by comparing cell counts in treated samples relative to cell counts in the non-treated control and converted to percentages.

Exposure of Mouse Melanoma Cells to CSE B16-BL6 cells (5×10⁶ cells) were exposed to 1% CSE for 30 min at 37°C. The cells were harvested with EDTA trypsin solution and subsequently resuspended in 1 mL of DPBS(−). Assay samples were prepared with a modification of the technique described in previous reports. Briefly, the cells were washed with 1 mL DPBS(−) by centrifugation at 200×g for 5 min. These cells were washed 3 times under the same conditions. Next, the cell pellets were collected and lysed with 50 μL of 70% methanol. The cell lysates were centrifuged at 17,400×g for 5 min at 4°C, and the resulting supernatants were collected and analyzed by LC/MS and LC/MS/MS.

In Vitro Reaction of GSH with CSE and Its Active Ingredients, CA and MVK To clarify the major reaction products of intracellular GSH with CSE, a 2 mM solution of GSH in DPBS(−) buffer was added to an equal volume of 2% CSE, 20 μM CA or 20 μM MVK in DPBS(−) buffer. After incubation for 30 min at 37°C, the chemical structures of the GSH adducts present in each reaction solution were analyzed and identified by LC/MS and LC/MS/MS.

Triple-Quadrupole Mass Spectrometer and HPLC Conditions A Quattro Premier triple-quadrupole LC/MS (Micromass, Manchester, U.K.) with an electrospray ionization (ESI) source was used for positive and negative ion mode Q1 scan and MS/MS analysis coupled to an Alliance HT 2795 Separations Module (Waters Co., Milford, MA, U.S.A.). The optimized conditions were as follows: source temperature, 120°C; desolvation temperature, 350°C; flow rate of cone nitrogen, 100 L/h and flow rate of desolvation nitrogen, 1000 L/h. Capillary and cone voltages were 3.0 kV and 20 V, respectively. The flow rate of argon collision gas for fragmentation in the product ion mode was 0.3 mL/min (3.37–3.39×10⁻⁷ mbar) by which the collisional energy was optimized for the fragment ions of GSH (5–25 eV). All chromatographic separations were performed using a Cosmosil 5C₁₈-AR-II column (4.6 mm × 150 mm). The mobile phase was composed of water containing 0.05% formic acid as solvent A and methanol as solvent B, and the flow rate was set at 0.3 mL/min. A linear gradient analysis was used for LC conditions in the separation. For the main gradient analysis, the initial elute solvent consisted of 1% solvent B until t=2 min, and using a linear gradient, it was raised to 40% solvent B at t=12 min. This condition was held for 3 min until t=15 min, then using a linear gradient, it was raised further to 95% solvent B at t=18 min. This condition was held for 3 min until t=21 min. Finally, the gradient solvent was lowered to 1% solvent B by 22 min and held for 2 min. The LC oven temperature was set at 27°C and the injection volume was 5 μL.

Statistical Analysis Results are expressed as means± standard error (S.E.). Statistical analyses were performed with the Dunnett and Tukey tests using the Graphpad Prism 4 software package (Graphpad Software, Inc., San Diego, CA, U.S.A.). Differences were considered significant at p<0.05.

Results and Discussion Comparative Effects of CSE, CA and MVK on Cell Viability Cytotoxic effects of CSE have been reported in various cells. In the next step, we are planning to investigate the effect of CSE and its ingredients on the function of the target cells. In these cases, B16-BL6 cells are suitable for evaluating the effect of CSE and its ingredients on the metastatic ability of cells, since this cell line is detectable easily in secondary metastasized organs in in vivo studies. In the present study, we clarified that CSE reduces cell viability in a concentration-dependent manner in B16-BL6 cells (Fig. 1a). In the presence of 1% CSE, cell viability was significantly reduced to about 45.3% at 24 h of incubation. To clarify the effective ingredients in CSE, we investigated the effect of α,β-unsaturated carbonyl compounds, CA and MVK, in CSE on cell viability. At a concentration of 30 μM, CA and MVK reduced cell viability to 54.2% and about 8.4%, respectively (Fig. 1b). It therefore seems that MVK has a stronger effect on the mechanism of cytotoxicity than CA. Furthermore, the cytotoxicity of CSE and MVK were antagonized significantly by the pre-addition of NAC, a GSH precursor (Figs. 2a, b).

LC/MS Analysis of Mouse Melanoma Cells Exposed to CSE B16-BL6 cells were exposed to CSE under a condition...
that has little effect on viability. We have previously reported that the growth curve of B16-BL6 cells is not affected by treatment with 1% CSE for 3 h.\(^{21}\) Therefore, the cells were exposed to 1% CSE at 37°C for 30 min and then deproteinized with 70% methanol/DPBS (−) solution. After ultracentrifuging, the supernatants were directly analyzed by LC/MS. We used positive and negative ion mode ESI Q1 scan (mass range: from \(m/z\) 52 to 720) for the mass spectral conditions and the gradient system for the HPLC conditions. Several new peaks were observed in the positive ion mode total ion current chromatogram of the sample obtained from cells exposed to CSE (Fig. 3a). The mass spectrum of the highest new sharp peak at a retention time of 16.1 min is shown in Fig. 4a. This new peak corresponded to the protonated ion \(m/z\) 378. On the other hand, in the mass spectrum of the round peak at a retention time of 10.5–13.6 min, which appeared in both Figs. 3a and b, a protonated ion at \(m/z\) 308 was observed as the base peak (Fig. 4b). The peak at \(m/z\) 308 is thought to correspond to the protonated GSH ion ([GSH+H]\(^+\)), because it is well known that GSH, a major antioxidant, is present in high concentra-

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**Fig. 1.** Viability of B16-BL6 Mouse Melanoma Cells Exposed to (a) Cigarette Smoke Extract (CSE), (b) Methyl Vinyl Ketone (MVK) or Crotonaldehyde (CA) for 24 h

Values are expressed as means±S.E. (n=4–6). *\(p<0.05\), **\(p<0.01\), statistical difference compared with the corresponding untreated control.

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**Fig. 2.** Effect of N-Acetyl Cysteine (NAC) on B16-BL6 Mouse Melanoma (B16-BL6) Cell Viability Inhibited by (a) CSE or (b) MVK for 24 h

Values are expressed as means±S.E. (n=4–6). **\(p<0.01\), statistical difference compared with the corresponding untreated control. *\(p<0.05\), **\(p<0.01\), statistical difference compared with (a) CSE 1% or (b) MVK 5 \(\mu\)M.
ions in cells (approximately 5 mM). In fact, we confirmed that the m/z 308 ion detected in the cells corresponds to the protonated molecule of GSH by showing that the product ion spectrum of the ion at m/z 308 matched that of authentic GSH (Fig. 5).

Also, we estimated the mass value m/z 70, by subtracting m/z 308 from m/z 378, to be two α,β-unsaturated carbonyl compounds, CA and MVK (M, 70 each), because these compounds are abundant in CSE. We have previously found, by using the selected ion monitoring mode of GC/MS, that the concentrations of CA and MVK in 100% CSE are approximately 450 µM and 550 µM, respectively.15) Other investigators have reported similar results.6,22) Van der Toorn et al.9) have recently demonstrated by MS analysis that a substantial
amount of GSH in epithelial cells exposed to the gaseous phase of cigarette smoke is irreversibly modified to GSH-CA derivatives, thereby depleting the total available GSH pool. Our experimental results on pre-addition of NAC confirmed the cytotoxicity of CSE caused by consumption of GSH. Moreover, Reddy et al.\textsuperscript{23} reported that the GSH-CA adduct is a major reaction product of the gas vapor phase of cigarette smoke with GSH. However, these reports do not mention formation of the GSH-MVK adduct. In the present study, we also found that the peak area of $m/z$ 308 (the protonated GSH ion) in B16-B16 cells treated with 1% CSE for only 5 min dramatically decreased to almost 40% of the control (data not shown).

Therefore, it seems reasonable that $\alpha,\beta$-unsaturated carbonyl compounds in CSE can readily produce GSH adducts via Michael addition. As for MVK, it has been reported that it induces apoptosis in neuronal cells through GSH depletion.\textsuperscript{14} In addition, GSH reacts with MVK to form the inactive GSH-MVK adduct.\textsuperscript{24} However, there is no report that exposure of CSE or cigarette smoke to cells results in the formation of GSH-MVK adducts. We therefore attempted to react CSE directly with GSH to identify the GSH-carbonyl adducts formed in B16-BL6 cells exposed to CSE.

**Identification of Reaction Products of GSH with MVK and CA** To clarify whether the ion at $m/z$ 378 detected in

![Fig. 6. Total Ion Current (TIC) Chromatogram and Extracted Ion Chromatograms of $m/z$ 308 and $m/z$ 378 Obtained from Reaction Products of GSH with (a) CA, (b) MVK, (c) DPBS(−) and (d) 1% CSE at 37°C for 30 min](image)

Extracted ion chromatograms of $m/z$ 378 of (b) and (d) have the same retention time. This indicates that GSH is modified by MVK in CSE. The chromatograms of (c), GSH with DPBS(−), are shown as controls.
the cells exposed to CSE is either the GSH adduct with CA or MVK, GSH (1 mM) was reacted with CA and MVK (10 µM each) at 37°C for 30 min. As a result, peaks at m/z 378 corresponding to CA and MVK appeared at retention times of 17.1 min and 16.1 min, respectively (Figs. 6a, b). When GSH was incubated with 1% CSE at 37°C for 30 min, a peak at m/z 378 was observed at a retention time of 16.1 min (Fig. 6c). These findings indicate that the peak at m/z 378 comes from the GSH-MVK adduct. Furthermore, in LC-MS/MS analysis, the product ion spectra of the ion at m/z 378 detected in CSE-exposed cells (Fig. 7a) agreed well with the spectra of the GSH-MVK adduct rather than the GSH-CA adduct (Figs. 7b, c). These product ion spectra of (a) and (c) have very similar fragmentation patterns. This indicates that GSH in the B16-BL16 cells is modified by MVK in CSE.

Fig. 7. Comparison with Product Ion Spectra of the Ion at m/z 378 Obtained from (a) the B16-BL16 Cells Exposed to 1% CSE (Fig. 3a) and Those of the Reaction Products of GSH with (b) CA and (c) MVK

The product ion spectra of (a) and (c) have very similar fragmentation patterns. This indicates that GSH in the B16-BL16 cells is modified by MVK in CSE.

Structural Elucidation of the GSH-MVK Adduct

The product ion spectra of GSH and the MVK-GSH adduct are shown in Fig. 8. Collision energy was optimized to the objective product ion. For product ion analysis, it was set at 10 eV because fragment ion y1 appeared in the product ion spectrum. For fragment ions, the accepted nomenclature is “b1” and “y1” ions. If the charge is retained on the N-terminal fragment, the ion is classed as a, b or c. If the charge is retained on the C-terminal, the ion type is x, y or z. The subscript indicates the number of residues in the fragment. The product ion at m/z 179 came from y1 cleavage of GSH, and the product ion at m/z 249 of the MVK-GSH adduct was increased by the addition of 70 units to the product ion at m/z 179. Another product ion at m/z 233 came from b2 cleavage of GSH. The product ion at m/z 303 of the MVK-GSH adduct was increased by the addition of 70 units to the product ion at m/z 233 of GSH. The MVK-GSH adduct was the 1,4 Michael-addition reaction product of MVK with the SH group of GSH (Fig. 8).

Conclusion

In conclusion, we demonstrated for the first time by LC/MS and LC/MS/MS analysis, that intracellular GSH is modified mainly by MVK in B16-BL6 mouse melanoma cells exposed to CSE. The major product formed was the GSH-MVK adduct via Michael-addition and was not the GSH-CA adduct.
In addition, GSH levels in the cells were markedly reduced by treatment with CSE. GSH depletion is a common feature of cell death. These findings suggest that depletion of cellular GSH with the formation of the GSH-MVK adduct may contribute to CSE-induced cytotoxicity.

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