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Short Communication

Inhibition of Centrosome Amplification in Human Osteosarcoma Cells (Saos2) by Antioxidants Glutathione and Trolox

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Abstract

Mustard gas has been used as a chemical weapon since 1917 during World War I. Since then, many reports have shown that survivors of attacks and those who make mustard gas exhibit a higher incidence of cancer than the normal population. We have recently shown that centrosomes may play a role in mustard gas-induced cancers since the mustard gas surrogate 2-chloroethyl ethylsulfide (2-CEES) increases centrosome amplification (more than two centrosomes per cell) in the Saos2 cell line (human osteosarcoma). To begin to delineate some of the mechanisms that might regulate 2-CEES-induced centrosome amplification, we exposed Saos2 cells to the antioxidants Trolox and glutathione (GSH) and 2-CEES in various combinations for 24 hours. We show that Trolox has a minimal effect on inhibiting centrosome amplification, whereas glutathione prevented 2-CEES-induced centrosome amplification below control levels. These data provide insight as to how 2-CEES can induce centrosome amplification, but whether or not that is more related to protein, lipid, or DNA damage is yet to be determined and should be investigated.

ABBREVIATIONS

MG: Mustard Gas; 2-CEES: 2-chloroethyl ethylsulfide; CIN: Chromosome instability; PCM: Pericentriolar material; MT: Microtubules; GSH: Glutathione; DMEM: Dulbecco's Modified Eagle's Medium; Trolox: (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; PBS: Phosphate buffered saline; TBS: Tris buffered saline; IgG: Immunoglobulin G; DAPI: 4',6-Diamidino-2-phenylindole; ROS: Reactive oxygen species; PARP: Poly (ADP-Ribose) polymerase; ex: excitation; em: emission

INTRODUCTION

Mustard gas (MG, β, β'-dichloroethyl sulfide) has been used as a chemical weapon since World War I when the Germans attacked British troops in Ypres, Belgium in July 1917, and continues to be a threat today due to its ease of manufacture and use [1,2]. MG is a vesicant (blistering agent) that causes acute damage primarily to the skin, eyes, respiratory, and digestive organs, resulting in a number of complications such as painful blisters on the skin and mucous membranes, respiratory distress, conjunctival irritation, corneal ulceration, nausea, vomiting, and diarrhea [3]. Over time, cancers of various organs can develop in individuals exposed during battle or during the manufacture of MG [4-10]. On a cellular level, MG damages DNA directly by forming monoadducts and interstrand crosslinks with the former being the most common [11,12] and via oxidative stress, forming 8-oxo-2-deoxyguanosine as the major product [13-15]. Chromosome instability (CIN) is a common phenotype of various cancer types and is thought to introduce multiple genetic lesions required for malignancy through the gain and/or loss of chromosomes during mitosis [16,17]. One mechanism that is thought to drive CIN is centrosome amplification. Centrosomes are small, non-membrane-bound organelles that are composed of two, orthogonally-arranged centrioles surrounded by an amorphous protein matrix called the pericentriolar material (PCM) [18]. They function to organize the microtubule (MT) network, the most notable being the bipolar mitotic spindle during mitosis, which directs the equal segregation of chromosomes during anaphase. After mitosis, daughter cells must duplicate their single centrosomes during S phase of the cell cycle to ensure that two and only two centrosomes are

present during the next mitosis [19]. If centrosome duplication is perturbed, centrosomes can fail to duplicate, resulting in a monopolar spindle. Alternatively, centrosomes could duplicate uncontrollably and produce a multipolar spindle, which has been shown to increase the instance of merotely (a single kinetochore attached to microtubules from opposite poles), thereby increasing the instance of lagging chromosome formation and chromosome instability [20]. Centrosome amplification can occur under a number of conditions. For example, inactivating mutations of p53 [21] or $p21^{Waf1}$ [22] or activating mutations of cyclin E [23] can cause centrosome amplification. Additionally, DNA damage and oxidative stress have been shown to induce centrosome amplification as well [24,25]. We recently showed that 2-chloroethyl ethylsulfide (2-CEES), a surrogate of MG, induces centrosome amplification in Saos2 cells [26]. Since 2-CEES damages DNA and induces oxidative stress [13,14], we wanted to further delineate the mechanisms that drive centrosome amplification in 2-CEES-treated cells using two different antioxidants that affect MG toxicity via two distinct mechanisms. Trolox, a water-soluble analog of Vitamin E [27], is known to inhibit oxidative stress, while glutathione (GSH) forms conjugates with MG directly and prevents both DNA damage and oxidative stress [14]. As a result, we show that Trolox does not significantly reduce 2-CEES-induced centrosome amplification in Saos2 cells, whereas GSH significantly reduces 2-CEES-indcued centrosome amplification to levels below that of control conditions.

MATERIALS AND METHODS

Cell Culture

Saos2 cells (human osteosarcoma) were obtained from the American Type Culture Collection (ATCC, Manassa, VA, HTB-85) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Hyclone/Fisher, Waltham, MA) for maintenance and experiments. Cells were incubated at 37°C and 10% CO¬2 in a humidified incubator for all experiments.

Cell Treatment

Saos2 cells were plated in 24-well plates containing glass coverslips and incubated overnight so that they were sub confluent after an overnight incubation. Cells were incubated in various combinations of 250 µM 2-chloroethyl ethylsulfide (2-CEES, Sigma, St. Louis, MO), 10 mM glutathione (GSH, Sigma, St. Louis, MO), and 800 µM Trolox (Sigma, St. Louis, MO) for 24 hours and then assayed for centrosomes using indirect immunofluorescent staining.

Centrosome Detection Assay

Cells were grown and treated on glass coverslips as described. After treatment, cells were washed in PBS and fixed in 4% formalin/methanol (FormaldeF resh, Fisher, Waltham, MA) for 20 minutes at room temperature followed by permeabilization with 1% Nonidet P-40 (Fisher, Waltham, MA) in PBS for 10 minutes at room temperature. Cells were blocked in 15% normal goat serum (Life Technologies, Carlsbad, CA) for 1 hour in a humidified chamber and then gently washed by dipping coverslips into three separate beakers of PBS. Cells were then incubated in rabbit-anti-γ tubulin antibody (Cell Signaling, Boston, MA) diluted in PBS for 45 minutes at room temperature. Cells were then washed with PBS for 15 minutes on a rocker and then exposed to AlexaFluor 594-conjugated goat-anti-rabbit IgG antibody (Life Technologies, Carlsbad, CA) diluted in PBS for 45 minutes at room temperature in the dark. Cells were washed in Tris buffered saline (TBS – 150 mM NaCl, 20 mM Tris, pH 7.4) for 15 minutes on a rocker in the dark followed by nuclear counterstaining with 500 nM 4',6-Diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) for 10 minutes in the dark at room temperature. Lastly, coverslips were washed again in TBS for 10 minutes on a rocker in the dark at room temperature and then mounted on coverslips using Fluoromount-G (Southern Biotech, Birmingham, AL). The number of centrosomes was determined for at least 100 cells in each condition.

Centriole Analysis

To identify centrioles, cells were incubated on coverslips and treated as noted above. After treatment, the 24-well plates containing cells were incubated on wet ice for 30 minutes. Coverslips were then transferred to wells containing cold PBS and washed one time with cold PBS. Coverslips were then incubated in cold extraction buffer (0.75% Triton X-100, 5 mM PIPES buffer, 2 mM EGTA) on wet ice for about 10-30 seconds per well. Coverslips were then washed 2-3 times with cold PBS and then incubated in cold fixative (4% formalin/methanol, FormaldeFresh, Fisher, and Waltham, MA) on wet ice for 10 minutes followed by 10 minutes in new, room temperature fixative. Coverslips were gently washed 2-3 times in room temperature PBS and then blocked in 15% normal goat serum (Life Technologies, Carlsbad, CA) for 1 hour in a humidified chamber, followed by a gentle wash in PBS by dipping coverslips into three separate beakers of PBS. Cells were then incubated in rabbit-anti-γ tubulin and mouse-anti-α tubulin antibodies (Cell Signaling, Boston, MA) diluted in PBS for 45 minutes at room temperature. Cells were then washed with PBS for 15 minutes on a rocker and then exposed to Alexa-Fluor 594-conjugated goat-anti-rabbit IgG and Alexa-Fluor 488-conjugated goat-anti-mouse IgG antibodies (Life Technologies, Carlsbad, CA) diluted in PBS for 45 minutes at room temperature in the dark. Cells were then washed in Tris buffered saline (TBS – 150 mM NaCl, 20 mM Tris, pH 7.4) for 15 minutes on a rocker in the dark followed by nuclear counterstaining with 500 nM 4',6-Diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) for 10 minutes in the dark at room temperature. Lastly, coverslips were washed again in TBS for 10 minutes on a rocker in the dark at room temperature and then mounted on coverslips using Fluoromount-G (Southern Biotech, Birmingham, AL).

Fluorescent Microscopy

Images were analyzed on a Zeiss AxioImager.A2 fluorescence microscope using 470/40 nm (ex) and 525/50 (em) for γ-tubulin (green), 565/30 (ex) and 620/60 (em) for α-tubulin (red), and 365+ nm (ex) and 445/50 (em) for DAPI (blue). Images were captured using an AxioCam MRm camera and AxioVision software (ver. 4.8.0.0). Figures were assembled using PowerPoint and

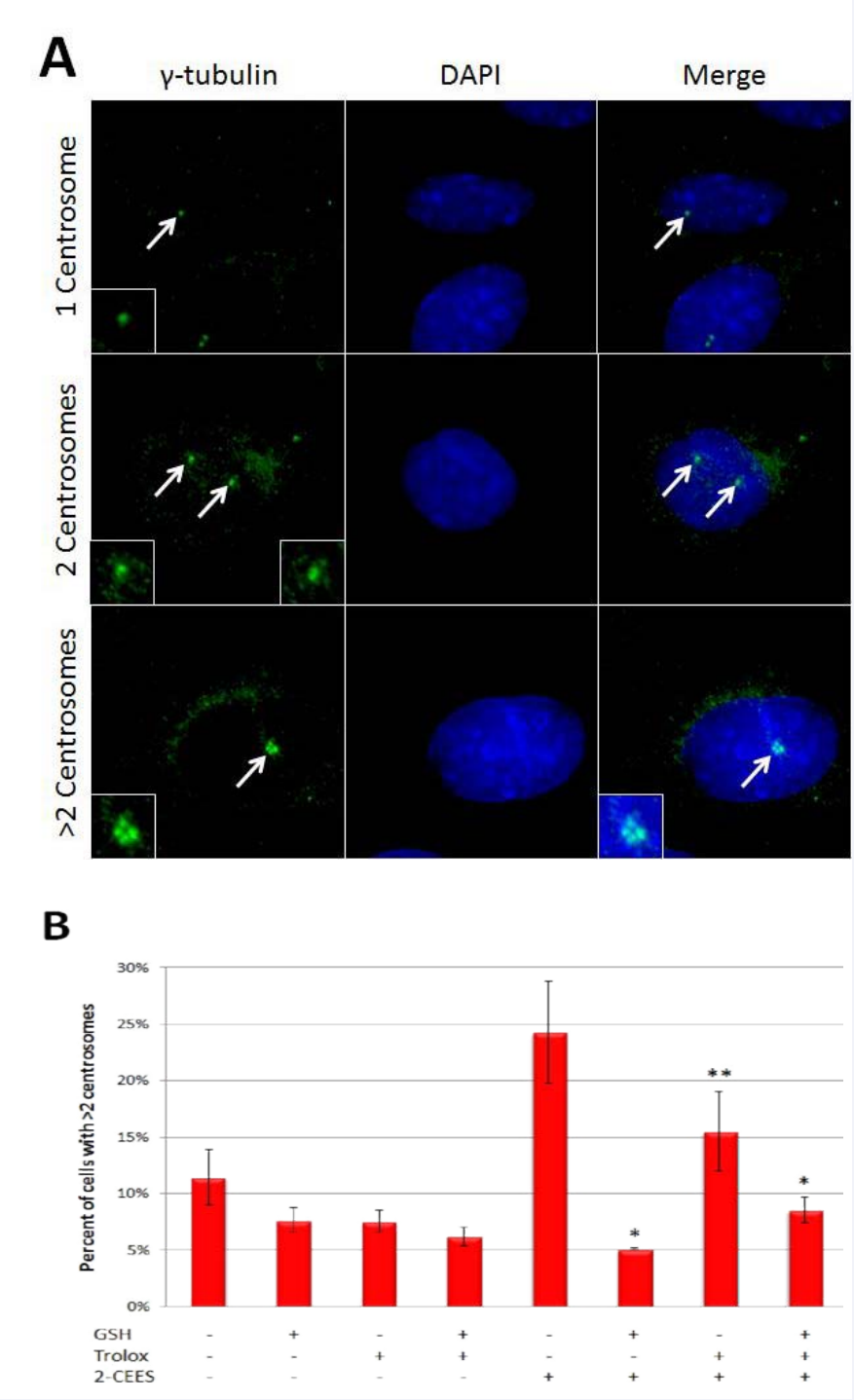

Figure 1 (A) Representation of micrographs from which data for Figure 1B was derived. Cells were treated and then immunostained for γ-tubulin (green) and costained with DAPI (blue). Cells were categorized as having one (1 centrosome), 2 (2 centrosomes), or more than two centrosomes (>2 centrosomes). (B) The percent of cells in each condition with centrosome amplification (>2 centrosomes). *p < 0.05 compared to cells treated with 2-CEES only (column 5).

Adobe Photoshop CS2 (ver. 9.0).

RESULTS AND DISCUSSION

GSH, but not Trolox, inhibits 2-CEES-induced centrosome amplification. We previously showed that 2-CEES can induce centrosome amplification in Saos2 cells after 24 hour incubation [26]. Additionally, previous work has shown that GSH and Trolox differentially prevent 2-CEES-induced DNA damage in JB6 cells (mouse skin epidermal cells) and dermal fibroblasts, with GSH being more effective [14]. To further determine how 2-CEES-

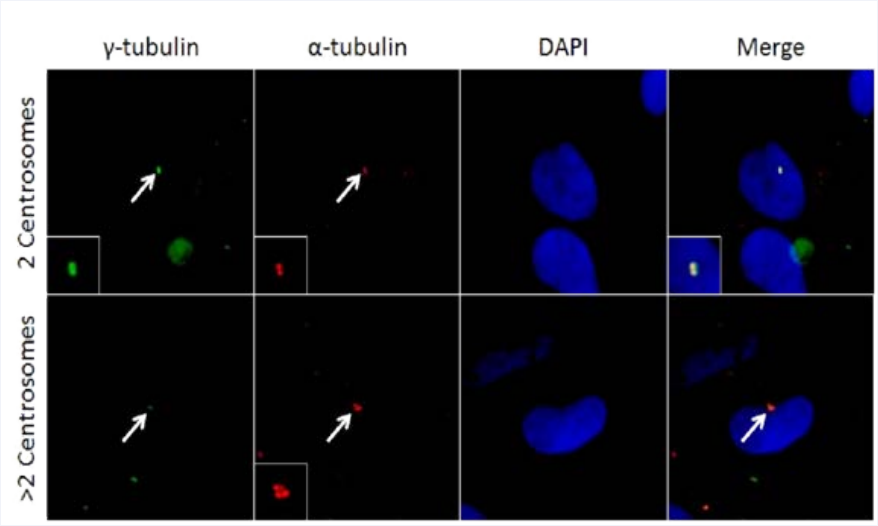

Figure 2 γ-tubulin spots are centrosomes. Cells were treated and then immunostained for γ-tubulin (green, PCM), α-tubulin (red, centrioles), and counterstained with DAPI (blue). Centrosomes contain centrioles.

induced centrosome amplification might occur, we treated Saos2 cells with 250 μM 2-CEES in the presence or absence of 800 μM Trolox, 10 mM GSH, both, or neither, along with the same 2-CEES untreated conditions, for 24 hours. Cells were then immunostained for γ-tubulin (a component of the PCM), to detect centrosomes, and counterstained with DAPI to detect nuclei. Centrosomes appeared as bright green dots (Figure 1A), the number of which was recorded as one, two, or more than two centrosomes per cell for at least 100 cells in each condition and then graphed (Figure 1B). Untreated, GSH- or Trolox-treated, and cells treated with both antioxidants showed low levels of centrosome amplification (6.2 – 11.4%). 2-CEES-treated cells exhibited high levels of centrosome amplification (24.3%), as expected. Interestingly, co-treatment of cells with 2-CEES and GSH significantly ($p < 0.05$) decreased centrosome amplification to less than control levels, while co-treatment of cells with 2-CEES and Trolox did not significantly decrease centrosome amplification ($p > 0.05$). Additionally, co-treatment of cells with 2-CEES, GSH, and Trolox together resulted in a significant decrease in centrosome amplification compared to 2-CEES only-treated cells (8.5% vs. 24.3%). These results indicate that GSH alone or GSH with Trolox is more effective in preventing centrosome amplification than Trolox alone. To ensure that γ-tubulin stained structures were indeed centrosomes, coverslips from untreated and 2-CEES-treated cells were immunostained for both γ- and α-tubulin. γ-tubulin is a major component of the PCM, while α-tubulin is found in both microtubules and centrioles. To specifically identify centrioles, cells were cold-treated, fixed, and immunostained with antibodies against α- and γ-tubulin (Figure 2). As shown, γ- and α-tubulin staining colocalized, indicating that the γ-tubulin staining used to determine centrosome amplification was indeed centrosomes.

CONCLUSION

Here, we have shown that GSH significantly inhibits 2-CEES-induced centrosome amplification in Saos2 cells, whereas Trolox does not. This differential effect on centrosome amplification provides some insight as to how 2-CEES can induce centrosome amplification in these cells. Both MG and 2-CEES form sulfonium ions in the body, which, due to its electrophilic nature, can bind to DNA, RNA, and proteins and cause cell toxicity and death [28-31]. Additionally, both MG and 2-CEES can lead to an increase in reactive oxygen species (ROS) through the depletion of cellular GSH and antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase [14,32-34]. Interestingly, both DNA damage and oxidative stress have been shown to induce centrosome amplification [24,25]. In this work, we wanted to investigate whether direct macromolecular damage or oxidative stress was responsible for the observed 2-CEES-indcued centrosome amplification from out previous work. To do this, we co-treated Saos2 cells with different combinations of GSH, Trolox, and 2-CEES. GSH is known to bind MG directly, forming conjugates, and has been shown to decrease 2-CEES-induced cell death and DNA damage [14,35,36]. Trolox is known to decrease ROS and oxidative DNA damage in cells, but not direct DNA damage by 2-CEES [14]. Thus, any decrease in centrosome amplification due to GSH treatment can be attributed to its ability to prevent both direct macromolecule damage and oxidative stress, while any reduction in centrosome amplification in Trolox-treated cells can be attributed to its ability to prevent only oxidative stress. Our data showed that both GSH and Trolox, individually, decreased 2-CEES-induced centrosome amplification, although the latter was statistically insignificant. Additionally, GSH and Trolox together significantly decreased 2-CEES-induced centrosome amplification in Saos2 cells. This indicates that 2-CEES-induced centrosome amplification likely occurs primarily due to its ability to directly interact with macromolecules. This supports observations that show that many proteins that regulate centrosome duplication are targets of MG toxicity, including p53, poly (ADP-ribose) polymerase (PARP), and NF-κB [21,37-40]. Although we show that GSH treatment significantly prevents 2-CEES-induced centrosome amplification, whether or not that is more related to protein, lipid, or DNA damage is yet to be determined and should be investigated.

Lastly, it is possible that the effects seen in our experiments may be the result of perturbations in cell cycle progression. Centrosome duplication is tightly coupled to DNA synthesis during S phase [41,42]. Thus, it is reasonable to think that cell cycle arrest in G_1 or S phase would concomitantly prevent centrosome amplification. However, it has been shown that centrosome duplication can still occur during G_1 and S phase arrest [17,43]. Trolox has been shown to not induce G_1 arrest [44]. Regardless, the effects of GSH and Trolox on cell cycle progression should be investigated in relation to centrosome duplication and amplification.

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