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Centrosome amplification and multinuclear phenotypes are Induced by hydrogen peroxide

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Abbreviations: DAPI, 4', 6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; MTOC, microtubule-organizing center; NAC, N-acetylcysteine; PDTC, pyrrolidinedithiocarbamate

Abstract

Multinucleated cells resulted from mitosis defect have been noted in pathophysiological states of the cells such as inflammation, senescence and cancer. Since oxidative stress has been known to correlate with these pathophysiological conditions, we tested the effect of H_2O_2 on the cell cycle progression and formation of multinucleated cells. H_2O_2 induced a significant delay in cell cycle progression in Chang liver cells. Interestingly, H_2O_2 actively induced hyperamplification of centrosomes ($n \geq 3$) and multipolar spindle formation during mitosis and subsequently increased the generation of multinucleated cells. A significant increase of the phospho-ERK level was observed upon H_2O_2 treatment but PD98059, an MEK1/2 inhibitor, didn't reduce the frequency of cells with hyperamplified centrosomes. On the other hand, treatment of either H_2O_2 or adriamycin increased intracellular ROS levels and multinucleated cells, which were significantly suppressed by antioxidants, N-acetylcysteine and PDTC. Thus, our results suggest that oxidative stress can trigger centrosome hyperamplification and multinucleated cell formation, which may promote pathophysiological progression.

Keywords: antioxidants; centrosome, giant cells, hydrogen peroxide, reactive oxygen species

Introduction

Centrosome is an important cellular organelle and plays a key role in cell division. The centrosome functions as a microtubule-organizing center (MTOC), which controls the polarity of microtubules in interphase and generates bipolar mitotic spindles during mitosis (Lange, 2002). Centrosome abnormalities with an excess number of centrosomes organize multipolar mitotic spindles, which pull the chromosomes in multiple directions, consequently increasing the chance of multinucleated cell formation and aneuploidy (D'Assoro *et al.*, 2002). Aneuploidy is a characteristic feature of cancer and found in virtually every type of human cancer. Interestingly, mutations of p53, Brca1 and Brca2 tumor suppressor genes and amplification of cyclin E and aurora A genes found in multiple origins of cancer are also highly correlated with supernumerary centrosomes (Bertwistle and Ashworth, 1999; Jeng *et al.*, 2004; Kawamura *et al.*, 2004).

Centrosome abnormalities are also described in some aging cells. Centrosome and microtubule network during meiosis were significantly altered in aging oocytes of Drosophila, resulting in aneuploidy or trisomic zygotes (Shatten *et al.*, 1999). Fibroblasts derived from Huntington's disease, a progressive neurodegenerative disorder, were found to have a high frequency of multiple centrosomes and aneuploidy (Sathasivam *et al.*, 2001). However, unlike in cancer, molecular features involved in centrosome abnormalities in aging have never been proposed. In the present study, we demonstrated that oxidative stress can induce supernumerary centrosomes and multinucleated cell formation, which may promote pathophysiological progression during aging.

Materials and Methods

Cell cycle synchronization and cell cycle analysis

Human Chang liver cells were originally purchased from ATCC and maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified CO_2 incubator. Cells were synchronized by the double

thymidine block (DTB) method at the G1/S boundary and released for cell cycle progression (Yun *et al.*, 2004). Briefly, 2 mM of thymidine was incubated for 18 h and followed by 6 h release. The 2nd incubation of thymidine was carried out for 18 h. H_2O_2 (0.1-0.2 mM) was added right after removal of 2nd thymidine. At each time point, cells were trypsinized, pelleted, and fixed with 70% cold ethanol for 30 min (Kim *et al.,* 2005). Cells were then resuspended in a solution containing propidium iodide and subjected to FACScan analysis using a FACS Vantage flow cytometer (Becton Dickinson).

Indirect immunofluorescence

Cells were synchronized by the DTB method and H_2O_2 was added right after removal of 2nd thymidine, followed by 12-24 h incubation. To obtain mitotic cells, 100 ng/ml of nocodazole was co-incubated with H_2O_2 for 8-12 h. The cells released from nocodazole arrest were grown on poly-L-lysine-coated cover glasses and allowed to further progress into mitosis. Cells were fixed with the mixture of methanol/acetone (1:1) solution and permeabilized with 0.5% Triton X-100. Fixed cells were preincubated in blocking solution (5% BSA in PBS), followed by incubation with primary antibodies for pericentrin, γ -tubulin or α-tubulin overnight at 4°C. Cells were then washed three times with shaking and probed with fluorescence-conjugated secondary antibody for 1 h at room temperature. After washing, cells were mounted in the mounting solution containing 4', 6-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscope (Zeiss) and analyzed with Aims software. For the accurate centrosome counting, antibodies against pericentrin were used and the number of centrosome was counted from 300-600 cells each time.

Western blotting

Western blotting was performed as described previously (Yun *et al.*, 2004). Briefly, harvested cell pellets were extracted with RIPA buffer, and the resultant extracts were subjected to a SDS-PAGE and probed with anti-cyclin A antibody (Santa Cruz Laboratory). The enhanced chemiluminescence (ECL) non-radioactive detection system was used to detect the antibody-protein complexes.

Measurement of intracellular ROS level

Intracellular ROS levels were determined by using an oxidative-sensitive fluorescence dye, dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes) as we previously described (Wang *et al.*, 2003). Cells were treated with 0.2 mM of H_2O_2 or 0.1 μg/ml of adriamycin for 24 h and further incubated with 10 μM of DCF-DA for 15 min. Unincorporated fluorescent dye was washed out with PBS and the intensities of fluorescence in cells were analyzed using FACS Vantage at the wavelength of 526 nm.

Results and Discussion

H_2O_2 Induces hyperamplification of centrosome and multipolar mitotic spindle formation

Multinucleated cells were often described in cells under pathophysiological conditions such as inflammation, senescence and cancer (Devaney *et al.*, 1992, Protzer *et al.*, 1996; Evans 2002; Ren *et al.*, 2005). Prolonged oxidative stress has been described as a common causative factor for these diseases. Since H_2O_2 has been most commonly used inducer for imposing oxidative stress or stress-induced premature senescence, we attempted to investigate whether centrosome abnormalities were involved in increasing genomic instability in cells under oxidative stress. Chang liver cells treated with 0.1 mM of H_2O_2, a subcytotoxic concentration, showed a significant delay in cell cycle progression (Figure 1A). In control Chang cells, cells G1 entered G2/M phase in 9 h after release and returned back to G1 phase in 12 h, completing another cell division in 18 h. On the other hand, Chang cells exposed to H_2O_2 entered G2/M phase in 12 h and slowly progressed to G1 phase in 30 h. These cells accumulated at G1 phase by 48 h and underwent senescence-like morphology such as enlarged and flattened cell shape (data not shown). A significant degradation of cyclin A level was accmpanied at 12 h-15 h where cells were staggered at G2/M phase in the presence of H_2O_2 (Figure 1B). When these flattened cells were stained with antibody for centrosome-specific pericentrin, some of them contained more than three centrosomes with tripolar directional chromosomal staining (Figure 1C, b). When the patterns of chromosome segregation with α-tubulin were overlaid, multipolar spindles radiating from the spindle poles as well as multidirectional segregation of chromosomes were increased in Chang cells exposed to H_2O_2 (Figure 1C, c). When these cells were further progressed into G1 phase, multinucleated cells with supernumerary centrosomes (Figure 1C, d) were found after staining cells with anti-γ-tubulin antibody. At least, 20% of Chang cells exposed to H_2O_2 were identified to contain supernumerary centrosomes whereas 5-7% of control Chang cells showed abnormal centrosome numbers. Similarly, the number of cells containing multiple nuclei was increased twice upon H_2O_2 treatment. The data here show that H_2O_2 actively induced

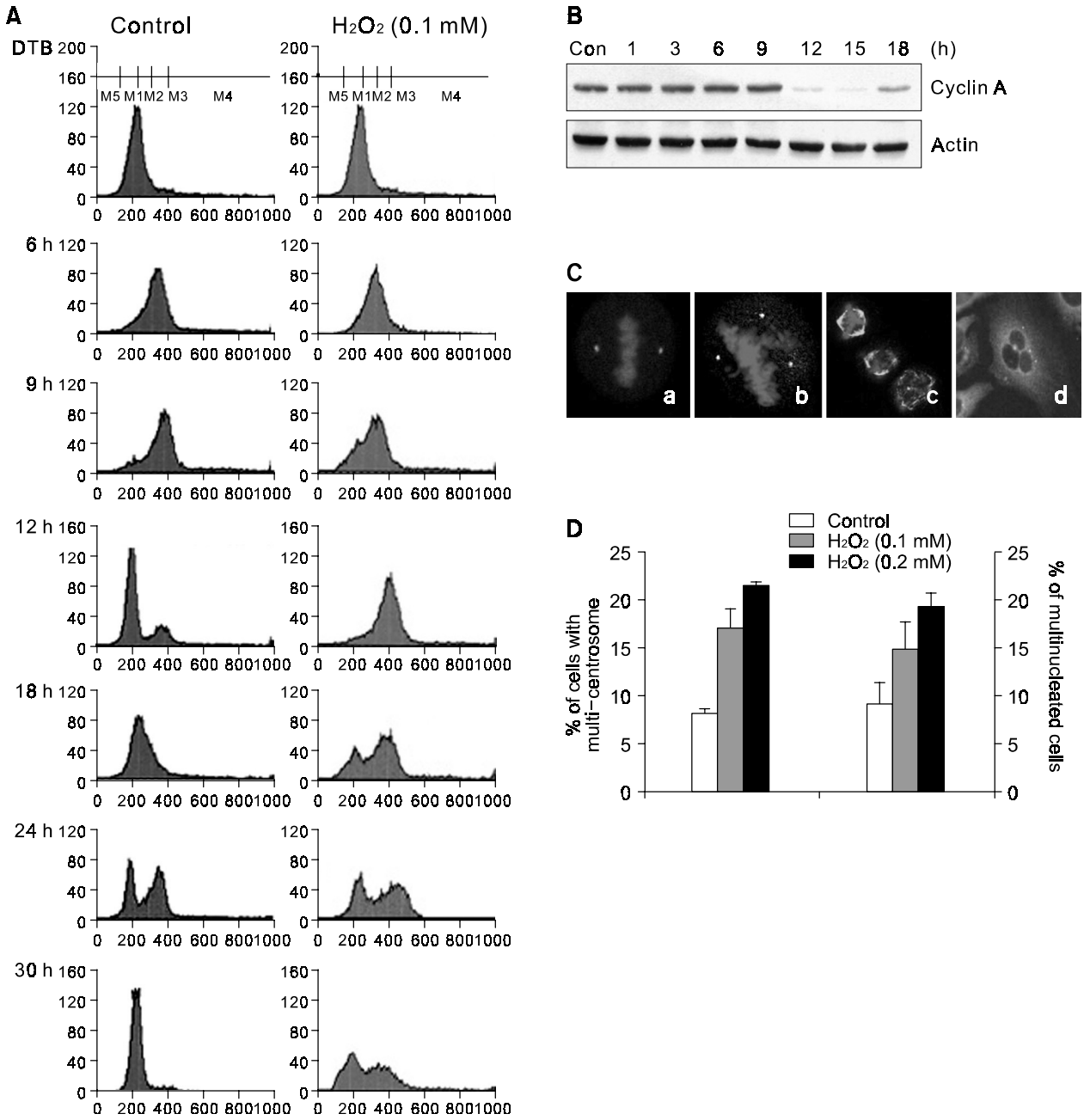

Figure 1. H_2O_2 induces hyperamplification of centrosome and multipolar mitotic spindle formation. (A) FACS analysis of cell cycle progression after H_2O_2 treatment. (B) Determination of cyclin A level upon H_2O_2 treatment. (C) Immunofluorescence staining of centrosome-specific pericentrin staining. The Chang cells released from the DTB were treated with 0.1 mM of H_2O_2 and subsequently with 100 ng/ml of nocodazole. Centrosomes were visualized by staining cells with anti-pericentrin antibody and DAPI (a, b). The overlaid image of mitotic spindles and chromosomal staining (c) and multinucleated cells visualized by anti-γ-tubulin staining (d). (D) Quantification of supernumerary centrosomes and multinucleated cells after H_2O_2 treatment. Bar represents mean ± standard deviation.

amplification of centrosomes ($n \geq 3$) and multipolar spindle formation during mitosis and subsequently increased the generation of multinucleated cells.

The ERK pathway does not mediate the formation of supernumerary centrosomes

Increase of intracellular reactive oxygen species (ROS) level can activate several stress signaling pathways, which may partly trigger senescence

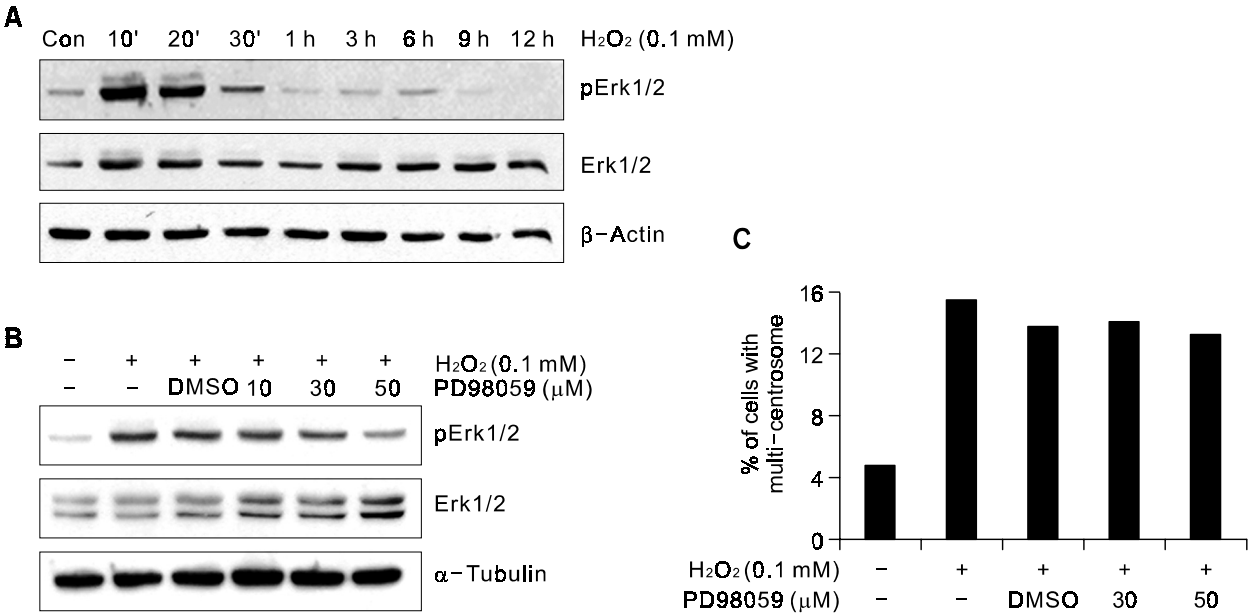

Figure 2. The ERK pathway does not mediate the formation of supernumerary centrosomes. (A) Immunoblotting of the phospho-ERK levels after H_2O_2 treatment. (B) Suppression of the phosphor-ERK by PD98059. (C) Suppression of the ERK pathway did not affect supernumerary centrosomes.

program (Leonard *et al.*, 2004). Ras/MAPK pathway is one of the most widely described signaling pathways, which were activated under oxidative stress (Hutter *et al.*, 2002). We and other group reported that the activation of Ras/ERK mediated hyperamplification of centrosomes in cells exposed to γ-radiation or overexpressing HBx viral oncoprotein (Saavedra *et al.*, 1999; Yun *et al.*, 2004). Therefore, the effect of ERK inhibitor on the centrosome amplification was examined using different concentrations of PD98059, an MEK1/2 inhibitor. When cells were exposed to 0.1 mM of H_2O_2, a significant induction of phopho-ERK level was observed in 10-20 min (Figure 2A). Preincubation of cells with 50 μM of PD98059 1 h before addition of H_2O_2 almost completely suppressed the increase of phospho-ERK level (Figure 2B). However, these concentrations of PD98059 did not reduce the frequency of supernumerary centrosomes in cells exposed to H_2O_2 (Figure 2C). Thus, the ERK pathway does not mediate supernumerary centrosomes in cells under oxidative stress.

Inhibition of intracellular ROS levels reduced the occurrence of multinucleated cells

In order to further delineate a correlation between oxidative stress and aberrant chromosome segregation and multinucleated cell formation, we employed adriamycin, a chemotherapeutic agent. It has been reported that adriamycin imposed a severe oxidative stress in cells (Childs *et al.*, 2002; Deepa and Varalakshmi, 2003). When we determined the intracellular ROS levels after treatment of 0.2 mM of H_2O_2 or 0.1 μg/m of adriamycin, a subcytotoxic concentration, evident increase of ROS levels was observed (Figure 3A). The median value of fluorescence intensities in control Chang was increased by ~ twice upon H_2O_2 treatment. Similarly, the median value of fluorescence intensities was also shifted to the right, showing ~ three times increase in ROS generation by adriamycin treatment. We found that adriamycin also significantly increased the generation of multinucleated cells in Chang cells (Figure 3B). We also found that pretreatment of antioxidants such as 20 mM of NAC or 50 μg/ml of PDTC significantly reduced the frequency of multinucleated cell generation. These results demonstrated oxidative stress increased generation of multinucleated cells, at least mediated through centrosome hyperamplification.

In the present study, we have demonstrated that H_2O_2 actively induced hyperamplification of centrosomes ($n \geq 3$) and multipolar spindle formation during mitosis and subsequently increased the generation of multinucleated cells. Multinucleated cells can be found in normal muscle cells and in pathophysiological states of cells such as cancer, aging and inflammation (Devaney *et al.*, 1992, Protzer *et al.*, 1996; Evans 2002; Ren *et al.*, 2005). Although, the exact role of multinucleated cells in these pathophysiological conditions is not clear, it certainly

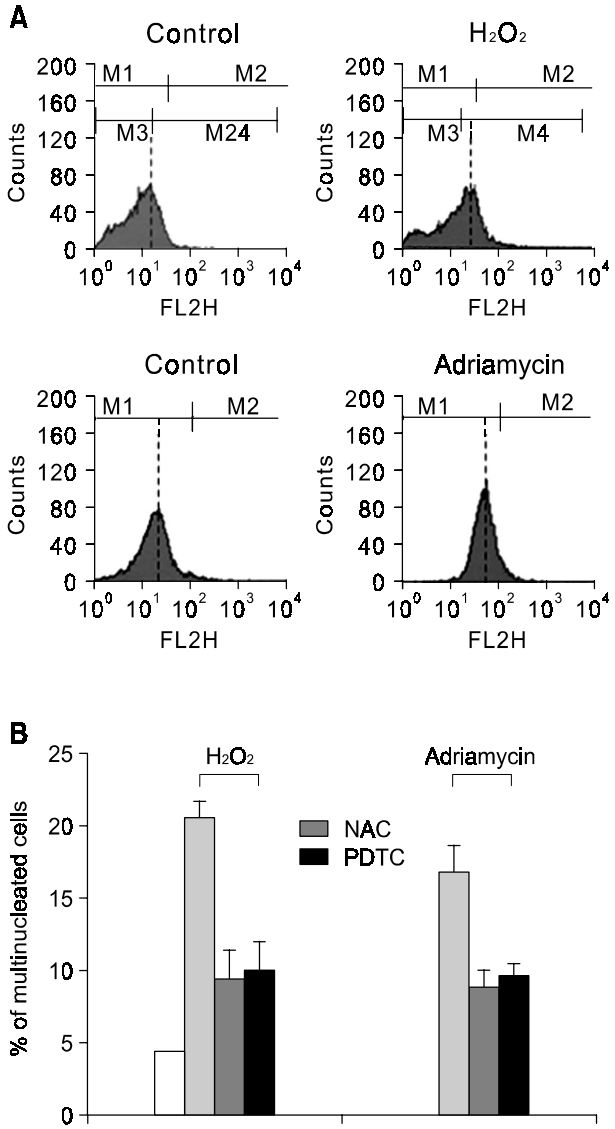

Figure 3. Inhibition of intracellular ROS levels reduced the occurrence of multinucleated cells. (A) Increase of intracellular ROS levels were determined by DCF-DA fluorescent dye and FACS Vantage. (B) Antioxidants, 20 mM of NAC or 50 μg/ml of PDTC, significantly suppressed the generation of multinucleated cells induced by 0.2 mM of H_2O_2 and 0.1 μg/ml of adriamycin. Bar represents mean ± standard deviation.

contributes to increase the genomic and morphological alterations during cell division. Importance of genomic instability in carcinogenesis can not be further emphasized (Park *et al.,* 2005). Interestingly, genomic instability is also considered as one of the major causative factors in aging. It has shown that genomic instability accumulated through DNA damage and DNA repair defect triggers aging (Beckman and Ames, 1998; Mattson 2003). Our findings here propose that oxidative stress, which is highly correlated with both cancer and aging, can trigger centrosome hyperamplification, which contributes to increase genomic instability. Interestingly, recent studies on telomere shortening and maintenance provided an insight in understanding a possible link between cancer and aging (Blasco 2005; Rodier *et al.,* 2005). Cells with telomere dysfunction do not divide and undergo senescence, accumulating genomic damage. However, those cells also encounter great risks of neoplastic transformation when cells acquired additional mutations in the p53 pathway or checkpoint function (Satyanarayana *et al.*, 2004). We propose that centrosome hyperamplification induced by hydrogen peroxide is another way to increase the genomic instability in aging and cancer.

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