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The effect of oxidative stress on breast cancer multicellular spheroids

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Abstract

Breast cancer is the most diagnosed cancer among women worldwide. Most cancer cells are reported with a high level of oxidative stress caused by generation of cellular reactive oxygen species (ROS). To investigate disease mechanisms, an *in vitro* multicellular tumor spheroid (MCTS) has gained more interest and been accepted to closely recapitulate *in vivo* cancer conditions. Thus, the aim of this study was to investigate the contribution of MCTS to the level of oxidative stress in breast cancer (MCF-7) cells. First, MCF-7 MCTS was successfully established with a significantly slower growth rate than monolayer. Contrast to the monolayer, MCF-7 MCTS showed no significant increase in a level of cellular ROS when induced with H_2O_2 from 0 – 250 µM. Additionally, gene and protein expressions of antioxidant enzymes especially superoxide dismutase (SOD2) in MCTS after oxidative stress induction were increased with less relative fold changes than the monolayer. Interestingly, the SOD2 up-regulation was also found in MCTS without H_2O_2 treatment. Moreover, the MCTS treated with H_2O_2 showed a higher apoptotic resistance. Altogether, we demonstrated that MCTS contributed to a lower H_2O_2 sensitivity and withstood higher level of the oxidative stress partly involving with a higher expression of the anti-oxidative enzymes. Also, we emphasized on an importance of using MCTS as an *in vitro* model for the study of oxidative stress in breast cancer.

Introduction

Breast cancer is the most generally diagnosed cancer in women worldwide including Thailand. The causes of breast cancer are still not clearly understood. Nowadays, an *in vitro* multicellular tumor spheroid (MCTS) cell culture system is widely accepted to recapitulate an *in vivo* condition more than monolayer cell culture as cancer cells in somatic growth lack oxygen, nutrients, and the ability of signal transduction from outside to inside of cells.[1] This ultimately influences cellular behaviors and gene expressions of cancer cells which cannot be observed from the monolayer cancer cell.[2] Free radicals are unstable molecules containing an unpaired electron in an atomic orbital that can donate or accept an electron from other molecules.[3] Major groups of free radicals are reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2). The ROS that is intrinsically generated from metabolism processes or extrinsically obtained from environments result in oxidative stress, cellular apoptosis, abnormal cell division, and cell senescence. Cells that are under the oxidative stress have an imbalance between a level of free radicals and anti-oxidative enzymes. Consequently, biomolecules including DNA, protein and cell membrane are damaged and not fully functioned. Furthermore, the damaged DNA leads to a genome instability leading to cancer. The ROS can damage double-stranded DNA molecules causing a mutation in tumor suppresser genes or increasing expression of proto-oncogenes.[4] In addition, a chronic oxidative stress

confers apoptosis resistance in breast cancer cells (MCF-7) due to the mutation of the p53 tumor suppresser protein and the up-regulation of anti-oxidative enzymes.[5] As a result, the cells are able to bypass program cell death or apoptosis.[6] Typically, the intracellular antioxidants are low molecular weight proteins such as glutathione, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).[7-9] Thus, the aim of this study was to investigate the contribution of MCTS to the level of oxidative stress in breast cancer (MCF-7) cells.

Research Methodology

Cell line and cell culture

The breast cancer, MCF-7 (HTB22) were obtained from American Tissue Culture ATCC Collection. MCF-7 cells were cultured in Minimum Essential Media (MEM) (Gibco, England) supplemented with 10 % fetal bovine serum (FBS) (Hyclone, USA) and 1 % penicillin-streptomycin. The cells were passaged at 80 % confluency. Media was changed every two days.

Preparation of multicellular tumor spheroid (MCTS) cell culture of breast cancer

MCTS were formed on a layer of Matrigel (Corning, USA). Briefly, Matrigel were mixed with serum free media at 1:1 ratio, placed at the bottom of a 96- or 24-well plates, and allowed to solidify at 37 °C for 1h. The cells in serum free media were seeded on the top of Matrigel and incubated at 37 °C for 1h and then 4% Matrigel in complete media was added on the top of cells.

Proliferation study of the cells

10,000 cells were seeded into a 96-well plate and incubated at 37 °C, 5% CO_2. The cells were then stained with 0.4% of trypan blue (sigma, Germany) and counted for viable cells every 24 hours until the cells growth reaches stationary phase. Then, the total cell numbers from each day were plotted against time to generate growth curve and doubling times were calculated.

Investigation of the level of cellular ROS and cell viability

The level of ROS was evaluated by using 6-carboxy-2', 7'-dichlorodihydrofluorescein (carboxy-H2DCFDA) dyes (Thermo, USA). 15,000 (monolayer) and 20,000 (MCTS) cells were seeded and allowed to grow overnight and 3 days, respectively. The cells were then stained with 10 µM carboxy-H2DCFDA, and incubated for 2 hours. Different concentrations of H_2O_2 were added ranging from 0 – 1.5 mM, followed by overnight incubation. The fluorescence intensity representing level of ROS was measured by micro plate reader at Ex/Em: ~492–495/517–527 nm. Then, the 0.5 mg/mL MTT solution were added into the cells and incubated at 37 °C, 5% CO_2 for 3 hr. The absorbance was measured by micro plate reader at 570 and 630 nm.

The effect of oxidative stress on breast cancer multicellular tumor spheroids

Apoptosis assay

The process of a program cell death or apoptosis was reported by using flow cytometer. The cells were seeded into 24 well plate (100,000 cells/well for monolayer and 70,000 cells/well for MCTS) and allowed to grow overnight for monolayer and 3 days for MCTS. The cells were treated with 500 µM H_2O_2 for 2 and 24 hours. The cells were then washed, trypsinized, and, resuspended with complete media. Next, 100 µl of cell suspension sample was incubated with 100 µl of Muse™ Annexin V & Dead Cell Reagent (Merck, Germany) for 20 minutes in the dark. The samples were analyzed by using Muse™ Cell Analyzer.

Determination of gene expression level

Total RNAs were extracted from cell pellets by using RNA E.Z.N.A total RNA kit (OMEGA, USA). Then, the RNA was reverse transcribed to cDNA by reverse transcriptase and the gene expression levels of glutathione synthetase (GSS), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase were determined by using a ddCT method.

Determination of gene expression level

Total proteins were extracted, quantified, and run on PAGE gel. Then, the proteins were transferred to the nitrocellulose membrane. Specific primary, followed by secondary antibodies to oxidative stress-related proteins, GPx1 and SOD2 were incubated on to the membrane. An enhanced chemi-luminescence method was used to develop the signals.

Results and Discussion

The MCF-7 MCTS successfully formed densely packed and round spheroids on Matrigel which was similar to the MCF-7 MCTS grew in agarose hydrogels previously reported[10] (Figure 1B). Also, the MCTS enlarged their size and cell numbers over the culture period. The size of MCTS was approximately double after four days of culture (data not shown). The proliferation of MCF-7 cells in MCTS demonstrated a slower growth rate than monolayer cells (Figure 1A) with doubling times of 57 and 46 hours, respectively (Figure 1C). This slower growth of MCTS was also observed in other types of cancer.[11]

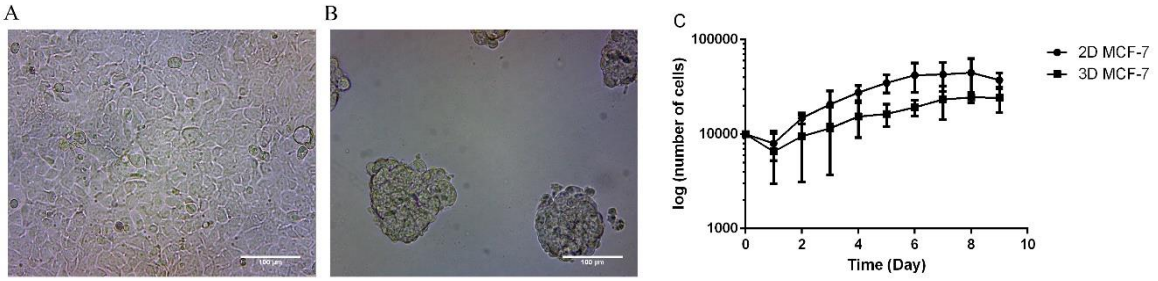

Figure 1. The morphology of MCF-7 monolayer cells (A) and multicellular tumor spheroid cells (B). The cell proliferation curves of MCF-7 monolayer cells (●) and MCTS cells (■) (C).

The effect of oxidative stress induced by H_2O_2 on cell viability of MCF-7 MCTS was further investigated by MTT assay (Figure 2A). At low level of oxidative stress (H_2O_2 from 0 -100 µM), there was no significant changes in the cell viability of both cells in MCTS and monolayer. However, at higher level of oxidative stress (H_2O_2 from 250 -1250 µM), the MCF-7 cells in MCTS exhibited a significant higher oxidative stress resistance than monolayer. This result confirmed the role of MCTS on diminishing a sensitivity to oxidative stress that was also observed in other study.[13] In addition, the level of ROS was examined by DCFDA fluorescence dyes. The level of ROS in MCF-7 monolayer cells were significantly increased at the concentration of H_2O_2 higher than 250 µM while no significant increase in ROS at the concentration of H_2O_2 up to 100 µM. The results were consistent with previous research that the level of ROS of the monolayer cells caused by H_2O_2 were increased in a dose-dependent manner.[12] In MCF-7 MCTS, the cells showed no statistically significant changes in ROS levels over the concentration of H_2O_2 from 0 to 1250 µM. This correlated with the high cell viability reported in figure 2A. It is possibly that the stress environment, H_2O_2 in this case, cannot reach the inner cells of MCTS in which the cells were shielded from harmful condition. Thus, the increase in the oxidative stress by H_2O_2 induction directly affected the MCF-7 cells in the monolayer, not in MCTS culture systems.

Next, the levels of apoptosis caused by the oxidative stress were determined. We found that the MCTS culture system resulted in a significant reduce in cell viability compared with the monolayer culture system (Figure 3A). This might due to a lack of essentials for viability such as nutrients and oxygen in the inner core of the MCTS[1] resulting in a previously reported centered luminal spaces[10]. However, the level of apoptosis, MCF-7 cells in the MCTS and monolayer and culture systems did not show any significant differences. As a result, the decrease in cell viability of MCTS was not mediated through the apoptotic pathway. Then, the oxidative stress was induced by treating the cells with 500 µM H_2O_2 for either 2 or 24 hours. We observed a significantly lower in the apoptotic degree on the MCF-7 cells in MCTS at 24 hr incubation (Figure 3B). Notably, the apoptotic rates of the MCF-7 cells in MCTS were indifferent whether the oxidative stress was induced correlating the high cell viability and low ROS generation in figure 2. Hence, the apoptotic results also confirmed that the cells in MCTS were significantly less sensitive to oxidative stress and apoptotic induction than that in monolayer.

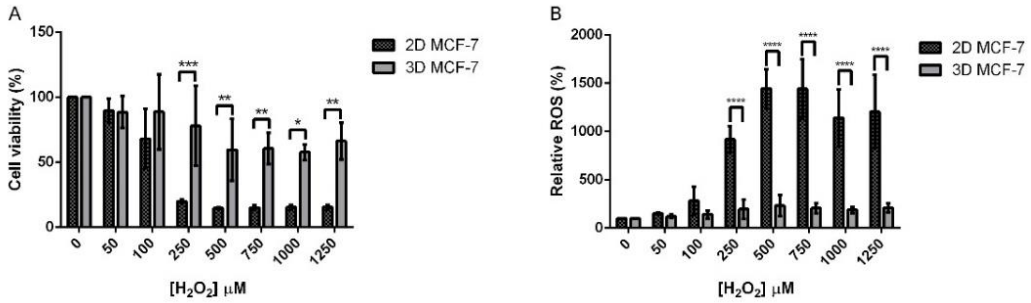

Figure 2. The viability of MCF-7 monolayer and MCTS cells in oxidative stress conditions (A). The level of ROS in MCF-7 monolayer and MCTS cells in oxidative stress conditions (B). (n = 3, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

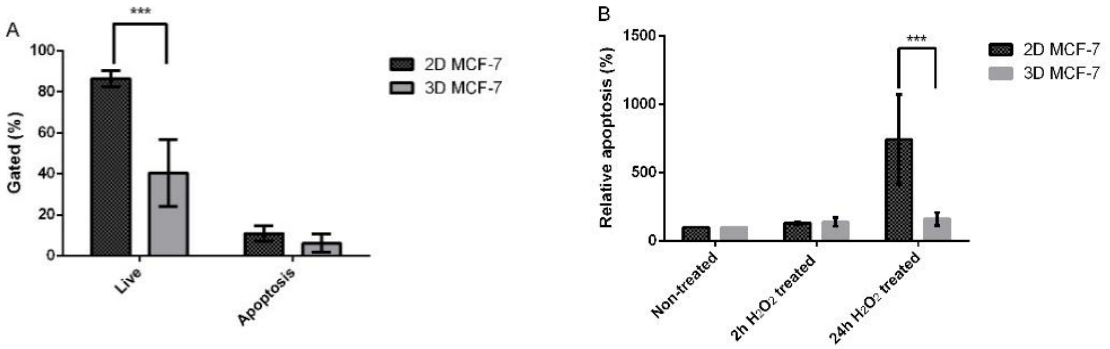

Figure 3. The level of apoptosis in the MCF-7 monolayer and MCTS cells in the absence of oxidative stress, normal condition (A) and an induction of oxidative stress (B). (*** p < 0.001).

To further explore mechanisms that are responsible for the oxidative stress resistance in MCF-7 MCTS, expressions of genes (Figure 4A and 4B) and proteins (Figure 4C) involved in the oxidative stress were determined. In non-oxidative stress condition, the MCF-7 cells in MCTS showed upregulations in antioxidant-related genes including GPx1, GPx4, SOD2, CAT and GSS ranging from 4- to 8-fold increases suggesting more antioxidation activity (Figure 4A). This could possibly explain the decrease in the sensitivity to the oxidative stress previously discussed. In the oxidative stress condition, the gene expressions of the antioxidant-related genes were upregulated in the monolayer cells; while, no significant change in the gene expression was observed in MCTS. This confirmed the sensitivity and resistance to the

oxidative stress condition in the monolayer and MCTS cells, respectively (Figure 4B). For example, the gene expression of SOD2 in the monolayer cells was significantly higher than that in the MCTS. As for other genes, the gene expressions were likely to also be upregulated in the monolayer cells but showed no statistical differences. Similar to the gene expression, the GPx1 and SOD2 proteins were also found to be upregulated in both monolayer and MCTS after the cells were induced by H_2O_2 (Figure 4C). Thus, in non-oxidative stress condition, the enzymes that involve in oxidative stress were upregulated in MCTS cells. However, such upregulation of gene expressions was not significantly observed after the oxidative stress induction. Unlike the MCTS, the monolayer cells responded to the oxidative stress by increasing in all antioxidant gene expressions.

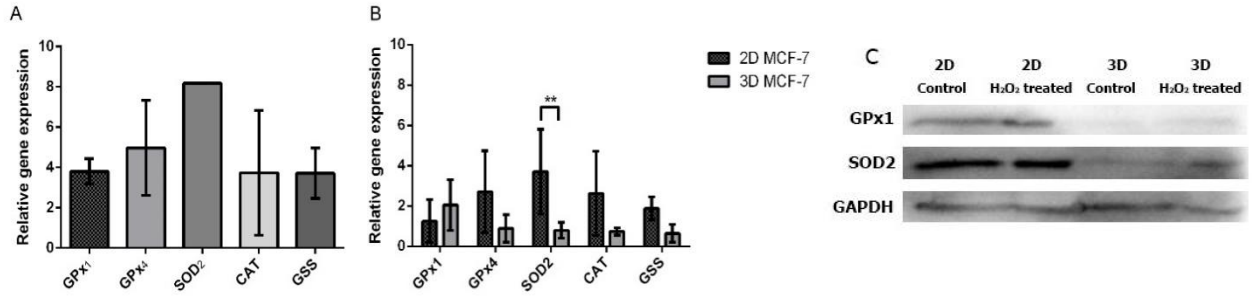

Figure 4. The overexpression of antioxidant-related genes in MCTS over monolayer (A) and the relative gene expression in oxidative stress condition of MCF-7 monolayer and MCTS (B). Protein expression levels of GPx1 and SOD2 in non-oxidative and oxidative stress induction (C). (**$p < 0.01$)

Conclusion

The MCF-7 cells in MCTS and monolayer were different in responding to the oxidative stress condition. The MCTS culture system itself caused the MCF-7 cells to be more resistant to the oxidative stress by lowering the degree of apoptosis and increasing the cell viability which was likely mediated through the upregulation of the antioxidant genes and proteins. Moreover, this study emphasizes on the importance of using MCTS as an *in vitro* oxidative stress-resistant model recapitulating in vivo environments for predicting tumor responses in breast cancer.

References

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