Cancer cell lines involving cancer stem cell populations respond to oxidative stress

Açelya Yilmazer\textsuperscript{a,b,*}

\textsuperscript{a} Biomedical Engineering Department, Engineering Faculty, Ankara University, Tandogan, Ankara, Turkey
\textsuperscript{b} Stem Cell Institute, Ankara University, Balgat, Ankara, Turkey

\section*{A B S T R A C T}
Cancer cells may be more prone to the accumulation of reactive oxygen species (ROS) than normal cells; therefore increased oxidative stress can specifically kill cancer cells including cancer stem cells (CSCs). In order to generate oxidative stress in various cancer cell lines including A549, G361 and MCF-7, cultured cells were exposed to H\textsubscript{2}O\textsubscript{2}. Incubation of cancer cells with H\textsubscript{2}O\textsubscript{2} results in concentration-dependent cell death in A549 and G361-7 cells, whereas MCF-7 cells showed higher sensitivity even at a lower H\textsubscript{2}O\textsubscript{2} concentration. H\textsubscript{2}O\textsubscript{2} treatment decreased the number of cells in G2/M phase and increased the number of apoptotic cells. Both CD24 negative/CD44 positive cells and CD146 positive cells were found to be present in all tested cancer cell lines, indicating that CSC populations may play role in the cellular response to oxidative stress. This study showed that inducing oxidative stress through ROS can offer a promising approach for anti-cancer therapy.

\section*{1. Introduction}
Oxidative stress occurs when there is an imbalance between generation of reactive oxygen species (ROS) and inadequate antioxidant defense systems. Oxidative stress can cause cell damage either directly or through altering signaling pathways. Oxidative stress is a consolidating mechanism of injury in many types of diseased and pathological conditions \cite{1}. During cancer therapy, it is well known that some chemotherapeutic agents and radiation therapy may result in the accumulation of reactive oxygen species (ROS) in patients. Free radicals, particularly ROS, have been reported to be common mediators for apoptosis. Recent studies have shown that the severity of the oxidative damage can determine the mode of cell death \cite{2}.

Low to moderate levels of ROS are indispensable to normal cellular proliferation, differentiation, and survival \cite{3}. Cancer cells produce more ROS than normal cells, therefore ROS is closely related to tumorigenesis \cite{4}. Although cancer cells regulated ROS levels by powerful antioxidant defense mechanisms, it is observed to remain higher than that in normal cells. Cancer cells may be more prone to the accumulation of ROS than normal cells; consequently, it has been suggested that increased oxidative stress by exogenous ROS generation therapy has an effect on selectively killing cancer cells without affecting normal cells \cite{3}. A recent study by Thanee et al. suggested that the redox status regulation of cancer cells depends on the expression of CD44, a cancer stem cell marker, to contribute the cysteine-glutamate transporter function and is a link to the poor prognosis of patients. Therefore they suggested that an inhibitor designed against this transporter could inhibit cell growth and activate cell death \cite{5}.

In 1994, Lapidot et al. discovered leukemia stem cells and since then researchers have shed light on the study of CSCs \cite{6}. CSCs have capacity to self-renew and differentiate into heterogeneous non-tumorigenic cancer cell types in accordance with their microenvironment and the status of the whole body \cite{7,8}. Although CSCs form a small proportion of the tumor, they play an important role to tumor formation and development. Furthermore, they have been reported to be closely related to chemotherapeutic and radiation resistance and disease recurrence \cite{7,9-13}. Therefore, CSCs are considered as important targets for cancer therapy \cite{14,15}. The study of intracellular ROS in CSCs remains an attractive field for research. Little is known about the biological effects and therapeutic implications of ROS in CSC subpopulations \cite{3}.

According to the Warburg effect, unlike normal cells, cancer cells gain energy primarily from glycolysis even under aerobic conditions, leading to increased ROS levels \cite{16}. In cancer cells, ROS levels are counteracted by elevated antioxidant defense

---

\textit{Abbreviations:} CSC, cancer stem cell; ROS, reactive oxygen species; qRT-PCR, quantitative reverse transcription polymerase chain reaction

* Corresponding author at: Biomedical Engineering Department, Engineering Faculty, Ankara University, Tandogan, Ankara, Turkey.

E-mail address: ayilmazer@ankara.edu.tr.

https://doi.org/10.1016/j.btre.2017.11.004
Received 28 September 2017; Received in revised form 14 November 2017; Accepted 15 November 2017
Available online 23 November 2017
2215-017X/ © 2017 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
mechanisms; however they are still higher than those observed in normal cells. Therefore, cancer cells may be more sensitive than normal cells to the accumulation of ROS, which offers an interesting therapeutic implication [3,17]. Hence, directly inducing oxidative stress by increased ROS to reach a level that is incompatible with cell viability and targeting the enhanced antioxidant mechanisms can selectively kill cancer cells, without affecting normal cells [3,18,19]. Despite the low level of ROS in CSCs and the active ROS detoxifying systems, elevating the concentration of ROS could still provide a potential treatment technology.

In this study, the main aim was to determine the anti-cancer activity of oxidative stress induced by the treatment of H$_2$O$_2$ on different human cancer cell lines including melanoma, lung and breast cancer. It was hypothesized that when cancer cell lines contain a CSC subpopulation, the anti-cancer effect of H$_2$O$_2$ will be much more pronounced and hence oxidative stress can be used as a potential treatment technology for these cancer types.

2. Materials & methods

2.1. Cell culture

Human lung carcinoma cell line A549, human melanoma cell line G361 and human breast cancer cell line MCF-7 were purchased from ATCC (Rockville, MD, USA). Human primary melanocytes were obtained from Lonza (UK). A549 cells, G361 cells and melanocytes were maintained in Dulbecco’s Modified Eagle Medium (DMEM), MCF-7 cells maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, 1% l-glutamine and 1% non-essential amino acids at 37 °C in 5% CO$_2$.

2.2. H$_2$O$_2$ treatment

In order to induce oxidative stress, cells cultures in 6 well- or 96 well- plates were incubated with H$_2$O$_2$ at the following concentrations 0.1, 0.5 and 1 mM; diluted in culturing media. H$_2$O$_2$ treatment was performed for 24 h.

![Fig. 1. H$_2$O$_2$ induced oxidative stress. A549 human lung carcinoma, G361 melanoma cells and MCF-7 human breast cancer cells were incubated with H$_2$O$_2$ at (0.5 mM); diluted in culturing media. Following 2h, cells were stained with CellROX Deep Red and imaged under fluorescence microscopy. Representative images were obtained at 10× magnification.](image-url)
2.3. Microscopy imaging for oxidative stress

Cells were treated with H₂O₂ and after 2 h, cells were washed with PBS and incubated with CellROX Deep Red (Life Technologies, UK) imaging in order to further visualize oxidative stress within the cells. After 15 min, cells were counterstained with DAPI and imaged under the fluorescence microscope (Evos Fl, Life Technologies).

2.4. Cell viability tests

The colorimetric MTT assay was used to measure cell viability. Yellow 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced by mitochondrial reductase in metabolically active cells to produce purple formazan. Stock MTT solution was prepared at 5 mg/ml in PBS, sterile filtered and stored in the dark. MTT was diluted 1:5 (v/v) into cell culture medium and added to the cells cultured in 96 well-plates. Cells were incubated for 2 h at 37 °C in 5% CO₂. MTT solution was then removed and DMSO was added to each well to solubilise formazan. Plates were read at 570 nm on a Clariostar Omega microplate reader (BMG Labtech, Germany).

2.5. Apoptosis assay

Treated or untreated cells were washed with PBS and stained with Annexin V-FITC/PI kit (Life Technologies, UK) according to the manufacturer’s protocol. In short, cells were incubated with Annexin V and PI for 15 min in the dark and then analyzed at a flow cytometer using BD Accuri Plus software (BD).

2.6. Cell cycle and flow cytometry analysis

Treated or untreated cells were collected and fixed in 70% ethanol for 1 h at 4 °C. For cell cycle analysis analysis, fixed cells were collected by centrifugation, washed with ice cold 1X PBS followed by staining with PI cell cycle reagent (BD). Cell cycle analysis was carried out by counting 5000 events and acquired data was analyzed using BD Accuri Plus software (BD).

For flow cytometry analysis of CSC markers, cells were collected by centrifugation, washed with ice cold 1X PBS followed by staining for CD24-PE (BD), CD44-APC (BD), CD-117-PerCp (BD), CD146-FITC (BD). Cells were analyzed in BD Accuri Plus Flow Cytometer (BD). For 20,000 events, percentages of positive populations were determined by using BD Accuri Plus software (BD).

2.7. Statistical analysis

Experiments were performed in triplicates on at least three independent occasions. Statistical analysis was performed by analysis of variance and Tukey’s pairwise comparison using SPSS software, version 16.0.

3. Results

In order to generate oxidative stress on cancer cell lines, cultured cells were exposed to different concentrations of H₂O₂ (0.1, 0.5 and 1 mM). Firstly, 2 h following H₂O₂ treatment, A549 human lung carcinoma, G361 melanoma cells and MCF-7 human breast cancer cells were stained with CellROX Deep Red in order to confirm the induction of oxidative stress. As seen in Fig. 1, red signal from the cytoplasm can be detected when cells were exposed to H₂O₂ which was concentration-dependent. After confirming that H₂O₂ at these concentrations can induce oxidative stress, cell viability was determined after 24 h of H₂O₂ treatment in different cell lines. According to the MTT assay (Fig. 2), A549 cells showed stepwise decrease in cell viability from 85% to 50% as H₂O₂ concentration increased from 0.1 mM to 1 mM. G361 cells also showed a similar trend, whereas MCF-7 cells showed around 40% viability at all H₂O₂ treatments. As a control experiment, human primary melanocytes were also incubated with varying concentrations of H₂O₂. According to Fig. S1, these cells also showed response to H₂O₂ treatment.

In order to assess apoptotic activity, treated or untreated cells were analyzed in an Annexin V-PI assay (Fig. 3). In A549 cells, 1 mM H₂O₂ treatment increased the apoptotic and death cell population to 35.1%, and necrotic cells to 6.3%. In G361 cells, H₂O₂ treatment resulted in 4.6% necrotic cells, 11.4% early-apoptotic cells and 47.7% apoptotic and death cells. Finally in MCF-7 cells, 1 mM H₂O₂ significantly increased both apoptotic and death cells and necrotic cells.

Cell cycle analyses were performed in order to further understand the effect of H₂O₂ and determine whether cells are directed to apoptosis. As represented in Fig. 4, H₂O₂ (0.1 mM) treatment decreased the number of cells in G2/M phase for all cell lines. Furthermore, there was an increase in the number of A549 and MCF-7 cells in the sub G1 phase, confirming that there are more cells...
Fig. 3. Apoptotic activity following H$_2$O$_2$ treatment. A549, G361 ad MCF-7 cells were incubated with H$_2$O$_2$ (1 mM) for 6 h. AnnexinV-FITC and PI staining were carried out. Cells were analyzed using BD Accuri Plus flow cytometer.
directed towards apoptosis.

Later, cell lines were analyzed for the expression of CSC markers by flow cytometry. Cancer cell lines were double stained for the expression of CD24 and CD44 markers, since CD24-/CD44+ cells have been reported to be cancer stem cells as discussed in previous studies [20]. Fig. 5 shows that A549 cells showed 5.4% CD24-/CD44+ cells, whereas G361 cells were 99.4% CD24-/CD44+. On the other hand, MCF-7 cells showed only 1.6% CD24-/CD44+ cells. In order to determine the expression of other CSC populations, cells were also stained for CD146 and CD117 markers. As shown in Fig. 5, none of the cell lines expressed CD117. CD146 showed 22.2%, 96.2% and 36.6% positivity in A549, G361 and MCF-7 cell lines, respectively.

4. Discussion

In this study, we investigated the effect of H$_2$O$_2$ treatment on melanoma, breast and lung cancer cell lines. According to the literature, A549 cells have been reported to be less aggressive than other lung carcinoma cell lines, for example H460 has been shown to nearly double the rate of A549 cells and they are highly aggressive due to their increased vascularity and ability to metastasize [21]. MCF-7 cells show low proliferation rates, oestrogen responsive and often chemotherapy responsive therefore they are not aggressive as other breast cancer cell lines [22]. Finally, G361 cell line is a malignant melanoma which has been shown to be one of the most aggressive phenotypes, with severe metastatic potential [23]. Incubation of these cancer cells having various aggressiveness with H$_2$O$_2$ results in oxidative stress as evidenced by fluorescence microscopy. Furthermore, H$_2$O$_2$ treatment induced concentration-dependent cell death in A549 and G361-7 cells, whereas MCF-7 cells showed high sensitivity even at a lower H$_2$O$_2$ concentration. This was confirmed both by MTT cell viability assay and Annexin V/PI staining. When cell cycle analyses were performed, H$_2$O$_2$ treatment decreased the number of cells in G2/M phase in general. H$_2$O$_2$ treated cells can increase the number of cell cycle arrest and it may result in cell death via apoptosis.

CD24-/CD44+ cells have been extensively studied in the CSC field and have been identified in various cancer types including breast, skin and lung cancer [24]. CD146 is a marker for mesenchymal stem cells and has been shown to induce stem cell properties in melanoma and breast and colorectal cancer [25,26], therefore it is another good candidate as a CSC marker. Finally, we have selected CD117 which has been identified as a CSC marker for different solid tumors including lung, melanoma, ovarian and etc [27,28]. When we analyzed the expression of CSC markers by flow cytometry, we found that both CD24-/CD44+ cells and CD146 cells were present in all tested cancer cell lines, whereas none expressed CD117. This data suggested that presence of different CSC populations may involve when cancer cells generate response to oxidative stress. Further studies are needed to elucidate which CSC populations are more important and deterministic during this process.

In this study, H$_2$O$_2$ treatment was used to mimic an oxidative stress environment on cancer cells bearing CSCs. We believe direct H$_2$O$_2$ treatment will not be suitable to be translated into clinical studies. Therefore this study is just an important proof-of-concept study that will support future experimentation focusing on different inducers of oxidative stress. One example could be photodynamic or photocatalytic activity based cancer therapy protocols. In these protocols, the agent or biomaterial to be tested can generate the formation of reactive oxygen species when exposed to light, which will result in the death of cancer cells including CSCs.

As a result of modulating redox systems, metabolic reprogramming, and reduced mitochondrial DNA levels, CSCs maintain lower levels of intracellular ROS [29,30]. It has been reported that a low concentration of ROS support the stemness of CSCs and contribute to tumorigenesis and development [3]. Therefore, HIF stabilization induced by ROS in CSCs plays a critical role. However, the underlying mechanisms in these processes have not been studied in depth. There exist various mechanisms for the regulation of oxidative stress in CSCs, such as through CSC surface markers, and these help maintain the ROS at a favorable level [1,15,17,19]. Consequently, inducing oxidative stress can offer a promising approach for the preferential killing of cancer cells, including CSCs.
Conflict of interests statement

The Author declares that there is no conflict of interest.

Funding

AY acknowledge support by the Scientific and Technological Research Council of Turkey (TUBITAK, grant number 113S897).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.btre.2017.11.004.

References

[1] G.W. Dryden, et al., Clinical implications of oxidative stress and antioxidant therapy, Curr. Gastroenterol. Rep. 7 (4) (2005) 308–316.
[2] T. Ozben, Oxidative stress and apoptosis: impact on cancer therapy, J. Pharm. Sci. 96 (9) (2007) 2181–2196.
[3] J. Liu, Z. Wang, Increased oxidative stress as a selective anticancer therapy, Oxid. Med. Cell. Longev. 2015 (2015) p12.
[4] T.P. Szatrowski, C.F. Nathan, Production of large amounts of hydrogen peroxide by human tumor cells, Cancer Res. 51 (3) (1991) 794–798.
[5] M. Thanee, et al., CD44 variant-dependent redox status regulation in liver fluke-associated cholangiocarcinoma: a target for cholangiocarcinoma treatment, Cancer Sci. 107 (7) (2016) 991–1000.
[6] T. Lapidot, et al., A cell initiating human acute myeloid leukaemia after transplantation into SCID mice, Nature 367 (6464) (1994) 645–648.
[7] M.F. Clarke, et al., Cancer stem cells—perspectives on current status and future direction: ACR workshop on cancer stem cells, Cancer Res. 66 (19) (2006) 9339–9344.
[8] L.V. Nguyen, et al., Cancer stem cells: an evolving concept, Nat. Rev. Cancer 12 (2) (2012) 133–143.
[9] M.F. Clarke, M. Fuller, Stem cells and cancer two faces of eve, Cell 124 (6) (2006) 1111–1115.
[10] T. Maraldi, et al., Reactive oxygen species in stem cells, Oxid. Med. Cell. Longev. 2015 (2015) 159080.