Endogenous and Induced Oxidative Stress in Multi-cellular Tumour Spheroids: Implications for Improving Tumour Therapy

Divya Khaitan and B S Dwarakanath*
Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Brig. S K Mazumdar Marg, Delhi 110054, India

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The endogenous oxidative stress in tumours is determined by the status of mitochondrial, metabolic, oxygen (hypoxia) and inherent enzymatic as well as non-enzymatic antioxidant defense systems, which influence tumour growth and respond to anticancer therapeutics. Induced oxidative stress is one of the important determinants of the outcome of treatment with certain chemotherapeutic drugs and ionizing radiation. The mild to moderate levels of reactive oxygen species (ROS) have often been found to trigger prosurvival responses, thereby contributing to the resistance against therapy. The higher levels of ROS stimulate multiple death pathways viz. typical and atypical apoptosis, necrosis etc, thereby enhancing the therapeutic efficiency. Therefore, approaches employing therapeutic agents that generate ROS efficiently in the tumour cells and enhance the antioxidant defense system in the normal cells could significantly enhance the therapeutic gain. Multi-cellular tumour spheroids (MCTS) offer an excellent in vitro system that mimics endogenous oxidative stress often observed in tumours, arising due to a number of factors (gradients of oxygen and nutrients, altered intercellular interaction and tumour necrosis factor), besides antioxidant defense systems similar to tumours in vivo. More importantly, MCTS resemble tumours in vivo with reference to the induced oxidative stress related responses, particularly following combinations of certain chemotherapeutic drugs and metabolic inhibitors and differs significantly from the responses in monolayer cultures. Therefore, MCTS appear to be excellent in vitro models, ideally suited for developing novel therapies that are based on the generation of oxidative stress in tumours. The present review provides a modest account on the utility of MCTS in understanding the role of oxidative stress in treatment-induced responses of tumours for designing therapies and therapeutics.

Keywords: Multi-cellular spheroids, Oxidative stress, Radiotherapy, Chemotherapy

Introduction

Mammalian cells continuously produce reactive oxygen species (ROS) in the mitochondria as a consequence of the use of oxygen in aerobic respiration\(^1\). Superoxide is generated within the mitochondria and is sequentially reduced to hydrogen peroxide and hydroxyl radicals. Neutrophils and macrophages produce ROS via a plasma membrane bound nicotinamide adenine nucleotide phosphate, reduced form (NADPH)-oxidase. The cytochrome P450 monooxygenase system of the hepatic and endoplasmic reticulum (microsomes) also generates a substantial amount of ROS in the process of metabolizing a chemically diverse group of compounds like drugs and other environmental substances. The plasma and nuclear membranes are less active site of ROS production, and enzyme systems, such as the xanthine-xanthine oxidase system, but can also generate ROS\(^2,3\).

Cells have a highly developed and regulated antioxidant defense system to maintain appropriate intracellular ROS levels and prevent oxidative damage. This system includes antioxidant enzymes such as (superoxide dismutase (SOD), catalase and various peroxidases and non-enzymatic systems (GSH, thioredoxin, uric acid, vitamins, coenzQ) that effectively remove ROS. Under normal conditions, antioxidant mechanisms scavenge ROS and protect the organism from the damaging effects of ROS. However, under conditions of excessive oxidative stress, cellular antioxidant mechanisms may be unable to prevent the adverse impact of ROS on critical
MAPK pathways drive cell cycle progression without the activation of oxidase. In production by activating Rac1 and the NADPH-oxidase. In the case in the tumour cells may exert differential effects of oxidative damage on cancer cells, whose binding sites located on the genes are directly involved in the pathogenesis of cancer. Tumour cell oxidative stress also promotes secretion of the matrix metalloproteinase-1 (MMP-1), a collagenase that aids vessel growth within the tumour microenvironment. Levels of the hypoxia inducible factor-1 (HIF-1) may be increased by oxygen radicals, implying that oxidatively stressed carcinoma cells might show increased HIF-1 induction during hypoxia and, therefore, produce more vascular endothelial growth factor (VEGF). Cells exposed to sub-lethal oxidative stress exhibit decreased attachment to immobilized laminin and fibronectin, thereby increasing the probability of metastasis and invasion.

Persistent oxidative stress in tumours may inactivate some tumour suppressor genes, or further increase expression of proto-oncogenes. Genetic instability due to persistent oxidative stress can lead to an increased mutation rate in the tumour and accelerate its progression. Tumour cells produce more ROS than normal cells, mainly due to alterations in metabolic pathways and inadequate tumour vascular network.

Tumour rapidly outgrows its blood supply, leading to glucose deprivation and hypoxia. Glucose deprivation rapidly induces cellular oxidative stress by depleting intracellular pyruvate, preventing the decomposition of endogenous oxygen radicals. Tumour growth is supported by stimulating blood vessel development (angiogenesis). Blood flow within these new vessels is often disordered, causing periods of hypoxia, followed by reperfusion, which causes the generation of ROS.

Increased intracellular concentration of ROS, as is the case in the tumour cells may exert differential effects on the regulation of genes, which are dependent on their concentration. Sub-lethal oxidative stress in the tumour cells is shown to promote cell proliferation in vitro by the activation of mitogen-activated protein kinases (MAPKs), extracellular signal related protein kinase, c-Jun amino-terminal kinase/stress-activated protein kinase, p38, and extracellular signal related protein kinase-2. In addition, ROS may trigger mitosis via MAPK independent mechanisms. Oncogenic Ras causes ROS production by activating Ras1 and the NADPH-oxidase. In Ras-transformed human fibroblasts, ROS drive cell cycle progression without the activation of MAPK pathways.

Certain transcription factors NF-κB and AP-1 are known to be regulated by the oxidative status of cells, whose binding sites located on the genes are inactivated in the pathogenesis of cancer. Tumour cell oxidative stress also promotes secretion of the matrix metalloproteinase-1 (MMP-1), a collagenase that aids vessel growth within the tumour microenvironment. Levels of the hypoxia inducible factor-1 (HIF-1) may be increased by oxygen radicals, implying that oxidatively stressed carcinoma cells might show increased HIF-1 induction during hypoxia and, therefore, produce more vascular endothelial growth factor (VEGF). Cells exposed to sub-lethal oxidative stress exhibit decreased attachment to immobilized laminin and fibronectin, thereby increasing the probability of metastasis and invasion.

Many of the antitumour therapies like radiotherapy, photodynamic therapy and chemotherapy using certain anticancer drugs generate a variety of highly reactive radicals (chemical and biomolecular) that is partly responsible for the toxic effects. Their antitumour activity is to a certain extent dependent on the induction of cell death by way of apoptosis in response to oxidative stress and oxygen radical-induced DNA damage. However, oxygen radicals have also been shown to increase drug resistance by increasing expression of P-glycoprotein (Pgp), the multi-drug-resistance efflux pump.

Various in vitro models have been used to study the effects of oxidative damage on cancer cells, and provide information about the biochemistry of cancer cells. However, in vivo tumour responses to therapeutic agents often differ from the responses of monolayer cultures, as these cultures cannot mimic realistically the complex environmental conditions in the tumours. This includes inadequate and heterogeneous vascular supply, leading to a heterogeneous cell population and signaling processes that influence the tumour response, thereby limiting therapeutic efficacy. It has long been recognized that the cell based models used in basic cellular research need to recapitulate both the 3-dimensional (3-D) organization and multi-cellular complexity of the tumours and also the normal tissue to translate findings from these studies into clinical applications.

3-D cultures have been utilized in biomedical research to gain deeper insight into the mechanisms of organogenesis and expression of malignancy. The MCTS model is one of the best-described 3-D in vitro tumour model systems, which depict many of the cellular processes. ROS can interact with cellular macromolecules, including DNA, protein and lipids, and interfere with vital cellular functions. Mutations caused by ROS can result in malignant transformation and the development of cancer.

Although it is rather difficult to unequivocally implicate the involvement of oxidative DNA damage in cancer etiology, evidences suggest that oxidative DNA base modifications are the main source of mutations that initiate carcinogenesis. Continuous oxidative stress, DNA damage and changes in signal transduction lead to cell mutation a expression of oncprotein on the cell surface. Further, the oxidative bursts releasing ROS during inflammation have been shown to facilitate tumour promotion.

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characteristics of tumour tissue and allow reproducible experiments. It shows strong similarities in morphology and mimics functional characteristics with in vivo solid tumours. This system was adapted in cancer research during early 1970s by Sutherland and colleagues and is widely used in many laboratories throughout the world today.

Spheroid models are sphere-shaped cell colonies that permit growth and functional studies of diverse normal and malignant tissues. Spheroid growth mimics the growth of naturally occurring human tumours, as they contain an extensive extracellular matrix and network of cell-to-cell and cell-to-matrix interactions that differ in the relative amount and assembly from the corresponding monolayer cultures. They are characterized by a high cell-density, close packing and 3-D tumour-like structure, which leads to severe diffusion limitations for molecules as small as glucose and oxygen, leading to a gradients in different zones of spheroids as observed in solid tumours. In addition, spheroids also generate ROS endogenously, similar to solid tumours that are involved in signaling cascades related to the regulation of tumour cell growth. Spheroids, therefore, serve as a good model bridging the condition between monolayers and in vivo tumours to gain further insight into the mechanisms involved in the response to various therapies that are mediated by oxidative stress.

**P-gp, HIF-1α expression and intracellular redox state**

The chemotherapy of cancer is often limited by either intrinsic or acquired expression of multi-drug resistance (MDR) transporters, including Pgp. The Pgp-mediated MDR phenotype is generally characterized by a decreased cellular drug accumulation, due to an enhanced drug efflux related to the over expression of drug transporters. The expression of Pgp is suggested to be redox-regulated, as down-regulation of Pgp and reversal of the MDR phenotype are achieved by the treatment of tumour cells with TNFα, known to utilize low levels of ROS in its signal transduction pathway. On the other hand, high levels of ROS, resulting in severe cellular oxidative stress have been reported to increase the expression of the MRP-1 and MDR-1b genes. The endogenously generated ROS has been demonstrated to regulate the expression of intrinsic Pgp in MCTS, because small, exponentially growing spheroids which robustly generate ROS display reduced Pgp levels, whereas up-regulation of Pgp in large, non-necrotic tumour spheroids correlates with decreased ROS levels.

The hypoxia in avascular micrometastases and avascular region of solid tumours are associated with increased resistance toward anticancer agents, although the molecular mechanisms underlying hypoxia-associated drug resistance are still unknown. ROS play a significant role in signaling pathways that regulate cell growth and differentiation. They mediate the proteolytic degradation of HIF-1α and down-regulate the intrinsic P-gp expression in multicellular prostate tumour spheroids, indicating that HIF-1 levels in the tissue are regulated by the intracellular redox state of the tissue. Incubation of spheroids with agents that raise intracellular ROS and stimulate cell proliferation are associated with reversal of MDR phenotype and down-regulation of Pgp (Fig. 1).

Pre-clinical models in vitro and in vivo have shown that hypoxia alters the malignant cell phenotype, selecting for p53 mutations, stimulating angiogenesis.

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![Fig. 1—Effects of pO2 gradients and ROS on the expression of HIF-1α and Pgp in different zones of spheroids](image.png)
and metastasis, besides markedly reducing the efficacy of radiotherapy and chemotherapy. Clinical studies have demonstrated that hypoxia confers a negative effect on local tumour control\cite{19,30} and disease-free overall survival. Therefore, knowledge of the association of P-gp-mediated MDR and hypoxia will help in fighting cancer by unconventional therapeutic approaches that alter the intra tumour oxygenation status.

**ROS and angiogenesis**

High levels of ROS (eg, the highly reactive hydroxyl radical) exert antiangiogenic effects\cite{30} and promote arteriosclerosis and endothelial cell death\cite{31,32}. On the other hand, low levels of ROS are involved in signal transduction cascades that regulate endothelial cell growth and migration\cite{33,34}. The nature of intercellular interactions and environmental conditions prevailing in monolayer cultures do not permit studies on this important aspect of tumour biology, which has been one of the main targets of anticancer therapies being developed currently. On the other hand, embryonic stem cells have been shown to differentiate into endothelial cell spheroids, which can form functional blood vessel-like structures. This *in vitro* model has been successfully used to screen various antiangiogenic agents for their efficiency\cite{35}. Changes in the intracellular redox state and matrix metallo-proteases (MMP) expression as well as angiogenesis studied in confrontation cultures of MCTS and embryoid bodies have shown an increase in the expression of MMP as compared to the tumour spheroids and embryoid bodies cultivated separately. This has been attributed to the elevated levels of ROS, as the effect is totally abolished in the presence of the free radical scavengers like vitamin E\cite{19,30}.

**Metabolic oxidative stress and cancer therapy**

The cells that undergo neoplastic transformation (cancer cells) often demonstrate altered metabolism when compared to normal cells. The most prominent changes are up-regulation of glucose and the loss of regulation between glycolysis and respiration\cite{6,37}. Further, mitochondria have been shown to be structurally abnormal in almost all malignant tumour cells and exhibit low level of antioxidant enzymes. Thus, tumour cells increase their glycolysis and pentose phosphate pathway as a compensatory antioxidant defence mechanism through the formation of pyruvate and NADPH for detoxifying cellular peroxide\cite{38}. Therefore, besides the energy production, glucose metabolism appears to be involved in defence against oxidative stress mediated by hydroperoxides as byproducts of oxidative metabolism, presumably via the formation of pyruvate and NADPH\cite{39}. In line with this suggestion, enhanced glucose concentration in the medium has been found to render CHO cells resistant to \(\text{H}_2\text{O}_2\)-induced cytotoxicity\cite{39}.

Many transformed cells exhibit more susceptibility toward glucose deprivation-induced cytotoxicity and oxidative stress, suggesting that there may be a fundamental defect in oxidative metabolism in cancer cells. Indeed, mere omission of glucose from the medium (glucose deprivation) induces cytotoxicity and oxidative stress in human tumour cells\cite{9,10,40}. Furthermore, transformed cells appear to be more susceptible to glucose deprivation-induced cytotoxicity and oxidative stress as compared to their normal matched pair cells\cite{9,10,40}. Moreover, glucose deprivation-induced cytotoxicity in transformed cell line (GM00637G) is shown to be dependent upon the metabolism of \(\text{O}_2\)\textsuperscript{\textsuperscript{2}}. Since most of \(\text{O}_2\) metabolism occurs in the mitochondria, this result is consistent with the speculation that a defect in transformed cell’s respiratory mechanism may lead to an increase in the ROS, responsible for glucose deprivation-induced cytotoxicity and oxidative stress. Therefore, a defect in oxidative metabolism of cancer cells can be used as a therapeutic advantage, to kill cancer cells sparing the normal tissues.

Studies using confocal microscopy with spheroids have shown that alterations in ATP level influence the generation of ROS\cite{41}. Spheroids generated from a human glioma cell line (BMG-1) do not show a significant difference in the ROS, as compared to monolayer cultures, although a decrease in the GSH levels is noted. A 2-3 fold increase in the glucose consumption and lactate production observed in MCTS suggest that the enhanced pyruvate could also function as an antioxidant. Further, TNF\(\alpha\) levels are also higher in MCTS, suggesting an increase in the ROS generation. Since the clonogenicity of MCTS cells are not significantly compromised as compared to the monolayer cultures, it appears that under unperturbed conditions, there is homeostasis between generation of oxidative stress and apoptotic defence and/or prosurvival factors. Further, the radiation-induced ROS in MCTS is significantly lower than the monolayer cultures that contribute to the radio-
resistance along with the enhanced glycolysis. However, the radiosensitization by the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) is reported to be significantly higher in MCTS, mainly due to the enhanced apoptotic cell death and correlates with the sustained elevation of ROS levels observed under these conditions. As tumour cells are generally associated with a disruption of intracellular oxidation/reduction reactions as well as glycolytic metabolism, results obtained using spheroids culture can be used for obtaining a near realistic estimate of the oxidative stress-related responses of tumours to the combined treatment of therapeutic agents, particularly metabolic modulators.

**Drug-induced oxidative stress**

Many antineoplastic drugs like chloroquin, quinine, mefloquine, primaquin, artemisinin, ciprofloxacin, etoposide, doxorubicin, etc act through the induction of oxidative stress. Recently, new therapeutic strategies that take advantage of increased ROS or inhibition of endogenous antioxidant defense, which produce a state of oxidative stress selectively in cancer cells have gained importance. Furthermore, intrinsic oxidative stress in the cancer cell is also suggested as a biochemical basis for therapeutic selectivity shown by 2-methoxyestradiol. Catalase, an important enzyme that catalyzes hydrogen peroxide, is inhibited by ceramide (generated during membrane damage induced by certain therapeutic agents), thereby increasing the oxidative stress leading to the induction of apoptosis. Multiple mechanisms contribute to the oxidative stress induced by many anti-cancer therapeutics that include generation of ROS, initiation of long range electron transfer (ET) reactions, metal chelation, DNA damage, and mitochondrial dysfunction.

Treatment with the DNA topoisomerase inhibitors etoposide, doxorubicin, camptothecin and with the alkylating agents cisplatin and melphalan causes peroxide accumulation and apoptosis in U937 human promonocytic monolayer cultures. The toxicity of antitumour drugs may largely depend on the intracellular level of GSH which is the main antioxidant system in the cell. Depletion of GSH by prolonged incubation with L-buthionine[S,R] sulfoximine (BSO), a specific inhibitor of \( \gamma \)-glutamylcysteine synthetase increases the lethality of the DNA topoisomerase I inhibitor CPT11 in V79 hamster lung fibroblasts cell line, DNA topoisomerase II inhibitor etoposide in K562 human erythroleukemia cells, and of the anthracycline doxorubicin in different monolayer cells. The influence of GSH is particularly evident in the case of alkylating agents, where BSO is occasionally able to change the mode of death from apoptosis to necrosis. These studies also point to the fact that GSH depletion facilitates ROS accumulation in cells treated with antitumour drugs, which, in turn, increases their lethality.

Oxidative stress induces apoptosis in a number of cell systems and also plays an important role as a mediator of apoptosis in diverse models. The functional importance of ROS generation for the activation of death mechanism has also been supported with studies using anti-oxidants and inhibitors of specific enzymes in monolayer cultures. Another mechanism of the induction of apoptosis by ROS involves activation of FAS receptors belonging to the TNF family, resulting in up-regulation of extrinsic apoptotic pathway. TNF is a strong inducer of oxidative stress and also NF-\( \kappa \)B-mediated prosurvival responses. While this signaling plays an important role in the response of solid tumours *in vivo*, it cannot be mimicked in monolayer culture models due to negligible levels of TNF\( \alpha \). On the other hand, MCTS are associated with varying levels of TNF\( \alpha \) depending on the extent of necrotic cells, which is influenced by the age and size of spheroids. Indeed, a significant difference in the levels of TNF\( \alpha \) and corresponding endogenous oxidative stress has been noted in the MCTS of a human glioma cell line (*BMG-1*) (Fig. 2).

Although induction of oxidative stress through depletion of GSH by BSO or addition of TNF\( \alpha \) results in an enhanced cell death in monolayer cultures, the extent of cell death through necrosis is significantly lower (<35%) in monolayer cultures, while necrosis is the major contributor for the cell death (>85%) under these conditions in MCTS (Fig. 2). These observations strongly suggest that the extent of cellular responses to certain anticancer treatments as well as the mechanisms underlying could significantly differ between the monolayer cultures and MCTS. Together with differences in the status of antioxidant defense between monolayer cultures and MCTS, this could give rise to quantitatively different responses in them, as has been found in the case of a human glioma, subjected to a combined treatment of etoposide and the glycolytic
Fig. 2—Relationship between the oxidative stress and different modes of cell death [Mitotic death (MD), apoptosis (Ap) and necrotic death (ND)] in monolayer cultures and spheroids treated with the topoisomerase II inhibitor etoposide and glycolytic inhibitor 2-deoxy-D-glucose.

Fig. 3—Schematic illustration showing differential mechanisms associated with ROS and cell signaling leading to cell death in monolayers (A & B) and spheroids (C & D).
inhibitor 2-DG (Fig. 3). These observations point out the likely shortcomings of approaches that employ inappropriate in vitro models, which may not adequately estimate the likely in vivo responses of a potential new therapy, thereby limiting the success.

Summary
Multi-cellular tumour spheroids offer an excellent in vitro system that mimics the endogenous oxidative stress in tumours as compared to the widely employed monolayer cultures in experimental oncology. Most importantly, the cellular responses to oxidative stress induced by combinations of certain chemotherapeutic drugs (viz. etoposide, a topoisomerase II poison) and metabolic inhibitors (viz 2-deoxy-D-glucose, a glycolytic inhibitor) in MCTS differ significantly from the monolayer cultures, but resemble in vivo tumour responses (Fig. 3). Therefore, MCTS appear to be excellent in vitro models, ideally suited for developing novel therapies that either exploit the endogenous oxidative stress in tumours or induce oxidative stress, specifically in tumours, thereby a promising differential response between tumour and normal tissues.

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References


