Review

Revisiting Sensitization Mechanisms in Cancer Thermochemotherapy : Does the Production of Radicals Hold the Key to Sensitization ?

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Abstract : The mechanism involved in the potentiation of cell killing and antitumor activity which result from combined treatments with hyperthermia and chemotherapeutic drugs is believed to be an increase in DNA damage caused by an increase in drug uptake. These events can be brought about by the disruption of membrane permeability by hyperthermia. It has recently been suggested that the contribution of reactive oxygen species (ROS) to the cytotoxic activity of anti-cancer drugs may be important in combined treatments utilizing hyperthermia and chemotherapeutic drugs. The present review examines the possibility that ROS, generated from chemotherapeutic drugs or by interactions between chemotherapeutic drugs and biomaterials or hyperthermia, may make a major contribution to the potentiation of cell killing and antitumor activity in thermochemotherapy.

Key Words : hyperthermia, chemotherapeutic drugs, reactive oxygen species (ROS)

Introduction

Since the first International Conference for Hyperthermic Oncology was held in Washington DC, USA, in 1975, interest in hyperthermic cancer therapy as a novel modality for cancer therapy has expanded around the world. Although both the number of articles and participants in meetings focusing on hyperthermic cancer therapy increased in the first decade after 1975, interest in hyperthermic cancer therapy was clouded by poor clinical outcomes. A renewed interest in hyperthermic cancer therapy as an adjuvant therapy before and after surgery, and as a combination therapy with radio- and chemo-therapy has been generated by improvements in heating devices, beneficial changes in systems for therapy planning, and by the elucidation of some of the mechanisms involved in cell killing and antitumor effects resulting from heating. Indeed, a reasonable efficacy for hyperthermic cancer therapy using a standard heating device has been demonstrated by local control rates for recurrent breast cancers and melanomas, by survival rates for head and neck cancers, and for glioma and cervical cancers in several phase III clinical trials designed to compare radiotherapy alone to combined treatments using radiotherapy and hyperthermia.

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It has been reported that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the mechanisms leading to cell killing and antitumor effects produced by chemotherapeutic drugs. Alkylating drugs (cyclophosphamide, melphalan, and BCNU), metal complex compounds (cisplatin and carboplatin), carcinostatic antibiotics (bleomycin, mitomycin C, and adriamycin), anesthetics¹⁾, and antioxidants²⁾ have revealed notable cell killing and antitumor effects in combination with hyperthermia. These drugs possess a common characteristic which is the fact that they can generate radicals through interactions with biological materials or hyperthermia (Table I). It has been reported that the production level of radical species increased in patients receiving cancer chemotherapy³⁾. In the present review, thermosensitization mechanisms will be examined from the viewpoint of the production of radicals.

Most anti-cancer drugs which are widely used for cancer patients produce cell killing effects which result from DNA damage induced directly by the drugs themselves. However, several anti-cancer drugs lead to the indirect induction of DNA damage, or to the modification of lipids and proteins by radicals produced from the compounds themselves, and show an ability to stimulate the production of radicals in combined treatments using chemotherapy and hyperthermia.

1. Alkylating agents

Alkylating agents demonstrate cell killing and antitumor effects which result from the alkylation of DNA or proteins. Cyclophosphamide and ifosfamide, which belong to the nitrogen mustard class of agents, and ACNU and BCNU of the nitrosourea class, have been well studied. ACNU and BCNU are used for radiochemotherapy after surgery for glioma, because they are able to pass through the blood-brain barrier. The pharmacodynamic action of alkylating agents is independent of the cell cycle phase in cancer cells.

Table I. Anti-cancer drugs that have their cytotoxic activities enhanced by hyperthermia.

Category	Anti-cancer drug
Alkylating agents	Melphalan
	Cyclophosphamide
	BCNU, CCNU
Anti-metabolites	5-Fluorouracil
Carcinostatic antibiotics	Bleomycin
	Peplomycin
	Mitomycin C
	Adriamycin
	Daunorubicin
	Actinomycin D
Metal complexes	Cisplatin
	Carboplatin

These anti-cancer drugs possess a common characteristic: they produce oxygen radicals through interactions with biomaterials and/or hyperthermia.

Several alkylating agents are involved in the production of radicals⁴. Cyclophosphamide is metabolized into acrolein by cytochrome P450 in the liver, and acrolein is cytotoxic. In this metabolic process, hydroperoxy or hydroxyethyl radicals ($\bullet\text{OOH}$ or $\bullet\text{CH}_2\text{CH}_2\text{OH}$) are produced by the reaction of cyclophosphamide with n-3 unsaturated fatty acids⁵. These radicals are also produced by the reaction of melphalan, BCNU, or CCNU with unsaturated fatty acids. Furthermore, since BCNU is an inhibitor of glutathione reductase, the generation of ROS by other agents or by hyperthermia seems to be enhanced by BCNU⁶. Therefore, the production of peroxy lipids from the interaction between these radicals in the plasma and mitochondrial membranes with hyperthermia provide a possible mechanism of sensitization in cancer thermochemotherapy (Fig. 1).

A synergistic enhancement of antitumor effects was found in transplanted Fsa-II tumors in the thighs of mice after combined treatment with ifosfamide and hyperthermia⁷, and also in experimental tumors after combined treatment with hyperthermia and BCNU or CCNU⁸. In addition, remarkable therapeutic efficacies were found in patients treated with a combination of alkylating agents and hyperthermia⁹.

Fig. 1. A possible mechanism for the potentiation of alkylating drug cytotoxicity resulting from the generation of ROS in a combined treatment with hyperthermia.

2. Anti-metabolites

Anti-metabolites, analogs of folic and nucleic acid, exhibit cell killing and antitumor effects which result from the inhibition of nucleotide metabolism and/or DNA replication, thus indicating a specificity for cells in S-phase.

5-Fluorouracil (5-FU) acts not only as an anti-metabolite for pyrimidine metabolism, but also as an inducer of ROS. Cardiac toxicity is a serious side effect observed in patients treated with 5-FU¹⁰. ROS generation during treatment with 5-FU, leading to lipid peroxidation and cell membrane damage, could

be one mechanism underlying the toxic effects of 5-FU on the endothelium¹¹⁾. Previous studies on cultured cells, comparing the toxicity of 5-FU on myocytes and endothelial cells from the rat heart, suggested that myocytes are less susceptible than endothelial cells to the direct toxic effect of 5-FU¹²⁾. Thus, peroxy lipid generated by ROS in the plasma membranes of endothelial cells in tumors can suppress the proliferation of cancer cells through the inhibition of angiogenesis, and can also directly induce cancer cell killing¹³⁾. Based on these observations, it has been suggested that ROS are involved in the cytotoxicity of 5-FU (Fig. 2). An enhancement of therapeutic efficacy was observed clearly in a combined treatment consisting of radiation and 5-FU¹⁴⁾, and also observed in combined treatments consisting of hyperthermia and 5-FU (chemohyperthermic peritoneal perfusion)¹⁵⁾.

Fig. 2. A possible mechanism for the potentiation of 5-fluorouracil cytotoxicity resulting from the generation of ROS in a combined treatment with hyperthermia.

3. Carcinostatic antibiotics

Research into carcinostatic antibiotics was initiated in Japan, and these are now widely used for cancer patients. Carcinostatic antibiotics include carzinophilin, mitomycin C, bleomycin, peplomycin, aclacinomycin A, chromomycin A3, and neocarzinostatin, all of which were discovered in Japan. In particular, mitomycin C and bleomycin are widely used around the world. Other carcinostatic antibiotics include actinomycin D, daunorubicin, and adriamycin (doxorubicin). Several carcinostatic antibiotics have the ability to produce ROS.

3.1 Bleomycin and peplomycin

Bleomycin and peplomycin each have a couple of binding sites for iron ions and DNA. When iron ions bind to specific sites on these drugs and capture oxygen, superoxide radicals ($\cdot\text{O}_2^-$) are produced which result in DNA cleavage (Fig. 3). An enhancement of cell killing and antitumor effects was clearly observed in combined treatments using hyperthermia and bleomycin or peplomycin¹⁶⁾.

Fig. 3. A possible mechanism for the potentiation of bleomycin/peplomycin cytotoxicity resulting from the generation of ROS in a combined treatment with hyperthermia.

3.2 Mitomycin C

The cell killing and antitumor effects of mitomycin C result from several mechanisms *via* the production of ROS, and include respiratory inhibition, inhibition of DNA replication, and DNA damage. Mitomycin C has a couple of binding sites for metal ions and DNA. When Zn (II), Cu (II), Mn (II), Cd (II), or Au (III) ions bind to the metal ion binding site in the drug, an irreversible complex (mitomycin C-Metal ion-DNA complex) is formed resulting in the inhibition of DNA replication (Fig. 4). In particular, Zn (II), Cu (II), and Mn (II) ions are highly effective. The induction of DNA damage by mitomycin C requires the protein to be activated by 1 or 2 electron reductions. Activated mitomycin C becomes a semiquinone or hydroquinone which reacts with oxygen molecules and produces ROS. These reaction processes are evoked in the mitomycin C-metal ion-DNA complex, and consequently DNA damage is induced by hydroxyl radicals ($\cdot\text{OH}$) produced through the Fenton reaction¹⁷⁾.

The cell killing effect of hyperthermia was enhanced by the simultaneous administration of mitomycin C in human non-small-cell lung carcinoma H1299 cells, and this enhancement was independent of the *p53* status of the cells¹⁸⁾. In spite of the requirement for oxygen in the production of hydroxyl radicals from mitomycin C, the cell killing effect of hyperthermia was further enhanced by the administration of mitomycin C under hypoxic conditions¹⁹⁾. This suggests that the changes in the intracellular microenvironment by hyperthermia affect the production of ROS by mitomycin C.

3.3 Anthracycline carcinostatic antibiotics

Some of the earliest studies describing the possible mechanisms of action of anthracyclines focus on the capacity of these drugs to inhibit DNA replication and RNA transcription caused by drug binding to DNA²⁰⁾. In particular, the inhibition of DNA topoisomerase II may be most closely associated with the lethal effects of anthracyclines in cancer cells and tumors. On the other hand, anthracycline carcinostatic antibiotics such as adriamycin and daunomycin have been shown to induce the generation of ROS²¹⁾. The quinone structure in these drugs acts as an electron acceptor in reactions catalyzed by

Fig. 4. A possible mechanism for the potentiation of anthracycline carcinostatic antibiotic cytotoxicity resulting from the generation of ROS in a combined treatment with hyperthermia.

oxoreductive enzymes including cytochrome P-450 reductase, NADH dehydrogenase, and xanthine oxidase²²⁾. When the quinones capture free electrons, they are reduced by one or two electrons, and can be converted into semiquinone radicals. The cell killing and antitumor effects of anthracyclines are believed to be caused by the reductive metabolic activation of these drugs into reactive intermediates including semiquinone free radicals²³⁾. The semiquinone form of these drugs rapidly autoxidizes and reduces molecular oxygen into the superoxide anion radical ($\cdot\text{O}_2^-$) and subsequently into other partially reduced species of oxygen, including hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot\text{OH}$) through the Fenton reaction in an antibiotic-metal ion-DNA complex²⁴⁾. These ROS from anthracycline antibiotics induce DNA damage²⁵⁾ (Fig. 4). However, unresolved questions remain of whether free radicals are generated at clinically relevant concentrations of anthracyclines and at normal (*i.e.* hypoxic) oxygen tension in cancer cells, and whether such free radicals could be responsible for anthracycline toxicity to the tumor.

The extent of potentiation of cell killing and antitumor effects caused by combined treatments of hyperthermia and adriamycin seems to depend on the temperature and period of heating, the type of cancer cells used in the experiments, and the type of tumors exposed to the treatments. Although a synergistic enhancement of thermosensitivity was induced by a combined treatment consisting of hyperthermia at 43°C and adriamycin in Chinese hamster HA-1 cells, only additive effects were found in prolonged treatments²⁶⁾. A stimulated thermotolerance was induced by a combined treatment with hyperthermia at 43°C and adriamycin, however, suggesting that the combined treatment may stimulate the induction of Hsp70^{27,28)}. Collectively, these observations suggest that the synergistic enhancement of thermosensitivity in cancer cells by a combined treatment of hyperthermia and anthracyclines can be found when semiquinone radicals and ROS are generated effectively and occur in addition to the cell killing and antitumor effects caused by binding of the drugs to DNA.

4. Alkaloid agents from plants

Several basic nitrogen compounds exist in plant tissues. These are collectively called plant alkaloids. Some alkaloids have cell killing and antitumor effects and they are widely used as cancer chemotherapeutic drugs. The cell killing and antitumor effects are believed to be due to the inhibition of cell division through the suppression of the formation of spindle fibers, and of the depolymerization of spindle fibers. Therefore, these drugs affect specifically cells in M phase.

4.1 *Vinca alkaloids*

Vinblastine and vincristine belong in this classification. These drugs block cell division through inhibition of the formation of spindle fibers made from microtubules by binding to tubulin. An enhancement of cell killing effects produced by interactive effects between these drugs and endogenous and exogenous *cis*-unsaturated fatty acids from both the n-6 and n-3 series was found to occur in human cervical carcinoma cells²⁹). The enhancement of cell killing effects by a combined treatment of hyperthermia with these drugs was also found in malignant glioma cells³⁰). However, a relationship between the enhanced cytotoxicity and radical generation has not been demonstrated yet.

4.2 *Taxane*

Paclitaxel belongs in this classification. Paclitaxel blocks cell division through the stabilization of microtubules by the stimulation of the polymerization of tubulin. Although paclitaxel itself has no capacity to generate radicals, the stimulation of ROS generation by treatment with paclitaxel was found to occur in human neutrophils³¹), suggesting that the antitumor effects of paclitaxel can be enhanced by the stimulation of ROS generation in lymphocytes which invade tumors.

4.3 *Etoposide (VP-16)*

Etoposide is an anti-cancer drug which is a specific inhibitor of DNA topoisomerase II, and topoisomerase II modifies DNA topology in an ATP-dependent manner. This allows it to cleave, rewind, and rejoin DNA double strands repeatedly. The antitumor activity of etoposide has been suggested to result from a covalent DNA topoisomerase II-DNA complex formed between the drug and the enzyme, which in turn produces double-strand DNA breaks in tumor cells³²). In addition, the dimethoxyphenol ring of etoposide has been implicated in cytotoxicity by leading to metabolic alterations of dihydroxy and *o*-quinone derivatives catalyzed by either cytochrome P-450s, peroxidases, or tyrosinase. Dihydroxy-etoposide promotes DNA damage in the presence of Cu (II) ions, indicating that hydroxyl radicals ($\bullet\text{OH}$) are responsible for the DNA damage. Hydroxyl radicals ($\bullet\text{OH}$) can be generated during redox-dependent complex formation between Cu (II) and etoposide in the presence of molecular oxygen (O_2), and it has been suggested that the hydroxyl radicals ($\bullet\text{OH}$) participate in the formation of single- and double-strand breaks in DNA³³) (Fig. 5). Enhanced thermosensitivity in combined treatments with hyperthermia and etoposide was observed in human colorectal cancer cells³⁴), suggesting that the generation of ROS may contribute to the potentiation of cell killing and antitumor effects in addition to the drug's effect on DNA topoisomerase II.

Fig. 5. A possible mechanism for the potentiation of etoposide cytotoxicity by the generation of ROS resulting from a combined treatment with hyperthermia.

5. Metal complex compounds

Chemical compound bound ligands around metal ions are collectively called metal complex compounds. The platinum-coordination complexes cisplatin (CDDP) and carboplatin (CBDCA) are potent anti-cancer chemotherapeutic drugs which exhibit a broad spectrum of activity against solid tumors. Carboplatin (CBDCA) has been introduced into clinical practice to overcome some of the toxic effects of cisplatin (CDDP)^{35,36}. The cytotoxic effects of cisplatin (CDDP) and carboplatin (CBDCA) result primarily from their interaction with cellular DNA, since these compounds form inter- and intra-strand crosslinks and DNA-protein crosslinks³⁷. It has been shown that these effects are mediated by the formation of common intermediate species, i.e. aquation products, in which the departing ligands, two chlorides and cyclobutanedicarboxylatochelate in cisplatin (CDDP) and carboplatin (CBDCA), respectively, are substituted by water³⁸. Recently, in addition to such cytotoxicity, the anti-cancer activities of cisplatin (CDDP) and carboplatin (CBDCA) have been associated with either the generation of ROS through binding to DNA, or with the stimulation of ROS generation in lymphocytes invading the tumors³⁹.

The most likely explanation, including the mechanism of ROS generation by a drug-DNA interaction, is as follows⁴⁰ (Fig. 6) :

- 1) $\bullet\text{O}_2^-$ is produced when solubilized molecular oxygen gains a single electron released by the drug-DNA interaction ;
- 2) H_2O_2 is produced by a dismutation of $\bullet\text{O}_2^-$
 $(2\bullet\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2)$;
- 3) $\bullet\text{OH}$ is produced by the Fenton reaction, probably in the presence of Fe (II) in the cytosol or chromatin
 $(\text{H}_2\text{O}_2 + \text{Fe (II)} \rightarrow \bullet\text{OH} + \text{OH}^- + \text{Fe (II)})$;
- 4) Single or double strand breaks in DNA are evoked by $\bullet\text{O}_2^-$ and $\bullet\text{OH}$.

In addition, the generation of ROS by interactions between these drugs and DNA is stimulated by the depletion of intracellular glutathione through crosslinking between the drugs and proteins⁴¹⁾.

There are many reports regarding the potentiation of cell killing and antitumor activity of hyperthermia by cisplatin (CDDP) and carboplatin (CBDCA)^{42–44)}. The mechanisms involved in the potentiation produced by a combined treatment consisting of hyperthermia and these drugs are believed to be related to an increase in drug uptake due to alterations of membrane permeability, and in an inhibition of the enzymes involved in the nucleotide excision repair of the drug-DNA complexes. Moreover, it has been suggested that hyperthermic stimulation of ROS generation in the drug-DNA complexes is also associated with the potentiation of cell killing and antitumor activity resulting from combined treatments of hyperthermia and these drugs⁴⁵⁾.

Fig. 6. A possible mechanism for the potentiation of metal complex compound cytotoxicity resulting from the generation of ROS in a combined treatment with hyperthermia.

6. Other anti-cancer drugs

Tirapazamine (3-amino-1,2, 4-benzotriazine 1,4-dioxide, SR-4233, TPZ) is a new anti-cancer drug which produces a specific activity in cells in the presence of low oxygen tensions. The mechanism involved in the preferential toxicity of tirapazamine in hypoxic cells is the result of an enzymatic reduction which adds an electron to the tirapazamine molecule converting it into a highly reactive radical. This radical causes cell killing by producing DNA damage leading to chromosomal aberrations. However, in the presence of oxygen, the radical is converted to nontoxic tirapazamine, suggesting a specificity of tirapazamine for targeting only hypoxic tumor cells but not aerobic cells in normal tissues⁴⁶⁾.

Although clinical trials of combined treatments using radiation or cisplatin and tirapazamine are currently underway^{47,48)}, current reports concerning a combined treatment of hyperthermia and tirapazamine are limited to experimental data. Synergistic enhancement of thermosensitivity was observed after a combined treatment with hyperthermia at 42°C and 43°C with tirapazamine in murine

Fsa-IIC cells, and a synergistic potentiation of antitumor effects was observed after a combined treatment with hyperthermia at 43°C and tirapazamine in transplanted tumors composed of murine Fsa-IIC cells⁴⁹⁾. Even combined treatments of hyperthermia at 40°C with tirapazamine showed an enhancement of cell killing activity in human lingual cancer cells⁵⁰⁾. Tirapazamine is the first anti-cancer drug reported to cause DNA damage by radicals under hypoxic conditions.

The possibility that ROS generated from anti-cancer drugs contributes to the cell killing and antitumor activities of these drugs was discussed in this review (Fig. 7). Further developments and studies of combined treatments with hyperthermia and anti-cancer drugs will hopefully bring about improvements and advances in the results delivered by hyperthermic cancer therapy.

Fig. 7. Summary of the present review.

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