Review

Hyperthermia Chemo-sensitization, Chemical Thermo-sensitization and Apoptosis

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Abstract : Hyperthermia (HT) which can be directly cytotoxic to cancer cells, impairs the synthesis of cellular proteins : if not properly chaperoned by heat shock proteins (HSP), this can lead to irreversible and toxic protein aggregates. Clinically, it is preferred to use HT in combination with radiation therapy and chemotherapy. Although the combination of thermoradiotherapy has been widely studied, much attention has recently been focused on the search for compounds which can sensitize tumor cells to HT damage, but at the same time lead to minimal damage to normal cells. Synergy between HT and drugs may be caused by the occurrence of multiple events such as HT damage to ATP-binding cassette transporters, intracellular drug detoxification pathways, and the reparability of drug-induced DNA adducts. This may be why cells with acquired drug resistance (often multi-factorial) can be made responsive to drugs again by the combination of a drug exposure in conjunction with HT. In this review, the mechanism of HT-induced apoptosis and compounds which can sensitize cancer cells to HT-induced apoptosis are discussed.

Key Words : protein denaturation, protein aggregation, heat shock proteins, apoptosis

Introduction

Despite tremendous progress in chemotherapy and radiation therapy for the treatment of cancer, there is still a significant fraction of malignancies which is refractory to all current treatment modalities. The main obstacle facing conventional therapeutics is that the 'therapeutic window' between normal and tumor cells cannot be widened easily. An in-depth understanding of the molecular and cellular biology of cancer cells which has been made from revolutionary advances in the past two decades can now be utilized to help, provide excellent opportunities and strategies for novel therapeutic approaches. Hyperthermia has been recognized as an effective and attractive tool, especially in combination with conventional therapies, to halt tumor growth. The significant advantage of this type of combination

therapy is the possibility of using lower doses of chemotherapy or radiation, and thus potentially widening the current ‘therapeutic windows’, leading to more effective treatment with fewer unwanted side effects, and a reduced resistance of cancer cells to the combination of drugs and radiation.

There are many studies which deal with the biochemical and biophysical mechanisms leading to cell death from HT or from a combination of HT and ionizing radiation^{1–5}. In this report, mechanisms are reviewed which could be involved in the enhancement of apoptosis induced by a combination of HT and drugs.

Effects of HT on cellular physiology

Hyperthermia induces numerous changes in cellular physiology (Table I). These cellular alterations make a combination of heat and drugs very attractive. Below some important cellular responses to potential therapeutic regimens are discussed in detail.

Table I. Molecular effectors of hyperthermia

Organelle	Functional changes	References
Cell membrane	Changes in fluidity/ stability	(65)
	Alteration in structure	
	Impairment of ion transport (Ca ²⁺ , Na ⁺ , Mg ⁺ , K ⁺)	
	Changes in membrane potential	
	Modulation of the transmembrane efflux pump	
Cytoplasm	Impairment of protein synthesis	(65)
	Denaturation of protein structure and function	
	Aggregation of proteins	
	Induction of HSP synthesis	
Mitochondria	Increase permeability of mitochondrial inner membrane.	(67)
	Depolarization of mitochondrial membrane potential	
	Depletion of ATP production	
	Production of reactive oxygen species (ROS)	
	Disruption of Ca ²⁺ transport across mitochondrial membrane.	
Endoplasmic Reticulum (ER)	ER stress due to excessive accumulation of misfolded proteins.	(68)
Nucleus	Impairment of RNA/DNA synthesis	(65)
	Inhibition of DNA repair enzymes	
	Alteration of DNA conformation	
	Changes of gene expression and signal transduction	

Changes in membrane permeability

Membranes are known to be extremely sensitive to heat stress because of their complex molecular composition of lipids and proteins. At a certain temperature, lipids change from a tightly packed gel phase to a less tightly packed crystalline phase, and permeability of the cell membrane (membrane fluidity) increases. Hyperthermia-induced cell membrane permeability leads to increased drug delivery into tumor cells. In addition, increased vascular permeability due to thermal increases in endothelial gap size also aids drug delivery into tumors. Alteration in membrane permeability also alters the cellular content of several ions (Na⁺, Mg²⁺, K⁺) in a number of cells⁶), although the changes in these ion

balances are not primarily responsible for hyperthermic cell death⁷⁻⁹. Another ion which might be involved in hyperthermic cell death is Ca^{2+} . Influxes of extracellular Ca^{2+} stimulates the activity of calmodulin-dependent protein kinases, inositol triphosphate production and other signaling cascades¹⁰.

Alteration of cytoskeletal systems

The response of cytoskeletal systems to HT varies depending on the cell type and HT dose¹¹. Hyperthermia-induced disassembly of the cytoskeleton enlarges the tumor pores which enables easier drug delivery¹¹. It also induces alteration of the mitotic spindles¹¹, centrosome organization, and protein denaturation which results in the formation of multinucleated non-clonogenic cells^{12,13}.

Inhibition of DNA repair

DNA appears to be the primary target for most of the currently adopted chemotherapeutic drugs¹⁴. The main obstacle in chemotherapy is the resistance of cancer cells to the cytotoxic effects of these drugs. Several possible mechanisms have been suggested to account for this. Examples of these mechanisms include intrinsic DNA repair capabilities, reduced drug diffusion into areas of vascular insufficiency, and cellular impermeability^{15,16}. Hyperthermia is reported to induce DNA double strand breaks due to the denaturation and dysfunction of heat-labile repair proteins such as DNA polymerases¹⁷ or to the precipitation of denatured proteins onto nuclear chromatin structures, generating a barrier which prevents repair enzymes from reaching damage sites¹⁸. HT-induced protein denaturation is also reported to alter multiple nuclear matrix-dependent functions [e.g., DNA replication, DNA transcription, mRNA processing and DNA repair]¹⁹.

It is thus hypothesized that hyperthermic treatment sensitizes tumor cells to other cancer treatments by altering cytoskeleton reorganization, enhancing membrane permeability and inhibiting DNA repair²⁰.

HT-induced signal transduction

a) Apoptotic signal transduction

Apoptosis is a genetically programmed and biochemically active mode of cell death in which the cell actively participates in its own destruction²¹. It is required for cell life span regulation and normal development²⁰. Apoptosis also aids in the self-deletion of injured cells, terminal differentiation of epithelial cells, and organ and tissue shaping²¹. Abnormalities in this process are implicated in several human diseases, including cancer. Radiation, cytotoxic drugs, viruses and hyperthermia can trigger this process.

Hyperthermia within a temperature range of 41-45°C induces apoptosis to varying degrees in many cell lines²¹. It induces apoptosis mainly through reactive oxygen species (ROS) generation, and a likely source of elevated ROS production is the mitochondria²². Mitochondria are believed to produce basal levels of ROS in the form of single-electron leakage to oxygen during normal metabolism²³. This can increase dramatically under conditions in which the mitochondria are damaged or exposed to certain toxic conditions²². In addition to this, HT can also alter the expression of the Bax and Bcl-2 genes, where such changes are dependent on the sensitivity of cell lines to HT²⁴. In thermo-resistant cell lines, HT by itself cannot change the expression of Bax and Bcl-2, but the combination of HT and

chemotherapy or radiotherapy can up-regulate Bax and down-regulate Bcl-2 expression²⁵⁾. Furthermore, hyperthermia-induced increases in intracellular Ca^{2+} ion ($[\text{Ca}^{2+}]_i$) concentration is also thought to be involved in cell death. However, evidence for the role of $[\text{Ca}^{2+}]_i$ in hyperthermic cell death is contradictory. Some investigators have stated that HT-induced increases in Ca^{2+} do not play a key role²⁶⁾, while others have concluded that thermal perturbations in $[\text{Ca}^{2+}]_i$ are among the primary events leading to heat-induced cell killing^{27–29)}. These observations suggest that the role of Ca^{2+} in HT induced cell killing may be dependent on cell type. HT is reported to increase the expression of IP3R1³⁰⁾ which may regulate the release of Ca^{2+} . Generally, Ca^{2+} can act on multiple targets to trigger apoptosis³¹⁾. Calpain, a calcium-dependent protease, has been considered as a possible target for calcium triggered apoptosis³²⁾. Lipid peroxidation due to ROS generation is also reported to alter Ca^{2+} distribution³³⁾ and to activate a Ca^{2+} -dependent apoptotic pathway³⁰⁾ (Fig. 1).

Fig. 1. Schematic summary of pathways involved in hyperthermic chemo-sensitization and chemical thermosensitization.

b) Anti-apoptosis signal transduction :

HT also simultaneously activates signal transduction pathways leading to anti-apoptosis activity and/or cellular proliferation. Key signaling factors, such as Akt, p38, extracellular signal-regulated kinase (ERK) and heat shock proteins (HSP) play important roles in anti-apoptosis or cellular proliferation pathways³⁴⁾. Such anti-apoptosis and cytoprotective signaling factors are known complications resulting from hyperthermia, and can lead to thermotolerance and chemoresistance. The presence of chemotherapeutic agents interferes with these pro-survival responses to hyperthermic monotherapy and can make cancer cells sensitive to HT.

Enhancement of hyperthermia-induced apoptosis by traditional and newly synthesized chemical agents

Hyperthermia alone plays no role in the curative treatment of human tumors³⁵⁾, therefore, much attention has been focused on combining HT with chemotherapy, and in searching for substances able to sensitize tumor cells to HT-induced damage^{36,37)}. An ideal sensitizer would be nontoxic at normal temperatures, but would become toxic at hyperthermic temperatures. Below, chemical agents which can act as heat sensitizers at non-toxic concentrations are briefly discussed.

a) Calcium channel blockers

Calcium channel blockers, such as verapamil or diltiazem, have been commonly used to treat arrhythmia and angina pectoris. Verapamil also has other pharmacologic actions, such as reversing multiple drug resistance and suppressing metastasis of cancer cells in BALB/c mice injected with mouse mammary adenocarcinoma F3II cells³⁸⁾. It also acts as a heat sensitizer in Chinese hamster ovary cells and U937 cells³⁹⁾. It sensitizes both normal and thermo-tolerant U937 cells to HT-induced apoptosis by increasing $[Ca^{2+}]_i$ concentration⁴⁰⁾.

b) Local anesthetics

Local anesthetics (LAs) belong to a class of clinically useful compounds which exert their pharmacological effect by blocking nerve impulse propagation. There are many reports showing that HT induced cell death is modified by LAs (e.g. potentiation by procaine in murine L5178Y lymphoma cells⁴¹⁾, by lidocaine in murine FM3A mammary carcinoma cells⁴²⁾ and in murine tumor models⁴³⁾, and by dibucaine, tetracaine, and procaine in hepatoma tissue culture cells⁴⁴⁾). Investigations of the molecular mechanism of apoptosis enhancement by LAs at hyperthermic temperatures, have produced evidence that the elevation of $[Ca^{2+}]_i$ is due to increased releases of Ca^{2+} from intracellular storage sites, and that this is caused by HT along with increased activity of the mitochondria caspase-dependent pathway (partly regulated by $[Ca^{2+}]_i$), and that this plays a crucial role in the enhancement of apoptosis induced by the combination of HT and lidocaine⁴⁵⁾.

c) Temperature-dependent free radical generator

A temperature-dependent free radical generator, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) acts as a heat sensitizer at non-toxic concentrations. It can sensitize hypoxic and thermo-tolerant cells, and enhances HT-induced apoptosis by increasing the $[Ca^{2+}]_i$ concentration. Hyperthermia combined with exposure to AAPH led to a loss of mitochondrial membrane potential (MMP), indicating that mitochondria function was damaged by this treatment³⁰⁾. Furthermore, AAPH has been reported to inhibit HT-induced anti-apoptosis signaling factors⁴⁶⁾.

d) Intracellular H_2O_2 generator

Another chemical agent which acts as a thermosensitizer is 6-formylpterin (6-FP). This compound generates intracellular H_2O_2 by transferring electrons from NAD(P)H to oxygen⁴⁷⁾, and at nontoxic concentrations, it enhances HT-induced apoptosis. Some investigations suggest that the increase in the $[Ca^{2+}]_i$ concentration, the activation of mitochondria-caspase dependent pathways, and the translocation of PKC δ to mitochondria play important roles in 6-FP⁴⁸⁾ enhancement of HT-induced apoptosis.

e) Spin trap : α -phenyl-tert-butyl nitron

Nitron spin trap, α -phenyl-tert-butyl nitron (PBN) has been used, not only for electron

paramagnetic resonance (EPR) -spin trapping studies, but also as an antioxidant *in vitro* and *in vivo* because PBN reacts with oxygen radicals to produce less reactive species^{49,50}. It has been reported that nitric oxide (NO) is released from PBN under oxidative stress conditions^{51,52}, and that HT induces oxidative stress in cells due to generation of superoxide (O_2^-). Consistent with these observations, it has been observed that PBN acts as a sensitizer for HT-induced apoptosis. When U937 cells were treated with a combination of HT and PBN, a significant enhancement of apoptosis was observed because NO reacts with O_2^- and produces $ONOO^-$, and this ROS enhances HT-induced apoptosis *via* stimulation of the mitochondria-caspase and the $[Ca^{2+}]_i$ -dependent pathways⁵³.

f) Furan-fused tetracyclic compounds

Recently, it was reported that a newly synthesized furan-fused tetracyclic compound, DF3 (a triisopropylsilyloxy (TIPS) derivative) acts as a heat sensitizer in U937 cells. DF3, at nontoxic concentrations with HT (44°C for 20 min), showed a significant enhancement of heat-induced apoptosis. DF3 induced a sustained elevation of O_2^- and a transient rise of H_2O_2 generation, and there was a further increase in these compounds when DF3 exposure was combined with HT. This enhancement in oxidative stress appears to be the key event in the improved apoptotic response of U937 cells when subjected to a combination of DF3 and HT⁵⁴.

Mild hyperthermia enhances chemical agent induced apoptosis

Hyperthermia produces synergistic results when combined with radiation or cytotoxic drugs at lower temperatures (40.5-43°C) (Table II). However, it is beyond the scope of this short review to discuss all of the observed interactions between these drugs and HT separately. Below is a summary of reports describing some chemical agents which showed a synergistic enhancement of apoptosis when combined with mild HT.

a) DNA-damaging agents

HT can enhance the cytotoxicity of various DNA damaging agents. Most impressive is data for a combined HT and cisplatin treatment. Hyperthermia enhances intra-cellular platinum accumulation, reduces intra-cellular detoxification which leads to more platinum-induced DNA adducts, and inhibits repair of these adducts⁵⁵. Interestingly, all of these processes are up-regulated in cells which have acquired platinum resistance⁵⁵. All of these enhancement effects appear to be due to HT-induced changes in cell metabolism, excretion, and membrane permeability or membrane transport⁵⁶. This has led to the suggestion that hyperthermia could be instrumental in reversing drug resistance, a major clinical problem in chemotherapy.

b) Macrosphelides

Recently, was reported that one of the synthetic diketone macrosphelides, specifically MS5 which is a 16-membered macrolide compound⁵⁷, led to the synergistic enhancement of apoptosis when combined with mild HT (41°C for 20 min) in U937 lymphoma cells. Significant increases in reactive oxygen species (ROS) generation were observed immediately after the combined treatment. Combination of MS5 and mild HT also led to a MMP loss, indicating that mitochondrial dysfunction occurred during this treatment. Furthermore, Fas expression, caspase-8 and caspase-3 activation were also observed at significantly increased levels after the combined treatment when compared with mild HT treatment alone.

Table II. Heat-induced enhancement of drug cytotoxicity in apoptosis

Drugs	Target for apoptosis	Cell line	Concentration/°C	Maximum Enhancement Ratio	Ref
Cisplatin (DNA damaging agent)	DNA	SW1573	5 μ M/41-43°C (60 min)	7.3-7.8*	(69)
Lobaplatin (DNA damaging agent)	DNA	SW1573	10 μ M/41-43°C (60 min)	1.6-2.9*	(69)
Oxaliplatin (DNA damaging agent)	DNA	SW1573	10 μ M/41-43°C (60 min)	2.0-2.1*	(69)
Paclitexal (Microtubule stabilizing agent)	Microtubules	FM3A	10 μ M/43°C (60 min)	2.1*	(70)
Etoposide (VP-16) (DNA damaging agent)	DNA	LU65A	8 μ M/43°C (45 min)	1.8*	(71)
5-Fluorouracil (Anti-metabolite)	DNA and RNA	CCRF-CEM	100 μ M/42°C (120 min)	1.0*	(72)
Verapamil (Ca ²⁺ Channel blocker)	Mitochondria	U937	100 μ M/42-44°C (30 min)	1.9-4.1 §	(37)
Lidocaine (Local anesthetic)	Mitochondria	U937	1 mM/44°C (10 min)	3.6 §	(44)
2-Amidinopropane dihydrochloride (Temperature dependent free radical generator)	Unknown	U937	50 mM/44°C (10 min)	5.0 §	(29)
6-Formylpterin (Intracellular hydrogen peroxide generator)	Mitochondria	U937	300 μ M/44°C (20 min)	4.4 §	(49)
α -phenyl-tert-butyl nitron (Spin trap agent, antioxidant)	Unknown	U937	10 mM/44°C (10 min)	5.7 §	(54)
Furan-fused tetracyclic compounds (Anti-viral agent)	Mitochondria	U937	20 μ M/44°C (20 min)	6.6 §	(55)
Macrosphelides (Anti-metastatic agent)	Mitochondria	U937	5 μ M/41°C (20 min)	2.1 §	(57)
Anisomycin (Protein synthesis inhibitor)	Proteins	U937	0.1 mM/41°C (60 min)	1.5 §	#
6-Dimethyl-a minopurine (Protein kinase inhibitor)	Unknown	U937	2 mM/41°C (60 min)	1.5 §	#

* Maximum enhancement ratio=Cell death (%) (measured with different methods) in the presence of the drug at elevated temperature/Cell death (%) in the presence of drug at a normal temperature (37°C).

§ Maximum enhancement ratio=DNA fragmentation (%) in the presence of a drug at elevated temperatures/DNA fragmentation (%) in the presence of a drug at a normal temperature (37°C).

Unpublished data.

Moreover, this combined treatment also altered the expression of apoptosis-related proteins, which was shown by the cleavage of Bid and the down-regulation of Bcl-2. The results have indicated that an early increase in ROS generation was primarily responsible for the synergistic enhancement of apoptosis after a combined treatment⁵⁸⁾.

c) Anisomycin

The effects of HT in combination with antibiotics were reported previously⁴³⁾. Some antibiotics such as Bleomycin, Doxorubicin and Actinomycin D showed more than an additive cell killing when combined with HT³⁵⁾.

Recently, the effect of a combination of mild HT (41°C for 60 min) and anisomycin was examined in U937 cells. This antibiotic is a reversible inhibitor of protein biosynthesis and also has been reported to inhibit DNA synthesis⁵⁹⁾. It also induces strong activation of JNK and P38 mitogen-activated protein kinase⁶⁰⁾. In preliminary studies, a significant synergistic enhancement of apoptosis was observed after a combined treatment when compared to anisomycin alone ($27.2 \pm 3.0\%$ and $17.8 \pm 4.1\%$, respectively). Mitochondrial dysfunction was also observed during this treatment indicating the involvement of mitochondrial apoptosis pathways.

d) 6-Dimethylaminopurine

Many alkylating agents, such as Melphalan, Ifosfamide and Mitomycin C³⁵⁾ have been reported to show additive cell killing when combined with HT. The alkylpurine, 6-dimethylaminopurine (6-DMAP) is a protein kinase inhibitor known to induce premature mitosis in S-phase arrested hamster cells^{61,62)}, and has been reported to induce apoptosis in HeLa cells⁶³⁾ and in epidermal growth factor and insulin treated G₀-phase Chinese hamster embryonic fibroblast (CHEF/18) cells⁶⁴⁾. The effects of 6-DMAP in combination with mild HT (41°C for 60 min) were examined in U937 cells. Preliminary data showed a synergistic enhancement of DNA fragmentation in cells treated with the combination when compared to 6-DMAP and mild HT alone ($43.4 \pm 8.2\%$, $28.6 \pm 6.2\%$ and $8.4 \pm 0.4\%$ respectively). However, further studies with anisomycin and 6-DMAP in combination with HT are still necessary to propose any detailed mechanism which can explain how these agents may be involved with apoptosis.

Conclusion

HT sensitizes cells to many cytotoxic agents, and even converts some innocuous drugs into highly toxic agents. HT alters the pharmacokinetics and pharmacodynamics of drugs⁶⁵⁾, increases DNA damage¹⁵⁾, decreases DNA repair^{8,12)} and alters membrane permeability⁶⁵⁾. Furthermore, HT can also lead to the modulation of cellular ROS concentrations, which could be responsible for thermal chemo-sensitization in cancer therapy. These HT-induced cellular changes may also have the potential to overcome some types of drug resistance⁶⁶⁾. Further detailed studies with combinations of drugs and HT are still required to identify effective heat sensitizers for cancer therapy.

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