Review

Chemical Sensitization of Apoptosis Induced by Hyperthermia

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Abstract: Hyperthermia (HT) has been recognized as an effective and attractive tool in anticancer therapy. However, the results obtained in therapy by HT alone have been only partially satisfactory. Consequently, attention has focused on the search for substances able to sensitize tumor cells to the damage caused by HT without reducing the capacity of normal cells. It has been reported that the tumor cell death in HT is mediated by apoptosis. Activation of a preprogrammed cascade of molecular events will result in DNA degradation and nuclear fragmentation, leading to apoptosis. However, the pathways which can induce apoptosis varies in different cells. If we could discover common stimuli which could induce apoptosis in all types of cells, we would obtain a new anticancer therapy against all types of tumor cells. In this report, we overviewed both mechanisms of HT-induced apoptosis, and the substances used to enhance HT-induced apoptosis. Moreover, we provide evidence for the role of intracellular Ca^{2+} concentration in HT-induced apoptosis.

Key Words: hyperthermia, apoptosis, sensitizer, intracellular Ca^{2+} concentration, free radical

1. Overview of Apoptosis Induced by Hyperthermia

HT has been recognized as an effective and attractive tool in anticancer therapy. However, little has been reported regarding the detailed mechanism of HT-induced anticancer effects¹⁾. It has been reported that tumor cell death in mild HT is mediated by apoptosis²⁻⁵⁾. Apoptosis is a suicidal mode of cell death, which is of critical importance for proper ontogeny and tissue homeostasis, as well as in the pathogenesis of a variety of diseases, including cancer. It represents the most frequent type of cell response to antineoplastic chemotherapy and radiotherapy⁶⁾.

The effectiveness of HT as an inducer of apoptosis has been reported⁷⁻¹³⁾, but little is known about the biochemical events underlying this phenomenon¹⁴⁾. It is evident that activation of a preprogrammed cascade of molecular events will result in DNA degradation and nuclear fragmentation, leading to cell

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death¹⁵⁾. The fate of a cell is determined by multiple genes. One group of gene products may promote apoptosis such as p53¹⁶⁾, whereas another group may induce survival, such as *bcl-2* family genes^{17) 18)}. Therefore, the final outcome of a trigger such as HT may be dependent on a balance between induction and reduction of various gene products.

(a) *Bax and the Bcl-2-dependent pathway*

It has been reported that DNA-damaging agents, such as ionizing radiation, induce nuclear accumulation of wild-type p53 protein and, in some cases, can cause apoptosis¹⁹⁾. Van Bree *et al.* studied the thermal response of transfectants of a human colon carcinoma cell line RKO, in which the function of p53 and of its down-stream effector in the G₁ arrest pRb were functionally inactivated²⁰⁾. Recently, it was reported that transfection of wild-type p53 into fibroblasts derived from p53-knockout mice enhanced thermal sensitivity and accelerated the apoptotic response²¹⁾. These results show that the *p53* gene is a factor in determining cellular thermosensitivity, and that wild-type *p53* contributes to thermosensitization resulting in enhancement of heat-induced apoptosis.

The role of p53 lies in its changing the balance of the two gene expressions. The p53 protein down-regulates *bcl-2* and up-regulates *bax* expression¹⁸⁾. Fuse *et al.* indicated that heat-induced apoptosis in A172 cells was accompanied by augmentation of the p53 protein, *bax* mRNA and protein²²⁾. Similar results were obtained in an other observation of *bax* expression using a cell line transfected with an expression plasmid encoding a temperature-sensitive version of p53²³⁾. Thus, these results suggest that the molecular mechanism which mediates heat-induced, p53-dependent apoptosis is the up-regulation of *bax* expression.

It has been demonstrated that *bcl-2* and *bax* genes and their family members are intimately involved in the regulation of apoptosis induced by various stimuli²⁴⁻²⁶⁾. The effect of *bcl-2* seems to be inhibitory, whereas *bax* expression seems to be required for the initiation of apoptosis. HT is in fact a powerful apoptotic inducer in HL-60 and U937 cells, which scarcely express antiapoptotic genes. On the contrary, the antiapoptotic *bcl-2* and *bcr-abl* genes, widely expressed in DoHH2 and K562 cells, respectively, are concomitant with very low apoptosis triggering or to strong cell resistance, as reported by a number of authors²⁷⁻³⁰⁾. One of the reported functions of Bcl-2 is inhibition of the activation of ICE-like cysteine proteases such as caspase 3, which is responsible for PARP cleavage³¹⁾. Furlong *et al.* demonstrated that intracellular acidification triggered apoptosis by directly or indirectly activating ICE-like proteases, while Bcl-2 overexpression delayed intracellular acidification and inhibited ICE-like protease activation³²⁾.

(b) *Bax and the Bcl-2-independent pathway*

On the other hand, Takasu *et al.* suggested the existence of apoptotic pathways independent of Bcl-2³³⁾. They observed the cleavage of poly (ADP-ribose) polymerase (PARP) in HL-60 cells after heating, particularly in those cells heated in a medium of pH 6.6, despite the lack of change in the expression of the Bcl-2 and Bax proteins. The optimal pH for PARP cleavage by caspase 3 is pH 6.5-7.0³⁴⁾. It is therefore probable that heating HL-60 cells in a pH 6.6 environment directly increased the proteolytic cleavage of PARP mediated by caspases without the involvement of upstream factors, *i.e.*, Bcl-2 and Bax, and triggered the downstream reaction, *e.g.*, fragmentation of DNA. Cleavage of PARP may lead to activation of the endonuclease responsible for DNA fragmentation in apoptosis³⁵⁾. It is also probable that the acidic

environment activates the endonuclease itself³⁶⁾. Recently, Pena *et al.* suggested that environmental stresses such as ionizing radiation, ultraviolet radiation, H₂O₂, and heat shock act directly on membranes and activate acid pH-dependent sphingomyelinase, generating ceramide and initiating signaling through a stress-activated protein kinase/c-jun-NH2-kinase pathway, leading to the activation of ICE/CED-3 proteases³⁷⁾. Therefore, the increase in heat-induced PARP cleavage in the pH 6.6 environment in their study may be attributed to the synergistic effect of acidic stress and heat shock activating acid pH-dependent sphingomyelinase, thereby activating ICE/CED-3 proteases. A temporal relationship between protein phosphatase, ICE/CED-3 proteases, intracellular acidification, and DNA fragmentation in apoptosis has been also proposed^{38) 39)}.

Katschinski *et al.* provided evidence for an autocrine effect of endogenously produced TNF- α that was responsible for heat-induced apoptosis in non-differentiated PLB-985 cells⁴⁰⁾. The ability to undergo heat-induced apoptosis was lost in neutrophilic-differentiated PLB-985 cells, which appeared to be related to the lack of heat-inducible TNF- α expression in differentiated cells. TNF- α -dependent apoptosis in nondifferentiated PLB-985 cells was mediated via the p55 TNF- α receptor. The expressions of Bcl-2 and Bax did not change after heat exposure, which implies that the heat-induced apoptotic process is independent of Bcl-2 or Bax. Heat-induced TNF- α mRNA expression and apoptosis were prevented by the addition of PDTC, which is able to block nuclear factor κ B activity by inhibition of reactive oxygen species formation. It is tempting to speculate that reactive oxygen intermediates are involved in the induction of TNF- α expression by heat. In general, TNF- α -induced effects are mediated by the p55 and p75 TNF receptors. Only the p55 receptor contains the intracellular death domain, which is critically important for recruiting several proteins involved in the TNF- α -mediated apoptotic program^{41) 42)}. Previous studies have shown that similar TNF- α -mediated self-inflicted apoptotic pathways play a significant role as negative feedback mechanisms which regulate the natural killer cell immune response after maximal stimulation with interleukin 12 and interleukin 15^{43) 44)}. Autocrine suicidal effects have also been demonstrated for other apoptotic death factors, such as the FAS ligand⁴⁵⁾.

(c) *Reactive oxygen species (ROS) and HT-induced apoptosis*

It has been repeatedly shown that HT can induce oxidative stress in tissues because of increased production of ROS and/or promotion of cellular oxidation events⁴⁶⁻⁵⁰⁾. Such an increased formation of ROS during HT can become a further major mechanism of cell injury and could in principle be exploited in hyperthermic cancer treatments⁴⁶⁾. ROS are in fact cytotoxic and have been shown to kill cells via apoptosis⁵¹⁾. In liver tissue exposed to hyperthermic perfusion, several markers of oxidative injury have determined, including the oxidation and depletion of reduced glutathione⁴⁸⁾ and the appearance of aldehydic products of lipid peroxidation^{49) 50)}.

In some studies, the activation of tissue xanthine dehydrogenase to its oxidase form has been implicated as a major mechanism in the oxidative stress caused by HT^{46) 47)}. Indeed, HT has been shown to induce xanthine oxidase (XO) activation^{46) 47)}, and it is known that the interaction of XO with its substrate (hypoxanthine) is associated with the generation of a superoxide anion as a by-product. XO⁵²⁾ and hypoxanthine concentrations⁵³⁾ are elevated in tumor tissues because of impaired energy metabolism leading to accumulation of ADP, as well as to degradation of nucleic acids after cell death, thus prompting the conditions for the production of significant amounts of superoxide during the XO reaction. The

superoxide dismutation product, hydrogen peroxide, is a well-established apoptotic agent⁵¹⁾. On the other hand, the reaction of reactive oxygen species with low molecular weight iron complexes could easily result in the onset of lipid peroxidation⁵⁴⁾, and indeed the release of such iron complexes is likely to occur during disaggregation of dead cells.

Autologous bone-marrow transplantation (ABMT) is widely used in the treatment of acute leukemias when a matched sibling donor is not available for allogeneic transplantation. In addition, there is no age restriction to its application, and morbidity and mortality are greatly reduced by the lack immunobiological effects of allogeneic grafts. A major drawback in the use of ABMT is the possibility that tumor cells re-infused with autologous marrow may contribute to a relapse⁵⁵⁾. Thus, *ex vivo* purging may be very important in preventing relapses after transplantation⁵⁵⁻⁵⁷⁾. A growing body of data indicates that HT could be used to purge residual leukemic progenitors from remission bone marrow or peripheral blood before transplantation^{58) 59)}, since leukemic progenitors are much more sensitive than normal hematopoietic progenitors to HT⁶⁰⁻⁶²⁾. However, the results obtained by HT alone have been only partially satisfactory. Consequently, attention has focused on the search for substances able to sensitize tumor cells to the damage caused by HT, without reducing the capacity of normal hematopoietic progenitors to reconstitute hemopoiesis^{63) 64)}.

2. Sensitization of HT-induced apoptosis by an inhibitor of HSP expression

It has been shown that the expression of human HSP-70 confers heat resistance to leukemic cells and solid tumors, suggesting that HSP-70 has a protective function against thermal stress^{65) 66)}. Furthermore, overexpression of the human *hsp-70* gene reduces, whereas expression of the human *c-myc* proto-oncogene enhances, heat-induced apoptosis⁶⁷⁾. Thus, substances able to inhibit the expression of HSP-70 could be important in enhancing the killing effect of HT.

Kim *et al.* reported that quercetin, one of the most widely distributed bioflavonoids in the plant kingdom, acted as an HT sensitizer in HeLa cells by inducing intracellular lactate accumulation and acidification⁶⁸⁾. Osman *et al.* also showed that quercetin significantly synergized the killing effect of HT on K562 and KU812 leukemic cell lines⁶⁹⁾. Moreover, quercetin is able to inhibit the heat-dependent overexpression of HSP-70 in myeloid- and lymphoid-leukemia cell lines, and to increase the number of leukemic cells undergoing apoptosis^{65) 70)}.

According to the observations of Chant *et al.*⁷¹⁾, acute myeloid leukemia (AML) blasts constitutively express varying amounts of cytoplasmic HSP-70, while acute lymphoid leukemia (ALL) blasts express barely detectable levels. Larocca *et al.* found that quercetin inhibited the heat-induced expression of HSP-70 in AML and ALL blasts, both at the protein and mRNA levels⁴⁾. Kinetic experiments revealed that quercetin inhibited heat-induced HSP-70, that this inhibitory effect appeared rapidly, and persisted for up to 24 h after treatment. Furthermore, there was good temporal correlation between the appearance of quercetin's inhibitory effect on heat-induced HSP-70 expression and the cell-killing action of the combined treatment with quercetin and HT.

Normal bone marrow or peripheral blood CD34+ cells did not express HSP-70 constitutively, and thermic shock induced large amounts of this protein in these cells. The heat-dependent up-regulation of HSP-70 in CD34+ cells was not inhibited by the presence of quercetin during HT. On the other hand,

these normal hematopoietic progenitors were resistant to the growth-inhibitory effect of quercetin⁷²⁾. The resistance of hematopoietic progenitors to combined treatment with HT and quercetin was probably due to the protective effect of HSP-70 not being modified by quercetin. As a consequence, heat-dependent apoptosis was much less marked in CD34+ cells than in leukemic blasts or more committed hemopoietic cells. In addition, the LTC-IC (long-term culture-initiating cell) population, representing the most primitive sub-set of hematopoietic progenitors⁷³⁾, appeared totally resistant to combined treatment with HT and quercetin. Unlike normal CD34+ cells, CD34+ leukemic blasts were sensitive to the combined treatment in terms of inhibition both of HSP-70 expression, and of clonogenic capacity. This difference in sensitivity between normal and leukemic progenitors could be a useful biological tool to define an optimal purging protocol that would eliminate leukemic progenitors while preserving sufficient normal hematopoietic progenitors to permit long-term recovery.

3. Sensitization of HT-induced apoptosis by free radical initiators

Recently, we found that 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), which is a temperature-dependent free radical generator^{74) 75)}, enhanced the apoptosis induced by HT in U937 lymphoma cells. The effects of HT on both lipid peroxidation (LPO), which was measured by the level of 2-thiobarbituric acid and malondialdehyde, and intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), were enhanced markedly by the combination with AAPH. HT combined with AAPH also led to loss of mitochondrial membrane potential ($\Delta\Psi_m$), indicating the function of the mitochondria was also damaged during this treatment. Although an intracellular Ca^{2+} ion chelator, BAPTA-AM, completely inhibited the DNA fragmentation induced by the combined treatment, a water-soluble vitamin E, Trolox, only partially suppressed DNA fragmentation and the increase in $[\text{Ca}^{2+}]_i$. In contrast, LPO was inhibited completely by Trolox, but no inhibition by BAPTA-AM was found. Of considerable interest is that addition of antioxidants could not effectively inhibit loss of $\Delta\Psi_m$, indicating LPO and loss of $\Delta\Psi_m$ appear to be independent features (Fig. 1). These results indicated the increase in $[\text{Ca}^{2+}]_i$ is the key event in the apoptosis of U937 cells induced by HT with AAPH, though LPO plays a partial role in this apoptosis.

Fig. 1. Schema of hyperthermia-induced apoptosis sensitization by AAPH in U937 cells.

The relationship between loss of $\Delta\Psi_m$ and the increase in $[Ca^{2+}]_i$ remains elusive. $\Delta\Psi_m$ represents the electrochemical forces used for ATP synthesis, as well as for other metabolic activities, including the maintenance of Ca^{2+} homeostasis within the mitochondria⁷⁶⁾. Due to the energy requirements of the different Ca^{2+} transport systems that control Ca^{2+} homeostasis, the loss of $\Delta\Psi_m$ and subsequent ATP depletion invariably result in the disruption of intracellular Ca^{2+} homeostasis, potentially triggering a series of Ca^{2+} -dependent events that ultimately lead to cell death.

4. Sensitization of HT-induced apoptosis by inhibitors of Ca^{2+} metabolism

Verapamil is pharmacologically classified as a calcium channel blocker, and has been commonly used to treat arrhythmia and angina pectoris. Verapamil also has other pharmacological actions, such as reversing multiple-drug resistance for vincristine⁷⁷⁾ and other anticancer agents⁷⁸⁾, and suppressing metastasis of cancer cells in BALB/c mice injected with mouse mammary adenocarcinoma F3II cells⁷⁹⁾. Additionally, Coss et al.⁸⁰⁾ found that verapamil acts as a hyperthermic sensitizer in Chinese hamster ovary cells exposed to HT.

We observed that combined treatment with nontoxic verapamil enhanced the DNA fragmentation and increase in $[Ca^{2+}]_i$ induced by HT in U937 cells (unpublished data). This enhancement was observed in both normal and thermotolerant U937 cells. The number of cells containing higher $[Ca^{2+}]_i$ (more than 200 nM) was significantly increased by HT, and further elevated by the addition of verapamil in both normal and thermotolerant cells. Moreover, when the intracellular Ca^{2+} chelator, BAPTA-AM, was used, it inhibited DNA fragmentation in a dose-dependent-fashion. These results indicate that $[Ca^{2+}]_i$ plays a major role in the regulation of prompt apoptosis induced by HT in U937 cells. Regulation of apoptosis by $[Ca^{2+}]_i$ appears to be universal for HT-treated cells.

Conclusion

The clinical application of HT has a strong biological rationale. Further elucidation of the molecular mechanisms responsible for the enhancement of heat-induced apoptotic cell death may provide a rational basis for the effective use of HT and HT-induced apoptosis sensitizers in cancer therapy.

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温熱誘発アポトーシスの化学的増感

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要 旨：温熱療法は癌治療において副作用が少なくかつ効率的な治療法となりうる可能性を秘めているが、現状の治療成績は必ずしも満足のものではない。それゆえ、温熱によって引き起こされる腫瘍細胞の損傷を増感する物質の探索が注目されはじめている。温熱による腫瘍細胞の死はアポトーシスが原因であるという報告が数多くなされている。細胞内の特定の情報伝達系が活性化されることによってアポトーシスは誘導されるが、その伝達経路は細胞種によって異なっている。もし、すべての腫瘍細胞にアポトーシスを誘導することのできる共通の経路をみつけだすことができれば、あらゆる癌に対抗できる新たな治療法が確立されるであろう。本論文では、現在までに解明されている温熱誘発アポトーシスの機序および温熱誘発アポトーシスを増強する物質について概観する。また、「すべての腫瘍細胞にアポトーシスを誘導することのできる共通の経路」の候補のひとつとしてわれわれが注目している“細胞内Ca²⁺”に焦点をあて、温熱誘発アポトーシスにはたすその役割についての最近の結果を報告する。