Modification of thermal effects • T. Takahashi

Review

Effects of Hyperthermia and Modification of Thermosensitivity

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Abstract: Hyperthermia is considered to be a treatment capable of a direct cytocidal effect, following 3 primary modalities for cancer treatment: surgery, radiotherapy, and chemotherapy. Usually, success in raising a tumor’s temperature to 43°C or above produces a strong anticancer effect both in vitro and in vivo, and a change of the inactivation energy of cells occurs at temperatures above 43°C. Although the cytocidal effect of mild hyperthermia (below 43°C) is negligible, such a treatment often appears to enhance the sensitivity of tumors to anticancer drugs, cytokines, and low-dose-rate irradiation. It has also been reported that mild hyperthermia, even when applied locally, increases immunological competence and enhances cytocidal effects via immunological responses. Heat shock proteins (HSPs), are molecular chaperones believed to be involved in thermoreistance events during hyperthermia, and also involved in the enhancement of immunological competence. Although immunotherapy and hyperthermia have fallen short of expectations, these two approaches, when combined appropriately, may provide synergistic treatments more effective than expected of either alone. The mechanism of hyperthermia action is also being studied at the genetic level, and the possibility of molecule-targeted treatments coupled with hyperthermia aimed at specific target genes is being studied. In this report, the present state of these approaches is described.

Key Words: mild hyperthermia, thermosensitivity, immune system, chemotherapy, radiation

Introduction

There have been a number of reports that hyperthermia at 43°C or above produces marked cytocidal effects, and that the direct anticancer effect of mild hyperthermia at less than 43°C is limited, but can enhance the sensitivity of tumors to anticancer drugs and cytokines. The effectiveness of mild hyperthermia in enhancing immunological competence is also being studied. This paper discusses the therapeutic mechanism of hyperthermia, problems of thermoresistance (particularly those related to HSPs), the mechanism involved in the enhancement of immunity, and effects of the concurrent use of hyperthermia with anticancer agents and radiation. Genetic approaches are also briefly discussed.

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Mechanism of hyperthermia

Hyperthermia, which can produce a strong cytocidal effect after heating at 43°C or above, is regarded as a non-surgical anticancer therapy following radiotherapy and chemotherapy. Details of the cytocidal effects of hyperthermia are not entirely clear, but these effects are not considered to be derived primarily from DNA double-strand breakage which leads to cell death after exposure to radiation. Various mechanisms, some of which target the cell membrane as well as nuclear DNA, have been suggested to be involved through intricate interactions. The heating of cells in culture shows a temperature- and time-dependent cytocidal effect, and the cell survival rate decreases rapidly above 42.5°C\(^1\). Usually, thermosensitivity subsequent to heating at less than 43°C is low. Arrhenius plots represent the activation energies of chemical reactions related to cell death, and these are obtained by determining D\(_9\) (h) from the cell survival curves after exposure to hyperthermia, and regraphing this in the form of 1/D\(_9\) (y-axis) and 1/T (horizontal axis)\(^1\). In such plots, a break point is observed around 43°C. Apoptosis and necrosis are both considered important in cell death mechanisms following hyperthermia as well as radiation\(^2,3\). Furthermore, there have been reports that apoptosis plays a major role at temperatures above 43°C, and that necrosis is more important than apoptosis at high temperatures.

Mechanism of thermoresistance and the enhancement of immunological responses

The development of thermoresistance after heating is a frequent problem with hyperthermia, and the induction of heat shock proteins (HSPs) in response to heating is thought to be involved in the development of thermoresistance\(^6\). Studies with various types of cells have also shown correlations between thermoresistance and the level of intracellular HSP expression\(^6\).

HSP expression increases in stressful environments. HSPs are considered to be molecular chaperones and are involved in the repair of defective proteins, and in the synthesis, transfer, and degradation of proteins. HSPs also protect cells from heat-induced apoptosis\(^6\). Clinically, an interval of 48 hours or longer is necessary between repeated hyperthermia exposures in order to avoid HSP induced thermoresistance\(^7\).

A recent report concerns the role of HSPs from a perspective different from the protection of cells from death: HSP70 and HSP90 are involved in immunological competence\(^6\). Yanase et al. noted that the expression of HSP70 enhanced anti-tumor immunity\(^6\). HSPs transfer antigen peptides and facilitate peptide presentation to CD8+ T cells\(^9\). Antigen presentation by antigen-presenting cells is an important step in generating an immune reaction with a sufficiently strong anticancer effect to occur, and the use of cytokines such as GM-CSF is useful in this situation\(^10\). When HSP binds to such cytokines, a CTL reaction is induced\(^12\). Thus, the HSP-peptide complex not only presents tumor-derived peptide antigens to dendritic cells but also provides immune cells with maturation signals. HSP-like proteins extracted from melanoma cells have demonstrated a vaccine-like reaction\(^13\). Tanaka et al. reported that dendritic cells activated by hyperthermia induced an increase in cytotoxic T cells and the enhancement of natural killer (NK) activity, resulting in high anticancer activity\(^14\). Terunuma et al.\(^15\) also reported that NK cell activity increased and the numbers of CD4+ T cells was markedly reduced by mild regional hyperthermia, and that both, the innate and adaptive immune reactions were activated. Therefore, mild hyperthermia (<43°C) may exert a lethal effect on tumor cells by activating immune reactions, although
its direct cytocidal effect on cancer cells is unremarkable.

Mukhopadhyay et al.\textsuperscript{19} reported \textit{in vivo} results indicating the effectiveness of a combination of localized hyperthermia and immunotherapy against prostate cancer. Heated cancer cells release HSPs, which prompts antigen presentation to dendritic cells (Fig. 1). They also reported that the administration of GM-CSF in addition to dendritic cells into the tumor, when combined with regional hyperthermia, markedly suppressed tumor growth. Since tumor antigens, which are usually undetectable, become detectable \textit{via} the action of HSPs obtained in response to hyperthermia, individualized vaccination therapies for cancer patients are theoretically possible\textsuperscript{17}. The clinical validation of such autologous HSP vaccines has commenced, and preliminary data have begun to be reported. Data suggesting that a combination of regional hyperthermia and immunotherapy may also be effective against non-immunogenic tumors are noteworthy, and advances in research in this field are awaited. This approach could theoretically become an effective local therapy against cancer recurrence after surgical treatment or radiotherapy.

To perform effective hyperthermia-immunotherapy, the clinical application of new heating technologies is anticipated, such as a targeting therapy for intracellular heating using magnetized microparticles, rather than the conventional heating methods (RF waves or microwaves). Cationic liposomes encapsulating magnetic particles accumulate efficiently in tumor tissues, and inductive heating then makes \textit{in vivo} cancer-selective hyperthermia possible. Recently, the relationship between the surface potential of liposomes and their antigenicity-enhancing effect in anticancer immunity has been evaluated, and cationic liposomes have been confirmed to induce antigen-specific immune responses\textsuperscript{19}. Many immunocompetent cells gather in tumors after hyperthermia. This may suggest that the antigenicity-enhancing effect of cationic liposomes is involved in the phagocytosis of tumor antigens by antigen-presenting cells such as macrophages. Anticancer therapy in which the two mechanisms of

\textbf{Fig. 1.} Mechanism of induction of antitumor immune response. HSPs play an important role in carrying tumor antigens and activating antigen-presenting cells.
regional hyperthermia and enhancement of immunity are effectively combined may become a clinical reality.

**Combination of anticancer drugs and mild hyperthermia**

Results of *in vitro* experiments to study the effects of various combinations of anticancer drugs and hyperthermia have been reported. The reported enhancement of thermosensitivity at 43°C or above is particularly noteworthy. *In vitro* experiments have demonstrated that the synergistic enhancement of cytocidal effects was observed when bleomycin or adriamycin was administered during hyperthermia at 42-43°C. Based on data from *in vitro* studies, the activation energies obtained from Arrhenius plots for cisplatin, bleomycin, and 5-fluorouracil used with mild hyperthermia below 41°C were found to be significantly smaller than for those above 41°C. Also, clear *in vivo* enhancement of the effects of cyclophosphamide, ifosfamide, and cisplatin after mild heating have been observed. While the increase in sensitivity to bleomycin may be related to repair mechanisms, that to adriamycin has been reported to be associated with an increase in its uptake by cells. Mild hyperthermia is reported to enhance the sensitivity to cisplatin due to an increase in DNA-cross-linking and a decrease in the efficiency of DNA repair mechanisms. Meyn et al. suggested that an increase in DNA-cross-linking at 43°C or above is the primary mechanism of the sensitivity enhancement.

We have observed a clear increase in sensitivity below 43°C, but no increase in sensitivity at 43°C or above by a combination of pirarubicin and hyperthermia. In the Arrhenius plot, the break point for thermosensitivity disappeared after the administration of pirarubicin, i.e., mild hyperthermia at less than 43°C markedly increased chemosensitivity (Fig. 2). Furthermore, this study confirmed that this effect is not dependent on the degree of pirarubicin uptake by cells. Ono et al. reported that mild hyperthermia at 42°C or less enhanced the therapeutic effect of anticancer drugs *in vivo*.

Although hyperthermia treatments at less than 43°C are clinically practical, it is not expected that it will be possible to control tumors with hyperthermia alone. It may be more important to combine hyperthermia with other therapies such as chemotherapy and immunotherapy to enhance the cytocidal effect of other therapies combined with hyperthermia. **Combinations of hyperthermia and anticancer drugs** which are expected to markedly enhance

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the sensitivity of tumors to drugs at temperatures below 43°C are worth reevaluating following further improvements in heating technologies.

Recently, anticancer taxanes have been widely used in the clinic for the treatment of diseases, including lung cancer, with satisfactory results. Improvements in therapeutic results for non-small cell carcinoma of the lung are particularly notable. The combination of hyperthermia with these new anticancer drugs has been evaluated, and enhancement of sensitivity at 41.5°C has been reported. Furthermore, an enhancement of sensitivity to CPT-11, which plays a central role in chemotherapy for colon cancer, has been recently reported. In addition, a strong cytotoxic effect of combination therapy with CPT-11 and MMC with hyperthermia at 42.5°C was reported, and this combination may be useful for the treatment of conditions such as peritoneal dissemination.

**Combination of mild hyperthermia with cytokines**

The quantity of antigen expressed on the surface of the cell membrane can increase by heating tumor cells *in vitro*. Wong et al. noted that CEA, a typical tumor marker, showed an increase in the amount of its expression on the cell surface after heating. CEA peak expression, which was 2-3 times the baseline level, was observed 3 days after heating at 43°C for 1 hour. These results suggest, for example, that an accumulation of radiolabeled antibody in the tumor may be enhanced *in vivo* by heating due to an improvement in blood flow and an increase in tumor antigen expression. In another study, enhancement of the expression of CEA on the surface of lung cancer cells was observed *in vitro* 3 days after heating at 43°C. According to this report, however, this enhancement of expression decreased after heating at 44°C or above, and the specific effects of cell damage cannot be clearly distinguished at such temperatures. Also, the level of CEA expression on the surface of the cell membrane increased synergistically with the concomitant use of interferon-γ and hyperthermia, and its time of its expression occurred earlier than after hyperthermia alone (Fig. 3). This suggests that cytokines can modify the effects of hyperthermia depending on the quantity and pattern of antigen expression. There have been reports concerning tumor markers other than CEA: for example, the expression of the 45-kDa glycoprotein present on cell membranes of the colon cancer cell line LS180 increased 2.7 times 2 days after heating at 43°C for 1 hour. With the further development of local heating technologies to permit a more selective heating of tumors, an effective immunotherapy targeted to cell membrane antigens and their increased expression is expected to become possible.

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**Fig. 3.** Time-course of the modification of CEA expression (carcinoembryonic antigen) by heat combined with IFN-γ. GLL-1 cells were heated at 43 degrees C for 3 hours. Heat and IFN-γ modified the expression of CEA on GLL-1 cells and showed a synergistic effect.
**Combination of radiation and hyperthermia**

Cells are highly sensitive to radiation in the G2M phase of the cell cycle, and to hyperthermia in the late S phase\(^{30}\), so that a combination of radiation and hyperthermia would theoretically be expected to be complementary. A combination of hyperthermia and X-rays shows a synergistic cytoidal effect exceeding the sum of the cytoidal effects of the two individual therapies in experiments using cells in culture. Also, although hyperthermia has been evaluated as a definitive treatment in combination with external beam radiation therapy for locally advanced tumors, the inability to achieve a high temperature uniformly within the tumor limits its application\(^{31}\). In addition, an effective combination therapy of hyperthermia and external beam radiation is difficult to design because of the occurrence of thermoresistance. Measures to clinically utilize the complementarity of the two therapies are still awaited. Tamaki et al. evaluated the combination effect of hyperthermia and radiation in cells with different radiosensitivities \textit{in vitro}, and found that enhancement of sensitivity was marked in radioresistant tumor cells\(^{32}\). This suggests the potential of hyperthermia against radioresistant tumors, but a more detailed evaluation of sensitivity-enhancing mechanisms other than p53 is necessary.

**Combination of radiation (low-dose-rate brachytherapy) and mild hyperthermia**

Sakurai et al. reported that an enhancement of sensitivity was observed \textit{in vitro} with a combination therapy of low-dose-rate irradiation and hyperthermia using \(^{137}\)Cs\(^ {33}\). They showed that a clear synergistic enhancement of cytoidal effects was observed after exposure to a combination of hyperthermia in a non-cytoidal temperature range (40-41.5°C) and low-dose-rate irradiation. In this situation, inhibition of the damage repair activity caused by low-dose-rate irradiation is thought to be the mechanism involved in sensitivity enhancement. This may also be related to the decrease in thermoresistance, which is usually observed in hyperthermia, by repeating hyperthermia applications with appropriate intervals between treatments. Clinically, hyperthermia was combined with low-dose-rate intraluminal irradiation against large cervical cancers, reportedly with satisfactory results. Raaphorst et al. also reported that mild hyperthermia enhanced the effectiveness of low-dose-rate irradiation\(^ {34}\). However, the combination of hyperthermia and low-dose-rate irradiation is performed less frequently today due to the increasing use of high-dose-rate brachytherapy using \(^{192}\)Ir.

**Recent findings concerning the mechanism of thermosensitivity and gene therapy**

Targets for thermotherapy probably include the cell membrane as well as the cell nucleus. Recent rapid developments in genetics have made it possible to evaluate biological phenomena through the detailed observations of gene and protein expression, attempts to learn more about the genetic mechanisms involved in thermosensitivity have begun. Hunt indicated that hyperthermia activated signaling pathways can overlap with pathways activated by ionizing radiation-induced DNA damage\(^ {35}\).

Roti Roti reported that heat shock (41-50°C) causes the unfolding of a number of nuclear proteins, and heat-induced changes in DNA replication complexes can be related to the killing of S-phase cells by heat\(^ {36}\). The reduced availability of the DNA repair protein MRE 11 for the repair of damaged DNA has been associated with thermal radiosensitization induced by mild hyperthermia\(^ {37}\). With future clarification of the target genes involved in hyperthermia, the possibility applying gene therapy methods
could also be examined. Onishi et al. reported that LY294002, which is a PI-3k inhibitor, inhibits anti-apoptosis signaling and is promising as a non-p53-dependent heat sensitizer\(^ {29}\). To date however, the target genes involved in hyperthermia, or the mechanism by which their function is only partially understood. During the past few years, many studies have been reported which focused on the relationship between p53 and thermosensitivity. Mitsuhashi et al. reported that NMT-1, which is a radiosensitive cell line expressing wild-type p53, is thermoresistant, but that radioresistant NMT-1R cells expressing mutant-type p53 were highly thermosensitive, showing an inverse relationship between radiosensitivity and thermosensitivity\(^ {29}\). This inverse relationship is interesting if it is observed universally, but, according to many other reports, cells in which wild-type p53 is functioning are highly thermosensitive\(^ {40}\). Moreover, in experiments performed under hypoxic conditions simulating an \textit{in vivo} environment, p53 accumulates, and apoptosis is readily induced in cancer cells expressing wild-type p53\(^ {41}\). Attempts to enhance thermosensitivity by artificially altering p53 from a mutant-type to wild-type phenotype have also been made: Onishi et al. reported that thermosensitivity can be changed \textit{in vitro} by treating cancer cells with Glycerol, which normalizes the function of mutant-type p53\(^ {42}\).

**Summary**

Hyperthermia, which can modify anticancer treatments and enhance immunological competence deserves researcher’s attention. \textit{In vitro} and \textit{in vivo} experimental results have not been readily reflected at the clinical level due to problems with heating technology, and clinical effects of hyperthermia have been unsatisfactory. However, with recent advances in research on heating technology, heat and microparticles, and at the level of single cells, progress in the molecular biological analysis of the mechanism of action of hyperthermia, and clarification of the mechanism involved in immunopotentiation, hyperthermia may well attract renewed clinical attention as a treatment for cancer.

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