Short-Term Hyperthermia Promotes the Sensitivity of MCF-7 Human Breast Cancer Cells to Paclitaxel

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As a physical adjuvant approach in the treatment of solid tumors, regional hyperthermia plays a synergistic role in enhancing the efficacy of simultaneous chemotherapy. Paclitaxel (PTX) is an anti-mitotic taxane drug that is widely used in chemotherapy for the treatment of various human malignancies such as lung, ovarian, breast, and head and neck cancers. Since the possibility that hyperthermia can enhance the antitumor effects of PTX has not yet been investigated, the present study was designed to evaluate the effects of short-term hyperthermia on PTX-induced antitumor activity in the human breast cancer line MCF-7. It was found that short-term hyperthermia promoted PTX-induced suppression of cell proliferation. The IC50 for PTX was reduced from 18.2 ± 1.0 to 15.0 ± 0.45 nm (p < 0.05). The level of PTX-induced cell apoptosis was increased from 8.5 ± 1.2 to 16.4 ± 2.4% (p < 0.05) and from 15.2 ± 1.4 to 34.9 ± 2.8% (p < 0.05), at the end of the first and second hyperthermia cycles, respectively; both the activity and expression of caspase-7 were enhanced. In addition, PTX-induced cell cycle arrest in the G2/M phase was further promoted by short-term hyperthermia, from 9.3 ± 0.7 to 12.5 ± 0.9% (p < 0.05). In contrast, short-term hyperthermia affected neither tumor cell migration nor invasion in the presence or absence of PTX. The presented data thus suggest that short-term hyperthermia may serve as a feasible approach in the promotion of breast cancer cell sensitivity to PTX.

Key words hyperthermia; paclitaxel; apoptosis; caspase-7; breast cancer

Epidemiological studies have revealed that breast cancer is not only the most frequently diagnosed cancer, but also the primary cause of cancer death among females, accounting for 23% of the total cancer burden and 14% of cancer deaths. In the United States, 230,480 new cases of invasive breast cancer and 39,520 deaths caused by breast cancer were predicted in 2011. Paclitaxel (PTX) has been used widely as a first-line chemotherapy drug for the treatment of breast cancer. A previous prospective study indicated that the antitumor activity of single-agent PTX was comparable to that of the three-drug combination of fluorouracil, doxorubicin and cyclophosphamide in patients with breast cancer. In addition, the combination of doxorubicin and PTX has been found to lead to superior overall response rates and a prolonged median time to treatment failure, as compared with sequential single-agent therapy. However, even though the addition of four cycles of PTX treatment to a standard combination program of cyclophosphamide with doxorubicin has been demonstrated to improve disease-free survival in postoperative patients with node-positive primary breast cancer, the combined treatment with PTX has been shown more recently not to improve overall survival. In addition, the side effects caused by the accumulation of PTX, such as peripheral neuropathy, myalgias and arthralgias, degrade patient quality of life. New approaches for amplifying the anti-tumor effects of PTX are being explored. The monoclonal antibody against epidermal growth factor receptor (EGFR) has been shown to improve the outcome of HER2-positive patients treated with PTX, and the monoclonal antibody against vascular endothelial growth factor (VEGF) has also been investigated. Nevertheless, for some breast cancer patients such as HER-negative patients who are being treated with PTX, not only to prolong their survival, but also to improve their quality of life, other effective and implementable approaches for promoting the efficacy of PTX with acceptable toxicity still need to be developed.

The delay of breast tumor growth in vivo caused by whole-body hyperthermia at 39.8°C has been reported. In addition to a direct effect, the enhancement of the cytotoxicity of chemotherapeutic agents at around 41°C has also been well characterized. The purpose of the present study was to determine the effects of short-term hyperthermia at 41°C for 2 h on PTX-induced anti-tumor activity using human breast cancer MCF-7 cells. The safety of the protocol for thermo treatment for hyperthermia used in this study was also evaluated.

MATERIALS AND METHODS

Cell Culture and Temperature Manipulation Human breast cancer cell line MCF-7 was maintained in our lab. Cells were routinely cultured at 37°C under 5% CO2 in Roswell Park Memorial Institute (RPMI) medium 1640 (Invitrogen Corp., Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (Invitrogen Corp.). The thermo treatment for cells was manipulated and monitored using a UHR-2000 microwave beam thermo device (Huayuan Medical Equipment Co., Ltd., Hunan, China). The cells grown on standard culture plates were subjected to thermo treatment by microwave emitters in a chamber of dimensions 16×16×5 cm3, into which 5% CO2 was pumped. The temperature inside the chamber was manipulated by modulating the microwave intensity according to the temperature, which was measured and recorded...
simultaneously. During the period of thermo treatment, temperature fluctuation was less than 0.2°C. The cells subjected to thermo treatment were maintained at 41°C for 2 h, followed by a temperature drop to 37°C, and were cultured routinely for 22 h, which constituted a standard short-term hyperthermia cycle.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay  Cell proliferation was determined using the MTT cell proliferation assay kit (Invitrogen Corp.). MCF-7 cells were seeded at a density of 5×10³ cells per well onto a 96-well plate. They were cultured in medium free of phenol red for 24 h before thermo treatment in the presence or absence of PTX (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.). The cell proliferation assay was performed according to the manufacturer’s protocol. Cell number was determined as the absorbance at 570 nm by a spectrophotometer. The results of the cell proliferation assay were expressed as the optical density (OD) values or the inhibition rates.

Cell Apoptosis Assay  Cell apoptosis was investigated using an Annexin V cell apoptosis assay kit (Keygen Inc., Nanjing, China). In brief, the cells grown on 6-well plates in each experimental group were subjected to respective treatment and then harvested. The cells were washed with phosphate buffered saline (PBS) solution and resuspended in Binding Buffer, preincubated with Annexin V-FITC and PI in the dark for 10 min. The fluorescence was detected by means of flow cytometry (Ex=488 nm; Em=530 nm). The status of the cells that were Annexin V-FITC-positive and PI-negative was defined as early apoptosis, and that of cells that were Annexin V-FITC-positive and PI-positive was considered to be late apoptosis. Cell apoptosis was estimated quantitatively by comparing the apoptosis rate in each experimental group, which was presented as the percentage of the apoptotic cells present.

Caspase Activity Assays and Western Blot  The activities of various caspases (caspase-7, 8 and 9) were elucidated separately using Homogeneous Caspase Assay Kits (Promega Corp., Madison, WI, U.S.A.). Cell lysates collected from each experimental group were used for caspase activity assays. Assays were performed on 96-well plates according to the manufacturer’s instructions; the activities were determined quantitatively as the fluorescence from each well. Pro- and cleaved caspase-7 expression was detected using Western blot (anti-pro-caspase-7 and anti-cleaved caspase-7 antibodies, both were 1:200 dilution, Abcam Corp., Cambridge, U.K.), according to the standard protocol; β-actin expression was also determined as internal controls (anti-β-actin antibody, 1:500 dilution, Abcam Corp.). Fifty micrograms of protein was loaded at per lane for the blotting. The blots were visualized using enhanced chemiluminescence detection (Pierce, Rockford, IL, U.S.A.).

Cell Cycle Analysis  The cell cycle stage was determined by means of flow cytometry using cell cycle assay kits (Keygen Inc.). Briefly, cellular DNA content was determined after PI staining. Cells were classified as being in the G0/G1, G2/M and S phases of the cell cycle based on fluorescence intensity, and the cell cycle distribution was analyzed using Cell Fit software (BD Biosciences, Franklin Lakes, NJ, U.S.A.).

Wound Healing  Wound healing assays were conducted as described previously. Cells were seeded onto 6-well plates to form a confluent monolayer. The wound was made by scraping a pipette tip across the monolayer and RPMI containing 20% serum was used to induce cell migration; three randomly selected fields were photographed. The selected fields were photographed again 24 h later and cell migration was quantified by evaluating the cell-covered area (%) in each field using quantitative wound healing image analysis software (Ibidi Inc., Martinsried, Germany).

Cell Tracking  The cell tracking assay was performed as described previously. MCF-7 cells were cultured in 35 mm dishes. At 12 h prior to track recording, cells were incubated with serum-free medium. RPMI medium containing 20% serum was added to induce cell migration, and the cells were maintained in a chamber supplied with 5% CO₂ throughout the experiment, in which the temperature could also be adjusted as needed (Biostation, Nikon Corp., Tokyo, Japan). Migrating cells were monitored for 6 h in randomly selected fields with a phase contrast microscope. The migration distances were measured using Image J software (NIH, U.S.A.).

Chamber Cell Invasion Assay  Cell invasion ability was investigated using a BD Matrigel Invasion Chamber (8 µm pore size; matrigel-coated; BD Biosciences). 1×10⁴ MCF-7 cells suspended in serum-free RPMI medium were added to each upper chamber, and the chamber was placed in 24-well dishes containing RPMI with 20% serum. Invasion assays were carried out for 24 h and cells were fixed with 3.7% formaldehyde and then stained with crystal violet staining solution, and the cells on the upper side of the insert were removed with a cotton swab. Three randomly selected fields were photographed, and the numbers of cells that crossed the membranes were counted. The level of invasion was evaluated as the average number of transmigrated cells in a field.

Statistics  All data were presented as the mean±S.D. The concentration-response data were fitted and IC₅₀ and Hill coefficient values were obtained using SigmaPlot 10.0 (Systat Software Inc., Munich, Germany). Student’s t-test was used for statistical analyses of the cell proliferation, ANOVA for the cell apoptosis rate, wound healing, cell tracking, and chamber cell invasion assays, and two way ANOVA for the caspase activity and cell cycle assays. Statistical analysis was conducted using Graph Pad Prism 5.0 (GraphPad Software Inc., San Diego, CA, U.S.A.). Statistical significance was assumed when p values were <0.05.

RESULTS

Enhancement of PTX-Induced Suppression of Proliferation in MCF-7 Cells by Short-Term Thermo Treatment  To investigate the effect of hyperthermia on the antineoplastic activity of PTX, we first performed the MTT assay to assess the proliferation rate of MCF-7 cells. In the absence of PTX, hyperthermia did not affect MCF-7 cell proliferation obviously. On the contrary, in the presence of 10 µM PTX there was significant suppression (p<0.05) of proliferation in MCF-7 cells at the end of second hyperthermia cycle, as compared with that in the PTX-alone treatment group (Fig. 1A). We further determined the rate of inhibition of proliferation within a range of concentrations of PTX from 2 to 100 nM, without or combined with two standard hyperthermia cycles. The thermo treatment failed to increase the maximal inhibition rate of cell proliferation. However, the IC₅₀ for PTX was significantly decreased by thermo treatment from 18.2±1.0 to...
out of all the doses that we used, PTX at 12.5 nM induced apoptosis in MCF-7 cells. To evaluate this hypothesis, we hypothesized that short-term thermo treatment enhances the mechanism by which it produces its anti-tumor activity, we considered the suppression of proliferation by promoting PTX- induced apoptosis has been considered to be the primary way that it produces its anti-tumor activity. Caspases are a family of cysteine proteases that play essential roles in apoptosis. The activation of caspase-8 and 9 have been demonstrated to be activated by short-term thermo treatment and the hyperthermia-induced activation of these enzymes is responsible for the enhanced PTX-induced suppression of proliferation in MCF-7 cells.

Promotion of PTX-Induced Apoptosis in MCF-7 Cells by Short-Term Thermo Treatment Since the apoptosis induced by PTX has been considered to be the primary mechanism by which it produces its anti-tumor activity, we hypothesized that short-term thermo treatment enhances the PTX-induced suppression of proliferation by promoting PTX-induced apoptosis in MCF-7 cells. To evaluate this hypothesis, cell apoptosis analysis was conducted using flow cytometry. Out of all the doses that we used, PTX at 12.5 nM hyperthermia exhibited its most significant enhancement of the level of inhibition of cell proliferation (from 62.8±1.2 to 74.4±0.74%), as presented above (Fig. 1B). Therefore the 12.5 nM concentration of PTX was selected for further investigation. The cells collected at the end of the first or second hyperthermia cycle showed a significant increase in the rate of apoptosis (Figs. 2A, B). Our results also show that short-term thermo treatment had no effect on the degree of cell apoptosis in the absence of PTX. These data suggest that the promotion of PTX-induced apoptosis by hyperthermia may be responsible for the enhanced suppression of proliferation in MCF-7 cells.

Promotion of PTX-Stimulated Activation of Caspase-7 in MCF-7 Cells by Short-Term Thermo Treatment The promotion of PTX-induced apoptosis in MCF-7 cells by short-term thermo treatment has been demonstrated in the present study. However, the pathway mechanism through which hyperthermia produces this effect remains unclear and is yet to be clarified. Caspases are a family of cysteine proteases that play essential roles in apoptosis. The activation of caspase-8 and 9 are reportedly responsible for the hyperthermia-induced enhancement of apoptosis in breast cancer cells, and both caspase-7 and 9 have been demonstrated to be activated in PTX-treated MCF-7 cells. Therefore, the activities of caspase-7, 8 and 9 were investigated in the present study. Immunoassays showed that the activity of caspase-7 and 9 was enhanced by up to 5.1 and 4.7-fold, respectively, after treatment with PTX for 48 h (12.5 nM). However, only the activity of caspase-7 was further promoted to 8.3-fold after combination with two cycles of thermo treatment (Fig. 3A). PTX had no effect on the activity of caspase-8, either in the presence or absence of thermo treatment. Furthermore, we performed immuno-blotting to confirm the result obtained from the caspase activity assay. Consistently, immuno-blotting revealed an increased expression of cleaved caspase-7, the activated form of caspase-7, in the MCF-7 cells which had been subjected to PTX and thermo treatments (Fig. 3B). Taken together, our data indicate that short-term thermo treatment enhanced PTX-induced suppression of proliferation by promoting apoptosis through a caspase-7-related pathway in MCF-7 cells.

Promotion of PTX-Induced Cell Cycle Arrest in MCF-7 Cells by Short-Term Thermo Treatment In addition to elevation in the degree of apoptosis, the PTX-induced cell cycle arrest also contributed to the anti-tumor activity of PTX. In the present study, an increased proportion of the cells in the G2/M phase of the cell cycle was detected at 48 h after PTX (12.5 µM) treatment, which was further promoted by combination of this chemotherapy treatment with two cycles of short-term thermo treatment (p<0.05), as was observed by cell cycle analysis with a flow cytometry (Fig. 4). Cell cycle analysis has also shown that hyperthermia without PTX did not affect the cell cycle stage. These findings reveal that the promotion of cell arrest in the G2/M phase may also be responsible for the enhanced PTX-induced suppression of growth in MCF-7 cells by hyperthermia.

Effect of Short-Term Thermo Treatment on the Migration and Invasion of MCF-7 Cells Even though short-term thermo treatment had been demonstrated to promote the anti-tumor activities of PTX, as indicated by the MTT assay, and cell apoptosis and cell cycle analysis, the safety of the protocol for hyperthermia that we used in the present study remains to be evaluated. It is possible that some unexpected side effects may occur during the period of thermo treatment. In particular, increased temperature could enhance the motility
of tumor cells and promote their migration and invasion, thus increasing the risk of metastasis. Therefore, whether or not the thermo treatment can enhance the migration and invasion of MCF-7 cells has yet to be clarified. The wound healing, cell tracking and cell invasion assays were employed to address this issue. As shown by these assays, short-term thermo treatment did not significantly affect serum-induced migration or invasion in MCF-7 cells (Figs. 5A–C).

DISCUSSION

The aim of the present study was to investigate the effect of hyperthermia on PTX-induced anti-tumor activity in MCF-7 breast cancer cells. Our findings revealed that short-term hyperthermia promoted the sensitivity of MCF-7 cells to PTX by: (1) reducing the IC\textsubscript{50} for PTX from 18.2±1.0 to 15.0±0.45 nm (p<0.05); (2) promoting PTX-induced apoptosis through the activation of caspase-7; and (3) increasing the proportion of cells arrested in the G2/M phase by PTX.

The IC\textsubscript{50} value for PTX varies under different practical experimental conditions. The IC\textsubscript{50} value of PTX for the MCF-7 cell line reported in a previous study was lower than the value obtained in our study. This inconsistency may be due to the longer period of PTX treatment (72 h).\textsuperscript{17} In addition, a higher IC\textsubscript{50} value than ours has also been reported recently.\textsuperscript{18} At around the IC\textsubscript{50} value obtained in our study (10 to 25 nm), thermo treatment was found to significantly enhance PTX-induced suppression of proliferation in MCF-7 cells. The dose of PTX administered clinically is generally calculated using body-surface area. At the standard recommended dose of PTX of 37°C 24h

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**Fig. 2. Effect of Short-Term Thermo Treatment on PTX-Induced Apoptosis in MCF-7 Cells**

Cell apoptosis was determined by double labeling with Annexin V-FITC/PI. The MCF-7 cells subjected to the indicated treatment were harvested at 24 h (A) and 48 h (B), which are denoted by arrows. The representative results are shown and the summaries of cell apoptosis in each experimental group are expressed as the percentage of apoptotic cells (right panels). Data are expressed as the mean±S.D. of four independent experiments. *p<0.05 as compared with PTX-treated alone.
175 mg/m², a peak plasma concentration of unbound PTX of up to 0.35 ± 0.02 µm was detected. However, due to continuous plasma clearance, it is meaningless to compare the in vitro and in vivo exposure level to PTX by merely calculating its concentration. Comparatively speaking, regarding the dynamic duration of unbound PTX, the evaluation of the area under the plasma concentration–time curve (AUC) appears to be a more reasonable and precise approach. In fact, by measuring
the AUC, the theoretical exposure level to PTX used in the present study (300 or 600 nm × h) seems more appropriate for comparison with that used for in vivo study. However, further in vivo investigation remains to be carried out to verify the hypothesis that hyperthermia promotes PTX-induced suppression of breast cancer progression. In addition, the optimal dose of PTX, as calculated by measuring body-surface area, at which the synergistic effect of PTX combined with hyperthermia could be maximized needs to be established.

Inhibition of the growth of human breast tumor xenografts caused by whole-body hyperthermia (39.8°C for 6–8 h) has been ascribed to the enhancement of tumor cell apoptosis mediated by the thermo-stimulated immune response. However, we found thermo treatment alone could not affect the level of apoptosis in MCF-7 cells in our in vitro study (Figs. 2A, B). These findings may indicate that the anti-tumor activity caused by single hyperthermia was mainly due to activation of the immune system, but not to a direct tumor-killing effect. In a previous study, the activation of both caspase-8 and 9 was found to mediate the enhancement of tumor necrosis factor (TNF)-induced apoptosis in MCF-7 cells. However, the data presented in our study argues that short-term hyperthermia promotes PTX-induced apoptosis in MCF-7 cells through the activation of caspase-7, as shown in the caspase activity assays and subsequently confirmed using western blotting (Figs. 3A, B). This would be explainable by referring to the specific pathway through which PTX induces apoptosis in MCF-7 cells. Actually, PTX (12 nm for 48 h) had no significant effect on the activity of caspase-3 (Fig. 3A). It is worth noting here that although caspase-3 is considered to participate in PTX-induced apoptosis in tumor cells, the human breast cell line MCF-7 that we used here has been proven to be a caspase-3 defective cell line. In fact, caspase-3 and caspase-7 share similar substrate specificity, and the induction of apoptosis by anti-cancer agents through the activation of caspase-7 independent of caspase-3 has been documented. The limitation of conventional approach for hyperthermia is the uneven thermal effect, for thermal energy was conducted from a heat source, and therefore the healing effect maybe different depended on the distance from the heat source. One of the advantages of microwave heating used in the present study is a precise manipulation of temperature. In
theory, this pattern of healing resulting from electromagnetic oscillation of molecules can overcome the weakness of uneven healing effect. However, microwave is also capable of affecting hydrogen bonding, hydrophobic bonding and Van der Waals’ force, thereby changing the conformation and activity of protein. The involvement of these effects in the promotion of PTX-induced apoptosis is poorly understood, and still needed to be clarified in future study.

In contrast to various anti-microtubule chemotherapeutic drugs, PTX exerts its antigen-related pharmacological activity by promoting the formation of unusually stable microtubules. It also inhibits the normal dynamic recognition of the microtubular network, which is required for mitosis and cell proliferation. As was shown in cell cycle analysis (Fig. 4), short-term hyperthermia significantly increased the number of cells arrested in the G2/M phase after exposure to PTX. It is well-known that breast tumor cells in the G2/M of the cell cycle exhibit the greatest sensitivity to radiotherapy. Accordingly, a future study should be conducted to assess whether the combination of PTX and hyperthermia could further improve the efficacy of anti-tumor radiotherapy.

The safety of short-term hyperthermia was also preliminarily investigated in the current study. We intended to evaluate the potential side effects associated with the enhancement of tumor cell motility by thermo treatment, which may result in an increased risk of metastasis. Despite the negative results in this regard, as indicated by wound healing, cell tracking and cell invasion assays, the safety of hyperthermia remains to be further determined in vivo.

In the present study we provided evidence that short-term hyperthermia promotes the sensitivity of MCF-7 human breast cancer cells to PTX. The fact that combination with modified hyperthermia can enhance the anti-tumor efficacy of selected cytostatic drugs has not only been demonstrated in laboratory investigations,[12] but has also been supported by clinical studies.[24,25] Furthermore, hyperthermia treatment has the capacity to overcome tumor cell resistance to chemotherapy drugs, such new approaches are urgently required.[15,26] With future in-depth studies, the development of hyperthermia would bring novel insights to the strategies for anti-cancer therapy.

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REFERENCES

19) Smorenburg CH, Sparreboom A, Bontenbal M, Stoter G, Nooter K,


