Effect of Heating Prior to Gemcitabine Exposure on Therapeutic Outcomes in Combination Therapies

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Abstract : Using chemotherapy alone, it is difficult to cure patients with solid tumors. However, chemotherapy combined with heat treatment enhances cytotoxicity by improving drug delivery to tumor tissues, leading to improvements in the cure rate.

Nuclear factor-kappa B (NF- κ B) has been reported to be activated by chemotherapy in some cancer cell lines, and NF- κ B activation is one mechanism through which tumors can become resistant to chemotherapy. Heat treatment-induced heat shock protein 70 (Hsp70) was reported to inhibit I kappa B (I κ -B) kinase (IKK), resulting in the inhibition of NF- κ B activation. In view of this observation, it appeared to be possible that activation of NF- κ B in a pancreatic cell line might be inhibited by heat treatment, leading to an enhancement of gemcitabine-induced cytotoxicity. However, the timing of the heat treatment is also very important in producing chemosensitization when combined with chemotherapy.

In this review, the timing of a heat treatment in relation to a gemcitabine treatments is discussed, along with the mechanisms leading to sensitization in combination therapy.

Key Words : heat treatment, gemcitabine, human pancreatic cancer, apoptosis, NF- κ B

Introduction

Although a heat treatment can produce a temperature-dependent cytotoxicity to help cure cancer, its therapeutic efficacy is limited. Recently, chemotherapy has made impressive progress, and the outcomes of therapy for inoperable cancer and recurrent cancer have improved dramatically due to the development of new anti-cancer agents such as docetaxel, paclitaxel, irinotecan, oxaliplatin, and gemcitabine. There is evidence that the anti-cancer effects of these newly developed drugs can be enhanced by a heat treatment in *in vivo* and *in vitro* studies (Table I). However, successful clinical response rates to chemotherapy are no more than 50%, and this rate is usually approximately 20-30% currently. Furthermore a major problem is that the effect of chemotherapy is impaired by the development of resistance to drugs over several treatment cycles, and it is difficult to treat patients and minimize therapeutic side-effects. In the clinic, one of the major purposes of heat treatment is to enhance chemotherapeutic and radiation

therapeutic sensitivity. However, it is possible to overcome these problems by using chemotherapy combined with heat treatments.

In this review, the effects of chemotherapy are discussed, and in particular, the effects of gemcitabine (30 μ M) on human pancreatic cancer cell lines when combined with a heat treatment (43°C, 1 h). The effect of the timing of the heat treatment in relation to the timing of chemotherapy is also considered.

Table I. Heat sensitization in combination with chemotherapy

Author	Year	Exp. type	Timing of chemotherapy	Ref.
Docetaxel				
Rietbroek RC	1997	<i>in vitro</i>	just before hyperthermia	(12)
Dumontet C	1998	<i>in vitro</i>		(13)
Mohamed F	2003	<i>in vitro</i>	just before hyperthermia	(14)
Mohamed F	2004	<i>in vivo</i>	before hyperthermia	(15)
Paclitaxel				
Cividalli A	2000	<i>in vitro</i>	different times in relation to hyperthermia	(17)
Othman T	2001	<i>in vitro</i>	1 h before hyperthermia	(18)
Mohamed F	2003	<i>in vitro</i>		(14)
Michalakakis J	2005	<i>in vitro</i>	simultaneous	(19)
Irinotecan				
Katschinski DM	1999	<i>in vitro</i>	simultaneous	(22)
Mohamed F	2003	<i>in vitro</i>		(14)
Le Page S	2006	<i>in vitro</i>		(23)
Makizumi R	2008	<i>in vitro</i>	24 h after hyperthermia	(24)
Oxaliplatin				
Rietbroek RC	1997	<i>in vitro</i>	1 h after hyperthermia	(25)
Urano M	2002	<i>in vitro</i>		(26)
Mohamed F	2003	<i>in vitro</i>		(14)
Atallah D	2004	<i>in vitro</i>		(27)
Makizumi R	2008	<i>in vitro</i>	24 h after hyperthermia	(28)
Gemcitabine				
Haveman J	1995	<i>in vitro</i>	simultaneous	(34)
Van Bree C	1999	<i>in vitro</i>	48 h before hyperthermia	(35)
Mohamed F	2003	<i>in vitro</i>		(14)
Vertrees RA	2005	<i>in vitro</i>	24 h after hyperthermia	(36)

The advantage of a heat treatment plus chemotherapy

An extensive review of efforts to combine heat treatment with chemotherapy was published in 1995¹⁾. Thermal enhancement of drug cytotoxicity was accompanied by cell death without increasing oncogenic potentials. The induction of genetically defined stress responses can deliver signals to activate the host's immune system. The positive results of randomized trials clearly established that heat treatment in combination with chemotherapy is a novel clinical modality for the treatment of cancer^{2–4)}. Heat treatment targets the action of chemotherapy within heated tumor regions without affecting systemic

toxicity⁵⁾.

Improved drug delivery relies on the fact that local heating results in the perforation of tumor blood vessels, microconvection in the interstitium, and in the perforation of cancer cell membranes. In summary, heat treatment enhances drug delivery from the blood into cancer cells with minimal thermal and mechanical damage to normal tissues. In addition, heat treatment can render tumor cells temporarily more sensitive to the damaging effects of radiation or chemotherapeutics⁶⁾. Furthermore it has been shown that mitomycin C, nitrosoureas, cisplatin, doxorubicin and mitoxantrone, in addition to heat treatment and chemotherapy, can counteract drug resistance⁷⁾. It was also found that some newly developed drugs such as docetaxel, paclitaxel, irinotecan, oxaliplatin and gemcitabine lead to thermal sensitization.

It was concluded that the mechanism responsible for thermal sensitization was the enhancement of damage to cellular membranes, proteins and DNA, improvements in the rate of drug uptake into the cells^{8,9)}, and the inhibition of DNA repair^{10,11)}.

Antitubulin agents such as docetaxel are especially interesting agents to consider for use in combination with therapeutic heat treatments: this is because their intracellular target, the soluble tubulin/microtubule complex, is a highly temperature dependent structure, at least *in vitro*^{12–15)}. The rationale for the use of a heat treatment with docetaxel is based on two factors: a mild heat treatment causes disorganization in the microtubule system, and docetaxel is considered to be a microtubule stabilizing agent¹⁶⁾. Furthermore, Mohamed *et al.*¹⁵⁾ suggested that heat treatment must be applied soon after this drug is administered.

The antineoplastic agent paclitaxel (PTX) is known to arrest cell cycle progression and induce apoptosis. Therapy consisting of PTX in combination with heat treatment may be useful^{14,17–19)}. Othman *et al.*¹⁵⁾ reported that exposure of a mouse mammary cancer cell line to PTX alone caused an increase in the number of cells in the G₂/M phase, while PTX applied concurrently with a 43°C heat treatment caused an increase in the number of cells in the G₂/M and S phases, and both treatments resulted in a decrease in the number of cells in the G₀/G₁ phase of the cell cycle. On the basis of these results it can be concluded that the mechanism of PTX action at the cellular level is associated with disturbances in the cell division process. This may be attributed to the effect of PTX on the microtubule system which is a target for anti-cancer drugs^{20,21)}.

An effect of heat treatment on the active metabolite of irinotecan, SN38, has been reported^{14,22–24)}. Katschinski *et al.*²²⁾ suggested that there is a temperature-specific (*i.e.* 41.8°C) effect on SN-38 induced Topoisomerase (Topo) I DNA cross-linking, and that cytotoxicity resulted from temperature-dependent changes in Topo I catalytic activity. An active Topo I enzyme function is a key requirement for Topo I DNA cross-linking.

Platinum-DNA adduct formation caused by oxaliplatin is also enhanced by heat treatment^{14,25–28)}. Rietbroek *et al.*²⁵⁾ confirmed that heat treatments combined with platinum drugs such as cisplatin and oxaliplatin not only increased cytotoxicity, but also led to an increase in platinum-DNA adduct formation. Whether this is the mechanism responsible for the observed thermal enhancement of cytotoxicity cannot currently be answered: other mechanisms, such as an inhibition of DNA repair, might be involved²⁹⁾.

In the clinic, the timing of drug administration and heat treatment is usually simultaneous, but with gemcitabine, it was reported that a heat treatment enhanced the cytotoxicity of gemcitabine, especially when the heat treatment was performed 24 h before exposure to gemcitabine (Fig. 1). When AsPC-1 cells were untreated, the apoptotic cell death rate was 1.5%. However, 30 μ M gemcitabine increased the apoptotic cell death rate to 3.1%. Moreover, the apoptotic cell death rate increased to 4.8% when a heat treatment was delivered before exposure to gemcitabine. In the case of MIA PaCa-2 cells, a heat treatment delivered 24 h before an exposure to gemcitabine also enhanced apoptosis (data not shown).

Gemcitabine, a chemotherapeutic agent with proven efficacy in the treatment of lung cancer and pancreatic cancer, is a deoxycytidine nucleoside analogue that affects several enzymes involved in DNA synthesis and repair. Once transported into the cell, Gemcitabine (dFdCyd) must be phosphorylated in order to become activated. The triphosphate form, dFdCTP, can directly inhibit DNA synthesis or inhibit replication through the addition of dFdCMP into the new DNA strand. The diphosphate form, dFdCDP, inhibits the formation of deoxynucleoside triphosphates needed for DNA synthesis by blocking the activity of ribonucleotide reductase^{30,31}. Gemcitabine has been shown to cause cell cycle arrest in S-phase leading to apoptosis³², or to be incorporated in RNA thereby inducing apoptosis due to poisoning of topoisomerase I in lung cancer cells³³.

Several investigators compared the effect of gemcitabine and heat treatment administered either simultaneously or sequentially^{14,34–36}. Studies have confirmed the results of Haveman

Fig. 1. Heat treatment (HT) enhanced the cytotoxicity of gemcitabine (Gem). Human pancreatic cancer cell lines AsPC-1 and MIA PaCa-2 received a heat treatment (43°C, 1 h) in combination with a gemcitabine treatment (30 μ M) at various relative times. The solid bars represent controls (no gemcitabine), and the open bars represent 30 μ M gemcitabine. The time when heat was delivered relative to gemcitabine is indicated at the bottom of the figures. Cell viability was measured by the WST-8 assay. Values represent mean $\pm$ SEM of four samples of a representative experiment; similar results were obtained in three independent experiments. * P < 0.01, compared with the control in AsPC-1 cells (A). ** P < 0.005, compared with the control in MIA PaCa-2 cells (B).

*et al.*³⁴⁾, Van Bree *et al.*³⁵⁾ and Vertrees *et al.*³⁶⁾: when gemcitabine and heat treatment were administered simultaneously, there was a reduced cytotoxicity. Based on these studies and the fact that heat could inhibit the cytotoxic effects of dFdC-metabolites, the effect of different temporal delivery patterns of dFdC and heat treatments on malignant human pancreatic cell lines were studied.

Nuclear factor-kappa B (NF- κ B) and a heat treatment delivered prior to gemcitabine

When human pancreatic cancer cell lines were exposed to gemcitabine (30 μ M), gemcitabine increased NF- κ B binding activity in these cells and this effect was reduced by a heat treatment (Fig. 2).

Fig. 2. Effects of gemcitabine on NF- κ B activity. Gemcitabine-induced NF- κ B levels were decreased after a combination therapy with heat treatment. The binding activity of NF- κ B was assessed after a 12 h gemcitabine treatment or 24 h after a heat treatment. AsPC-1 cells were exposed to four kinds of stimuli. The results for no treatment (control), treatment with gemcitabine alone (30 μ M), treatment with heat alone (43°C, 1 h), and heat treatment combined with gemcitabine treatment are shown.

The level of proteins which are associated with NF- κ B was examined with western blotting in AsPC-1 and MIAPaCa-2 cells. Increasing levels of Hsp70 were induced by heat treatment, and this effect was further increased by a combination of gemcitabine and heat treatment in MIAPaCa-2 cells. In contrast, gemcitabine alone did not affect the protein levels of Hsp70. In AsPC-1 cells, increased levels of Hsp70 protein were also induced by heat treatment (data not shown). However, western blotting showed that the combination of gemcitabine and heat treatment reduced the levels of vascular endothelial growth factor (VEGF), and cyclin D1 compared to the controls (Fig. 3).

In general, many types of anti-cancer agents, including gemcitabine, target DNA and activate apoptotic pathways. However, NF- κ B is often activated by anti-cancer agents, such as 5-FU and CPT-11, and NF- κ B activation is one of the mechanisms through which tumors become resistant to chemotherapy^{37,38)}. Therefore, blocking NF- κ B activation may enhance the anti-tumor effects of chemotherapy and may help prevent chemo-resistance. In this study, gemcitabine activated NF- κ B binding activity in both AsPC-1 and MIAPaCa-2 pancreatic carcinoma cell lines, and exposure to heat significantly attenuated gemcitabine-mediated NF- κ B activation. These observations together with previous reports^{39,40)} strongly suggest that combination therapies consisting of a heat treatment in conjunction with gemcitabine might lead to enhanced cell cytotoxicity in carcinoma cells by blocking the activation of NF- κ B. Although the inhibition of NF- κ B activation by a heat treatment is established^{41,42)}, an understanding of the detailed mechanisms through which heat treatments block the activation of NF- κ B is lacking. Previous studies from this laboratory have demonstrated that Hsp70 and Hsp32 induced by heat treatment play important roles in the inhibition of NF- κ B activation. If heat

Fig. 3. The expression levels of Hsp70, cyclin D1, survivin, and VEGF. Cells were treated with heat 24 h before a treatment with gemcitabine (Gem, 30 μ M). Whole cell extracts were analyzed with western blotting. The results for no treatment (normal or control), treatment with gemcitabine alone (30 μ M), heat treatment (HT) alone (43°C, 1 h), and heat treatment combined with gemcitabine treatment are shown. Significant levels of Hsp70 were induced by heat treatment in MIAPaCa-2 cells. In contrast, gemcitabine treatment alone did not affect the protein levels of Hsp70. The levels of cyclin D1, VEGF were decreased after a heat treatment combined with a gemcitabine treatment.

sensitive proteins such as Hsp70 and Hsp32 induced by heat treatment inhibit NF- κ B activation and result in the enhancement of gemcitabine-mediated cytotoxicity in AsPC-1 and MIAPaCa-2 cell lines (Fig. 1), the timing of the heat treatment relative to the gemcitabine treatment can be important. Thus, a protocol was used in which the two pancreatic cancer cell lines were treated with gemcitabine and heat with four different intervals between the two agents. AsPC-1 and MIAPaCa-2 cells were treated with heat 24 h before gemcitabine (30 μ M), simultaneously with gemcitabine, 24 h after gemcitabine, and 48 h after gemcitabine. The treatment with gemcitabine alone showed significant cytotoxicity. Heat combined with gemcitabine enhanced cytotoxicity, especially when heat was introduced 24 h before gemcitabine. These observations and previous reports^{41,42)} suggest that heat treatment enhances the cytotoxicity of gemcitabine through the inhibition of NF- κ B. The time course of Hsp70 expression after heat treatment has been examined in AsPC-1 and MIAPaCa-2 cells. Heat induced Hsp70 expression 3 h after heat was applied, and this induction continued for at least 72 h. Figure 3 shows Hsp70 expression for 72 h after heat treatment. However, activation of NF- κ B was initiated between 3 h and 24 h after cancer cells were exposed to gemcitabine. It was reported that NF- κ B is activated by chemotherapy in some cancer cell lines, and NF- κ B activation is one of the mechanisms through which tumors become resistant to chemotherapy. Heat treatment-induced heat shock protein 70 (Hsp70) was reported to inhibit I kappa B (I κ -B) kinase (IKK), resulting in the inhibition of NF- κ B activation. Therefore, it

Fig. 4. Gemcitabine actually activates NF- κ B in pancreatic carcinoma cells. The activation of NF- κ B was inhibited, probably by heat treatment-induced Hsp70. As a result, the death signal was emphasized over the survival signal.

appeared possible that activated NF- κ B could be inhibited by a heat treatment in a pancreatic cell line, resulting in the enhancement of gemcitabine-induced cytotoxicity. However, the inhibitory effect of Hsp70 on NF- κ B activation by gemcitabine must occur at an optimal time.

Western blotting analysis (Fig. 3) indicated that the expression of NF- κ B-regulated proteins was inhibited by heat treatment combined with gemcitabine, and suggests that the heat-treatment-mediated inhibition of NF- κ B may be effective, not only to inhibit increases in cancer cell numbers *in vitro* (Fig. 1), but also to inhibit tumor growth *in vivo*.

Survivin has multiple functions including cytoprotection, inhibition of cell death, and cell-cycle regulation, especially during the mitotic stages, and all of these functions favor cancer cell survival. Many studies on clinical specimens have shown that survivin over-expression is invariably up-regulated in human cancers, is associated with resistance to chemotherapy or radiation therapy, and is linked to a poor prognosis. This suggests that cancer cells survive with the help of survivin⁴³⁾ expression. *Survivin* promoter activity is basically silent in normal cells, but is strongly expressed in tumor cells⁴⁴⁾, and this occurs independently of cell types, mitotic status, or genetic makeup. Supporting this model, complementary studies have identified a number of oncogenic gene expression pathways, initiated by activated NF- κ B⁴⁵⁾ and STAT3⁴⁶⁾, which target the survivin promoter to stimulate vigorous transcription selectively in cancer cells. Evidence suggests that these mechanisms include direct, and discrete binding sites for various oncogenic transcriptional activators, and NF- κ B and STAT3 have been identified at the proximal survivin promoter. On the basis of these findings, survivin has been suggested as an attractive target for new anticancer interventions, and survivin inhibitors have recently entered clinical trials⁴⁴⁾. In this study, there was a decrease in the activation of NF- κ B after a treatment with heat plus gemcitabine

(Fig. 2); survivin levels were decreased after a treatment with this combination therapy. This could result in enhancing the induction of apoptosis in AsPC-1 and MIA PaCa-2 cells.

Among proteins related to the G0/G1 phase of cell cycle progression, the expression of cyclin D1 was significantly down-regulated by treatment with gemcitabine alone or a heat treatment in combination with gemcitabine in AsPC-1 and MIA PaCa-2 cells (Fig. 3). A recent study reported that cyclin D1 regulates cell cycle progression and also mitochondrial function and size⁴⁷⁾. It acts through apoptosis in the carcinoma cell lines, and also through cell cycle delays. According to the data shown in Figure 3, the expression of cyclin D1 was down-regulated by a treatment with gemcitabine alone, and down-regulated to a larger extent by a treatment with heat plus gemcitabine. This suggests that the heat treatment-mediated inhibition of NF- κ B lead to the decrease in cell numbers through the induction of apoptosis and the attenuation of cell proliferation.

There is compelling evidence that vascular endothelial growth factor (VEGF) is a major regulator of tumor growth and metastasis^{48,49)}. VEGF is secreted at high levels in numerous tumor types, and its expression is associated with a poor prognosis⁵⁰⁾. From Western Blotting analysis, the decrease of VEGF after exposure to heat plus gemcitabine may help lead to an inhibition of tumor growth *in vivo*. This scenario is supported by Solorzano *et al.*⁵¹⁾, which shows that VEGF plays a key role in regulating tumor vascularization, and this is further supported by other studies which indicate that over-expression of NF- κ B is a key component of the angiogenic cascade. Such a cascade contributes to VEGF-induced angiogenesis through the up-regulation of VEGF mRNA expression in many tumors⁵²⁾. Although the role of VEGF in tumor angiogenesis is well recognized, it is also a key factor in promoting and sustaining the immune system's lack of response to growing tumors^{50,53)}. Tumor-derived VEGF binds to the VEGFR1/FLT1 receptor on CD34+ bone marrow progenitor cells, decreasing the ability of these cells to differentiate into functional dendritic cells⁵⁴⁾. Therefore it is possible this combination therapy might be improve cancer immunotherapy results in patients.

Conclusion

In clinical practice, heat treatment is frequently applied simultaneously with an intravenous infusion of anti-cancer agents, or immediately after an intra-arterial infusion of anti-cancer agents. However, in the case of gemcitabine, in order to inhibit the activation of NF- κ B, it is concluded that heat treatments should be applied before chemotherapy.

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