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Preclinical study

Modulation of 9-nitrocamptothecin-induced apoptosis by hyperthermia in human leukemia HL-60 cells

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We have investigated whether hyperthermia (HT) treatment at 43 C for 15–60 min can affect the extent of apoptosis induced in human leukemia HL-60 cells by the anticancer drug 9-nitrocamptothecin (9NC). Quantitative changes in the apoptotic (Ap) fraction in the cell cultures were monitored by flow cytometry. The results showed that (i) heating for 15 min prior to or concurrently with 9NC exposure had no effect on the Ap fraction generated by the drug alone, whereas 60 min heating resulted in an increase in the Ap fraction; and (ii) heating of the cells at 6–24 h after exposure to the drug enhanced the Ap fraction. These results indicate that appropriate scheduling of HT and 9NC treatments may lead to thermochemotherapy protocols that will result in increased 9NC-induced death of human leukemia cells.

Key words: Leukemia cells, hyperthermia, 9-nitrocamptothecin, thermochemotherapy.

Introduction

Temperatures of 41–45 °C are selectively lethal for cancer cells both experimentally and clinically,[1,2] and various studies have established that hyperthermia (HT) in the temperature range of 41–45 °C induces programmed cell death (apoptosis) in many cell types *in vitro* and *in vivo*.[3–8] Following mild HT treatment, subsequent incubation at 37 °C is necessary to induce apoptosis as assessed by extensive double-stranded DNA fragmentation.[9] In contrast, heating at 46 and 47 °C produces only necrosis.[10]

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9-Nitrocamptothecin (9NC) is a semisynthetic derivative of the plant alkaloid product camptothecin (CPT) that, when added to cultures of human cells, elicits either apoptosis in cells traversing the S phase of the cell cycle or their arrest at the boundary of late S/G_2 phases.[11–13] In general, 9NC exerts its action via the nuclear enzyme topoisomerase I (Topo I) by interfering in the mechanism of breakage-reunion of single-stranded DNA. *In vivo*, 9NC inhibits growth and induces regression of various human tumors established as xenografts in nude mice, in the absence of detectable 9NC-induced side toxic effects. Structural features, molecular, cellular, antitumor and antiviral activities, and metabolism of 9NC have been extensively reported.[11,12,14,15]

Application of HT combined with anticancer drugs, i.e. thermochemotherapy, has been explored as a clinical strategy to enhance the therapeutic effect of several anticancer drugs even in drug-resistant cells.[16–23] However, there is quite a discrepancy in the results of studies dealing with CPT derivatives. In this context, there was no significant increase in the effectiveness of CPT *in vitro* when mouse mammary tumor cells were exposed to the drug at 42 °C compared with the same drug exposure at 37 °C.[24] However, CPT-induced killing of human melanoma SK-Mel-3 cells was slightly enhanced by subsequent heating of the cells at 45 °C for 15 min, but not at 41 °C for 8 h, whereas heating at 45 °C for 15 min prior to CPT treatment almost completely abrogated the CPT-induced toxicity.[25] In addition, diverse results were obtained in studies of combination treatments of HT and CPT-11, a water-soluble CPT derivative, in inducing DNA damage in mammary carcinoma cells.[26] Thus, HT combined with low or high CPT-11 concentrations, respectively, resulted in increased or decreased DNA damage.[26]

In this report we have investigated effects of various combinations of HT and 9NC treatments on cells. We have used a mild temperature of 43 C because prolonged treatments with temperatures of 45 C or higher result in necrosis rather than apoptosis. The studies were conducted with HL-60 cells, and the combined effects of HT and 9NC treatments on the cells were determined by quantification of the apoptotic (Ap) fraction in the cell culture. This way we have identified parameters for maximization of 9NC-induced apoptosis in heat-treated HL-60 cells.

Materials and methods

Cells

Human leukemia HL-60 cells,[27] at passages 44–52, were grown in RPMI 1640 media supplemented with 10% fetal calf serum and antibiotics in a 5% CO_2 atmosphere at 37 C. For drug and/or HT treatments, fresh cell cultures were prepared at 2×10^5 cells/ml in media made of 80% fresh and 20% cell-conditioned media. We have assessed that use of this media-mixture abrogates the lag growth period frequently observed when cells are transferred in fresh media. In all experiments, the culture volume was 10 ml per 75 cm^2 flask (Falcon). Cell proliferation was monitored by counting the number of cells/ml, while viability was detected by the Trypan blue exclusion method.

Drug and hyperthermia treatments

9NC was prepared according to published procedures[28] and used as a fine suspension in polyethylene glycol (PEG 400; Aldrich, Milwaukee, WI). At the desired 9NC concentrations in the cultures the final PEG concentrations were 0.2–0.4% (v/v). Cell cultures were heated in T-75 flasks with the caps tightly closed. Each flask was hermetically sealed in a plastic freezer-bag, then immersed and kept in the water of a circulating water-bath (Model 260; Precision, Chicago, IL) with the aid of a lead-brick. The circulating water had a constant temperature of 43 ± 0.1 C as monitored by the built-in platinum temperature probe. The temperature in the cell culture was monitored by a microthermometer probe (SensorTek, Clifton, NJ) emerged in a duplicate mock cell culture and connected to a TM-10 digital thermometer (SensorTek). The cell culture reached 43 C at about 4 min after the flask was immersed in the heated water, and remained at this temperature for the desired period, then removed from the sealing bag, the cap was loosened up to allow for gas equilibration and incubation continued in a 37 C cell culture incubator. Culture aliquots were removed at desired times, and cells were counted and processed immediately for flow cytometry studies. In general, heating of cells at 43 C for 2 h resulted in a large number of necrotic cells and therefore all hyperthermia treatments were for 60 min or less.

Flow cytometry

Cell cycle perturbations in the cultured cells were monitored by determining the relative DNA content of the cells using an Epics Elite laser flow cytometry (Coulter Counter, Hialeah, FL) and analyzed with the aid of the Multicycle program (Phoenix Flow Systems, Dan Diego, CA). Flow cytometry is the methodology of choice to detect and quantify apoptosis because: (i) it distinguishes Ap from necrotic cells; (ii) it detects apoptosis at early stages that cannot be detected by other methodologies; (iii) it is a rapid method for the analysis of apoptosis from heterogeneous cell populations; (iv) it requires 10 000–20 000 cells to accurately estimate the apoptotic fraction in a cell culture; and (v) quantification of the apoptotic fraction is highly reproducible.[29–32] In general, flow cytometry analysis has been applied to study cell cycle specificity and the extent of effectiveness of chemotherapeutic agents including 9NC.[13,32–34] The flow cytometry findings described in this report were derived from triplicate experiments, but only the results (histograms) of a representative experiment are shown.

Results

9NC treatment precedes heat treatment

We have investigated the extent of apoptosis in cultured cells that were exposed to 9NC for 24 h before they were heated. Three of four identical cultures received 20 nM 9NC, while the fourth culture was left untreated and served as the control. All cultures were placed in the 37 C incubator and after 24 h two of the 9NC-treated cultures were transferred to the 43 C water-bath. One culture remained at 43 C for 30 min and the second for 60 min. Immediately after heating at 43 C, the cultures were transferred to 37 C and cells were removed from the cultures after 24 h for various analyses. Control cells that received no drug or HT treatment were actively proliferating as assessed by counting of cells and their distribution in various phases of the cell cycle as determined by flow

cytometry. Monitoring of viability by the Trypan blue dye indicated that only 2% of the cells were dead and this finding is in good agreement with the presence of 1% Ap cells in the culture detected by flow cytometry (Fig. 1A). 9NC alone in the culture for 48 h resulted in an increase in the number of cells in the S and G_2 phases, and the appearance of an Ap fraction of 16% (Fig. 1B), while the cell viability was 96%. However, when the 9NC-treated cells were heated for 30 min after 24 h of drug exposure, and then transferred to 37°C for another 24 h, more than 30% of the cultured cells were in the Ap fraction (Fig. 1C), while 91% of the cells excluded Trypan blue. Further, heating of the cells for 60 min instead of 30 min resulted in a larger Ap fraction of about 70% (Fig. 1D), while about 75% of the cells still appeared alive by excluding Trypan blue. Finally, heating for 15 min had no significant effect on the 9NC-induced Ap fraction and dye-assessed viability (results not shown).

In conclusion, the results indicated that heating of cells pre-exposed to 9NC increases the Ap fraction; moreover, the effect was more pronounced when HT treatment was for 60 min instead of 30 or 15 min.

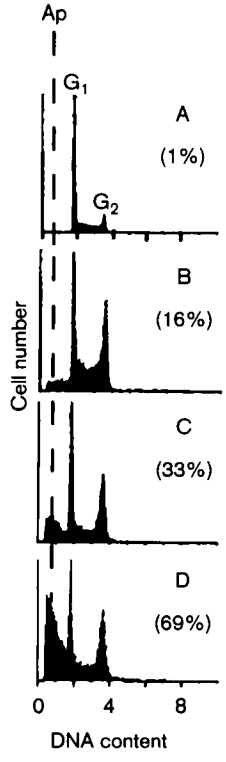

Figure 1. Histograms of cells exposed to 9NC for 24 h prior to heating. Cultures of HL-60 cells were exposed to 20 nM 9NC for 24 h (B–D) before they were heated for 30 (C) or 60 (D) min, or received no heat treatment (B). A control culture (A) received neither 9NC nor heat treatment. G_1=G_0+G_1; G_2=G_2+M; Ap=apoptotic cells. Cells were collected for analysis 24 h after heating. Numbers in parentheses indicate the percent of Ap cells (i.e. Ap fraction) in the culture.

Concurrent treatment of cells with 9NC and hyperthermia

In this study the cell cultures received 10 or 20 nM 9NC and then were immediately subjected to HT treatment. Untreated cells and cells that received HT alone or 9NC alone served as controls. We investigated the effect of heating for 15 and 60 min. Following heating at 43°C for 15 min, the cell cultures were transferred to 37°C for 24 h, when the cells were collected for flow cytometry analysis (Fig. 2). A small but reproducible increase in the Ap fraction was observed after HT treatment alone (Fig. 2B). On the other hand, 10 nM 9NC alone (Fig. 2D) or 20 nM alone (Fig. 2E) resulted in a dramatic accumulation of cells at the S/G_2 boundary with no significant changes in the Ap fraction. Further, the combination of 10 nM 9NC/15 min HT generated an Ap fraction that was similar to the Ap fraction generated by 10 nM 9NC alone (*cf.* Fig. 2D and F). Also, the combination of 20 nM 9NC/15 min HT generated an Ap fraction similar to that generated by 20 nM 9NC alone (*cf.* Fig. 2E and G). Taken together these results indicate that, in the protocol used in this study, 15 min heating had no inhibitory effect on induction of apoptotic cells in cultures concurrently treated with 10 and 20 nM 9NC.

In the same study, we also studied concurrent treatment with 9NC and heating for 60 min instead of 15 min. The 60 min HT treatment alone (Fig. 2C) resulted in an Ap fraction larger than the 15 min HT treatment (Fig. 2B), but there was not any indication that heat targeted a particular cell cycle phase in these cells. The combined treatment of 10 nM 9NC/60 min HT and 20 nM 9NC/60 min HT generated Ap fractions of 16 and 69%, respectively (Fig. 2H and I). These Ap fractions are much larger than those generated by 10 or 20 nM of 9NC alone (Fig. 2D and E). Thus, the results indicate that concurrent heating for 60 min, unlike heating for 15 min, enhances the 9NC-induced apoptosis, particularly when the drug is used at 20 nM.

Discussion

In this report, we have described experimental findings to show that HT treatment can modulate the apoptosis-inducing action of the anticancer drug 9NC in the human leukemia HL-60 cells. Modulation of the apoptotic effect of CPT and CPT-11 by HT has been

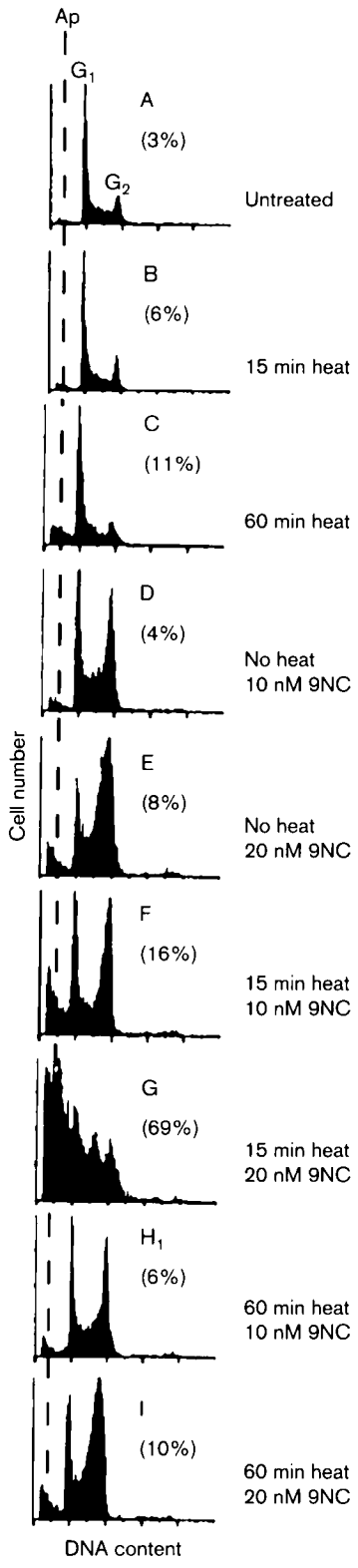

Figure 2. Histograms of cells subjected to HT treatment and immediately (near concurrently) exposed to 9NC. Cultures of HL-60 cells were either untreated (A); only heated at 43 C for 15 (B) or 60 (C) min; exposed to 10 (D) or 20 (E) nM of 9NC; or heated at 43 C for 15 or 60 min and near concurrently exposed to 10 (F) or 20 (G) nM 9NC. Cells were collected 72 h after heating. Ap fractions are indicated in parentheses.

previously reported, but results have been contradictory and/or quantitatively varied, apparently reflecting the variability in the experimental parameters applied in those studies including cells of diverse tissue origin, different temperatures and durations of HT treatments, different concentrations of drugs used, different schedules of HT and drug treatments, etc. Therefore, in this study we have used a single cell line, HL-60, in all experiments. HL-60 cells are sensitive to both HT treatment and exposure to the Topo I-directed drug CPT.[35-37]

First, we demonstrated that the outcome of the combination treatment with HT and 9NC depends on the sequence of each treatment, i.e. the drug effectiveness can be enhanced or decreased by HT depending on whether HT is applied *after*, *before* or *concurrently* with exposure of the cells to the drug. It has already been demonstrated that 9NC, or HT alone, can induce apoptosis in cells by a metabolic process that requires active endonuclease and other effectors.[36,38,39] Further, 9NC is cell cycle phase specific,[37,39,40] whereas HT-induced apoptosis is cell cycle phase non-specific[35] or the phase targeted may depend on the cell line.[41] Our results show that HL-60 cells that are pre-exposed to 9NC are more sensitive to HT than non-exposed cells. Therefore, it is possible that heat results in deactivation or reduced activity of an enzyme or other effector important for religation or repair of the DNA damaged by the drug. It is also possible that heat increases the stabilization of already existing Topo I-DNA-9NC complexes thus resulting in enhanced apoptosis. Finally, heating may up-regulate the expression of Topo I,[42] which in turn generates more Topo I-DNA-9NC complexes and, subsequently, enhanced apoptosis. Also, other possibilities exist to explain the heat-enhanced apoptotic action of 9NC, but none of these possibilities has been investigated yet.

The killing efficiency of 9NC was decreased when the HL-60 cells were concurrently treated with HT and 9NC or were exposed to 9NC several hours after heating. Further, our results showed that HT alone does not interfere with a specific phase of the cell cycle in HL-60 cells, in agreement with a previous report.[35] However, this interference delays or slows down cell progression through the S phase that is targeted by 9NC. Our hypothesis is supported by a report that heat induces G_0/G_1 arrest in several human cell lines and this arrest correlates with increases in the

levels of p16,[41] a protein that acts as a negative regulator of the cell growth. Therefore, heat may induce diverse effects on key regulatory molecules of the cell cycle depending on the cell type. In turn, cell heating prior to drug treatment may de-sensitize or reduce the cell response to the drug treatment.

Since heat alone elicits responses that depend on the cell type, it is conceivable that the same protocol of heat/drug treatment may generate interactions that are cell-type dependent. Therefore, it may be necessary to separately study and subsequently develop various thermochemotherapy protocols, using 9NC, for the treatment of various types of tumors. At any rate, the results described in this report indicate that specific scheduling of HT and chemotherapy with 9NC can result in increased drug-induced toxicity in the leukemic cells. In turn, this means that HT may potentiate low 9NC concentrations to be as effective as higher concentrations of 9NC when this drug is used alone in the treatment of leukemia in the absence of toxicity for the normal hematopoietic cells.

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