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Increased cytotoxicity of low-dose, long-duration exposure to 5-fluorouracil of V-79 cells with hyperthermia

Yuichiro Kido*, Hirioyuki Kuwano, Yoshihiko Maehara, Masaki Mori, Hideo Matsuoka, and Keizo Sugimachi

Department of Surgery II, Kyushu University, Fukuoka 812, Japan

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Summary. We examined the cytotoxic effects of combined low dose and long exposure to 5-fluorouracil (5-FU) and hyperthermia on Chinese hamster V-79 cells with reference to timing and sequence of administration. The survival rate following hyperthermia at 42° C for 2 h alone was 95.4%, and that after exposure to 1.0 μg/ml/5-FU alone for 48 hours, 94.2%. With respect to the combination of 5-FU and heat, the survival rate of cells exposed to hyperthermia at 42° C for 2 h followed by 1.0 μg/ml 5-FU treatment for 48 h was 52%, while 1.0 μg/ml 5-FU treatment for 48 h followed by hyperthermia led to a survival rate of 10%. Flow cytometric analysis of V-79 cells after exposure to 1.0 μg/ml 5-FU for 48 h revealed an accumulation of cells in the S-phase; the percentage of S-phase exponential growing cells was 65% and the plateau phase was 38%. The former were more sensitive to heat than the latter cells according to the MTT assay. V-79 cells pretreated with 5-FU were more sensitive to hyperthermia than were those not pretreated with 5-FU. Therefore, when 5-FU plus heat is to be used to treat a patient with a malignancy, the sequence of 5-FU followed by hyperthermia may be more effective than the reverse.

Key words: Hyperthermia – 5-Fluorouracil – Timing

Introduction

The antimetabolite, 5-fluorouracil (5-FU), an extensively used chemotherapeutic agent, is often given in combination with other drugs or irradiation [3, 14]. 5-FU seems to exert antitumor effects through a metabolic conversion to 5-fluorouridine-5′-triphosphate (FUTP) with subsequent incorporation into RNA, and through the formation of 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), the well-recognized inhibitor of thymidylate synthase (TS) (EC 2.1.1.45) [7, 12].

Hyperthermia augments the action of chemotherapeutic agents such as bleomycin [10], Adriamycin [4], cisplatin and mitomycin C [21]. Much less is known of the interaction between 5-FU and hyperthermia. When cells in culture were exposed for short periods to 5-FU, there was no enhanced effect of the combination of heat and 5-FU [19], and Hahn classified 5-FU with drugs showing no thermal enhancement of cytotoxicity between 37° C and 45° C [11]. Lange et al. reported that the cytotoxicity of 5-FU was substantially enhanced in the presence of hyperthermia (41.8° C), both in vitro and in vivo [14]. Adwankar et al. reported that 1 h exposure to hyperthermia along with 10 μg/ml 5-FU resulted in synergistic cell killing action against P388 leukemic cells using the in vitro/in vivo bioassay method [1]. Despite several in vitro and in vivo studies on the therapeutic efficacy of thermochemotherapy, data concerning optimal scheduling of hyperthermia and 5-FU are still sparse, and the data recorded with these combinations are often conflicting.

For these reasons, we examined the combined effect of 5-FU and hyperthermia, in particular the most effective timing and sequence of administration of these agents, and the influence of prolonged 5-FU exposures on cell cycle kinetics at low (non-lethal) doses in vitro.

Materials and methods

Cell culture. Exponentially growing V-79 cells originating from Chinese hamster lung fibroblasts [9] were cultured in Dulbecco's modified Eagle medium (DMEM) (Nissui, Osaka, Japan) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Hazleton, Lenexa, USA) and 5 μg/ml of gentamicin (Essex, Osaka, Japan). The cells were inoculated at 37° C with 95% air and 5% CO_2 in culture medium with pH of 7.4. After preparation of a single cell suspension (0.25% trypsin for 5 min at 37° C), 1×10^2 or 1×10^3 cells were plated in a 60-mm plastic culture dish (Corning 25010, Tokyo, Japan). The drug and heat treatments were initiated at least 12 h after plating.

* *Present address:* Departent of Medical Oncology, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 52 Houston, Texas 77030 USA

Offprint requests to: Y. Kido at the present address

Effect of 5-FU alone on growth of V-79 cells. Exponentially growing V-79 cells were inoculated into 60-mm dishes (2×10^5 cells/dish) on day 0. On day 2, 5-FU (Kyowa Hakko, Tokyo, Japan) was added to the dishes at the appropriate concentrations (0.1–10 μg/ml). Cells were washed once with phosphate-buffered saline (PBS), trypsinized and counted daily, using a Coulter counter.

Sensitivity of V-79 cells in either the exponential or plateau phase of growth to heat. To determine the sensitivity of exponential and plateau phase cells to heat, 2 h heat 42° C treatment was given to these cells (inoculated into 60-mm dishes at a density of 1×10^3 or 1×10^5 cells/well 3 days earlier), after which cell number and cytotoxicity were measured for in triplicate using the MTT assay on day 5, as described elsewhere [2, 16].

Heat and drug treatment. 5-FU was dissolved in and diluted to the appropriate concentration with complete medium. Exponentially growing cells were exposed to graded concentrations of 5-FU from 2 to 48 h. For hyperthermia, culture dishes were placed in a 42° C humidified incubator during the first or last 2 h of drug exposure. After these treatments, the cells were washed twice with fresh medium and then incubation was carried out for 7 days at 37° C. The colonies were rinsed twice with PBS, fixed with methanol, stained with Giemsa, and counted under a stereoscopic microscope. Only colonies with over 50 cells were scored, and the cloning efficiency was expressed as the percentage of colony number in the treated plates divided by the number in the untreated controls. All experiments were performed at least twice.

Flow cytometry. The DNA distribution pattern was examined by means of flow cytometry (FACS can Becton Dickinson Immunocytometry Systems, San Jose, USA) and the percentage of S-phase cells was assessed planimetrically (DNA Cell-Cycle Analysis Software, Ver C 5/87 Polynomial Model, San Jose, USA). Approximately 2×10^5 V-79 cells were inoculated into 60-mm dishes for 12 h without drugs, growing exponentially and then cultured in appropriate concentrations of 5-FU containing medium for 24 and 48 h. Cells were washed twice with PBS, stained with propidium iodide [17], then analyzed by flow cytometry.

Statistical analysis. Significant differences between the means were analyzed using Student's *t*-test ($P < 0.05$).

Results

Growth of V-79 cells

The average cell population doubling time for the V-79 cells was approximately 12 h (Fig. 1, left).

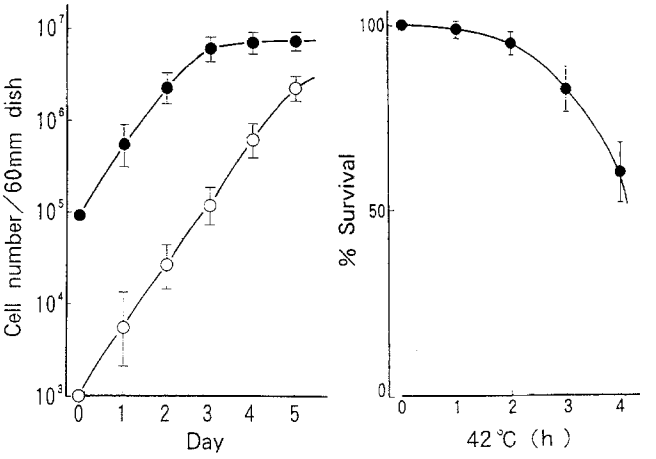

Fig. 1. *Left*, growth curves of V-79 cells: 1×10^3 (○) or 1×10^5 (●) cells were inoculated into 60-mm dishes. Media were changed on day 3. The average cell population doubling time for the V-79 cells was approximately 12 h. On day 3, growth in the dish with 1×10^5 cells first inoculated plateaued, while the 1×10^3 cells inoculated in the other dish were still growing exponentially. *Right*, effect of hyperthermia on V-79 cells. *Points*, means of at least two separate experiments; *bars*, standard errors

Effect of heat or 5-FU alone

The survival of cells exposed to 42° C for 0–4 h is shown in Fig. 1 (right). Growth inhibition of the cells in the presence of 5-FU was not concentration-dependent in the range of 0.1–1.0 μg/ml. At a concentration of 10 μg/ml, 5-FU had a cytotoxicity effect (data not shown).

Combined effects of 5-FU and hyperthermia

Figure 2 shows the interaction between 42° C hyperthermia for 2 h and 5-FU. The survival rate of combined treatment decreased with the duration of 5-FU exposure. When the cells were exposed to 42° C hyperthermia for over 24 h after the 5-FU exposure, the hyperthermic potentiation increased. Cell survival rate after exposure to 1.0 μg/ml of 5-FU for 48 h was 94.2% while the rate of survival of cells exposed to hyperthermia following 5-FU treatment was 52%. The survival rate in case of exposure to 5-FU for 48 h following exposure to hyperthermia (42° C 2 h) was 10%.

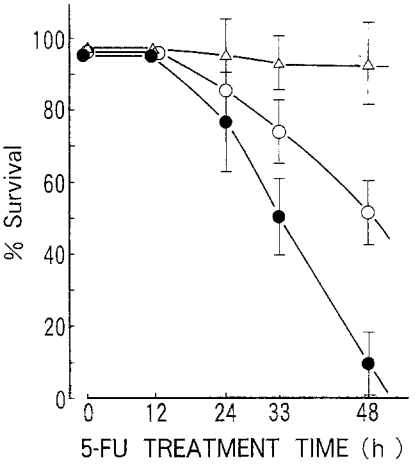

Fig. 2. Effect of 5-FU in combination with hyperthermia. V-79 cells were exposed to 5-FU with or without hyperthermia at 42° C for 2 h. *Points*, means of at least two separate experiments; *bars*, standard errors. △, 5-FU 1.0 μg/ml only; ○, heat, 42° C for 2 h then 5-FU 1.0 μg/ml; ●, 5-FU 1.0 μg/ml then heat, 42° C for 2 h

Flow cytometry

Effects on the DNA content distribution of 48 h exposure to 5-FU at 1.0 μg/ml are shown in Fig. 3. The 2C and 4C peaks correspond to cells in the G_1- and G_2-M phases, respectively. After exposure of 1.0 μg/ml 5-FU for 48 h, the percentage of S-phase cells increased from 28% to 49%, and that of cells in G_1-phase decreased.

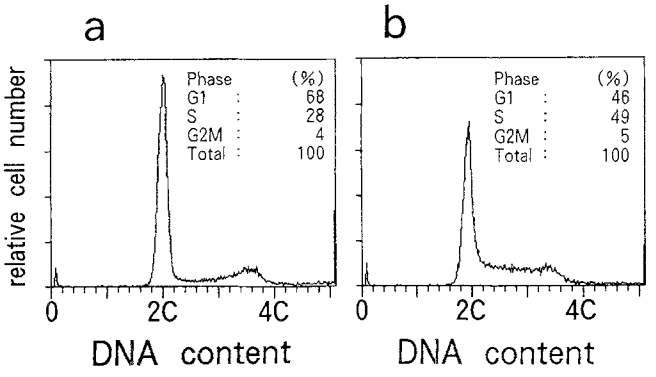

Fig. 3a, b. DNA distributions of V-79 cells: **a** control (5-FU, 0 μg/ml); **b** 5-FU, 1.0 μg/ml for 48 h. *Ordinate*, frequency of a given DNA content, expressed in cell numbers per 1×10^4 cells. After exposure to 1.0 μg/ml 5-FU for 48 h, the percentage of S-phase cells increased from 28% to 49%

Sensitivity of V-79 cells in exponential or plateau phase of growth to heat

Table 1 shows the results of experiments to compare the effect of heat on cells in exponential and plateau phases. Changes in succinate dehydrogenase (SD) activity were determined following exposure to 42°C for 2 h. The SD activity decreased to a greater extent in the exponentially growing cells, as shown in Table 1. Significant differences ($P < 0.05$) were noted between the SD activities of the exponential and plateau phase cells. Exponentially growing cells were more sensitive to hyperthermia than were the plateau phase cells.

The percentage of S-phase cells was greater among the exponentially growing cells than among plateau phase cells.

Discussion

Time-dose relationships for 5-FU cytotoxicity against a variety of human epithelial cancer cells were described by Calabro-Jones et al. [8]. They stated that when 5-FU is administered at lower doses and for longer time periods it is more effective in producing direct cytotoxic effects in human tumor cells than when administered at higher doses for shorter times. Link et al. also reported that at a high concentration of 1000 μg/ml, a 1-h exposure to 5-FU was followed by considerable regrowth after removal of the drug, resulting in a tumor cell kill of only about 70% [15]. Therefore, we determined the effect of low-dose, long-time exposure to 5-FU. Net cytotoxicity was increased in a supraadditive manner by combining hyperthermia and 5-FU, especially when hyperthermia was applied in the last 2 h of 5-FU treatment.

Bhuyan et al. showed the effect of combinations of drugs that take advantage of partial synchronization of L-1210 cells obtained with non-lethal doses of 5-FU [5]. When L-1210 cells were exposed for 8 h to a non-lethal dose of 5-FU (0.25 μg/ml), the percentage in the S-phase increased from 74.9% to 93%. Our experiment also showed an increase in the proportion of V-79 cells in the S-phase from 28% to 49% after a 48-h exposure of 5-FU at the concentration of 1.0 μg/ml. V-79 cells treated with the sequence of hyperthermia following exposure to 5-FU revealed more positive effects than when hyperthermia was applied first.

Table 1. Sensitivity of exponential or plateau phase cells to heat (42°C, 2 h)

Phase	Cells in S-phase (%)	SD activity (% of control)	Cell number (% of control)
Exponential	65±4.2[a]	39.8± 9.3*	68±2.2**
Plateau	38±4.2	97.8±13.3*	93±6.5**

Similar results were observed in replicate experiments
[a] Mean ± SE
* $p < 0.05$; ** $p < 0.1$

Other investigators showed that drug exposure time and the sequence of administration of hyperthermia and 5-FU can be important [13, 18]. Mini et al. reported combined effect of hyperthermia and 5-FU on human leukemia CCRF-CEM cell line. They reported that partial synchronization after 2 h hyperthermia at 42°C causes increased sensitivity to subsequent exposure to FUra. Hyperthermia causes a decrease in DNA synthesis, followed by a rebound increase 12 h later that was associated with increased proportion of S-phase cells. They also observed additive effect of the exposure to FUra (4 h and 8 h) immediately before hyperthermia. We did our experiment with lower concentration and longer exposure of 5-FU combined with 42°C hyperthermia. In our experiment, the enhanced effect of combined 5-FU and hyperthermia was recognized in over 24-h exposure to 5-FU (Fig. 2). The pretreatment with 5-FU induces accumulation of cells in S-phase by blocking cells at the G_1/S boundary, and subsequent treatment with hyperthermia may selectively kill cells in the S-phase. Cells in this phase are more sensitive to hyperthermia than are cells in the G_1-, M-, and G_2-phases [6, 20]. We found that exponentially growing V-79 cells (65% S-phase) were more sensitive to hyperthermia than were those in the plateau phase (38% S-phase). Hyperthermia after long-term treatment with low-dose 5-FU might also potentiate cytotoxicity by inhibiting the repair of DNA.

The findings in this study have clinial relevance. We conclude that both the duration of 5-FU treatment and the 5-FU-heat sequence appear to be critical factors in the cytotoxicity of the combined modalities. The sequence of 5-FU followed by hyperthermia may be more effective than the reverse.

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