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5-Fluorouracil's cytotoxicity is enhanced both in vitro and in vivo by concomitant treatment with hyperthermia and dipyridamole

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Summary. We obtained evidence that the cytotoxic effect of 5-fluorouracil (5-FU) is augmented when the drug is given in combination with hyperthermia (HYP) and dipyridamole (DP). Nontoxic levels of DP enhanced the combined cytotoxicity of 5-FU and HYP against B16 melanoma and human tumor cells in vitro as measured by the succinate dehydrogenase inhibition (SDI) test. Growth of B16 melanoma that had been subcutaneously implanted into the feet of C57 BL mice was inhibited by treatment with the combinations of 5-FU and HYP, of 5-FU and DP, and of 5-FU, HYP and DP as compared with the administration of 5-FU alone. Treatment with HYP plus DP did not alter the body weight of mice that received 5-FU. The administration of DP plus HYP seemed to render the tumor cells more sensitive to 5-FU. The combination of 5-FU, HYP and DP shows promise for the treatment of patients suffering from malignant disease.

Introduction

Hyperthermia (HYP) enhances the selectivity of antitumor drugs by increasing cell killing within the region of elevated temperature; therefore, doses of antitumor drugs can be reduced and minimized systemic toxicity ensues [9]. The combination of HYP and antitumor drugs has been prescribed for the treatment of patients presenting with cancer. Studies have also been carried out on the combined effect of 5-fluorouracil (5-FU), a widely prescribed pyrimidine analogue, and HYP [1, 20, 24].

Dipyridamole (DP), which is usually prescribed clinically as a vasodilator and as an antiplatelet agent in various countries, is a potent inhibitor of membrane nucleoside transport [14, 35]. The clinical pharmacology of DP has been given much attention, and no severe toxicity has developed in humans receiving this drug [36]. DP has been found to augment the cytotoxicity of anti-metabolites [7, 12, 13, 34], anthracyclines [3, 22, 38], *Vinca* alkaloids [3, 18] and etoposide [18] under experimental conditions. We have found synergy in the cytotoxic effects of 5-FU, HYP and DP against HeLa and B16 melanoma cells in vitro [31]. In an attempt to improve the clinical effects of 5-FU treatment, we investigated the combined effects of 5-FU, HYP and DP against B16 melanoma cells in vitro and in vivo and against human solid-tumor cells in vitro.

Materials and methods

Chemicals. 5-FU was obtained from the Kyowa Hakko Co., and DP was obtained from the Boehringer Ingelheim Japan Co.

Materials. Tissues used in this study were obtained from ten Japanese patients who had undergone gastric resection and from seven who had undergone resection of the colon or rectum in the Department of Surgery II, Kyushu University Hospital (Fukuoka, Japan), between 1988 and 1989. All tissues were placed in McCoy's 5A solution immediately following their excision. Informed consent to partipate in chemosensitivity testing was obtained from all patients prior to surgery.

Cells. B16 melanoma cells were routinely cultured in monolayers on plastic dishes using minimal essential medium (MEM; Nissui Seiyaku Co., Japan) supplemented with L-glutamine (292 mg/ml), 10% fetal calf serum (Gibco Laboratories, USA), penicillin (100 IU/ml), streptomycin (100 μg/ml) and gentamycin (40 μg/ml). Parent B16 melanoma cells, the origin and properties of which have been described by Fidler [10], were kindly provided by Dr. S. Taniguchi (Medical Institute of Bioregulation, Kyushu University, Fukuoka).

SDI test. The SDI test was done as described elsewhere [26, 29, 32]. Tumor tissues were cut with scissors, passed through number 32 stainless steel mesh into McCoy's 5A solution containing antibiotics, and washed three times with this solution. The fragments were then suspended in MEM solution, plated in plastic dishes, and then incubated with 100 μg 5-FU/ml [28] and/or 2.5 μg DP/ml [22] at 37° C in a humidified atmosphere containing 5% CO_2 for 72 h. In the HYP-treated group, the cells were incubated at 43° C for 10 h and then incubated at 37° C [29]. These fragments were then assayed for succinate dehydrogenase (SD, EC 1.3.99.1) activity. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) [30] was used as the hydrogen acceptor to

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Table 1. Changes in the SD activity of B16 melanoma cells exposed to 5-FU, HYP and/or DP

Treatment[a]	SD activity (%)
5-FU[b]	78.7±4.1
HYP[c]	61.6±3.4
DP[d]	97.3±2.6
5-FU + HYP	55.6±2.9*1, *2
5-FU + DP	63.2±3.7*1, *3
5-FU + HYP + DP	41.8±5*1-*5

Data represent mean values ± SD
[a] Five plates were assayed for each group
[b] 100 μg/ml for 72 h
[c] 43° C for 10 h
[d] 2.5 μg/ml for 72 h
*1 Significantly different from 5-FU (P <0.05); *2 Significantly different from HYP (P <0.05); *3 Significantly different from DP (P <0.05); *4 Significantly different from 5-FU + HYP (P <0.05); *5 Significantly different from 5-FU + DP (P <0.05)

Table 2. Changes in the SD activity of human tumor tissues exposed to 5-FU, HYP and/or DP

Treatment[a]	SD activity (%)
5-FU[b]	86.1±12.4
HYP[c]	73.2±7.8
DP[d]	98.6±3.6
5-FU + HYP	69.1±9.2*1, *2
5-FU + DP	65.3±13.1*1, *3
5-FU + HYP + DP	51.8±11.7*1-*5

Data represent mean values ± SD
[a] 3 plates were assayed for each of 17 tissues
[b] 100 μg/ml for 72 h
[c] 43° C for 10 h
[d] 2.5 μg/ml for 72 h
*1 Significantly different from 5-FU (P <0.05); *2 Significantly different from HYP (P <0.05); *3 Significantly different from DP (P <0.05); *4 Significantly different from 5-FU + HYP (P <0.05); *5 Significantly different from 5-FU + DP (P <0.05)

measure the SD activity. The formazan formed from MTT was extracted with acetone containing 0.5% trichloroacetic acid and the absorbance of formazan was measured at 565 nm. The chemosensitivity was estimated as a percentage of the SD activity in controls. Three to five plates were assayed for each group.

Effect on B16 melanoma solid tumors. C57 BL mice aged 6 weeks were housed under conditions of constant temperature and humidity. In all, 3×10^5 B16 melanoma cells in 10 μl were implanted subcutaneously into the foot tissue of these mice on day 0, and the animals were treated with 5-FU, HYP and/or DP on days 2, 4, 6, 8 and 10. The drugs were given orally at doses of 13.2 mg/kg 5-FU and 100 mg/kg DP [22]. 5-FU was sonicated in 0.5% acasia gum solution [33]. HYP was applied by immersing the foot of the mouse into a circulating water bath at 43° C for 15 min. As measured using a needle probe (type NST, Shibaura Electronics Co., Japan) and a thermometer (MGA III-219, Nihon Kohden Co., Japan), the temperature of the tumor was stabilized at 42.6° ± 0.3° C. In another group of mice, 5-FU and DP were given concomitantly, followed 60 min later by HYP. On day 12, the size of the tumors and the animals' body weights were recorded. The tumor weight in milligrams was estimated from measurements of its longest diameter (length) and its shortest diameter (width) in millimeters using the formula [tumor weight = length × $(\text{width})^2/2$] [11].

Statistical analysis. The significance of each difference between the mean values obtained for the various treatment groups was determined using Student's t-test. A value of P <0.05 was considered to be statistically significant.

Table 3. Effects of combination treatments with 5-FU, HYP and/or DP on B16 melanoma solid tumors

Treatment[a]	Tumor weight (mg)[b]	Body weight change (%)[c]
Control	40.6±13.9	105.1±5.7
5-FU	34.2±5.9	100.6±7.4
HYP	34.5±12.7	104.3±6.3
DP	38.9±8.6	103.2±8.1
5-FU + HYP	26.5±4.2*1, *2	101.6±6.7
5-FU + DP	24.3±7.6*1, *3	102.6±5.8
5-FU + HYP + DP	13.7±4.3*1-*5	99.8±4.2

Data represent mean values ± SD
[a] Eight mice were included in each group
[b] Tumor weights were estimated from tumor dimensions
[c] The mean body weight on day 12 is expressed as a percentage of that on day zero for each group
*1 Significantly different from 5-FU (P <0.05); *2 Significantly different from HYP (P <0.05); *3 Significantly different from DP (P <0.05); *4 Significantly different from 5-FU + HYP (P <0.05); *5 Significantly different from 5-FU + DP (P <0.05)

Results

Combined effects in vitro

The cytotoxic effect of 5-FU combined with HYP and DP was assessed using the SDI test. DP (2.5 μg/ml) was nontoxic to B16 melanoma and human tumor cells. HYP and DP enhanced the cytotoxicity of 5-FU against B16 melanoma cells (Table 1) and human tumor tissues (Table 2). Decreases in SD activity were more prominent following treatment with the combination of 5-FU, HYP and DP than after exposure to other single or combination treatments.

Combined effects in vivo

The effect of combination treatment with 5-FU, HYP and DP on solid B16 melanoma tumors was examined in groups of eight mice each. The tumor weights estimated on day 12 are shown in Table 3. When given alone, DP had no effect on tumor growth. Although tumor growth was slightly inhibited by treatment with 5-FU or HYP as single-agent therapy, exposure to 5-FU plus DP and to 5-FU plus HYP were more effective. The antitumor effect of 5-FU was markedly enhanced when the drug was given in combination with HYP and DP, and the tumor weights resulting from this treatment were significantly lower than those seen following the administration of any other combination. The mean body weight on day 12, expressed as a percentage of that on day 0, did not decrease in any group.

Discussion

5-FU has widely been prescribed both as a single agent and in combination with other drugs for the treatment of gastrointestinal, breast and ovarian cancers and squamous-cell

carcinomas of the head and neck [6]. As the effect of 5-FU varies with the patient treated and rates of objective response to the drug range between 20% and 25%, combination therapy is needed to enhance the cytotoxic effect of 5-FU [4, 15].

5-FU is phosphorylated to 5-FU metabolites via three enzymatic pathways that involve pyrimidine nucleotide synthesis [21, 37]; thymidine monophosphate (dTMP) synthase (EC 2.1.1.45) [16, 17], DNA synthesis [40], and RNA synthesis [42] are inhibited and the cells die. We have found that the cytotoxic effect of 5-FU against HeLa or B16 melanoma cells in vitro is enhanced by concomitant treatment with HYP and DP [31]. As shown by Grem and Fischer [12] and by Kusumoto et al. [23], no increase in the concentration of 5-FU was seen at the intracellular level following treatment with any combination of the drugs. DP does not alter the influx of 5-FU but does reduce the efflux of fluoro-nucleosides, and a concomitantly marked enhancement of intracellular levels of 5-fluorodeoxyuridine 5′-monophosphate has been noted in a colon cancer cell line after its exposure to 5-FU and DP [13]. HYP improves the selectivity of drugs that are used to treat malignant lesions [29] because 5-FU and fluoro-nucleosides are more likely to be phosphorylated in tumors than in normal tissues during HYP [25]. In the present investigation, the phosphorylating activity of 5-FU was higher at 43° C than at 37° C in homogenates of murine and human tumor tissues. 5-FU is expected to be more rapidly metabolized to active forms when it is used in combination with both HYP and DP than when it is given alone or in combination with either HYP or DP.

In the present study, we used the SDI test (MTT assay) [30] to determine the effect of different combinations of these drugs. This test was introduced to examine chemosensitivity as judged by the correlation of succinate dehydrogenase (EC 1.3.99.1) activity with cell viability using tetrazolium salt (MTT) as a hydrogen acceptor. The results of the SDI test were found to correlate with those obtained using the clonogenic assay [5], the ATP assay [2, 27] and the nude-mouse assay [41]. The SDI test clearly revealed the combined effect of 5-FU, HYP and DP on B16 melanoma and human tumor tissues in vitro.

We found that the antitumor effect of 5-FU is also enhanced in vivo when the drug is given in combination with both HYP and DP. Neither DP nor DP plus HYP enhanced the adverse effects of 5-FU in vivo as represented by changes in body weight. Thus, local HYP combined with DP enhances the antineoplastic effect of 5-FU in the region of the local tumor. The preoperative use of this treatment can reduce the dissemination of tumor cells. The clinical response of gastrointestinal cancers to antitumor drugs, including 5-FU, has thus far been unsatisfactory [15]. Previous findings in human tissues prompted us to examine the efficacy of these three combinations in the treatment of patients presenting with gastric and colorectal cancers. Since DP binds extensively to protein in human plasma, it shows low activity as a modulator [8, 19]. As the DP concentration that is required for the inhibition of nucleoside uptake is low [14], concentrations of DP that are sufficient to result in synergism between this drug and 5-FU can safely be attained [39]. DP is quite suitable for clinical application, and the combined clinical treatment of malignancies with 5-FU, HYP and DP is being given serious attention.

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