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In vivo Antitumor Effects of Fluoropyrimidines on Colon Adenocarcinoma 38 and Enhancement by Leucovorin

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Antitumor effect and active metabolites of fluoropyrimidines were examined in mice with transplantable colon adenocarcinoma 38 (Co 38). 5-Fluoro-2′-deoxyuridine (FUdR) treatment resulted in a much higher level of free 5-fluoro-2′-deoxyuridine-5′-monophosphate in the tumor than 5-fluorouracil (5-FU) did, and thymidylate synthase was almost completely inhibited after FUdR treatment, but FUdR showed weaker antitumor activity than 5-FU did. Moreover, 5-fluorouridine (FUR) also hardly inhibited tumor growth. A more marked tumor inhibition was obtained when FUdR and FUR were administered together. The antitumor activity of 5-FU was similar to that of the combination of FUdR and FUR. In combination with 2,2′-anhydro-5-ethyluridine, a uridine phosphorylase inhibitor, FUdR lost its antitumor activity, but that of FUR was somewhat potentiated. On the other hand, in combination with leucovorin (LV), 5-FU showed markedly potentiated antitumor activity, while the antitumor activity of FUdR or FUR was not potentiated. Addition of LV to the combination of FUdR and FUR enhanced the inhibitory effect of the drugs. From these results, the combination of FUdR and FUR together with LV, and the combination of 5-FU and LV seem to be highly efficaceous against Co 38.

Key words: Fluoropyrimidine — 5-Fluorouracil — 5-Fluoro-2′-deoxyuridine — Antitumor activity — Colon adenocarcinoma 38

5-Fluorouracil (5-FU) is still the drug of preference for chemotherapy of colorectal cancers in spite of the agent's limited effectiveness; the reported response rates are 10–20%. In recent years biochemical modulation of 5-FU with leucovorin (LV, 5-formyltetrahydrofolate) has attracted much interest for chemotherapy of advanced colorectal cancer since this combination has been proven more effective than 5-FU alone.[1–5] Houghton *et al.*[6] observed that, in some 5-FU-resistant xenograft tumors, endogenous pools of reduced folates were too low to allow complete inhibition of thymidylate synthase (TS) by 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), and maximum binding of FdUMP could only be achieved with an excess concentration of LV.[7] In the colon adenocarcinoma 38 (Co 38) tumor system, the antitumor activity of 5-FU is markedly potentiated by addition of LV.[8] However, we found that the optimal dose of 5-fluoro-2′-deoxyuridine (FUdR), that produced a large amount of FdUMP in the tumor, was less effective on Co 38 compared to the optimal dose of 5-FU. Furthermore, the antitumor activity of FUdR was not potentiated by addition of LV. Thus, we investigated the optimal conditions to get maximum antitumor effect of fluoropyrimidines in the Co 38 tumor system.

MATERIALS AND METHODS

Drugs 5-FU, 5-fluorouridine (FUR), FUdR and FdUMP were obtained from Sigma Chemical Co., St. Louis, MO. *dl*LV was kindly supplied by Lederle (Japan) Ltd., Tokyo. 2,2′-Anhydro-5-ethyluridine (ANEUR) was generously provided by Dr. Szinai.

Animals Groups of six specific-pathogen-free male BDF_1 mice with body weights of 21–23 g (SLC Japan, Hamamatsu) were housed in plastic cages with woodchip bedding and received CA-1 pellet diets (CLEA Japan, Inc., Tokyo) and water *ad libitum*. All experiments were performed in an animal laboratory with controlled temperature (25°C).

Treatment and antitumor activity Co 38 tumor cells (15 mg/mouse) were implanted subcutaneously (s.c.) on day 0. The tumors had been maintained by s.c. transfer every 3 weeks into syngeneic C57BL/6 mice kept in our laboratory. As soon as the tumors were sufficiently developed (5×5 mm) (about day 10–14), the mice were randomized before being divided into groups of 6 animals each and the drugs were administered intraperitoneally (i.p.). 5-FU was administered at its maximum tolerated dose for 5 daily or 5 weekly injections, 20 or 100 mg/kg/day, respectively. FUdR and FUR were administered weekly at doses of 300 and 20 mg/kg/day, respectively. The growth of the implanted Co 38 was monitored by measuring the perpendicular diameters with calipers, and the tumor volume (mm^3) was calculated by means of the formula $(1/2) \times (\text{major diameter in mm}) \times (\text{minor diameter in mm})^2$.

Measurement of intratumor FdUMP pool following fluoropyrimidine treatment A single dose of 5-FU (100 mg/kg), FUdR (300 mg/kg) and 5-FU plus LV (200 mg/kg) was given i.p. to mice bearing a twelve-day-old

tumor. The mice were killed 30 min, 1, 3, 6, 15 and 24 h later (three mice/group) and the tumors were removed and chilled as quickly as possible in dry-ice acetone. Free FdUMP in the tumors was determined as reported by Moran *et al.*[9]

TS level determination To quantitate free TS (not binding with FdUMP) in the tumor, the amount of [^{3}H]FdUMP bound to the enzyme in the presence of 5,10-methylenetetrahydrofolate was determined by the procedure of Moran *et al.*[9]

Statistical analysis The *t* test for small samples was used to determine the statistical significance; $P \leqq 0.05$ was considered significant.

RESULTS

Antitumor effect of FUdR and FUR on Co 38 tumor growth In this tumor system, the maximum tolerated dose of FUdR (300 mg/kg) showed weaker antitumor activity than 5-FU (100 mg/kg) as shown in Fig. 1. Moreover, FUR at 20 mg/kg, that is, the maximum dose in this schedule, hardly inhibited the tumor growth. However, administration of FUdR plus FUR significantly inhibited the tumor growth as compared to the effect of each drug when given alone (Fig. 2). When combined with ANEUR, which is a potent inhibitor of uridine phosphorylase,[10] FUR showed enhanced antitumor activity, while FUdR lost its activity (Fig. 3).

Effect of LV on antitumor activity of fluoropyrimidines in Co 38-bearing mice Simultaneous administration of LV (740 mg/kg) and 5-FU enhanced the antitumor activity as compared with 5-FU alone in a 5 weekly injections regimen. Moreover, treatment with half the dose of LV (370 mg/kg) 1 h before and the other half together with 5-FU resulted in more antitumor activity than 5-FU alone or the simultaneous treatment with LV plus 5-FU (Fig. 4). Following this treatment schedule, tumor-bearing mice were given various doses of LV plus 5-FU (100 mg/kg). Better results were obtained when mice were treated with more than 100 mg/kg (twice/day) of LV. However, the antitumor activity of FUdR or FUR was not significantly potentiated by addition of LV (Figs. 2 and 5). On the other hand, when LV was added to the combination of FUdR and FUR, the tumor growth was more markedly inhibited than when the combination of just FUdR and FUR was used (Fig. 2).

Free FdUMP level and TS inhibition in the tumor after treatment with fluoropyrimidines The level of free FdUMP following treatment with 5-FU plus LV was 301 ±93 pmol/g, i.e., higher than after administration of 5-FU alone, when free FdUMP reached a peak of 254± 82 pmol/g at 15 min posttreatment. Moreover, the level of FdUMP remained higher in the tumors of mice given 5-FU plus LV than in those given 5-FU alone, i.e., the levels were 179±57 and 56±20 pmol/g at 3 h posttreatment, respectively. TS inhibition in the tumor following administration of 5-FU plus LV was also higher (84%) than after administration of 5-FU alone (75%) at 3 h posttreatment (Fig. 6). When FUdR was administered to tumor-bearing mice, the free FdUMP level in the tumor

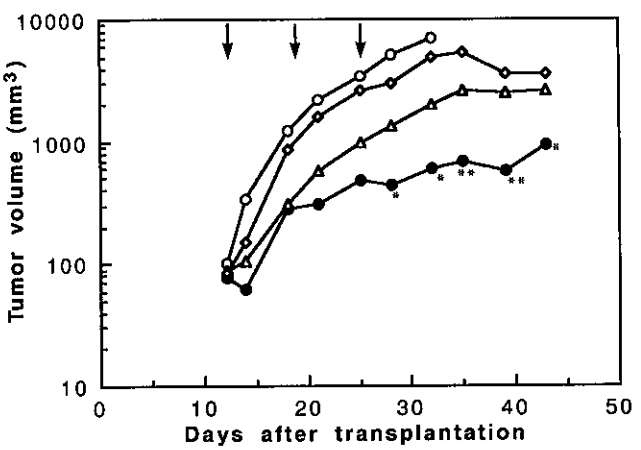

Fig. 1. Effect of fluoropyrimidines on growth of colon adenocarcinoma 38. ○, Control; ◇, FUR (20 mg/kg/day); △, FUdR (300 mg/kg/day); ●, 5-FU (100 mg/kg/day). Arrows indicate the i.p. injections. *, Compared with FUdR, $P \leqq 0.05$; **, $P \leqq 0.01$.

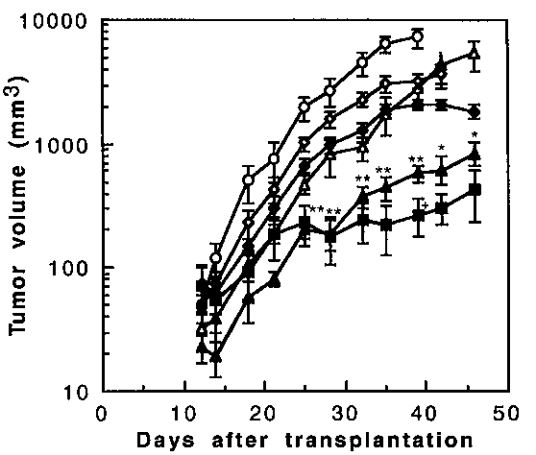

Fig. 2. Effect of LV on the antitumor activity of FUR and FUR plus FUdR against colon adenocarcinoma 38. The doses of FUdR and FUR were 300 and 20 mg/kg/day, respectively. LV (370 mg/kg) was administered twice 1 h before and together with the fluoropyrimidines. The drugs were administered i.p. on days 12, 19, 26, 33 and 40. Bars indicate SE. ○, Control; ◇, FUR; ◆, FUR+LV; △, FUdR; ▲, FUdR+FUR; ■, FUdR +FUR+LV. *, Compared with FUdR alone, $P \leqq 0.05$; **, $P \leqq 0.01$. +, Compared with FUdR+FUR, $P \leqq 0.05$.

was much higher than after administration of 5-FU, and TS was almost completely inhibited (Fig. 7), i.e., FdUMP level and TS inhibition 30 min after treatment with FUdR were 4600±267 pmol/g and 98%, respectively. The free FdUMP level rapidly decreased and was only 65.0 pmol/g at 6 h posttreatment, though strong TS inhibition (95%) was still observed at this time. Thus, the FdUMP level and TS inhibition after administration of FUdR were much higher than after 5-FU. These results suggest that disruption of RNA by incorporation of FUTP into RNA is required to produce the antitumor effect of 5-FU.

DISCUSSION

When Co 38-bearing mice were treated with FUdR, larger amounts of FdUMP (more than 10-fold compared to 5-FU treatment) were produced and inhibition of TS in the tumor was greater than when 5-FU was administered. Spears *et al.*[11] reported that TS inhibition is an important correlate of 5-FU cytotoxicity. However, in this tumor system the maximum tolerated dose of FUdR showed weaker antitumor activity than that of 5-FU. Moreover, the *in vivo* antitumor activity of FUdR was

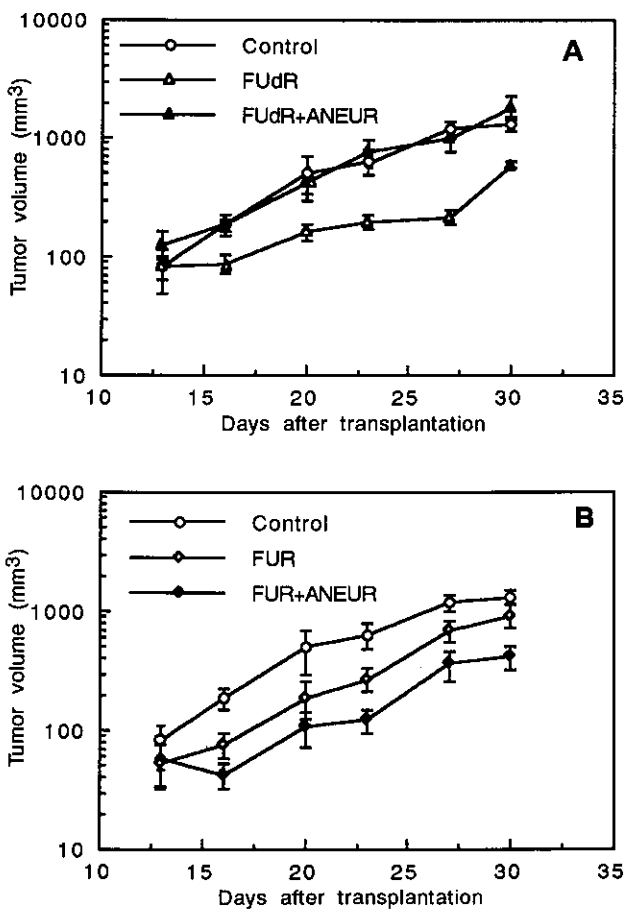

Fig. 3. Effect of ANEUR on antitumor activity of FUdR (A) and FUR (B). The doses of ANEUR, FUdR and FUR were 100, 300 and 20 mg/kg/day, respectively. The drugs were administered i.p. on days 13, 20 and 27. Bars indicate SE.

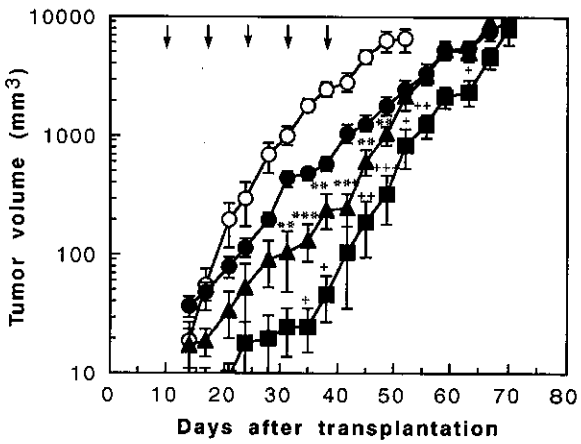

Fig. 4. Effect of LV and 5-FU given once a week on tumor growth. ○, Control; ●, 5-FU (100 mg/kg/day); ▲, combination of 5-FU and LV (740 mg/kg/day); ■, LV (370 mg/kg) was administered 1 h before and together with 5-FU. Bars indicate SE. Arrows indicate injections. *, Compared with 5-FU alone, $P \leqq 0.05$; **, $P \leqq 0.01$; ***, $P \leqq 0.001$. +, Compared with 5-FU+LV, $P \leqq 0.05$; ++, $P \leqq 0.01$; +++, $P \leqq 0.001$.

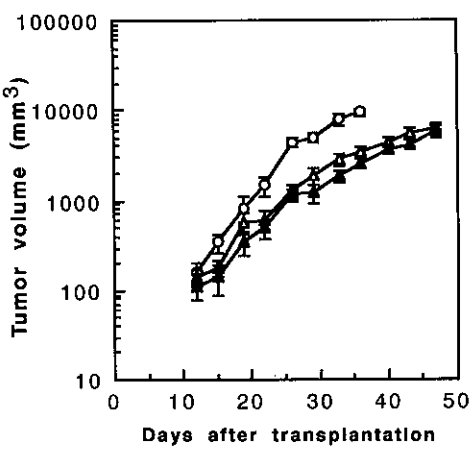

Fig. 5. Effect of LV on the antitumor activity of FUdR against colon adenocarcinoma 38. LV (370 mg/kg) was administered twice 1 h before and together with FUdR (300 mg/kg). The drugs were administered i.p. on days 12, 19, 26, 33 and 40. Bars indicate SE. ○, Control; △, FUdR; ▲, FUdR+LV.

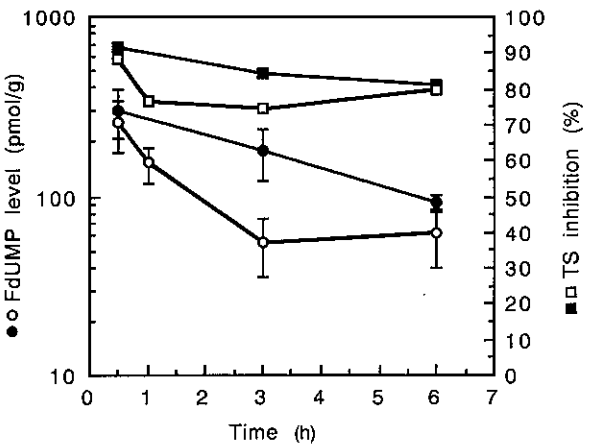

Fig. 6. Free FdUMP levels and TS inhibition following administration of 5-FU (○, □) and its combination with LV (●, ■). The doses of 5-FU and LV were 100 and 200 mg/kg, respectively. LV was administered 1 h before and together with 5-FU. Bars indicate SE.

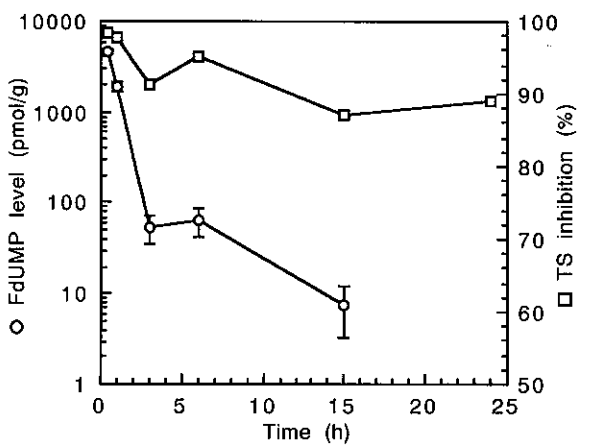

Fig. 7. Free FdUMP levels and TS inhibition following administration of FUdR (300 mg/kg). Bars indicate SE.

enhanced little by the addition of LV, though numerous cell culture studies have clearly demonstrated that LV can enhance the growth-inhibitory effect of 5-FU or FUdR.[12-14] On the other hand, antitumor activity of 5-FU was markedly enhanced when a large dose of LV was administered weekly 1 h before and together with the optimal dose of 5-FU, in agreement with the data of Nadal *et al.*[8] The combination of 5-FU and LV resulted in higher TS inhibition than that seen after administration of 5-FU alone.

ANEUR blocks the metabolism of FUR or FUdR to 5-FU because of the inhibitory effect of ANEUR on uridine phosphorylase. Plasma concentration of FUR or FUdR is markedly increased following administration of FUR or FUdR together with ANEUR.[10] In the combination of ANEUR and fluoropyrimidines in this tumor system, antitumor activity of FUdR was annihilated and that of FUR was somewhat potentiated. These observations suggest that TS inhibition may not be critical to produce antitumor activity of fluoropyrimidines. Thymidine administration, that bypasses the *de novo* block to synthesis of thymidylate, does not always relieve the cytotoxic effects of 5-FU,[15,16] and these results suggest the chemotherapeutic importance of RNA disruption by incorporation of FUTP into RNA. Recently, Nord and Martin reported that thymidine kinase activity from Co 38 was severely inhibited when a high dose of 5-FU (100 mg/kg) was administered, and its effect appears to correlate with incorporation of FUTP into RNA.[17]

However, in the tumor system described here, FUR showed weak antitumor activity, while ANEUR somewhat increased the antitumor activity of FUR. In contrast to FUdR or FUR alone, combined treatment with these drugs caused marked inhibition of the tumor growth. Moreover, the tumor growth inhibition by the combination of FUdR and FUR was enhanced by LV.

In the case of murine adenocarcinoma 755, ANEUR markedly potentiates the antitumor activity of FUR but abolishes that of FUdR.[10] LV does not potentiate the antitumor activity of 5-FU in this tumor system. On the other hand, FUdR remains active against human colon carcinoma LS174T when its conversion to 5-FU is impeded by ANEUR,[10] and LV potentiates the antitumor activity of 5-FU. In contrast to these tumors, LV markedly enhanced the activity of 5-FU against Co 38. However, the weak inhibition of the tumor growth by FUdR in spite of the dramatic reduction of TS activity and the diminution of the antitumor activity of FUdR by ANEUR suggest that in addition to the inhibitory effect of FdUMP on TS, RNA disruption following incorporation of FUTP into RNA may also be important to enhance antitumor activity against Co 38. Thus, the mechanism of natitumor activity of fluoropyrimidines may be different in different tumors. The combination of FUdR and FUR together with LV, as well as the combination of 5-FU and LV, may be a good way to get maximum antitumor effect on Co 38.

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