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5′-Deoxy-5-fluorouridine improves cachexia by a mechanism independent of its antiproliferative action in colon 26 adenocarcinoma-bearing mice

Hiroyuki Eda, Yutaka Tanaka, and Hideo Ishitsuka

Nippon Roche Research Center, Dept. of Oncology and Immunology Kajiwara 200, Kamakura City, Kanagawa-247, Japan

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Summary. The cytostatic agent 5′-deoxy-5-fluorouridine (5′-dFUrd) improves cachexia and prolongs survival, suppressing tumor growth in mice bearing large burdens of colon 26 adenocarcinoma. To investigate the mode of this anticachectic action, we isolated colon 26 variants that were resistant to the anticachectic activity in vivo in tumor-bearing mice that initially responded to 5′-dFUrd in terms of tumor growth and cachexia but again became cachectic and refractory to the drug after prolonged treatment. The original line and variants were equally susceptible to the antiproliferative action of 5′-dFUrd, and their growth was stopped. However, 5′-dFUrd given to cachectic mice exhibiting large burdens of these variants could not reverse wasting and only slightly prolonged the survival period. These results indicate that the anticachectic activity of 5′-dFUrd is independent of its antiproliferative action and that the survival of colon 26-bearing mice is shorter when the size of the tumors is not reduced to levels below those that cause cachexia.

Introduction

We have reported that murine colon 26 adenocarcinoma causes progressive wasting and physiological changes associated with tumor cachexia such as hypoglycemia, hormonal imbalance, hepatic malfunction, and elevation of acute-phase reactants when the tumor size reaches 2–3 g [14]. In addition, we have found that the cytostatic agent 5′-deoxy-5-fluorouridine (5′-dFUrd) improves these abnormalities in mice bearing this tumor [15]. Peters et al. [12] have also observed that 5′-dFUrd improves weight loss in this tumor model.

5′-dFUrd is a prodrug that is converted to the active metabolite 5-fluorouracil (5-FUra) by uridine and thymidine phosphorylases, which are more abundant in tumors than in normal tissues except for the intestinal tract [8–10]. Consequently, 5′-dFUrd has been shown to be both more effective against many murine transplantable tumors in terms of therapeutic indices and less toxic, particularly less immunosuppressive, than 5-FUra [1–3, 11]. Since 5-FUra and its derivatives 2′-deoxy-5-fluorouridine (2′-dFUrd) and tegafur, which share the same antitumor mechanism, were not significantly capable of improving colon 26-mediated cachexia, it was suggested that 5′-dFUrd exerts its anticachectic activity by a mechanism different from that previously reported for 5-FUra [15].

To investigate the mode of this anticachectic action, we isolated colon 26 variants that were resistant to the anticachectic activity of 5′-dFUrd in mice that initially responded to the drug in terms of tumor growth and cachexia but again became cachectic and refractory to 5′-dFUrd after prolonged treatment. In the present study, we compared various responses of these variants to 5′-dFUrd with those of the original line and found that the anticachectic activity of 5′-dFUrd is independent of its antiproliferative action.

Materials and methods

Animals and tumors. Male CDF_1 (BALB/c × DBA/2)F_1 mice aged 4 weeks were obtained from Japan SLC (Hamamatsu, Japan) and were used after they had reached the age of 5 weeks. Six animals per cage were bred at 22°±2°C and 55%±5% humidity. They were given the F1 breeding diet of Funabashi Farm (Funabashi, Japan) and water ad libitum [14]. Murine colon 26 adenocarcinoma cells, kindly supplied by Dr. T. Kataoka at the Cancer Chemotherapy Center (Cancer Research Foundation, Tokyo, Japan), were cultured in vitro with RPMI 1640 containing 10% fetal calf serum. A single-cell suspension of these cells (10^6 cells) obtained by treatment with trypsin was inoculated s.c. into the CDF_1 mice.

Drug treatment. 5′-dFUrd (Nippon Roche, Tokyo, Japan) was dissolved in a sterile solution of carboxymethyl cellulose (0.5%) and given orally to mice bearing colon 26 adenocarcinoma. Drug treatment was commenced at approximately 22 days after inoculation of the tumor, at which time the difference between the carcass weight of these tumor-bearing mice and that of age-matched controls was reduced by >7 g, which

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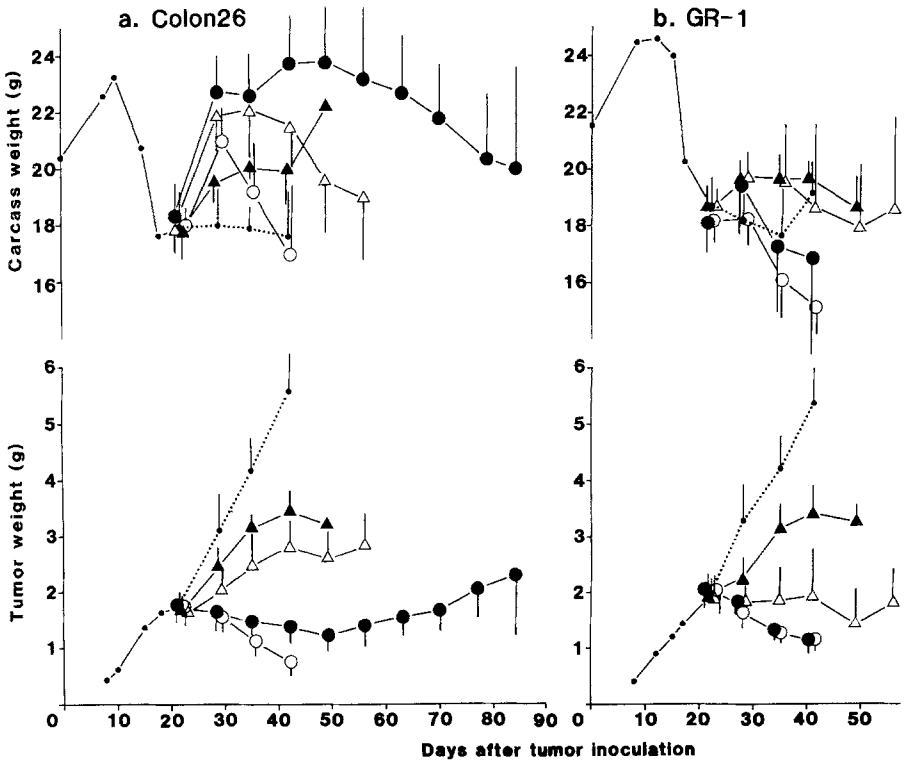

Fig. 1 a, b. Antitumor and anticachectic activities of 5′-dFUrd in mice bearing the original and variant lines of colon 26 adenocarcinoma. **a** Groups of 7 mice were inoculated with colon 26 cells (10^6 cells) on day 0. Then, the mice were given daily oral doses of vehicle or 5′-dFUrd from day 22 until their death. The tumor size and carcass weight were measured weekly and included in the analysis for as long as half of the mice survived. **b** At day 122 after inoculation of the original colon 26 line, cells of the variant colon 26 GR-1 tumor were harvested from a regrowing solid tumor in one mouse that was treated with 5′-dFUrd (0.5 mmol/kg daily). Colon 26 GR-1 cells were inoculated into mice on day 0. The animals were then given oral doses of vehicle or 5′-dFUrd from day 22 until their death. •, Vehicle; ▲, 0.125 mmol/kg 5′-dFUrd; △, 0.25 mmol/kg 5′-dFUrd; ●, 0.5 mmol/kg 5′-dFUrd; ○, 1 mmol/kg 5′-dFUrd. A significant improvement in wasting occurred between days 22 and 43 during treatment with 5′-dFUrd at doses of 0.125–0.5 mmol/kg (Fig. 1 a; $P < 0.0001$, Tukey-Kramer test)

corresponds to 25% of the weight of normal mice; this was defined as cachexia in the present study.

Isolation of colon 26 variants. After 2–4 months of daily treatment with 0.25 or 0.5 mmol/kg 5′-dFUrd, the tumor began to regrow and some of the tumor-bearing mice again became cachectic. Colon 26 variant cells were harvested from the regrowing solid tumors. The variant tumor cells were passaged once in vivo and then in vitro. For this purpose, tumor tissues that exhibited no necrotic portions were cut into small pieces and suspended in RPMI 1640 containing 10% fetal calf serum. The cells grown in in vitro cultures were harvested and passaged. A variant of the colon 26 tumor (DR-2P) isolated described as above was further passaged twice in vivo. Then, single cell lines of colon 26, including the variant DRC-3 line, were cloned from DR-2P cells in tissue culture.

Measurement of tumor, body, and tissue weight. The mice were weighed and the length (a) and width (b) of the tumor were measured twice a week. The tumor volume was estimated using the equation $ab^2/2$, and the tumor weight was then estimated as described elsewhere [14]. The carcass weight was calculated as the difference in weight between whole-body and tumor tissues. Tumor growth inhibition was calculated using the equation $(1\text{-}T/C) \times 100$ (expressed in percent), where T and C represent the tumor weight gain in mice that were treated with drugs and with vehicle, respectively. The left epididymal adipose tissue was dissected and weighed.

Assay. For determinations of antiproliferative activity, tumor cells (1×10^4 cells/well) were cultured in the presence of various doses of 5′-dFUrd, 2′-dFUrd, and 5-FUra in RPMI 1640 containing 10% fetal calf serum at 37°C in an atmosphere containing 5% CO_2. After 3 days, the number of cells was counted and the 50% inhibitory dose (ID_{50}), i. e., that at which cell growth was inhibited by 50%, was estimated. For determinations of glucose concentrations, blood samples were collected from the orbital veins and the concentration of glucose in the serum was measured by a color-reaction method using o-toluidine [7].

Statistical analysis. Differences in tumor size, carcass weight, and other biological parameters were compared using the Mann-Whitney U-test, Student's t-test, the generalized linear model for analysis of variance, or the Tukey-Kramer method for multiple comparisons, whereas differences in survival were compared using the SAS Life Test procedure. Differences exhibiting a value of $P < 0.05$ were considered to be significant.

Results

Isolation of colon 26 variants

Figure 1 a shows the growth curves for colon 26 and the fluctuation of the carcass weight of mice bearing the tumor. Carcass weight loss started at around days 10–14 and reached its maximum at around day 21 after tumor inoculation. When mice bearing large burdens of the tumor were given 5′-dFUrd daily from day 22 until their death, the drug reversed the carcass weight loss and inhibited the tumor growth. The weight loss was improved even at the lowest dose given (0.125 mmol/kg), following which there was some increase in the tumor size. At 0.5 mmol/kg, 5′-dFUrd increased the median survival of mice to 61 days, which represented an increase of 160% as compared with the survival of 23.5 days observed in controls, and maintained the carcass weight for longer periods. After 2 months of daily treatment, the response of the tumor gradually shifted from no change to regrowth, and the tumor-bearing mice lost weight and again became cachectic at around day 80. At 1 mmol/kg, 5′-dFUrd improved the wasting of animals only transiently and failed to prolong their survival, probably because of its toxicity, although it suppressed tumor growth during the first 2 weeks of treatment.

Colon 26 tumor variant GR-1 was obtained from tumor tissue in one of the mice that survived until day 122 due to

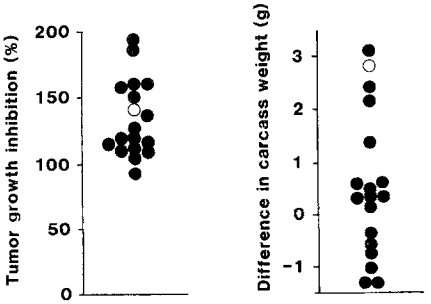

Fig. 2. Tumor growth inhibition and the change in the carcass weight of each mouse bearing the original (○) and 17 DR-2P variant (●) lines of colon 26 adenocarcinoma. 5′-dFUrd (0.5 mmol/kg) was given daily for 7 days starting at around day 22, at which time colon 26-bearing mice ($n = 6$) became cachectic. At 1 day after the last treatment, carcass weight gain and tumor growth inhibition were calculated

5′-dFUrd treatment at 0.5 mmol/kg in the experiment shown in Fig. 1a; the mouse subsequently became refractory to the antitumor action of the drug. The variant tumor was then examined for its susceptibility to 5′-dFUrd in vivo (Fig. 1b). The growth potential and cachexia-inducing capability of colon 26 GR-1 were similar to those of the original line. 5′-dFUrd given to cachectic mice exhibiting large burdens of colon 26 GR-1 suppressed the growth of this tumor at doses somewhat lower than those at which it inhibited that of the original line. However, the drug did not improve the weight loss in mice at of the any doses tested (0.125–1 mmol/kg), and it prolonged their survival only slightly at a dose of 0.25 mmol/kg. However, 5′-dFUrd given at 0.5 mmol/kg daily to the same tumor-bearing mice starting at 1 day after tumor inoculation inhibited the tumor growth and prolonged the survival of mice as long as the tumor remained smaller than the size that causes wasting (data not shown). The colon 26 variant tumor GR-1 that was isolated after long-term treatment with 5′-dFUrd induced 5′-dFUrd-resistant cachexia but remained susceptible to the antiproliferative action of the drug.

In two of four additional experiments in which cachectic mice bearing the original line were treated with 5′-dFUrd daily for periods of up to 3 months, colon 26 variant cell lines (DR-2P and HR-1) exhibiting characteristics similar to those of colon 26 GR-1 were obtained. One of the variants (DR-2P) was cultured in vitro, and 17 single cell lines, including variant DRC-3 derived from the DR-2P variant, were then randomly cloned and characterized for their susceptibility to the in vivo antiproliferative and anticachectic actions of 5′-dFUrd. All 17 clones grew and caused weight loss (7–10 g; compared with the body weight of age-matched normal mice). At 0.5 mmol/kg, 5′-dFUrd suppressed the growth of all of the variant lines as well as that of the original lines (Fig. 2, left). However, in mice that had been inoculated with 13 of the 17 clones, 5′-dFUrd could not reverse weight loss by >1 g as compared with that in animals that were treated with vehicle (Fig. 2, right). The tumor tissues from which the colon 26 variant lines were isolated after long-term treatment with 5′-dFUrd appeared to consist mainly of tumor cells that cause 5′-dFUrd-resistant cachexia yet also contained either

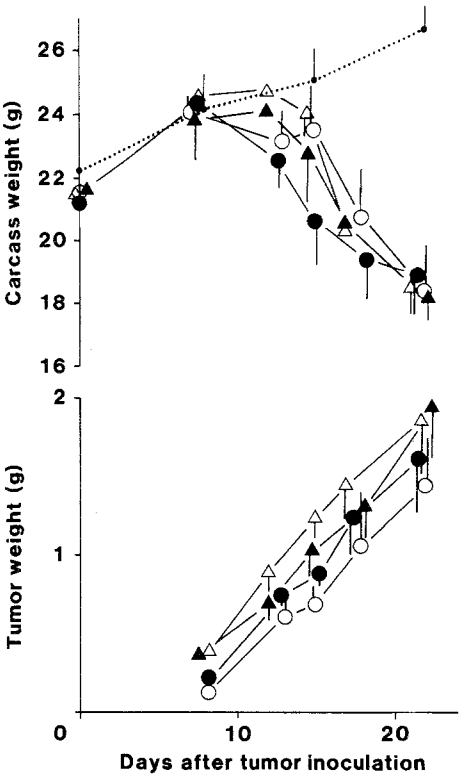

Fig. 3. Tumor growth and carcass weight change in mice bearing the original and variant lines of colon 26 adenocarcinoma. Colon 26 variant cells (10^6 cells) were inoculated s.c. into mice ($n = 6$), and subsequent changes in tumor size and carcass weight were determined. ○, Original line; ●, DRC-3 line; △, GR-1 line; ▲, HR-1 line; •, non-tumor-bearing controls

the parent tumor cells or those exhibiting characteristics similar to those of the parent line.

Characteristics of colon 26 variants

Figure 3 compares tumor growth and carcass weight in mice bearing original colon 26 adenocarcinoma and the three variant lines DRC-3, GR-1, and HR-1. These colon 26 lines grew equally and caused cachexia corresponding to a weight loss of >7 g as compared with the weight of non-tumor-bearing mice. Thus, the original line and the variants display similar characteristics in both their growth rate and their capability to cause cachexia. These tumor lines were further characterized by their susceptibility to the anticachectic and antitumor activity of 5′-dFUrd (Fig. 4). When 5′-dFUrd was given at doses ranging from 0.125 to 1 mmol/kg daily for 1 week to cachectic mice bearing the original tumor, it improved weight loss by 1.7–4.4 g and suppressed tumor growth by 47%–110% as compared with the values observed in tumor-bearing animals that were treated with vehicle. On the other hand, 5′-dFUrd could not reverse the weight loss in mice bearing the variant lines at of the any doses tested, whereas it suppressed the growth of all of the variants as well as that of the original line. The in vitro tests confirmed that the susceptibility of the variants to the antiproliferative action of 5′-dFUrd, 5-FUra, and 2′-dFUrd remained unchanged

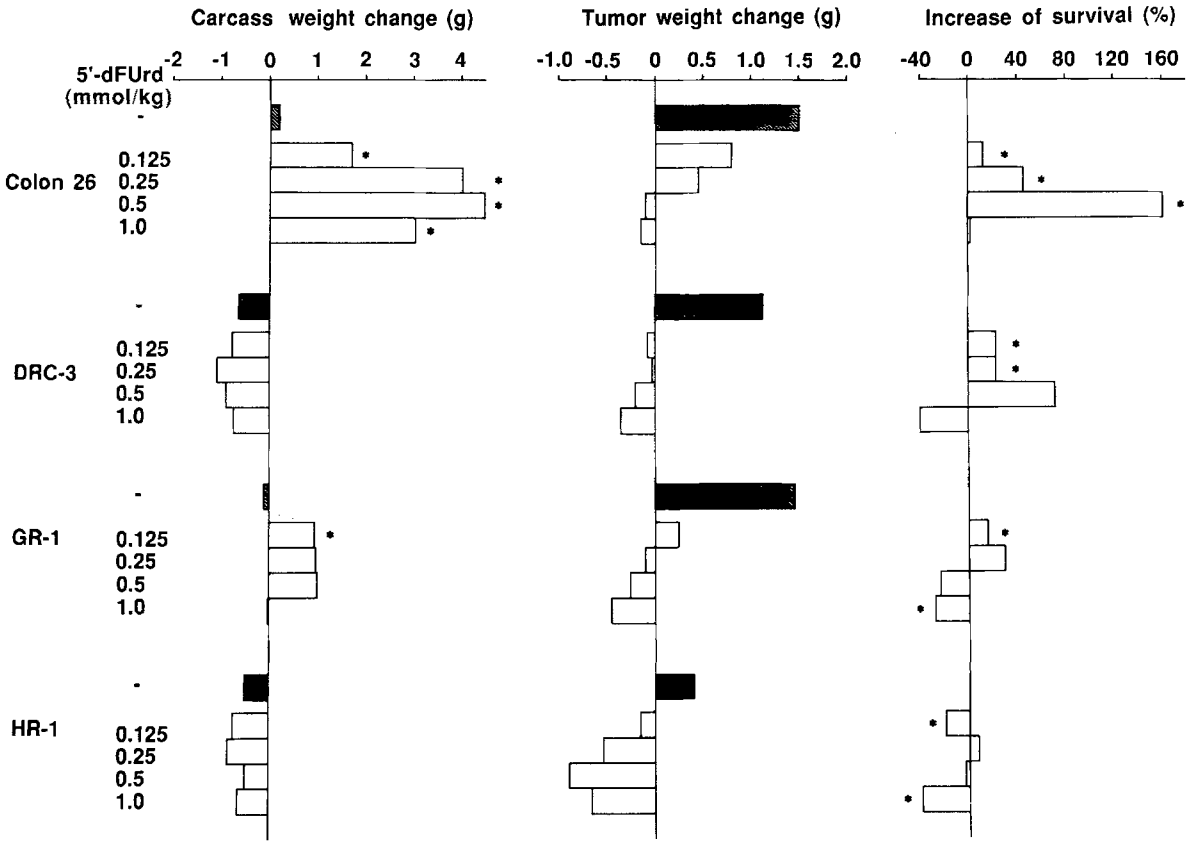

Fig. 4. Effects of 5′-dFUrd on the carcass weight, tumor size, and survival of mice bearing the original and variant lines of colon 26 adenocarcinoma. 5′-dFUrd was given daily to tumor-bearing mice on day 22, at which time the carcass weight and tumor weight were 17.9 and 1.66 g, respectively, for the original line; 20 and 1.48 g, respectively, for the DRC-3 line; 18.4 and 1.97 g, respectively, for the GR-1 line; and 19.2 and 2.14 g, respectively, for the HR-1 line. The treatment was continued until half of the mice in each group had died. The carcass weight gain and the inhibition of tumor growth were estimated by determining the difference between the carcass weight and tumor size measured on day 22 and those measured on day 29. The median survival was calculated from the initiation of treatment on day 22. * $P < 0.05$ vs untreated mice (Mann-Whitney U-test or SAS Life Test procedure for the increase in survival)

(data not shown). Thus, we could isolate colon 26 variants that caused 5′-dFUrd-resistant cachexia but remained susceptible to the antiproliferative action of the drug.

In the same experiments shown in Fig. 4, we also determined the survival of cachectic mice that were treated with 5′-dFUrd until their death (Fig. 5). 5′-dFUrd increased the median survival of mice bearing original colon 26 by up to 160% at 0.5 mmol/kg daily, the highest tolerated dose for long-term treatment. On the other hand, it did not substantially prolong the survival of mice bearing the three variants at of the any doses tested, although it suppressed the tumor growth. The only exception was that 5′-dFUrd given at 0.5 mmol/kg prolonged the survival, albeit not significantly, of mice bearing the variant DRC-3 line.

Resistance of the variants to the anticachectic activity of 5′-dFUrd was also confirmed by measuring other parameters associated with tumor cachexia, that is, hypoglycemia and adipose tissue wasting (Fig. 6). 5′-dFUrd improved these abnormalities as well as carcass weight loss in cachectic mice bearing original colon 26 adenocarcinoma when given at daily doses of 0.25 and 0.5 mmol/kg, at which the drug failed to produce any sign of toxicity or cause any change in these parameters in age-matched normal mice (data not shown). However, 5′-dFUrd did not improve these parameters in mice bearing the variant lines.

Discussion

In the present study, we isolated colon 26 variants that were resistant to the anticachectic activity of 5′-dFUrd but remained susceptible to its antiproliferative action. These findings revealed that the anticachectic activity of 5′-dFUrd is not merely associated with the antiproliferative action of 5-FUra, the active metabolite of this drug. The identification of the variants suggests that 5′-dFUrd directly affects the tumor cells, thus improving cachexia. 5′-dFUrd may either suppress the production of mediators in colon 26 cells that trigger the cachexia process or kill a particular tumor cell population that is likely to trigger the cachexia process. The involvement of a mechanism by which the toxicity of 5′-dFUrd in mice bearing the resistant variants might have interfered with its anticachectic activity, thus decreasing the appetite of the animals, is unlikely because all doses of 5′-dFUrd that were tested in cachectic mice bearing the variant lines, including those that produced no sign of toxicity (0.125–0.5 mmol/kg), failed to improve the wasting and hypoglycemia.

After long-term treatment with 5′-dFUrd, mice bearing the original colon 26 line became refractory to the antiproliferative and anticachectic actions of the drug. However, the variant lines isolated from the mice were suscep-

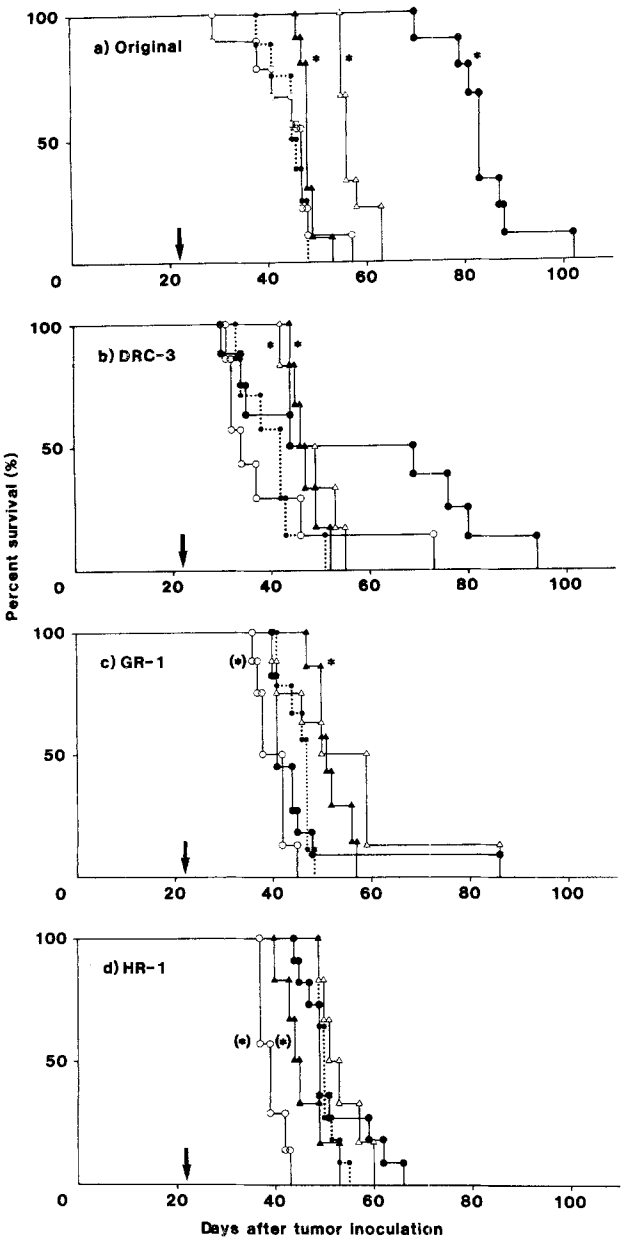

Fig. 5 a–d. Effects of 5′-dFUrd on the survival of mice bearing the original and variant lines of colon 26 adenocarcinoma. Colon 26 original and variant cells (10^6 cells) were inoculated s. c. into mice ($n = 6-12$). 5′-dFUrd was given daily to the animals from day 22 after tumor inoculation until their death. •, Vehicle; ▲, 0.125 mmol/kg 5′-dFUrd; △, 0.25 mmol/kg 5′-dFUrd; ●, 0.5 mmol/kg 5′-dFUrd; ○, 1 mmol/kg 5′-dFUrd. * $P < 0.05$ vs untreated mice (SAS Life Test procedure)

tible to either the in vitro or the in vivo antiproliferative action of 5′-dFUrd, although they were resistant to its anticachectic action. The change in the in vivo susceptibility was not attributable to selection of the sensitive cell lines during the isolation of the variants. The colon 26 tumor that had become refractory to 5′-dFUrd was also susceptible to the drug after young mice (5 weeks old) had been inoculated with the tumor and treated with the drug 1 day thereafter (data not shown). Following long-term treatment, unknown 5′-dFUrd toxicity or tumor-induced disorders that were not completely reversed by the drug may accumulate and disturb the efficacy and pharmacokinetics of 5′-dFUrd. In fact, a hepatic malfunction such as a decrease in the activity of P450 drug-metabolizing enzymes has been observed in cachetic colon 26 adenocarcinoma-bearing mice [15]. Moreover, the possible cachexia mediators tumor necrosis factor and interleukin-1 are known to reduce the activity of P450 drug-metabolizing enzymes [5, 6]. Hepatic malfunction and other disorders disturb drug pharmacokinetics, which may result in a change in susceptibility to the drug.

Cachexia is associated not only with a deterioration in the quality of life but also with a shorter survival. DeWys et al. [4] have reported that the survival of cancer patients who had experienced prechemotherapy weight loss was significantly shorter than that of those who had not. Using the colon 26 variants, we obtained results that lead us to suggest that tumor cachexia is associated with shorter survival. 5′-dFUrd given to cachectic mice bearing large burdens of original colon 26 adenocarcinoma improved cachexia and prolonged the survival of mice. However, it failed to improve cachexia or prolong the survival of cachectic mice bearing the colon 26 variants, although it inhibited the growth of the tumors. Furthermore, we have reported that indomethacin treatment increases the survival of mice bearing a large burden of colon 26 by improving the cachexia caused by the tumor, although the growth of the tumor was enhanced as compared with control values [13]. Survival seemed to be shorter as long as the size of the tumors was not reduced to levels below those that cause cachexia.

Mice suffering from cachexia-associated disorders of homeostasis, such as hypoglycemia, hypercorticism, hepatic malfunctions, and elevation of acute-phase reactants [14], may be vulnerable to either endogenous or exogenous stimuli. Another possibility may be that the growth of variant tumors metastasizing to a site that is inaccessible to 5′-dFUrd causes cachexia and results in shorter survival. However, this possibility is unlikely, because excision of primary tumors of both the original and the variant colon 26 lines resulted in the cessation of weight loss followed by a rapid weight gain, the and metastatic potential of these colon 26 lines was not extremely different (data not shown). Disorders that are not directly associated with cachexia may also account for the reduction in survival. The mechanisms that cause shorter survival in cachexia remain to be investigated.

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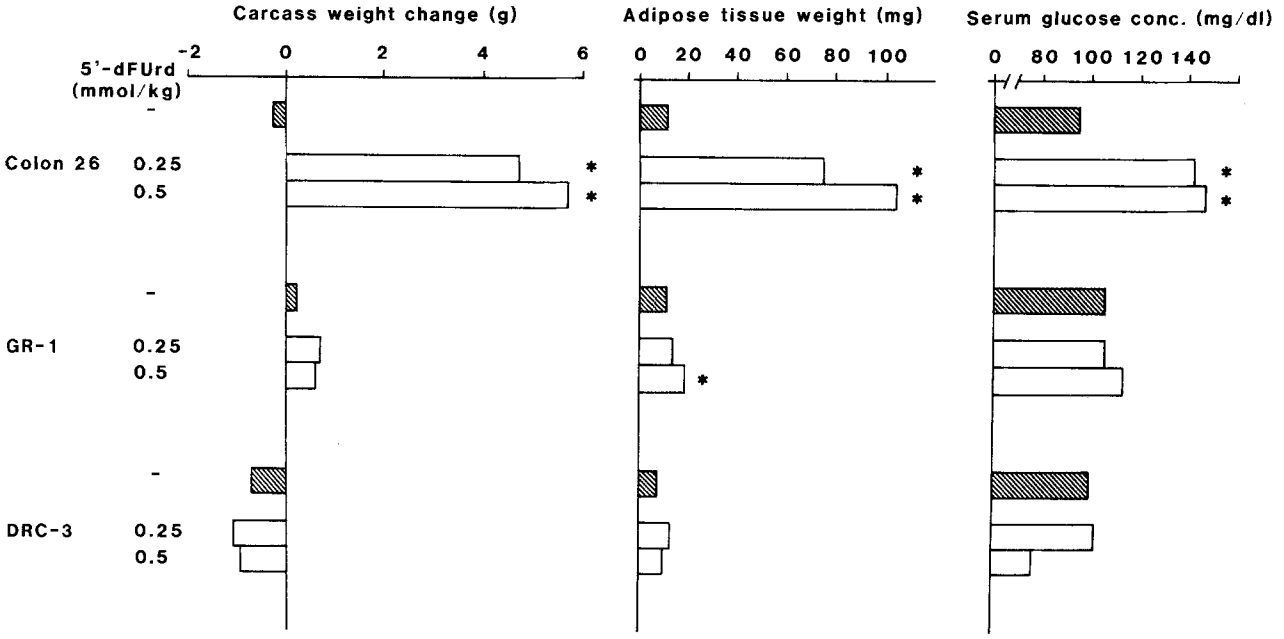

Fig. 6. Recovery of 5′-dFUrd from the carcass, adipose tissue wasting, and hypoglycemia in cachectic mice bearing the original colon 26 line as compared with those bearing variant lines DRC-3 or GR-1. Mice (n = 3–6) bearing the original colon 26 cell line or the DRC-3 or GR-1 variant lines were given 5′-dFUrd daily for 7 days beginning on day 22 after tumor inoculation. On day 29, the body weight gain, the adipose tissue weight, and the serum glucose concentration were measured. * P <0.05 vs untreated mice (Student's t-test)

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