Jpn. J. Cancer Res. **90**, 454–459, April 1999

Enhancement of the Anti-tumor Effect of 5′-Deoxy-5-fluorouridine by Transfection of Thymidine Phosphorylase Gene into Human Colon Cancer Cells

Hiroki Kanyama, Naohiro Tomita, Tomoki Yamano, Yasuo Miyoshi, Masayuki Ohue, Yoshiyuki Fujiwara, Mitsugu Sekimoto, Isao Sakita, Yasuhiro Tamaki and Morito Monden

Department of Surgery II, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871

Thymidine phosphorylase (dThdPase) is an enzyme that converts 5′-deoxy-5-fluorouridine (5′DFUR) to the toxic substance 5-fluorouracil (5-FU); it is also known to be a platelet-derived endothelial cell growth factor. In order to investigate the feasibility of suicide gene therapy against colorectal cancer by means of the combination of 5′DFUR and the converting enzyme dThdPase, we transfected the dThdPase gene into the human colon cancer cell line SW480 and analyzed the growth pattern as well as the sensitivity to 5-FU or 5′DFUR of the dThdPase-transfected cells. The 50% inhibition (IC_{50}) values of 5-FU against the SW480 parental cells, control vector-transfected cells SW480/V1, and dThdPase-transfected cells SW480/dThdPase were approximately 4.9, 6.3, and 2.9 μM, respectively. The IC_{50} of SW480/dThdPase was lower than that of SW480 or SW480/V1, although the differences were not statistically significant. The IC_{50} values of 5′DFUR for SW480, SW480/V1, and SW480/dThdPase were approximately 300, 330, and 3.2 μM, respectively. The sensitivity to 5′DFUR of SW480/dThdPase was increased by about 100-fold compared with that of SW480 or SW480/V1. With only 10% transfection efficacy, a high enough sensitivity to 5′DFUR was obtained to suppress the cell growth, indicating that a strong bystander effect was induced by this system. The *in vivo* growth of the s.c. transplanted SW480/dThdPase tumor in nude mice was significantly suppressed by i.p. injection of 5′DFUR compared with that in control mice that received phosphate-buffered saline (PBS) treatment. These results suggest that gene therapy using the combination of 5′DFUR and the dThdPase gene may be a useful approach for treatment of colon cancer.

Key words: dThdPase — Converting enzyme — 5′DFUR — Colon cancer — Suicide gene therapy

In line with the recent westernization of Japanese dietary habits, the incidence of colon cancer has been increasing in Japan. Although the prognosis for colon cancer patients who have undergone curative surgical resection is relatively fair compared with that for malignancies of other organs, e.g., esophagus or pancreas, chemotherapy with anti-cancer drugs remains essentially the only option for treating far-advanced colon cancer. 5-Fluorouracil (5-FU) is the chemotherapeutic agent which has been most widely used for colon cancer, and it has proved to be of some value in individuals with tumors sensitive to this agent. Its use, however, is sometimes limited by side effects, when the drug is given systemically. Among various strategies to decrease the side effects, suicide gene therapy[1–3] using molecular biological techniques seems to be one of the most promising. The strategy of suicide gene therapy basically involves the introduction into tumor cells of nonmammalian genes encoding enzymes that convert nontoxic prodrugs into toxic anti-metabolites, together with the systemic application of the prodrugs. By this means, it could be feasible to achieve high local concentrations of 5-FU in the target tumor tissue.

Thymidine phosphorylase (dThdPase) catalyzes the reversible phosphorolytic cleavage of thymidine, deoxyuridine and their analogues to their bases and deoxyribose 1-phosphate. Thus, it converts 5′-deoxy-5-fluorouridine (5′DFUR) to 5-FU.[4, 5] Furthermore, dThdPase catalyzes the conversion of the pyrimidine antimetabolite 5-FU to 5-fluoro-2′-deoxyuridine.[6] This enzyme is widely expressed in human tissues, including leucocytes and platelets.[7–9] The levels of dThdPase expression vary up to 15-fold in different human tissues and between different individuals.[7, 10, 11] As for expression in cancer tissues, dThdPase levels have been reported to be elevated compared with the levels in non-neoplastic counterparts. In colon cancer, however, the dThdPase level is not as high as it is in other cancers.[10–12] Therefore, if it were possible to increase the level of dThdPase in colon cancer tissue, we could reasonably expect a greater anti-tumor effect of 5′DFUR. Here, we report our *in vitro* and *in vivo* investigations into the feasibility of suicide gene therapy for colorectal cancer using 5′DFUR and the converting enzyme dThdPase.

E-mail: cba77840@pop06.odn.ne.jp

MATERIALS AND METHODS

Cells line Human colon cancer cell line SW480 was maintained in Dulbecco's modified minimal essential medium (DMEM) (Nikken Biomedical Laboratory, Tokyo) containing 10% fetal bovine serum (FBS) and antibiotic-antimycotics consisting of 10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B as Fungizone R in 0.85% saline (GIBCO BRL, Grand Island, NY).

Expression vector dThdPase expression plasmid (pRc/CMV/dThdPase) and control plasmid (pRc/CMV) were the kind gifts of Drs. Eda and Watanabe, Nippon Roche K.K. Research Center, Kamakura. Originally, pRc/CMV/dThdPase was constructed by the insertion of dThdPase cDNA at the *Hin*dIII site of the pRc/CMV plasmid.

Transfection Cells (5×10^3) were placed in 96-well microplates and incubated at 37°C with 5% CO_2 until 40–60% confluency. pRc/CMV or pRc/CMV/dThdPase (0.08 μg/well) and Lipofectin reagent (GIBCO BRL) (0.3 μl/well) were mixed at room temperature for 15 min. Each well was washed twice with phosphate-buffered saline (PBS), then inoculated with liposome/DNA complexes and incubated at 37°C for 12 h. Cells were selected with medium containing the neomycin analogue G418 (1 mg/ml) (GIBCO BRL). After G418 selection, control vector pRc/CMV transfectant (SW480/V1) and pRc/CMV/dThdPase transfectant (SW480/dThdPase) were obtained.

Confirmation of dThdPase gene transduction To confirm the transduction of dThdPase cDNA, genomic DNA was extracted from the transfected cells, and polymerase chain reaction (PCR) was performed with dThdPase-specific primers: sense, ATGGCAGCCTTGATGACCCCGG; antisense, TCCCACGCCAACCAGCGTCTT.

Expression of dThdPase To confirm dThdPase protein expression, western blotting was performed. Lysates were prepared from transfectant cell pellets as described previously.[13] The lysates (total protein 30 μg) were electrophoresed on 10% polyacrylamide gel. The electrophoresed proteins were transferred onto an Immobilon poly-(vinylidene difluoride) membrane (Millipore, Bedford, MA). After overnight treatment with blocking buffer at 4°C, the membranes were incubated with anti-human dThdPase mouse monoclonal antibody (from Nippon Roche K.K. Research Center) (final concentration, 1.5 mg/ml) at room temperature for 2 h. The filters were then incubated for 1 h with alkaline phosphatase-conjugated rabbit anti-mouse IgG at a dilution of 1:3000 and developed with ProtoBlot NBT and the BICP color development system (Promega, Madison, WI).

Growth inhibition assay Aliquots of 5×10^3 cells/well were placed in 96-well microplates and incubated overnight at 37°C with 5% CO_2. Then, the medium was changed to that containing various concentrations of 5′DFUR or 5-FU. After incubation for 5 days, WST1 assay was performed as described previously.[14]

Bystander effect To examine the bystander effect of dThdPase-transfected cells, nontransfected cells were co-cultured with SW480 parental cells at various ratios of SW480/dThdPase cells (0, 5, 10, 20, 30, 50, 70, 100%) in the presence of various concentrations of 5′DFUR for 5 days, then WST1 assay was performed.

***In vivo* growth inhibition** To examine the growth inhibition *in vivo*, SW480, SW480/V1 and SW480/dThdPase (1×10^7 cells) were inoculated s.c. into athymic BALB/c *nu*/*nu* mice (6–8-week-old females; CLEA Japan, Inc., Tokyo) (each n=3). One week after inoculation, 5′DFUR was administered at the dose of 120 mg/kg body, daily for 2 weeks by i.p. injection. The tumor size was measured in a blind fashion with calipers, and was calculated as [length (mm)×width $(mm)^2$]/2.

RESULTS

dThdPase gene induction dThdPase gene induction was confirmed by the PCR method using dThdPase-specific primers. Fig. 1 shows that a 777 bp PCR product was recognized in SW480/dThdPase, but not in SW480 or SW480/V1.

Expression of dThdPase dThdPase expression was confirmed by western blot analysis. Fig. 2 shows that the band at the 45 kD position was recognized in SW480/dThdPase, but not in SW480 or SW480/V1.

***In vitro* growth of transfectants** There were no differences in morphology or in growth rate between SW480/dThdPase, SW480, and SW480/V1 *in vitro* (data not shown).

Sensitivity of transfected cells to 5-FU and 5′DFUR The sensitivities to 5-FU of SW480, SW480/V1 and

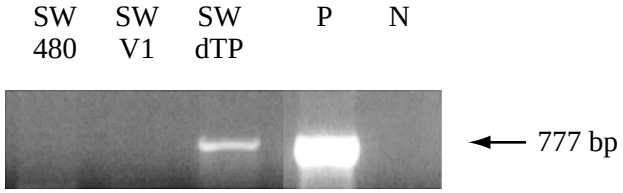

Fig. 1. Confirmation of transfection of dThdPase cDNA. Genomic DNA was extracted from parental cells SW480, control vector-transfected cells SW480/V1, and dThdPase-transfected cells SW480/dThdPase, and PCR using dThdPase-specific primers was performed. PCR products were electrophoresed in 0.8% agarose gels and then visualized with ethidium bromide staining; SW480, parental cells; SW480/V1, control vector-transfected cells; SW480/dTP, dThdPase-transfected cells; P, expression vector pRc/CMV/dThdPase as a positive control; N, pRc/CMV as a negative control.

SW480/dThdPase were studied in the presence of various concentrations of 5-FU. The number of viable cells was estimated by means of the WST1 assay. Fig. 3A shows the sensitivity to 5-FU. The 50% inhibition (IC_{50}) values of SW480, SW480/V1, and SW480/dThdPase were approximately 4.9, 6.3, and 2.9 μM, respectively. The IC_{50} value of SW480/dThdPase was lower than that of SW480 or SW480/V1, although the differences were not statistically significant. As for the sensitivity to 5′DFUR, the IC_{50} values of SW480, SW480/V1, and SW480/dThdPase were approximately 300, 330, and 3.2 μM, respectively (Fig. 3B). The sensitivity to 5′DFUR of SW480/dThdPase was approximately 100-fold higher than that of SW480 or SW480/V1.

Bystander effect SW480 were co-cultured for 5 days with various ratios of SW480/dThdPase in the presence of various concentrations of 5′DFUR. As shown in Fig. 4, when SW480/dThdPase was added to the cultures at ratios of 0, 5, 10, 20, 30, 50, 70, 100%, the IC_{50} values were approximately 300, 150, 50, 35, 25, 15, 10, 3 μM, respectively. These results demonstrate that dThdPase-transfected cells induce a strong bystander effect.

***In vivo* growth inhibition** SW480, SW480/V1, and SW480/dThdPase were inoculated s.c. into BALB/c *nu/nu* mice. Beginning 1 week after the inoculation, 5′DFUR (120 mg/kg body) or PBS was i.p. administered daily for 2 weeks. Fig. 5 shows the resulting changes in the tumor growth. First, SW480/ dThdPase gave significantly faster-growing tumors as compared with SW480 or SW480/V1. Then, the tumor growth of SW480/dThdPase treated with 5′DFUR was remarkably suppressed, whereas PBS was ineffective. Similarly, the growth of SW480 and SW480/V1 tumors treated with 5′DFUR was suppressed compared with that of the tumors treated with PBS. The degree of the suppression, however, was higher in the SW480/dThdPase-inoculated group, although the difference was not statistically significant.

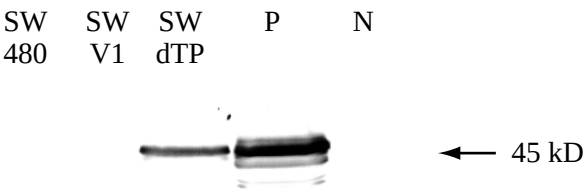

Fig. 2. Expression of dThdPase in SW480, SW480/V1, and SW480/dThdPase. Western blot analysis was performed as described in the text; SW480, parental cells; SW480/V1, control vector-transfected cells; SW480/dTP, dThdPase-transfected cells; P, recombinant dThdPase protein as a positive control; N, DW as a negative control.

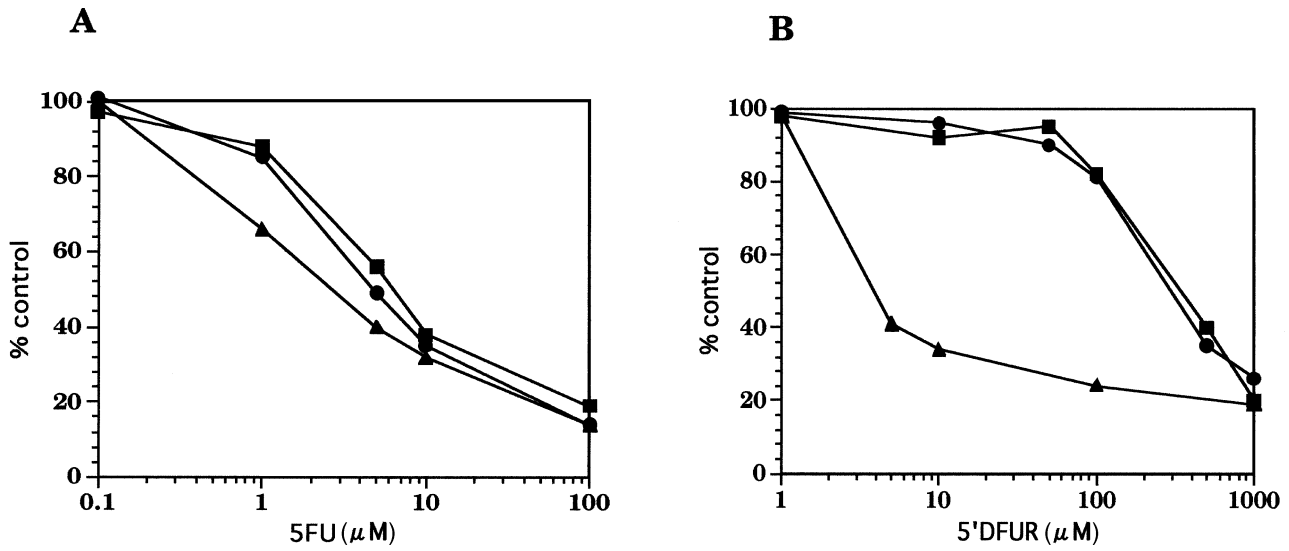

Fig. 3. Sensitivity of SW480, SW480/V1, and SW480/dThdPase to 5-FU or 5′DFUR *in vitro*. A: The sensitivities of SW480, SW480/V1, and SW480/dThdPase to 5-FU were analyzed in the presence of various concentrations of 5-FU. Viable cell counts were estimated by WST1 assay. B: The sensitivity of SW480, SW480/V1, and SW480/dThdPase to 5′DFUR. ● SW480, ■ SW480/V1, ▲ SW480/dThdPase.

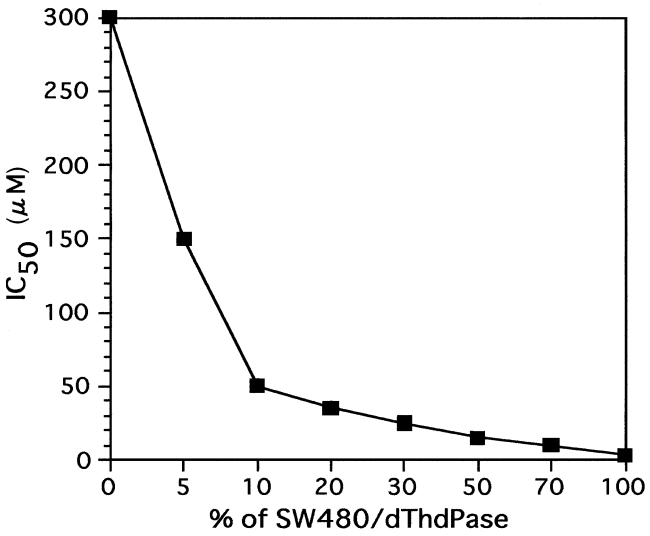

Fig. 4. Bystander effect of dThdPase-transfected cells on untransfected parental cells. SW480 cells were cultured with and without various ratios of SW480/dThdPase for 5 days in the presence of various concentrations of 5′DFUR. The IC_{50} values were calculated at various ratios of SW480/dThdPase.

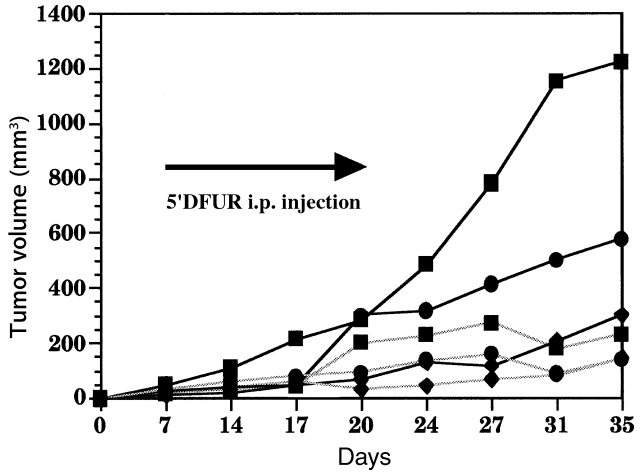

Fig. 5. *In vivo* tumor growth of SW480-transfected clones with or without 5′DFUR injection. SW480, SW480/V1, and SW480/dThdPase were inoculated s.c. into BALB/c *nu*/*nu* mice. One week after the inoculation, 5′DFUR (120 mg/kg body) or PBS was administered i.p. daily for 2 weeks. SW480-PBS, SW480/dThdPase-PBS, SW480/V1-PBS, SW480-5′DFUR, SW480/dThdPase-5′DFUR, SW480/V1-5′DFUR.

DISCUSSION

5-FU is an antimetabolic chemotherapeutic agent that is widely used to treat various types of malignant neoplasms. In patients whose tumors have sufficient sensitivity to this agent, the anti-cancer cytotoxic effect mainly depends on the local concentration of 5-FU in the tumors. However, the systemic application of 5-FU sometimes exerts a similar cytotoxic effect on normal cells, e.g., mucosal epithelial cells of the gastrointestinal tract or bone marrow cells, and causes unfavorable side effects which require the cessation of 5-FU administration. Several strategies have been reported to diminish the side effects of 5-FU. During the course of these trials, several non-toxic prodrugs for 5-FU have been developed. Among them, 5′DFUR is a prodrug which can be activated to 5-FU by the converting enzyme dThdPase in cancer cells themselves.[4, 5] This enzyme is known to be widely expressed in many human tissues, and also in various carcinomas.[7–9] dThdPase levels were elevated in tumors of various organs compared with the levels in non-neoplastic regions of the same organs, but the level and activity of dThdPase vary among different tissues and among different individuals. In recent years, several *in vitro* studies have shown that human cancer cells transfected with dThdPase gene acquire increased sensitivity to 5′DFUR[15, 16]; however, the effect of 5′DFUR on *in vivo* growth of dThdPase-transfected cancer cells was not clarified in those studies. In the present study, we used colon cancer cells as the recipient for dThdPase overexpression and examined the effect of systemically applied 5′DFUR on the growth of these cells *in vivo* as well as *in vitro*. The reason why we selected colon cancer cells was that the level of dThdPase activity in colorectal cancer has been reported to be relatively low compared with that in cancers in other organs.[12] From the clinical standpoint, we might reasonably expect that overexpression of this enzyme in the tumor tissue of colorectal cancer patients would yield a greater anti-cancer effect with systemic administration of 5′DFUR than it would in tumors with low expression of the enzyme.

First, we transfected the dThdPase gene into human colon cancer cell line SW480 to assess the effects on sensitivity to 5′DFUR and cell growth *in vitro*. There was no difference in growth rate between the dThdPase-transfected cells and the control cells. The dThdPase-transfected cells showed about a 100-fold reduction of the IC_{50} value for 5′DFUR compared with the control cells. The sensitivity of the dThdPase-transfected cells to 5-FU itself was slightly increased compared with the control cells, although the increase was not statistically significant. One possible explanation is that dThdPase enhanced the production of 5-fluorodeoxyuridine monophosphate (FdUMP). It has been reported that dThdPase can enhance the formation of FdUMP through the reversible addition of deoxyribose 1-phosphate to the enzymatically released

5-FU. FdUMP forms a ternary complex with thymidylate synthetase and CH_2THF, and this inhibits DNA synthetase.[17] Therefore, it is probable that the sensitivity of dThdPase-transfected cells to 5-FU is increased.

In this system, dThdPase-transfected cells were shown to be capable of killing neighboring untransfected cells in the presence of 5′DFUR. This phenomenon is called the bystander effect, and has been reported in the herpes simplex virus thymidine kinase (HSV/TK) and ganciclovir system as well as in the *Escherichia coli* cytosine deaminase (CD) and 5-fluorocytosine system. Although the mechanism has not yet been completely elucidated, some reports on the HSV/TK and ganciclovir system have proposed that phosphorylated ganciclovir passes into adjacent tumor cells via gap junctions, because the phosphorylated ganciclovir was not able to pass through the cell membrane.[18] In the *E. coli* CD and 5-fluorocytosine system, 5-FU generated from 5-FC can pass through the cellular membrane and exhibit cytotoxic effects.[19] In our study, even with only 10% transfection efficacy, the sensitivity to 5′DFUR was sufficient to suppress the cell growth, indicating that a strong bystander effect was induced by this system. This result is consistent with the report of Patterson *et al*.,[16] who found a significant bystander effect of dThdPase gene transfection in a human breast cancer cell line, MCF7.

To examine the anti-tumor effect *in vivo*, dThdPase-transfected cells were inoculated s.c. into nude mice, and, after tumor formation was confirmed, 5′DFUR was administered i.p. The growth of the dThdPase-transfected cell-containing tumor treated with 5′DFUR was suppressed compared with that of the cells treated with PBS. Similarly, the tumor growth of the parental cells or the control vector-transfected cells treated with 5′DFUR was suppressed as compared with the case of PBS treatment. The degree of suppression was higher in the SW480/dThdPase group, although the difference was not statistically significant. In the present study, we used the nude mouse tumor generated by dThdPase-transfected colon cancer cells to check the efficiency of this suicide gene therapy system *in vivo*. However, from the viewpoint of clinical application, the most important thing is how to deliver the suicide gene to solid tumor efficiently. Among several methods which have been reported so far, we tried the direct injection method. Contrary to our expectations, however, the nude mouse tumor generated by SW480/dThdPase cells showed a faster growth rate than the control tumor (Fig. 5). One possible explanation is clonal variation of transfectants in this experiment. The cultured tumor cell lines usually diverge into various types of cells with heterogeneous characteristics during serial passages. Also, we have already reported that the SW480 cell line has heterogeneous subpopulations and contains distinct subtypes having completely different growth rates.[20] Using a subclone of SW480 cells, we performed the *in vivo* experiment again and could not find any difference in growth rate *in vivo* between the dThdPase-transfected cells and control cells (data not shown). However, this result did not entirely exclude the possibility of some growth-related activity of dThdPase. Another possible explanation is the angiogenic property of dThdPase. Although there was no difference of cell growth between SW480/dThdPase and SW480 or SW480/V1 *in vitro*, SW480/dThdPase gave rise to significantly faster-growing tumors *in vivo*. The growth enhancement *in vivo* may arise as a result of an angiogenic advantage, although this remains to be proven.[21]

Interestingly, recent studies show that the platelet-derived endothelial-cell growth factor (PD-ECGF) is identical to an internal sequence of human dThdPase.[22] PD-ECGF was originally isolated from human platelets, and it is known to stimulate endothelial cell growth and chemotaxis *in vitro* and angiogenesis *in vivo*.[23, 24] Takebayashi *et al*.[25] reported that the expression level of dThdPase in tumor specimens was statistically correlated with the depth of tumor invasion, frequency of metastasis, and a shorter survival time. Therefore, an elevated expression of dThdPase predisposes the patients to both aggressive disease and improved response to fluoropyrimidine-based chemotherapy.

In conclusion, our results suggest that gene therapy using dThdPase gene transfer to cancer cells coupled with systemic injection of 5′DFUR may have potential as a therapeutic strategy for colon cancer. It is now important to elucidate the details of the angiogenic activity of dThdPase in tumors.

ACKNOWLEDGMENTS

We thank Dr. Ohnishi for valuable advice and Ms. R. Matuyama for secretarial assistance. We also thank Drs. Eda and Watanabe of Nippon Roche K.K. Research Center for their gift of the dThdPase expression plasmid.

(Received October 30, 1998/Revised January 26, 1999/Accepted February 10, 1999)

REFERENCES

1) Mullen, C. A., Kilstrup, M. and Blaese, R. M. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. *Proc. Natl. Acad. Sci. USA*, **89**, 33–37 (1992).
2) Mullen, C. A., Coale, M. M., Lowe, R. and Blaese, R. M.

Tumors expressing the cytosine deaminase suicide gene can be eliminated *in vivo* with 5-fluorocytosine and induce protective immunity to wild type tumor. *Cancer Res.*, **54**, 1503–1506 (1994).
3) Sugaya, S., Fujita, K., Kikuchi, A., Ueda, H., Takakuwa, K., Shoji, K. and Tanaka, K. Inhibition of tumor growth by direct intratumoral gene transfer of herpes simplex virus thymidine kinase gene with DNA-liposome complex. *Hum. Gene Ther.*, **7**, 223–230 (1996).
4) Bodycote, J. and Wolff, S. Metabolic breakdown of [^{3}H]thymidine and the inability to measure human lymphocyte proliferation by incorporation of radioactivity. *Proc. Natl. Acad. Sci. USA*, **83**, 4749−4753 (1986).
5) Birine, G. D., Kroeger, H. and Heidelberger, C. Studies of fluorinated pyrimidines. XVIII. The degradation of 5-fluoro-2′-deoxyuridine and related compounds by nucleoside phosphorylase. *Biochemistry*, **2**, 566−572 (1963).
6) Krenitsky, T. A. and Koszalka, J. V. Purine nucleoside synthesis, an efficient method employing nucleoside phosphorylases. *Biochemistry*, **20**, 3615–3621 (1981).
7) Zimmerman, M. and Seidenberg, J. Deoxyribosyl transfer. I. Thymidine phosphorylase and nucleoside deoxyribosyltransferase in normal and mucosal tissues. *J. Biol. Chem.*, **239**, 2618–2621 (1964).
8) Marsh, J. C. and Perry, S. Thymidine catabolism by normal and leukemic human leucocytes. *J. Clin. Invest.*, **43**, 267–278 (1964).
9) Shaw, T., Smillie, R. H., Miller, A. E. and Macphee, D. G. The role of blood platelets in nucleoside metabolism: regulation of platelet thymidine phosphorylase. *Mutat. Res.*, **200**, 117–131 (1988).
10) Yoshimura, A., Kuwazuru, Y., Furukawa, T., Yoshida, H., Yamada, K. and Akiyama, K. Purification and tissue distribution of human thymidine phosphorylase: high expression in lymphocytes, reticulocytes and tumors. *Biochim. Biophys. Acta*, **1034**, 107–113 (1990).
11) Luccioni, C., Beaumatin, J., Bardot, V. and Lefrancois, D. Pyrimidine nucleotide metabolism in human colon carcinomas; comparison of normal tissues, primary tumors and xenografts. *Int. J. Cancer*, **58**, 517–522 (1994).
12) Peters, G. J., van Groeningen, C. J., Laurensse, E. J. and Pinedo, H. M. A comparison of 5-fluorouracil metabolism in human colorectal cancer and colon mucosa. *Cancer*, **68**, 1903–1909 (1991).
13) Haba, A., Monden, T., Sekimoto, M., Ikeda, K., Kanyama, H. and Monden, M. PyNPase expression in human colon cancer. *Cancer Lett.*, **122**, 85–92 (1998).
14) Ishikawa, M., Shiga, M., Sasamoto, K. and Mizoguchi, M. A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. *Chem. Pharm. Bull.*, **41**, 1118–1122 (1993).
15) Haraguchi, M., Furukawa, T., Sumizawa, T. and Akiyama, S. Sensitivity of human KB cells expressing platelet-derived endothelial cell growth factor to pyrimidine antimetabolites. *Cancer Res.*, **53**, 5680–5682 (1993).
16) Patterson, A. V., Zhang, H., Moghaddam, A., Bicknell, R., Talbot, D. C., Stratford, I. J. and Harris, A. L. Increased sensitivity to the prodrug 5′-deoxy-5-fluorouridine and modulation of 5-fluoro-2′-deoxyuridine sensitivity in MCF-7 cells transfected with thymidine phosphorylase. *Br. J. Cancer*, **72**, 669–675 (1995).
17) Schwartz, E. L., Baptiste, N., O'Connor, C. J., Wadler, S. and Otter, B. A. Potentiation of 5-fluorouracil in colon carcinoma cells by the combination of interferon and deoxyribonucleotides results from complementary effects on thymidine phosphorylase. *Cancer Res.*, **54**, 1472–1478 (1994).
18) Freeman, S. M., Abboud, C. N. and Whartenby, K. A. The bystander effect: tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.*, **53**, 1503–1506 (1994).
19) Kuriyama, S., Matui, K., Sakamoto, T., Nakatani, T., Ikenaga, K. and Tsujii, T. Bacterial cytosine deaminase suicide gene transduction renders hepatocellular carcinoma sensitive to the prodrug 5-fluorocytosine. *Int. Hepatol. Commun.*, **4**, 72–79 (1995).
20) Tomita, N., Jiang, W., Hibshoosh, H., Warburton, D., Kahn, S. M. and Weinstein, I. B. Isolation and characterization of a highly malignant variant of the SW480 human colon cancer cell line. *Cancer Res.*, **52**, 6840–6847 (1992).
21) Moghaddam, A., Zhang, H. T., Fan, T. P., Hu, D. E., Lees, V. C., Turley, H., Fox, S. B., Gatter, K. C., Harris, A. L. and Bicknell, R. Thymidine phosphorylase is angiogenic and promotes tumor growth. *Proc. Natl. Acad. Sci. USA*, **92**, 998–1002 (1995).
22) Furukawa, T., Yoshimura, A., Sumizawa, T., Haraguchi, M. and Akiyama, S. I. Angiogenic factor. *Nature*, **356**, 668 (1992).
23) Miyazono, K., Okabe, T., Urabe, A., Takaku, F. and Heldin, C. H. Purification and properties of an endothelial cell growth factor from human platelets. *J. Biol. Chem.*, **262**, 4098−4103 (1987).
24) Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W. and Heldin, C. H. Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature*, **338**, 557–562 (1989).
25) Takebayashi, Y., Yamada, K., Sumizawa, T., Miyadera, K., Aikou, T. and Akiyama, S. Clinicopathological significance of an angiogenic enzyme, thymidine phosphorylase (dThdPase), in colorectal carcinoma. *Proc. Am. Assoc. Cancer Res.*, **36**, 90 (1995).