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

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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 (IC50) 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 IC50 of SW480/dThdPase was lower than that of SW480 or SW480/V1, although the differences were not statistically significant. The IC50 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 therapy1-3 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.
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/CJV) 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 HindIII site of the pRc/CMV plasmid.

Transfection  Cells (5x10⁵) were placed in 96-well microplates and incubated over-night at 37°C. Aliquots of 5x10³ cells/well were plated in 96-well microplates and incubated over-night at 37°C with 5% CO₂. 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.¹⁰

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, and SW480/V1.

In vivo growth inhibition  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.

SW480, SW480/V1 and SW480/dThdPase (1x10⁷ 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)xwidth (mm)²]/2.

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/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 $\mu$M, 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 $\mu$M, 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 $\mu$M, 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.

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**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.
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

**DISCUSSION**

**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.
5-FU. FdUMP forms a ternary complex with thymidylate synthetase and CH₂THF, and this inhibits DNA synthetase. 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. 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. 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., 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. 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.

Interestingly, recent studies show that the platelet-derived endothelial-cell growth factor (PD-ECGF) is identical to an internal sequence of human dThdPase. 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. Takebayashi et al. 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.

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