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Overexpression of pyrimidine nucleoside phosphorylase enhances the sensitivity to 5′-deoxy-5-fluorouridine in tumour cells *in vitro* and *in vivo*

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Abstract

5-Fluorouracil (5-FU) and 5′-deoxy-5-fluorouridine (5′-DFUR), a prodrug of 5-FU, are representative of the chemotherapeutic agents for colorectal adenocarcinomas. Pyrimidine nucleoside phosphorylase (PyNPase) catalyses the conversion of 5′-DFUR to 5-FU, the activated form. Murine adenocarcinoma CT26 cells were transfected with human *PyNPase* cDNA. The engineered transfectants producing PyNPase augmented the response to 5′-DFUR *in vitro* and *in vivo*. Animals were administered by means of intraperitoneal (i.p.) injection, and not orally, in order to obtain a better efficiency of absorption. The tumours of the transfected cells nearly all disappeared, even following treatment with quite a small amount of the anticancer agent. The animals injected with the tranfected cells were protected against subsequent challenge with the parental tumour cell line. These findings demonstrate that *PyNPase* gene transfection increases the sensitivity to 5′-DFUR, and thereby decreases the toxicity of the agent.

Keywords: Pyrimidine nucleoside phosphorylase (PyNPase); 5′-Deoxy-5-fluorouridine (5′-DFUR); Gene therapy

1. Introduction

It has been difficult to find suitable medical treatments against advanced colorectal adenocarcinomas, including those with invasion, dissemination and metastasis with multiple lesions.

5-Fluorouracil (5-FU) is representative of the anticancer agents that are most effective [1–7], although only 5–20% of the patients respond in advanced colorectal cancer [8]. The application of 5-FU is limited due to side-effects including gastrointestinal toxicity and myelosuppression. 5′-Deoxy-5-fluorouridine (5′-DFUR) is a derivative of 5-FU and results in less toxicity. It has a higher therapeutic index [9–14], and is associated with less immunosuppression [15,16] and also induces a lower grade of leucopenia than 5-FU [17]. It is a prodrug of 5-FU that is cleaved into 5-FU.

Pyrimidine nucleoside phosphorylase (PyNPase) catalyses the reversible phosphorolysis of pyrimidines in the nucleotide synthesis pathway [18]. PyNPase accepts both thymidine and uridine in the bacterium, whereas it is specific for thymidine in mammalian cells [18]. It also enzymatically converts 5′-DFUR to 5-FU [8].

A potent strategy for cancer treatment is the transfer of a gene into cancer cells that encodes an enzyme that renders them susceptible to a prodrug such as 5′-DFUR. This can result in a reduced toxicity associated with the prodrug in untransfected cells and the transfected tumour cells are more susceptible to the anticancer drug. A bystander effect also contributes to the success of the gene therapy [19].

PyNPase shares 40% sequence similarity with human thymidine phosphorylase (TP), which has been implicated as a growth factor in tumour angiogenesis. TP is identical to a tumour angiogenic factor, platelet-derived endothelial cell growth factor (PD-ECGF) [20,21]. TP phosphorolytically cleaves thymidine to yield thymine and 2-deoxyribose-1-phosphate. TP only promotes angiogenesis in the presence of thymidine [22]. The control of angiogenesis could occur either by regulating extracellular thymidine levels or by the amount of TP activity which produces 2-deoxyribose-1-phosphate followed by the formation of 2-deoxyribose as an angiogenic factor by dephosphorylation [23,24].

We determined here a strategy of PyNPase overexpression in CT26 colon adenocarcinoma cells and studied its effectiveness *in vitro* and *in vivo*.

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2. Materials and methods

2.1. Cell lines and mice

The CT26 tumour cell line a poorly immunogenic murine colon carcinoma established from a N-nitroso-N-methylurethane-induced transplantable tumour was used [4]. This cell line was obtained from Hoffmann-La Roche (Kamakura, Japan) and was maintained in Dulbecco's Modified Eagle medium (Nissui Pharmaceutical, Co., Japan) supplemented with 10% fetal bovine serum (Gibco BRL, USA), 2×10^{-5} M 2-mercaptoethanol, 2M-L-glutamine, and antibiotics at 37 °C in a 5% CO_2-incubator. 6-week-old, pathogen-free BALB/c female mice were used for the *in vivo* model.

2.2. *Human* PyNPase *cDNA sub-cloning*

The pBluescriptSK + vector containing a full-length human *PyNPase* cDNA was kindly provided by Dr Eda (Hoffmann-La Roche, Kamakura, Japan) The full length human *PyNPase* cDNA was obtained by digesting the pBluescriptSK + vector with *EcoRI*. This cDNA was then ligated into the multi-cloning site of the mammalian expression vector PCI (Promega, Madison, USA) (PyNPCI). The PyNPCI vector was transformed into competent cells, *Escherichia coli* (DH5α), and the plasmid DNA was purified by using a Plasmid Kit (Qiagen, Courtaboeuf, France). Digestion of the PyNPCI with *BamHI* and *EcoRI* was carried out to make sure that the *PyNPase* cDNA was inserted into the plasmid in the correct orientation.

2.3. *Human* PyNPase *expression in CT26 cells*

CT26 cells were co-transfected with the PyNPCI and the pSV_2*neo* vector by using a lipofection method. 5 μg of the PyNPCI and 0.66 μg of the pSV_2*neo* were added to 30 μl of DOTAP Liposomal Transfection Reagent (Boehringer, Mannheim). The DOTAP/nucleic acid mixture was gently mixed with 6 ml culture medium. Culture medium was removed and the culture medium containing the DOTAP/nucleic acid mixture was added to the CT26 cultured dish. The cells were incubated with the DOTAP/nucleic acid mixture for 9 h. The medium was replaced with fresh culture medium and the cells were further incubated. Stable transfectants were then selected in medium containing 1 mg/ml of the neomycin analogue Geneticin (active concentration of 687 μg/mg of G418, GIBCO). The transfection efficiency was determined by using an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (MAbs) specific to human PyNPase as previously described in Ref. [25]. These MAbs were kindly provided by Dr Eda (Hoffmann-La Roche, Kamakura, Japan).

2.4. *MTT assay for the detection of sensitivity to anticancer drugs*

Chemosensitivity was assessed by using the tetrazolium salt 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma No. M2128) to measure the viability of the tumour cells [26,27]. 100 μl of tumour cell suspension (1×10^5 cells/ml) was added to 100 μl of each drug solution in 96-well flat-bottomed microtitre plates (Corning No. 23860), and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 96 h. The chemosensitivity assay was assessed in triplicate. Three microtitre wells containing tumour cells suspended in 200 μl of complete medium (total tumour cell number was equivalent to that in the sample wells) were used as controls for cell viability, and three wells containing only complete medium were used as controls for non-specific dye reduction. After that incubation, the plates were centrifuged, the supernatants removed, and 15 μl/well of MTT solution with 10 μM of sodium succinate was added to all of the wells [28]; the mixture was pipetted thoroughly to dissolve the dark blue crystals. The plates were then read on a microplate reader (Corona Electric, MTP-32) using a test wavelength of 550 nm and a reference wavelength of 630 nm. The control wells without tumour cells had an Optical Density (OD) of less than 0.001. The background of tumour cells (including dead cells) without the addition of MTT had an OD of less than 0.02 after 96-h incubation, and therefore the influence of the dead tumour cells could be discounted in the present study.

2.5. In vivo *experiments*

Tumours were generated by subcutaneous (s.c.) injection of the CT26 parental or transfected cells (1×10^6 in 200 μl of normal saline) into each back of the syngeneic BALB/c mice. The mice were treated intraperitoneally (i.p.) with 5′-DFUR (5 consecutive days in the first and second weeks); control animals received an injection of saline solution only. The mice inoculated with the tumour cells had no body-weight loss compared with mice inoculated with 200 μl of normal saline alone (data not shown). Tumour volume was measured every few days for 22 days after transplantation.

3. Results

3.1. *Construction of plasmid and expression of human PyNPase*

Transfectants were selected in the neomycin analogue G418, and more than 20 clones of approximately equal size of the G418-resistant line (CT26-PyNPase$^+$) were chosen for further study. These cell lines were evaluated

for PyNPase activity by ELISA assay with MAbs specific to human PyNPase, and a selected CT26-PyNPase$^+$ line was found to contain 41.6 U/mg protein of PyNPase activity in the cytosols of the cells, indicating that the CT26-PyNPase$^+$ cells contained significant quantities of PyNPase. No detectable PyNPase activity was found in the cytosols from the parental CT26 cells.

3.2. *The effect of human* PyNPase *expression* in vitro

The chemosensitivity of CT26 cells expressing human *PyNPase* to 5-FU and 5′-DFUR was studied by using the tetrazolium salt MTT assay. In Fig. 1, the growth ratio was determined as (absorbance at 550 nm of samples with 5-FU or 5′-DFUR/absorbance at 550 nm of controls without 5-FU or 5′-DFUR) using the MTT assay. 5-FU and 5′-DFUR lowered the growth ratio of the CT26 parental cells in a concentration-dependent manner with IC_{50} values of 2.3 ± 0.16 and 70 ± 6.8 μM, respectively. The sensitivity of CT26 cells transfected with pSV$_2$*neo* was almost the same as that of the CT26 parental cells; the IC_{50} values were 2.3 ± 0.16 μM for 5-FU and 51 ± 2.8 μM to 5′-DFUR, respectively. However, the CT26-PyNPase$^+$ cells were more sensitive to the cytotoxic effect of 5-FU and 5′-DFUR than the parental cells: the respective IC_{50} values were 0.39 ± 0.02 μM for 5-FU and 0.98 ± 0.09 μM for 5′-DFUR. The difference in sensitivity was significant (Student's *t*-test, $P<0.001$ and $P<0.004$, respectively). Notably, the sensitivity to 5′-DFUR was significantly higher than that to 5-FU; the CT26-PyNPase$^+$ cells were found to be approximately 71 times more sensitive to 5′-DFUR than the parental cells, and 6 times more sensitive to 5-FU.

3.3. *The effect of human PyNPase expression* in vivo

Either CT26 or CT26-PyNPase$^+$ cells were inoculated into the left flank of syngeneic Balb/c mice by s.c. injection as described in the methods. Tumours were generated and grew exponentially over the studied period in the control mice. The rate of growth and size of the tumours were almost the same between the CT26-bearing mice and the CT26-PyNPase$^+$-bearing mice without 5′-DFUR treatment (Fig. 2). Moreover, they were also similar between the CT26-bearing mice and mice bearing CT26 cells containing the pSV$_2$*neo* vector without 5′-DFUR treatment (data not shown).

However, the sensitivity to 5′-DFUR was significantly higher in the CT26-PyNPase$^+$-bearing mice than in the CT26-bearing mice (Fig. 2). The rate of growth decreased in a concentration-dependent manner in both cases. Over the time of the experiment (22 days), the tumour volume of the CT26 cells increased steadily, except following treatment with the highest dose (200 mg/kg) of 5′-DFUR (Fig. 2a). In contrast, the tumours of CT26-PyNPase$^+$ cells almost disappeared at day 22 in all of the mice treated with 5′-DFUR; the tumour volume initially increased then decreased after treatment with 5′-DFUR (Fig. 2b). When CT26-PyNPase$^+$-bearing mice treated with 5′-DFUR were re-challenged by an injection of CT26 cells at tumorigenic doses into the right flank, there was no further occurrence of tumours.

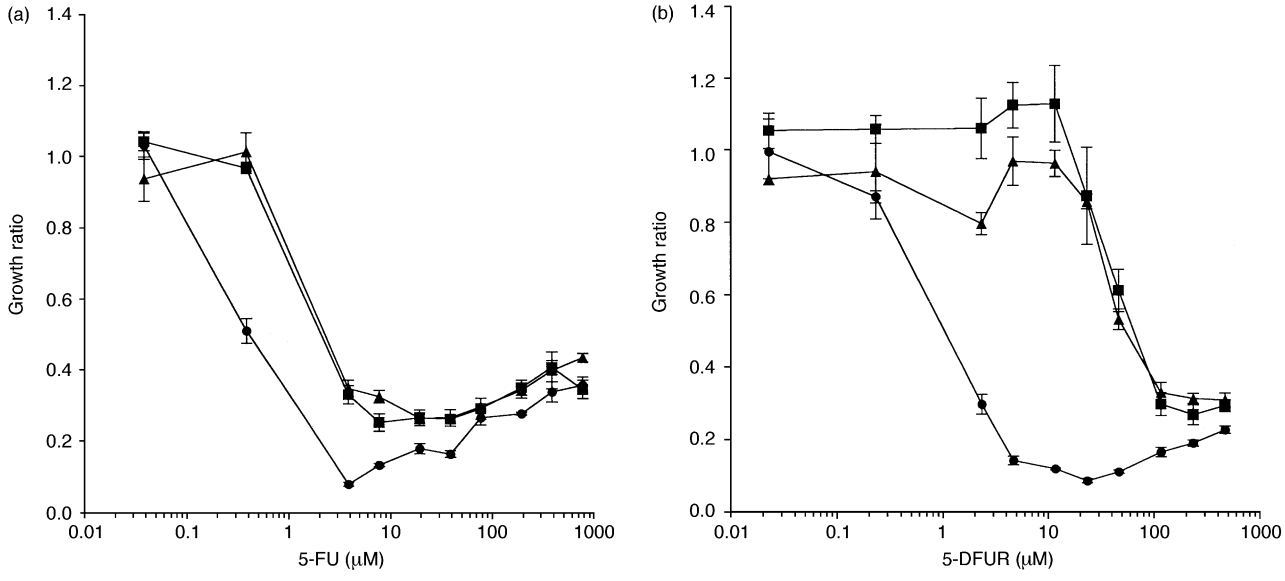

Fig. 1. Effects of pyrimidine nucleoside phosphorylase (PyNPase) expression on the *in vitro* response to (a) 5-fluorouracil (5-FU) and (b) 5′-deoxy-5-fluorouridine (5′-DFUR). Cells were incubated at 37 °C for 96 h in the absence of (control) or in the presence of increasing concentrations (a) (0.04–782 μM) of 5-FU or (b) (0.02–467 μM) of 5′-DFUR. The growth ratio was assessed by means of the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *Y*-axis values were determined as (absorbance at 550 nm of treated cells/absorbance at 550 nm of non-treated cells (controls)). Data is presented as means±standard error of the means (S.E.M.). Symbols: CT26 parental cells (closed squares); CT26 cells containing the pSV$_2$*neo* vector, thus neomycin-resistant (closed triangles); the *PyNPase* gene transduced CT26 cells (closed circles).

4. Discussion

Colorectal adenocarcinoma is often resistant to medical treatments. We have chosen here to use a murine colorectal adenocarcinoma cell line, CT26, which is undifferentiated and of low immunogenicity; s.c. injection of a small number of these cells into syngeneic (BALB/c) mice result in a lethal tumour and do not induce detectable tumour-specific CTLs [29].

5-FU and 5′-DFUR, a prodrug of 5-FU, are representative of the anticancer drugs used to treat cancers of digestive organs. 5-FU has side-effects that should not be ignored, commonly myelosuppression, and gastrointestinal and skin toxicity [30]. 5′-DFUR shows less toxicity than 5-FU in experimental [31,32] and clinical studies [13,14] and generates its efficacy by being converted to 5-FU [8]. PyNPase is involved intracellularly in that conversion [8]. CT26 cells were reported to have a high level of PyNPase and to be relatively susceptible to 5′-DFUR, resulting in the generation of high concentrations of 5-FU in the cells [11]. Meanwhile, normal intestine has higher endogenous levels of PyNPase activity than other normal tissues [8,33]. This might explain the high frequency of gastrointestinal side-effects seen in patients treated with 5′-DFUR [34,35].

In order to obtain the advantages and overcome the disadvantages of treatment with 5′-DFUR, we designed a system of PyNPase overexpression in the tumour CT26 cells.

In this study, we produced a CT26-PyNPase$^+$ cell line by transfecting PyNPCI into CT26 cells. The CT26-PyNPase$^+$ cells were examined to see how they would respond to 5-FU and 5′-DFUR treatment *in vitro*; the sensitivity to 5′-DFUR was much higher than that to 5-FU, compared with the sensitivity of parental CT26 cells to these anticancer drugs. This indicates that the CT26-PyNPase$^+$ cells could increase the activity of 5′-DFUR, by the conversion of 5′-DFUR to 5-FU, the active form in the cells. The results correspond to a high titre of human PyNPase activity in CT26-PyNPase$^+$ cells, whereas this activity was not detected in the parental cells.

Following confirmation of the high susceptibility to 5′-DFUR of the CT26-PyNPase$^+$ cells *in vitro*, we investigated the effects *in vivo*. We employed 5′-DFUR through an i.p. injection, and not through an oral administration.

In our study, the CT26-PyNPase$^+$-bearing mice showed steady growth of their tumour initially by 2 weeks, however, the tumour disappeared just after finishing the treatment with the highest dose of 5′-DFUR (200 mg/kg). The size of their tumour was so reduced that the tumours were hardly seen even with the lowest dose (25 mg/kg) at around 3 weeks from the inoculation of the tumour cells.

The i.p. injection could be more reliable than the oral administration because the former could bring much more stable and higher efficiency of absorption of the agent than the latter. Furthermore, we could expect a

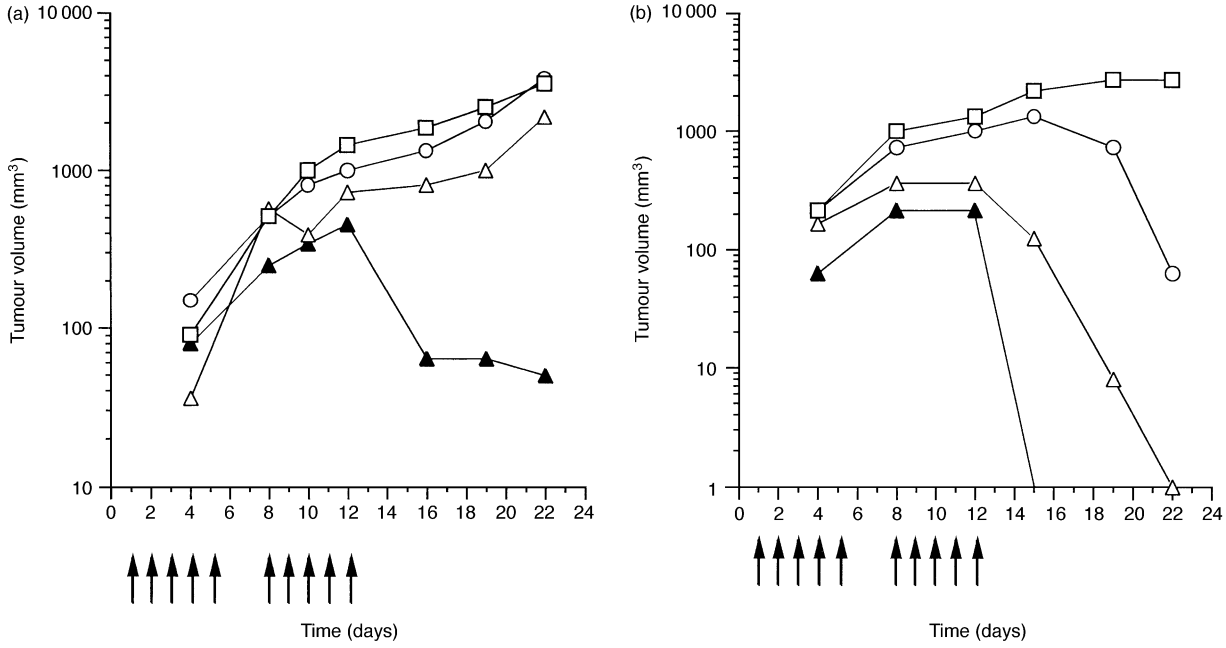

Fig. 2. Effects of PyNPase expression on the *in vivo* response to 5′-deoxy-5-fluorouridine (5′-DFUR). Balb/c mice were injected subcutaneously (s.c.) in the left flank with cells (1×10^6) of either CT26 (a) or CT26-PyNPase$^+$ (b). The animals were then treated daily for 5 days a week in the first and second weeks with 5′-DFUR at doses of 25 mg/kg (open circles), 100 mg/kg (open triangles), 200 mg/kg (closed triangles) or with saline alone (open squares). Tumour volumes were assessed every few days by palpation and measurement. In (b), the tumour completely disappeared following treatment with 200 mg/kg, therefore the data are not shown after 2 weeks.

direct effect at the focus of the cancer such as peritonitis and pleuritis carcinomatosa through the i.p. administration of 5′-DFUR. It is possible that this study will be useful as a reference point when alternative agents given either i.p. or intravenously (i.v.) become available in the future.

PyNPase has been known to have some angiogenic effect due to the partial similarity with TP [18, 20,21,23,24]. TP has a highly restricted target cell specificity acting only on endothelial cells. It promotes angiogenesis *in vivo* and stimulates the *in vitro* growth of a variety of endothelial cells. TP does not promote tumour growth of colorectal-adenocarcinoma cells [39]. Our study showed similar results in that PyNPase did not affect the growth of the tumour.

The PyNPase-transfected CT26 tumour-bearing mice rejected the parental, non-transfected cells over 30 days after the first injection of the transfected cells. This is possibly due to shedding and the subsequent presentation of tumour antigen to the immune system, thereby inducing protective immunity against the parental CT26 cells in the PyNPase-transfected CT26 tumour-bearing mice. Additionally, the activity of 5-FU might facilitate the delayed immune response: pretreatment with 5-FU rendered a human renal cancer cell line more susceptible to lymphokine-activated killer cells [36]. Furthermore, 5-FU stimulated target tumour cells and these were subsequently more sensitive to NK cell-mediated cytotoxicity [37].

5-FU has been reported to have a bystander effect that does not require cell–cell contact [38]. The drug diffuses readily across cell membranes and this effect should be beneficial for the therapy, not only for solid tumours, but also for peritonitis and pleuritis carcinomatosa that are not joined by gap junctions.

The combined therapy with 5′-DFUR and PyNPase overexpression leads to a selective susceptibility of the CT26 tumour cells to 5′-DFUR, thereby gaining efficacy in the tumour and reducing toxicity in normal tissues. Additionally, a protective effect may be expected to follow, thereby preventing the formation of another tumour.

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