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ORIGINAL ARTICLE

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Biomodulation of 5-fluorouracil by interferon-α in human renal carcinoma cells: relationship to the expression of thymidine phosphorylase

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Abstract *Purpose*: To provide the basis for improved therapeutic benefit in combination chemotherapy with interferon (IFN) and 5-fluorouracil (5-FU), we investigated the modulatory actions of human recombinant IFN alfa-2a on 5-FU in five renal cell carcinoma (RCC) cell lines in vitro, in particular focusing on thymidine phosphorylase (TP) expression. *Methods*: The sensitivity of RCC cell lines to the drugs was evaluated using an AlamarBlue assay. An enzyme-linked immunosorbent assay (ELISA) was used to determine TP expression. *Results*: IFN-α enhanced the sensitivity of three of five RCC cell lines to 5-FU in a dose- and schedule-dependent manner. When IFN-α was given prior to 5-FU, sensitivity to 5-FU was significantly higher than when IFN-α was given simultaneously ($P < 0.05$). IFN-α enhanced TP expression in a dose-dependent manner in three of five RCC cell lines ($P < 0.05$). The relative IFN-α-induced increase in sensitivity to 5-FU correlated with the relative IFN-α-induced increase in TP expression ($P < 0.05$). In addition, two of three RCC cell lines with more than a twofold increase in sensitivity to 5-FU induced by IFN-α showed a higher TP expression without IFN-α stimulation. *Conclusions*: These results suggest that IFN-α upregulates TP expression and modulates 5-FU anabolism thus enhancing 5-FU cytotoxicity in a dose- and schedule-dependent manner in some RCC cells. The results imply that TP expression measurement in RCC could identify subgroups of metastatic RCC that may respond to IFN-α/5-FU combination therapy, and sequential administration of IFN-α followed by 5-FU may be beneficial in such cases.

Key words Renal cell carcinoma · Interferon · 5-Fluorouracil · Thymidine phosphorylase · Biomodulation

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Introduction

Renal cell carcinoma (RCC) is a common disease and one-third of patients have metastatic disease at the time of first diagnosis [4]. Conventional therapies with cytotoxic agents, hormones and cytokines have a poor success rate against metastatic RCC [3, 12, 28]. Although interferon (IFN) as a single agent is widely used to treat metastatic RCC, its efficacy is not sufficient: objective response rates of 15% to 20% are mainly partial responses and approximately 2% are complete responses [28]. Thus, effective therapy is urgently needed.

Fluoropyrimidines such as 5-fluorouracil (5-FU), inhibitors of pyrimidine synthesis, are cytotoxic to tumor cells. There are three potential cytotoxic mechanisms [27]: (1) inhibition of thymidylate synthesis (TS), (2) incorporation of fluorodeoxyuridine triphosphate (FdUTP) into DNA, and (3) incorporation of fluorouridine triphosphate (FUTP) into RNA. Recent studies have shown that IFN synergistically potentiates the antitumor activity of 5-FU; this is known as biomodulation. Although the precise mechanism of the synergy is unclear, the following mechanism has been suggested: IFN augments the anabolism of 5-FU to its active metabolite, fluorodeoxyuridine monophosphate (FdUMP) by inducing thymidine phosphorylase (TP) [25], and increased levels of FdUMP inhibit TS activity resulting in an increase in DNA double-strand breaks [36]. Based on these studies, clinical trials with an IFN-α/5-FU combination have shown good response rates in patients with colorectal carcinoma [34]. Recently, several clinical trials in metastatic RCC have shown response rates of 12.5% to 43% [8, 10, 11, 26] with the regimen [34] used by Wadler et al. with or without modification. Elias et al. pointed out that dosage and scheduling are probably important and might be further optimized in IFN-α/5-FU combination chemotherapy [8]. On the other hand, Atzpodien et al. reported that the combination of 5-FU, IFN-α and interleukin-2 had significant efficacy against metastatic RCC with an overall response rate of 48.6%

[2]. These reports prompted us to elucidate the mechanism underlying the IFN-α-induced enhancement of sensitivity of RCC to 5-FU to provide the basis for improved therapeutic benefits of IFN-α/5-FU combination chemotherapy against metastatic RCC. We investigated the modulatory actions of IFN-α on 5-FU in human RCC cell lines in vitro, and in particular focused on TP expression.

Materials and methods

Cell lines

The human RCC cell lines used were JMSU3 [18], Caki1, KU2, OS-RC-2, and VMRC-RCW. The last four of these cell lines were supplied by the Japanese Cancer Research Resources Bank (JCRB). These cell lines were maintained in vitro in RPMI-1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 m*M* L-glutamine at 37 °C in an incubator under an atmosphere containing 5% CO_2.

Reagents

5-FU was provided by Kyowa Hakko Kogyo Co. (Tokyo, Japan). Human recombinant IFN alfa-2a (specific activity, 6×10^6 U/mg protein) was supplied by Takeda Pharmaceutical Co. (Osaka, Japan).

Drug sensitivity

Rapidly growing cells were seeded in 96-well flat-bottomed plates at a density of 1000 cells/well. In preliminary experiments, seeding densities were determined to ensure that cultures did not become confluent before conducting the assay (data not shown). After a 24-h incubation to allow cell attachment, cells were treated with one of two schedules. In a simultaneous treatment, a solution of 5-FU and IFN-α (50 μl each) at various concentrations was added to appropriate wells. In a sequential treatment, an IFN-α solution (50 μl) at various concentrations was dispensed into appropriate wells, and then a 5-FU solution (50 μl) at various concentrations was added 24 h later. In both schedules, the plates were incubated at 37 °C in an incubator under an atmosphere containing 5% CO_2 for an additional 4 days after the 5-FU addition. The AlamarBlue assay [1] was used to evaluate cell viability. AlamarBlue solution (20 μl) was added to each well and the mixture incubated for 6 h. Absorbances at 540 nm and 650 nm were measured with a microplate reader Model 450 (BioRad Laboratories, Richmond, Calif.) and the percent survival was calculated using the formula: % survival = [sample ($OD_{570} - OD_{600}$) − blank ($OD_{570} - OD_{600}$)]/[control ($OD_{570} - OD_{600}$) − blank ($OD_{570} - OD_{600}$)] × 100. Dose-response curves were plotted and the 50% inhibitory concentration (IC_{50}) was determined graphically as the dose of drug causing a 50% reduction in absorbance compared to the control. The AlamarBlue assay was performed in quadruplicate and repeated at least three times.

Enzyme-linked immunosorbent assay (ELISA) for TP

Cells were scraped and homogenized in phosphate-buffered saline (PBS) in a plastic pestle at 4 °C. After centrifugation at 20 000 *g* for 20 min, supernatants were frozen and stored at −80 °C until analysis. The protein levels were determined by the DC Protein Assay (BioRad). TP protein expression was analyzed using a two-step sandwich ELISA developed by Nishida et al. [19]. They reported that TP levels measured by ELISA correlate well with TP activity using a conventional TP enzyme assay [19]. Briefly, samples and standard were dispensed into antihuman TP monoclonal antibody (mAb) 104B-coated microplate wells. After incubation for 2 h at 37 °C, the plates were washed with PBS. Antihuman TP mAb 232-2 was added to each well and the plates were incubated for 2 h at room temperature. These antihuman TP mAbs were a gift from Nippon Roche K.K. (Tokyo, Japan). After washing with PBS, goat antimouse IgG serum conjugated with peroxidase was added and the plates were incubated for an additional 1 h at room temperature. A substrate reaction was performed with a substrate solution containing 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 (KPL Inc., Maryland, USA). Absorbance after terminating the peroxide reaction with 19 *M* phosphoric acid solution was measured at 450 nm, and the TP level was determined with reference to a standard curve obtained with the crude homogenate of human colon cancer xenograft HCT 116 (TP activity, 432 U/ml) and expressed as units per milligram protein where one unit was defined as the TP level that phosphorylated 5′-deoxy-5-fluorouridine (5′-DFUR) to 5-FU at the rate of 1 mg 5-FU/h [19, 22]. Each assay was performed in triplicate and repeated at least three times.

Statistics

Student's two-tailed *t*-test was used to assess the statistical significance of differences between data. Spearman's rank correlation coefficient was used to analyze the correlation between data. Differences were considered to be statistically significant when a probability (*P*) value was less than 0.05.

Results

Effect of IFN-α on the sensitivity to 5-FU in RCC cell lines

The modulatory effect of IFN-α on the sensitivity to 5-FU was evaluated in five RCC cell lines using the AlamarBlue assay. IFN-α alone had no significant growth inhibitory effect on any of the RCC lines examined at concentrations up to 10 000 U/ml, suggesting that the RCC cell lines used were not sensitive to IFN-α under the conditions employed in the present study. Based on this finding, IFN-α concentrations of less than 1000 U/ml, which is clinically achievable by administration of 3–6×10^6 U of IFN-α, were used in subsequent experiments. We then evaluated the effect of IFN-α on the sensitivity to 5-FU in the RCC cell lines. The IC_{50} for 5-FU without IFN-α stimulation ranged from 22.5 to 310 μ*M* (Table 1). Simultaneous treatment with IFN-α (100 U/ml) enhanced the sensitivity to 5-FU significantly in JMSU3, KU2 and VMRC-RCW cells but not in Caki1 and OS-RC-2 cells (Table 1). Representative dose-response curves for VMRC-RCW cells are shown in Fig. 1 when 5-FU and IFN-α at concentrations of 1, 10, 100, and 1000 U/ml were given simultaneously. The dose-dependent IFN-α-induced enhancement of sensitivity to 5-FU was observed not only in VMRC-RCW cells but also in JMSU3 and KU2 cells but not in Caki1 and OS-RC-2 cells (data not shown).

Next we examined the schedule-dependency of the modulatory effect of IFN-α on the sensitivity to 5-FU at a fixed concentration of 100 U/ml given in two schedules as described in Materials and methods (Table 1). In both

Table 1 Modulatory effect of IFN-α on the sensitivity to 5-FU of RCC cell lines. Cells were cultured with 5-FU alone or with combinations of 5-FU and IFN-α (100 U/ml) in two schedules as described in Materials and methods, and the viability was evaluated using the AlamarBlue assay. Values are mean ± SD of IC_{50} (μ*M*); values in parentheses indicate the increase in sensitivity to 5-FU induced by IFN-α calculated as IC_{50} for 5-FU alone/IC_{50} for the combination of 5-FU and IFN-α

Schedule	Caki1	JMSU3	KU2	OS-RC-2	VMRC-RCW
5-FU alone	310 ± 96	22.5 ± 8.7	75.0 ± 20	255 ± 33	110 ± 27
Simultaneous treatment	267 ± 76 (1.2)	11.0 ± 3.0*[1] (2.0)	30.0 ± 4.1*[1] (2.5)	238 ± 63 (1.1)	33.8 ± 7.5*[2] (3.3)
Sequential treatment	250 ± 89 (1.2)	5.8 ± 1.3*[1,*3] (3.9)	17.5 ± 2.9*[1,*4] (4.3)	220 ± 48 (1.2)	16.3 ± 4.8*[2,*4] (6.7)

*[1] $P < 0.5$, *[2] $P < 0.01$ vs 5-FU alone, *[3] $P < 0.05$, *[4] $P < 0.01$ vs simultaneous treatment

schedules, IFN-α enhanced the sensitivity of JMSU3, KU2 and VMRC-RCW cells to 5-FU significantly more when it was given prior to 5-FU than when it was given simultaneously. These findings indicate that sequential administration of IFN-α followed by 5-FU may be the effective schedule for this combination in some RCC cell lines in terms of direct antiproliferative effect in vitro.

Effect of IFN-α on TP expression in RCC cell lines

To elucidate the mechanism underlying the IFN-α-induced enhancement of sensitivity of RCC to 5-FU, we examined, with the use of an ELISA, whether IFN-α would enhance TP synthesis in RCC cell lines. As shown in Table 2, TP expression levels without IFN-α stimulation, varied among RCC cell lines; JMSU3 had the highest TP expression level among the five RCC cell lines. Table 2 also shows the TP expression level of each cell line when cultured with IFN-α (100 U/ml) for 24 h. In Caki1 and OS-RC-2 cells there was no significant change in the TP expression as a result of IFN-α stimulation as compared with the control. On the other hand, TP expression in JMSU3, KU2 and VMRC-RCW cells was significantly enhanced by IFN-α stimulation ($P < 0.05$). TP expression in JMSU3, KU2 and VMRC-RCW cells was enhanced by IFN-α stimulation in a dose-dependent manner (as shown in Fig. 2 for VMRC-RCW cells) as compared with the control ($P < 0.05$) while a dose-dependent increase in TP expression following IFN-α stimulation was not observed in Caki1 and OS-RC-2 cells (data not shown).

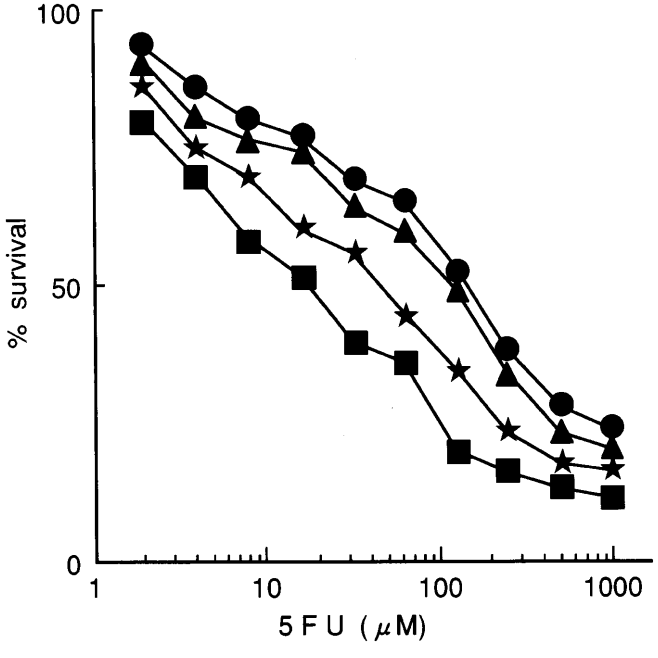

Fig. 1 Representative dose-response curves for the combination of IFN-α and 5-FU in VMRC-RCW cells. VMRC-RCW cells were cultured with 5-FU in the absence (●) or presence of IFN-α at concentrations of 1 (▲), 10 (★), and 100 U/ml (■) for 4 days. Percentage survival was measured using the AlamarBlue assay. Each point represents the mean value from quadruplicate experiments. The SD of the means were less than 15% and are omitted

Relationship between the sensitivity to 5-FU and TP expression

The sensitivity to 5-FU (IC_{50}) was compared with TP expression in the RCC cell lines. There was no significant correlation between sensitivity to 5-FU (Table 1) and TP expression (Table 2) without IFN-α stimulation. However, a significant correlation was observed between the relative IFN-α-induced increase in sensitivity to 5-FU and the relative IFN-α-induced increase in TP expression ($P < 0.05$). That is, VMRC-RCW cells, which had the highest increase in TP expression following IFN-α stimulation, showed the highest IFN-α-induced increase in sensitivity to 5-FU among the RCC cell lines examined. On the other hand, in Caki1 and OS-RC-2 cells, which showed no significant increase in TP expression following IFN-α stimulation, IFN-α did not significantly affect the sensitivity to 5-FU. In addition, two (JMSU3 and VMRC-RCW) of three RCC cell lines which showed a more than a two-fold increase in sensitivity to 5-FU following IFN-α stimulation (Table 1) showed higher TP expression without IFN-α stimulation (Table 2).

Discussion

TP, the first enzyme in the salvage pathway for the direct conversion of 5′-DFUR to 5-FU to deoxyribonucleo-

Table 2 Modulatory effect of IFN-α on TP expression in RCC cell lines. Cells were cultured in the absence or presence of IFN-α at a fixed concentration of 100 U/ml for 24 h, and TP expression was evaluated using an ELISA. Values are mean ± SD of TP expression level (U/mg protein); values in parenthesis indicate the increase in TP expression as a result of the presence of IFN-α calculated as: TP expression for IFN-α +/ TP expression for IFN-α −

	Caki1	JMSU3	KU2	OS-RC-2	VMRC-RCW
IFN-α −	2.3 ± 1.6	38.4 ± 5.8	2.5 ± 1.1	1.4 ± 0.6	13.7 ± 2.5
IFN-α +	2.7 ± 0.7 (1.2)	55.9 ± 6.9** (1.5)	4.5 ± 0.9* (1.8)	1.6 ± 0.6 (1.2)	27.4 ± 3.6** (2.0)

* $P < 0.05$, ** $P < 0.01$ vs IFN-α −

tides, is inducible by IFN-α [23, 24, 31] and other cytokines [5] and is identical to platelet-derived endothelial cell growth factor (PD-ECGF) [14] which has angiogenic activity [9]. Recent studies have shown that TP expression increases in such cancers as esophageal, gastric, pancreatic, colon, lung, bladder, ovarian, breast, and RCC compared with the surrounding normal tissues [13, 16, 17, 20, 21, 29, 30, 32, 37]. Imazono et al. have found that TP expression in RCC is ninefold higher than in non-neoplastic kidney tissue and is correlated with microvessel density and tumor grade [13]. These reports indicate that elevated TP expression is associated with a predisposition to aggressive disease and/or a poor prognosis. Therefore, it is a reasonable strategy to target TP in the treatment of RCC using fluoropyrimidines. The present study was designed to investigate the modulatory actions of IFN-α on 5-FU in RCC cell lines in vitro, in particular focusing on TP expression.

In the present study, IFN-α, at noncytotoxic concentrations, enhanced the sensitivity of some RCC cell lines to 5-FU in a dose-dependent manner. This biomodulation of 5-FU by IFN-α, independent of immunomediated or host-protective effects, has been reported in some tumors including a human colon cancer cell line, the human salivary gland adenocarcinoma 38 cell line, and the human promyelocytic leukemia cell line HL-60 [33]. The interaction of IFN-α and 5-FU in human colon cancer cell lines (HT-29 and SW480) has been examined using a clonogenic assay [23, 35]. It was demonstrated that IFN-α exhibits synergy with 5-FU accompanied by an increase in uridine phosphorylase and pyrimidine nucleoside phosphorylase activities. Eda et al. have [6] reported that a mixture of IL-1α, TNF-α and IFN-γ strongly enhances the cytotoxicity of 5-FU and 5′-DFUR and the expression of uridine phosphorylase, which predominantly exists in rodents, in the mouse colon 26 carcinoma cell line. Furthermore, they have found that the increase in the cytotoxicity of 5-FU and 5′-DFUR induced by the cytokine mixture is abolished by an inhibitor of uridine phosphorylase (2,2′-anhydro-5-ethyluridine). Direct evidence has been found by Schwartz et al. [25]: transfection of the TP gene into human colon carcinoma cells (HT-29) greatly increases the sensitivity of the cells to 5-FU, and this action is mediated by an enhancement of the metabolic activation of 5-FU by increased TP expression.

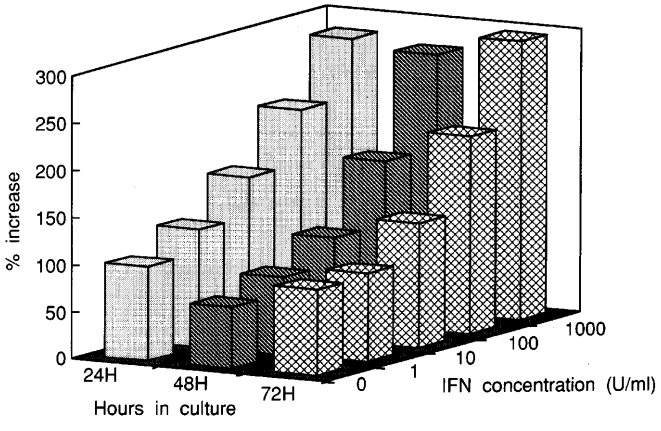

Fig. 2 Enhancement of TP expression by IFN-α stimulation in VMRC-RCW cells. VMRC-RCW cells were cultured in the absence or presence of IFN-α at concentrations of 1, 10, 100, and 1000 U/ml for up to 72 h. Points are the means from triplicate experiments expressed as the percentage of the TP expression level at 24 h without IFN-α (14.0 U/mg protein). The SD of the means were less than 15% and are omitted

These reports support the following proposed mechanism of biomodulation of 5-FU by IFN-α. IFN-α increases TP activity resulting in increased levels of the active 5-FU metabolite, FdUMP. Increased levels of FdUMP reduce TS activity, and finally the inhibition of DNA synthesis is enhanced [27, 33], suggesting an important role of TP in the biomodulating action of IFN-α on the sensitivity to fluoropyrimidines. Based on these reports, we evaluated TP expression in RCC cell lines, and showed that TP is constitutively expressed without IFN-α simulation in RCC cell lines and the relative IFN-α-induced increase in sensitivity to 5-FU correlates with the relative IFN-α-induced increase in TP expression.

Furthermore, IFN-α alone did not inhibit TS activity but enhanced the inhibition of TS activity by 5-FU significantly, accompanied by enhanced sensitivity to 5-FU in VMRC-RCW cells (data not shown). These findings suggest that TP is involved in the IFN-α-induced enhancement of sensitivity to 5-FU in RCC. However, we found no significant correlation between TP expression and sensitivity to 5-FU without IFN-α stimulation, indicating that factors other than TP expression, such as TS and dihydropyrimidine dehydrogenase (DPD) [27, 34] contribute to sensitivity to fluoropyrimidines. The finding that the cell lines with higher TP expression without IFN-α stimulation showed higher IFN-α-induced increases in sensitivity to 5-FU suggests that TP expression could be a marker for

identifying RCC that may respond to IFN-α/5-FU combination therapy. Since the present study used a limited number of long-term cultured cell lines, further studies with freshly isolated cells are needed to confirm our results.

The optimal timing for administration of IFN-α in combination with fluoropyrimidines is of critical importance. Elias and Crissman [7] have examined the effect of various schedules of IFN-α and fluoropyrimidines on the growth of murine colon adenocarcinoma MCA-38 cells in vitro. They found that sequential treatment produced greater synergy when IFN-α preceded 5-FU or 5′-DFUR rather than the reverse protocol. The present study showed that the enhancement of sensitivity to 5-FU was more marked when IFN-α was given prior to 5-FU rather than simultaneously. It seems likely that pretreatment with IFN-α for 24 h enhances TP expression before 5-FU administration, probably resulting in increased levels of active 5-FU metabolites and a marked enhancement of sensitivity to 5-FU. This finding has important practical implications for the design of clinical treatment regimens: sequential administration of IFN-α followed by 5-FU would be beneficial in some metastatic RCC patients.

In conclusion, these in vitro results suggest that IFN-α upregulates TP expression and modulates 5-FU anabolism resulting in an enhancement of 5-FU cytotoxicity in a dose- and schedule-dependent manner in some RCC cell lines. Recently, capecitabine, which is a new 5-FU prodrug that is converted finally to 5-FU by TP, has been assessed in clinical trials and has been shown to have significant antitumor efficacy against human cancer xenografts with high TP levels and low DPD levels [15]. Further studies of the biomodulation of fluoropyrimidines including capecitabine by cytokines are needed to improve the therapeutic efficacy against metastatic RCC.

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