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European Journal of Cancer 38 (2002) 167–176

European Journal of Cancer

www.ejconline.com

Potentiation of the sensitivity of renal cell carcinoma cells to TRAIL-mediated apoptosis by subtoxic concentrations of 5-fluorouracil

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Received 9 April 2001; received in revised form 13 September 2001; accepted 20 September 2001

Abstract

Cytotoxic chemotherapy has shown little antitumour activity against renal cell carcinoma (RCC). Although immunotherapy is relatively effective against RCC, the response rate is approximately 20%. Therefore, there is an urgent need to increase this response rate. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo-2L) is one member of the tumour necrosis factor ligand family that selectively induces apoptosis of cancer cells. Since several cytotoxic anticancer drugs including 5-fluorouracil (5-FU) also mediate apoptosis, we reasoned that combined treatment of cancer cells with TRAIL and drugs might result in synergy and overcome the resistance of the cancer cell. This study has examined whether TRAIL can synergise with 5-FU in both cytotoxic and apoptotic assays against drug-resistant RCC cells. Cytotoxicity was determined by an 1-day microculture tetrazolium dye assay. Synergy was assessed by isobolographic analysis. Treatment of Caki-1 cells with TRAIL in combination with 5-FU resulted in a synergistic cytotoxic effect. Synergy was also achieved in freshly derived RCC cells from 3 patients. The enhanced cytotoxicity was obtained irrespective of the sequence of the treatment, but the highest cytotoxicity was observed when Caki-1 cells were treated with TRAIL and 5-FU simultaneously. The synergy achieved in cytotoxicity with TRAIL and 5-FU was shown to be due to apoptosis. The mechanisms responsible for the synergistic cytotoxicity and apoptosis were examined. Treatment of Caki-1 cells with 5-FU enhanced the expression of p53 and bax, but had no effect on the expression of bcl-2. Incubation of Caki-1 cells with TRAIL enhanced the intracellular accumulation of 5-FU and 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP). Treatment of Caki-1 cells with TRAIL downregulated the expression of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) modestly, and upregulated the expression of orotate phosphoribosyltransferase (OPRT). However, the expression level of thymidine phosphorylase (TP) was not affected by TRAIL. This study demonstrates that combined treatment of RCC cells with TRAIL and 5-FU overcomes their resistance. The sensitisation obtained with freshly isolated RCC cells required low subtoxic concentrations of 5-FU. These findings support the potential application *in vivo* of a combination of TRAIL and 5-FU in the treatment of TRAIL/5-FU-resistant RCC. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: TRAIL; Apo-2; Apoptosis; 5-FU; Renal cell carcinoma

1. Introduction

Cytotoxic chemotherapy, an integral part of the therapeutic approach for treatment of many of the solid tumours, has shown little or no antitumour activity against renal cell carcinoma (RCC) and has played no role in either an adjuvant or a neoadjuvant support therapy [1]. Immunotherapy including interleukin-2 and interferon-α is relatively effective against RCC, however, the response rate is approximately 20% [2]. Hence, alternative approaches are necessary for patients with RCC.

The cloning of biologically active cytotoxic molecules has been considered as potential new therapeutics for drug-resistant cancer cells. For example, some members of the tumour necrosis factor (TNF) superfamily are characterised by their ability to induce apoptosis in cancer cells. TNF-α is the first molecule to be tested for

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PII: S0959-8049(01)00339-2

its anticancer activity, followed by Fas ligand. These two molecules are efficient in killing a variety of cancer cells, however, they cause significant damage to normal tissues that results in life-threatening toxicities [3,4]. The search for a cytotoxic molecule that is selective for cancer cells has thus continued until the recently discovered new member of the TNF superfamily, namely the TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L) [5,6]. TRAIL has been shown to selectively induce apoptosis in cancer cells and has minimal or no toxicity against normal tissues, as examined both *in vitro* and *in vivo* in mice [7,8]. Therefore, TRAIL may be effective *in vivo* as an anticancer agent, provided the cancer cells are sensitive to TRAIL. However, most cancer cells are not sensitive to TRAIL-mediated apoptosis.

Several anticancer chemotherapeutic drugs, as well as TRAIL, mediate apoptosis and may share common intracellular signalling pathways leading to apoptosis. We have reasoned that cancer cells which are resistant to TRAIL/drugs can be sensitised by combined treatment with TRAIL and anticancer drugs. Indeed, several studies tested the hypothesis and corroborated the drug-mediated sensitisation of resistant cancer cells to TNF family members such as TNF-α and Fas ligand [9,10]. This study has investigated whether the resistance of RCC cells to TRAIL/5-fluorouracil (5-FU) can be overcome by a combination of TRAIL and 5-FU. Furthermore, this study explored possible underlying mechanisms involved in the reversal of drug resistance.

2. Materials and methods

2.1. Tumour cells

The Caki-1 human RCC cell line [11,12] was maintained in monolayers on plastic dishes in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Biocult, Glasgow, Scotland, UK) supplemented with 25 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 1% non-essential amino acid (Gibco), 100 units/ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco) and 10% heat-inactivated fetal bovine serum (Gibco), hereafter referred to as complete medium.

Fresh RCC cells derived from 3 patients were separated from surgical specimens as previously described in Refs. [12,13]. The histological diagnosis revealed that all patients had clear cell carcinoma. Their histological classification and staging according to the TNM classification were: patient no. 1: T1N0M0, grade 1; patient no. 2: T1N0M0, grade 1; patient no. 3: T3N0M1, grade 2. Briefly, cell suspensions were prepared by treating finely minced tumour tissues with collagenase (3 mg/ml, Sigma Chemical Co., St. Louis, USA). After washing three times in RPMI-1640 medium, the cell suspensions were layered on discontinuous gradients consisting of 2 ml of 100%, 2 ml of 80% and 2 ml of 50% Ficoll-Hypaque in 15-ml plastic tubes and were centrifuged at 400*g* for 30 min. Lymphocyte-rich mononuclear cells were collected from the 100% interface, and tumour cells and mesothelial cells from the 80% interface. Cell suspensions enriched with tumour cells were sometimes contaminated by monocyte-macrophages, mesothelial cells or lymphocytes. To eliminate further contamination of host cells, we layered the cell suspensions on a discontinuous gradient containing 2 ml each of 25, 15, and 10% Percoll in complete medium in 15-ml plastic tubes and centrifuged them for 7 min at 25*g* at room temperature. Tumour cells depleted of lymphoid cells were collected from the bottom, washed and suspended in complete medium. To remove further contamination from mesothelial cells and monocyte-macrophages, we incubated the cell suspension in plastic dishes for 30–60 min at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, non-adherent cells were recovered, washed, and suspended in complete medium. Usually, the non-adherent cells contained mainly tumour cells with less than 5% contaminating non-malignant cells, as judged by morphological examination of Wright-Giemsa-stained smears, and were more than 93% viable according to the trypan blue dye-exclusion test. Cells having less than 5% contamination with non-malignant cells were accepted for use as cancer cells.

2.2. Reagents

Recombinant human TRAIL was purchased from Pepro Tech, Rocky Hill, NJ, USA. 5-Fluorouracil (5-FU) (lot. no. 469ABG) was supplied by Kyowa Hakkou Co. Ltd., Tokyo, Japan.

2.3. Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine tumour cell lysis as previously described in Refs. [12,13]. Briefly, 100 μl of the target cell suspension (2×10^4 cells) were added to each well of 96-well flat-bottom microtitre plates (Corning Glass Works, NY, USA), and each plate was incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, 100 μl of drug solution or complete medium for control were distributed in the 96-well plates and each plate was incubated for 24 or 72 h at 37 °C. Following incubation, 20 μl of MTT working solution (5 mg/ml, Sigma Chemical Co.) was added to each culture well and the cultures were incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was removed from the wells and replaced with 100 μl of isopropanol (Sigma Chemical Co.) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture plate reader (Immunoreader, Japan Intermed

Co. Ltd., Tokyo, Japan) at 540 nm. The percent cytotoxicity was calculated by the following formula: Percentage cytotoxicity = [1−(absorbance of experimental wells/absorbance of control wells)]×100.

2.4. Chromatin staining with Hoechst 33258

Apoptosis was observed by chromatin staining with Hoechst 33258 as previously described in Ref. [14]. Caki-1 cells in a chamber/slide (Miles Scientific, IL, USA) were incubated with TRAIL at 100 ng/ml in the absence or presence of 5-FU at 100 μg/ml for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, the supernatant was discarded and Caki-1 cells were fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, washed four times with PBS, and exposed to Hoechst 33258 at 10 μM for 30 min at room temperature. The cell preparations were examined under ultraviolet (UV) illumination with an Olympus fluorescence microscope. Apoptosis was defined as the presence of apoptotic bodies, chromatin condensation and/or fragmented nucleus.

2.5. DNA ladder assay

Apoptosis was also observed by a DNA ladder assay as previously described in Ref. [15]. Caki-1 cells were treated with TRAIL at 100 ng/ml and/or 5-FU at 100 μg/ml for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. The cells were lysed in 200 μl of DNA lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM ethylene diamine tetra acetic acid (EDTA), 0.5% sodium dodecyl sulphate (SDS) and 0.5 μg/ml proteinase K) and incubated for 3 h at 37 °C. Lysates were extracted with an equal volume of 1:1 phenol:chloroform and precipitated with two volumes of isopropanol. Cell pellets were resuspended in 100 μl of TE with 50 μg/ml RNase A and incubated for 15 min at 37 °C. Sample buffer (25 μl of 5×buffer, 50% glycerol, 0.1 M Tris, 0.1% SDS, 0.1 M EDTA, 0.01% xylene cyanol and 0.01% bromophenol blue) was added, and samples were incubated for 10 min at 68 °C. Samples were loaded onto a 1% agarose gel, electrophoresed overnight at 20–30 mA and stained with ethidium bromide to visualise the DNA.

2.6. Immunocytochemical detection of p53, bcl-2 and bax

The expression of p53, bcl-2 and bax in Caki-1 cells was examined by immunocytochemical staining using DAKO LSAB Kit (lot. no. 117-4, Dako Corporation, CA, USA). The monoclonal antibodies for bcl-2 and bax were purchased from MBL, Nagoya, Japan. The monoclonal antibody for p53 was purchased from Oncogene Science Inc., Cambridge, MA, USA.

Caki-1 cells incubated with 5-FU at 100 μg/ml for 24 h were treated with trypsin-EDTA, followed by attachment to glass slides with centrifugation in a cytospin. The cells were fixed with 10% formaldehyde for 1 h and dehydrated in 70% ethanol. The streptoavidin biotinylated immunoperoxidase method was used for the immunocytochemical staining.

2.7. Determination of 5-FU

Determination of 5-FU in Caki-1 cells was made by GLC-mass fragmentography and GLC-mass spectrometry using a JMS-D 300 mass spectrometer with a JGC-20KP gas chromatograph (JEOL, Tokyo, Japan) as described in detail elsewhere [12,16].

2.8. 5-Fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) determination

Intracellular FdUMP concentrations in Caki-1 cells were determined by using high performance liquid chromatography (HPLC) and [^{3}H]5-FU as previously described in Ref. [17].

2.9. Measurement of thymidylate synthase (TS) expression

The expression of TS was determined by the FdUMP binding assay combined with gel filtration as previously described in Refs. [17,18]. Caki-1 cells were sonicated in homogenate buffer (50 mM Tris–HCl, 1 mM EDTA and 5 mM $MgCl_2$ pH 7.4) at maximum output (Sonifier cell disruptor 350: SmithKline), and centrifuged at 105,000*g* at 4 °C for 60 min in a Beckman ultra-centrifuge (model TL-100). The supernatant was divided into several tubes and frozen at −80 °C until use.

The supernatant was incubated with [^{3}H]-FdUMP and 5,10-CH_2–FH_4 at 30 °C for 20 min, then the mixture was gel-filtered using a PD-10 column (Pharmacia Biothech, Uppsala, Sweden) to separate TS-bound from free [^{3}H]-FdUMP. The sample was eluted with PBS (–) and the total radioactivity of the fractions containing protein was measured. Protein content of the supernatant was measured using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL, USA).

2.10. Measurement of dihydropyrimidine dehydrogenase (DPD) activity

Caki-1 cells were homogenised in 4 volumes of 50 mM Tris–HCl (pH 8.0) containing 5 mM 2-mercaptoethanol, 25 mM KCl and 5 mM $MgCl_2$. The homogenate was centrifuged at 105,000*g* for 1 h at 4 °C, and the supernatant fluid was used for the measurement of DPD activity as described before in Ref. [19]. Briefly, the assay mixture, in a final volume of 0.25 ml, consisted of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM NaF, 50 mM nicotinamide, 5 mM adenosine triphosphate

(ATP), 1 mM NADPH, [6-^{3}H] 5-FU (0.2 μCi, 20 μM) and the enzyme extract (0.1 ml). The mixture was incubated for 30 min at 37 °C and the reaction was stopped by heating at 100 °C in a water-bath. After centrifugation at 3000 rpm, the supernatant (0.1 ml) was treated with 0.01 ml of 2 M KOH for 30 min at room temperature. Then, the mixture was treated with 0.005 ml of 2 M PCA and centrifuged. An aliquot (20 μl) of the supernatant was spotted onto a thin layer chromatography plate (Merck silica gel 60F254 precoated plate, 2.5×10 cm, thickness 0.25 mm) and developed with a mixture of chloroform, methanol and acetic acid (17:3:1, v/v/v). The spots of 2-fluoro-β-alanine and 2-fluoro-β-ureidopropionic acid, 5-FU degradation products, were scraped into vials and mixed with 10 ml of ACS-II scintillation fluid (Amersham). The radioactivity was measured in a Wallac 1410 liquid scintillation counter (Pharmacia).

2.11. Assay of 5-FU-anabolising enzymes

Activities of thymidine phosphorylase (TP) and orotate phosphoribosyltransferase (OPRT) were measured as previously reported in Ref. [17].

2.12. Statistical analysis

All determinations were made in triplicate, and the results were expressed as the mean±standard deviation (S.D.). Statistical significance was determined by Student's *t*-test. A *P* value of 0.05 or less was considered significant.

Calculations of synergistic cytotoxicity were determined by isobolographic analysis as described by Berenbaum [20,21]. Whether the combination is additive, synergistic or antagonistic is shown by whether the point lies on, below or above the straight line joining the doses of the two drugs that, when given alone, produce the same effect as that of the combination in isobolographic analysis.

3. Results

3.1. Synergistic cytotoxicity against RCC cells following combination treatment with TRAIL and 5-FU

When Caki-1 cells were treated with a combination of TRAIL and 5-FU, significant potentiation of cytotoxicity and synergy were obtained (Fig. 1a and b). The synergistic cytotoxicity was observed with subtoxic concentrations of 5-FU. Furthermore, synergy was also obtained in freshly derived RCC cells from three patients, irrespective of the baseline sensitivity of the cancer cells to either 5-FU or TRAIL when used alone (Fig. 2a–d). The synergistic cytotoxic effect was observed in a 3-day MTT assay as well as a 1-day MTT assay. The degree of synergy obtained in a 3-day MTT assay is similar to that in a 1-day MTT assay.

3.2. Effect of the sequence of treatment with TRAIL and 5-FU on synergy

The findings above demonstrate that simultaneous treatment of RCC cells with the TRAIL and 5-FU resulted in synergy. The effect of sequential treatment with TRAIL and 5-FU was examined and was compared with treatment using both agents added together. The Caki-1 RCC cells were treated for 6 h with one agent, the medium was aspirated and the Caki-1 cells were washed twice with RPMI medium, and the second agent was subsequently added for 18 h, and the cells were tested for viability. The results show that synergy was obtained irrespective of the sequence of the treat-

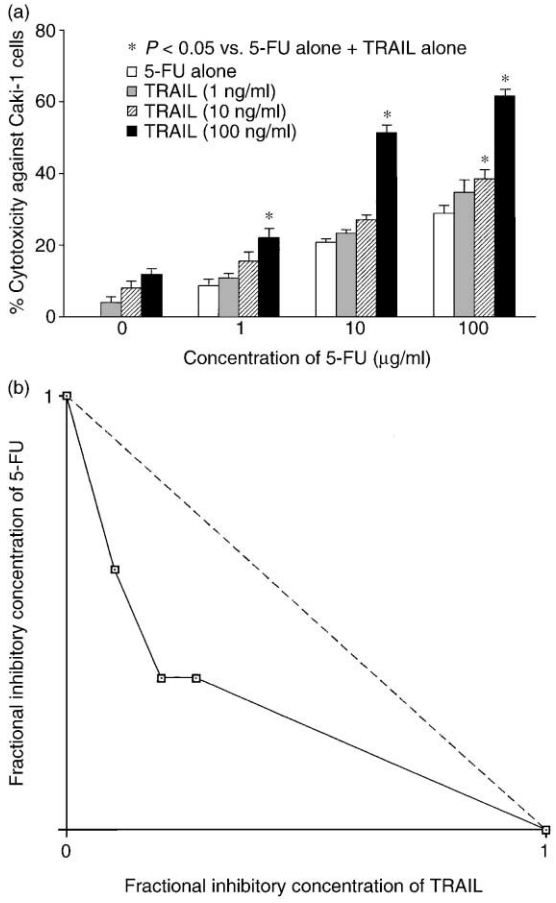

Fig. 1. The synergistic cytotoxic effect of TRAIL and 5-fluorouracil (5-FU) used in combination against Caki-1 cells. The cytotoxic effect of TRAIL and 5-FU used in combination on Caki-1 cells was assessed in a 1-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (a) and synergy was estimated by isobolographic analysis (b). The results are derived from three different experiments. *Values in the combination treatment are significantly higher than those achieved by treatment with 5-FU alone plus those with TRAIL alone at $P<0.05$.

ment, but the highest percent cytotoxicity was obtained when Caki-1 cells were treated with both agents simultaneously (Table 1). Similar results were obtained when TRAIL and 5-FU were used at different concentrations (data not shown). These findings demonstrate that the sequence of treatment with TRAIL and 5-FU might be critical to obtain maximal synergy in the cytotoxicity against the RCC cells.

3.3. Mechanisms of synergistic cytotoxicity achieved with TRAIL and 5-FU

3.3.1. Induction of apoptosis

Since both TRAIL and 5-FU mediate apoptosis, we examined by Hoechst 33258 staining whether the synergy achieved in cytotoxicity with TRAIL and 5-FU, as detected by MTT assay, also resulted in apoptosis. Apoptosis was defined microscopically by the presence of apoptotic bodies, chromatin condensation and/or fragmented nucleus. No apoptosis was seen in the Caki-1 cells cultured in medium. Treatment of the cells with 5-FU at a concentration of 100 μg/ml or TRAIL at a concentration of 100 ng/ml resulted in modest apoptosis (approximately 10 and 15%, respectively). However, when TRAIL and 5-FU were used in combination, fragmented nuclei of Caki-1 cells were observed and almost all Caki-1 cells were apoptotic, thus synergy in apoptosis was observed (Fig. 3a–c). These findings demonstrate that synergy in cytotoxicity was paralleled by synergy in apoptosis.

Apoptosis was also examined by a DNA ladder assay. 5-FU mediated minimal apoptosis. When Caki-1 cells were treated with TRAIL at a concentration of 100 ng/ml, a DNA ladder was observed. Treatment of the Caki-1 cells with a combination of TRAIL and 5-FU resulted in more apoptosis as represented by increased laddering (data not shown).

These results indicated that there was a good correlation between cytotoxicity in the MTT assay and apoptosis after treatment of Caki-1 cells with a combination of TRAIL and 5-FU.

3.3.2. Enhanced p53 and bax expression in Caki-1 cells following 5-FU treatment

We have examined whether treatment of Caki-1 cells with 5-FU regulates the expression of p53, bcl-2 and bax. Although immunocytochemical analysis failed to detect p53 in the Caki-1 cells, p53 expression was observed after treatment with 5-FU (Fig. 4). Treatment of Caki-1 cells with 5-FU did not change the expression of bcl-2 (data not shown). However, bax expression in the Caki-1 cells was upregulated following their treatment with 5-FU (Fig. 5). These findings suggest that 5-FU-mediated sensitisation to the TRAIL-mediated

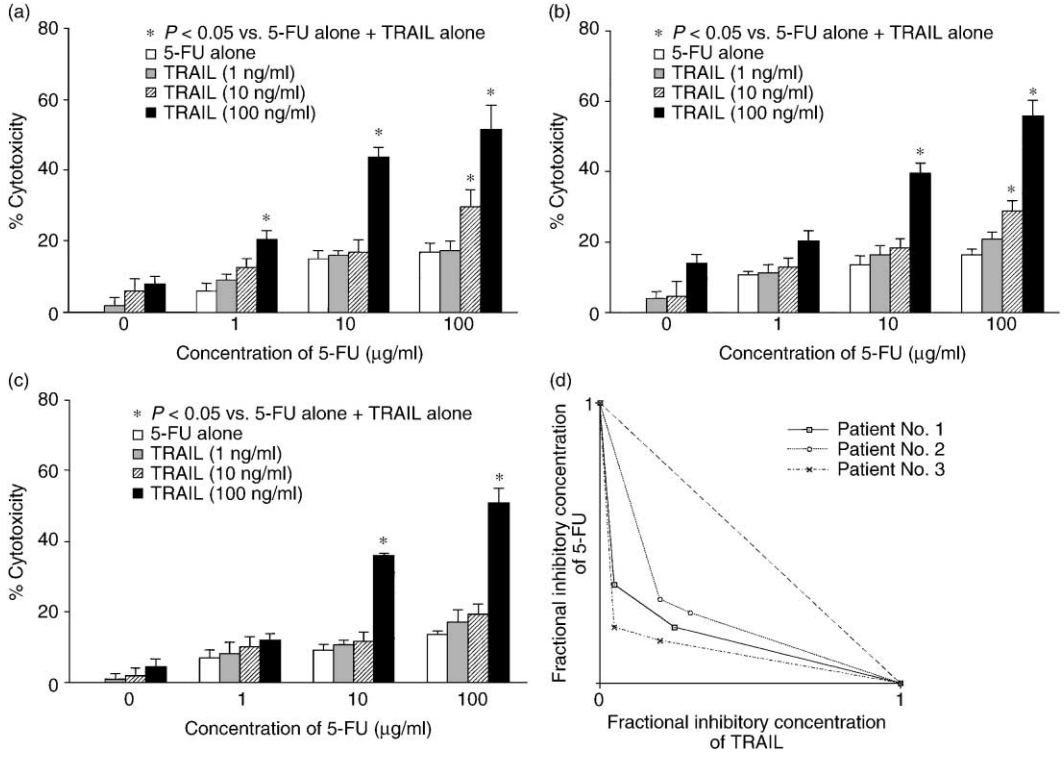

Fig. 2. The synergistic cytotoxic effect of TRAIL and 5-fluorouracil (5-FU) used in combination against freshly isolated RCC cells. The cytotoxic effect of TRAIL and 5-FU used in combination on fresh RCC cells derived from 3 patients ((a) patient no. 1, (b) patient no. 2; (c) patient no. 3) was assessed in a 1-day MTT assay and (d) synergy was assessed by isobolographic analysis. The results are derived from triplicate samples. *Values in the combination treatment are significantly higher than those achieved by treatment with 5-FU alone plus those with TRAIL alone at $P<0.05$.

apoptotic signalling pathway may be due, in part, to the enhanced expression of the proapoptotic p53 and bax proteins.

3.3.3. Enhanced intracellular accumulation of 5-FU and FdUMP

The synergistic cytotoxicity may be due to an increased accumulation of 5-FU and FdUMP in RCC cells treated with TRAIL. When Caki-1 cells were treated with a combination of 5-FU and TRAIL, 5-FU and FdUMP accumulation inside the cells increased modestly (Table 2). These findings suggest that the synergistic cytotoxicity might be due to the enhanced 5-FU and FdUMP accumulation in the RCC cells.

Table 1
Effect of the sequence of treatment with TRAIL and 5-fluorouracil (5-FU) on the cytotoxic activity against Caki-1 cells

First treatment[a] (6 h)	Second treatment[a] (18 h)	% Cytotoxicity (mean±S.D.)[b]
Medium	TRAIL	13.2±1.8
Medium	5-FU	24.5±4.1
Medium	TRAIL plus 5-FU	64.6±6.6[c,d]
TRAIL	5-FU	48.6±5.2[c]
5-FU	TRAIL	47.7±4.8[c]

S.D., standard deviation.
[a] 100 ng/ml TRAIL or 100 μg/ml 5-FU were used. Cytotoxicity was assessed in a 1-day MTT assay.
[b] The results are expressed as the mean±S.D. of three separate experiments.
[c] Values in the combination treatment are significantly higher than those achieved by treatment with 5-FU alone plus those with TRAIL alone at $P < 0.05$.
[d] Values in the combination treatment are significantly higher than those achieved when either TRAIL or 5-FU was given first at $P < 0.05$.

Table 2
Effect of TRAIL on the intracellular accumulation of 5-fluorouracil (5-FU) and 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) in Caki-1 cells

Treatment	Intracellular accumulation (mean±S.D.)	
	5-FU (ng/10^6 cells)[a]	FdUMP (pmol/10^6 cells)[a]
Control (medium)	7.3±0.6	3.1±0.8
TRAIL	11.7±1.5[b]	7.1±0.7[b]

S.D., standard deviation.
[a] Caki-1 cells were treated with 5-FU (100 μg/ml) in combination with medium or TRAIL (100 ng/ml) for 24 h. The medium was aspirated and Caki-1 cells were washed three times with Roswell Park Memorial Institute (RPMI) medium. The intracellular concentrations of 5-FU and FdUMP were measured by a gas chromatographic-mass fragmentographic method and a thymidylate synthase (TS) binding assay, respectively. The results are expressed as the mean±S.D. of three different experiments.
[b] $P < 0.05$ versus control.

3.3.4. Effect of TRAIL on the expression of enzymes involved in the metabolism of 5-FU

Since increased intracellular accumulation of 5-FU and FdUMP by TRAIL was observed, we examined the effect of TRAIL on the expression of enzymes involved in the metabolism of 5-FU. When Caki-1 cells were

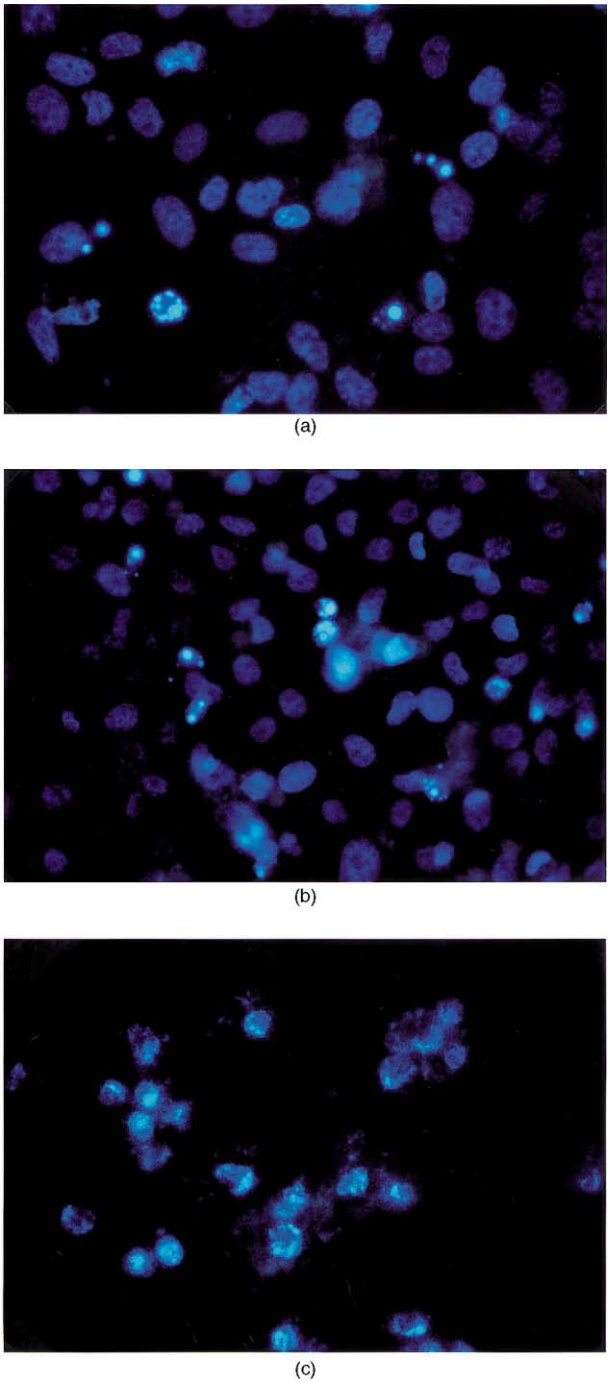

Fig. 3. Apoptosis in Caki-1 cells treated with TRAIL and 5-FU by Hoechst staining. Apoptosis in Caki-1 cells was determined by chromatin staining with Hoechst 33258. The figure is a fluorescent microscopic view of chromatin staining with Hoechst 33258 (×200) after the following treatments: (a) 5-FU at 100 μg/ml; (b) TRAIL at 100 ng/ml; (c) TRAIL at 100 ng/ml and 5-FU at 100 μg/ml.

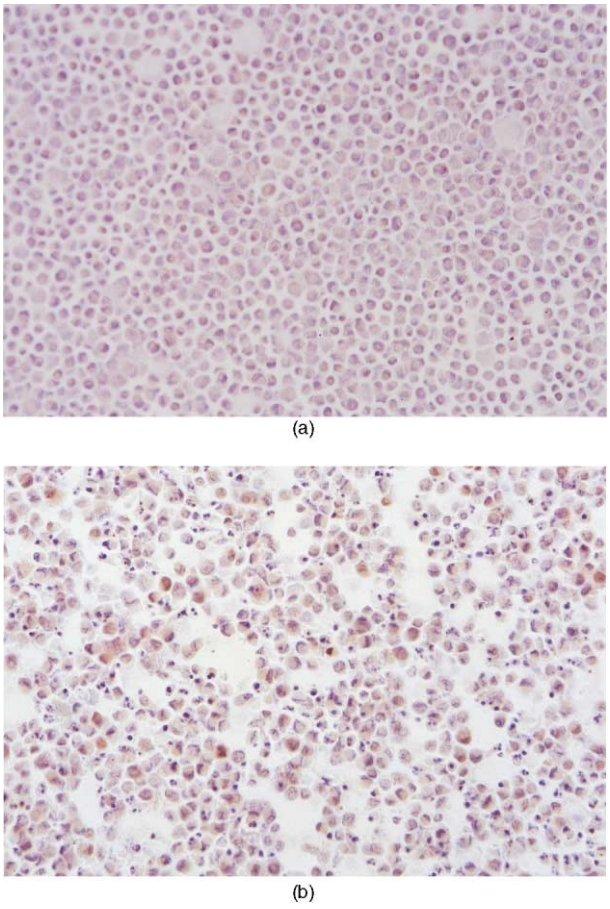

Fig. 4. Immunocytochemical staining for p53. The expression of p53 in Caki-1 cells was enhanced following treatment with 5-FU: (a) medium only; (b) 100 μg/ml 5-FU (magnification ×200).

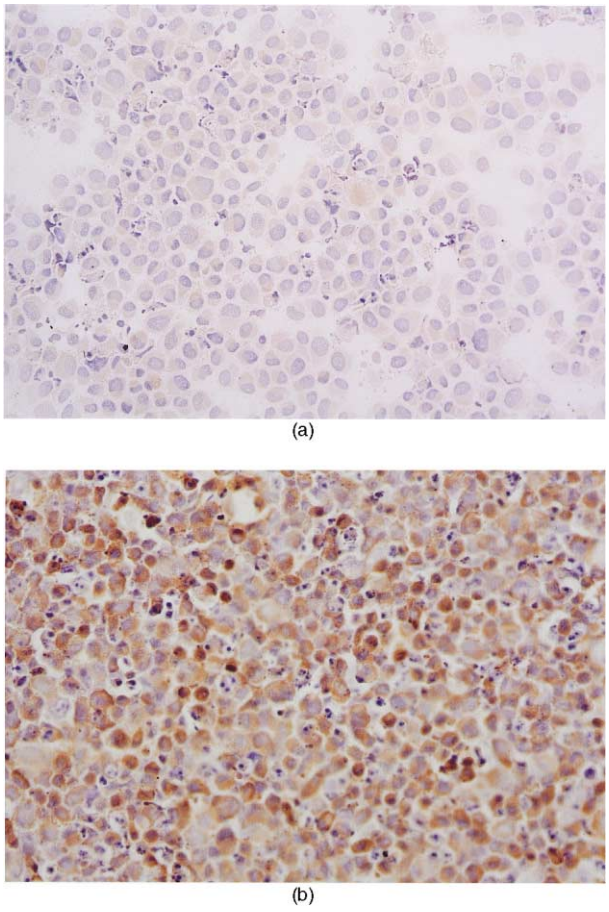

Fig. 5. Immunocytochemical staining for bax. The expression of bax in Caki-1 cells was enhanced following treatment with 5-FU: (a) medium only; (b) 100 μg/ml 5-FU (magnification ×200).

treated with TRAIL, TS and DPD expression was slightly reduced, whereas the expression of OPRT was upregulated. However, the treatment had no effect on TP expression (Table 3). These findings suggest that the enhanced intracellular accumulation of 5-FU and FdUMP might be due, in part, to the modified expression of at least some of these enzymes by TRAIL.

Table 3
Effect of TRAIL on the expressions of enzymes involved in the metabolism of 5-FU

Enzymes	Treatment[a]	
	Control (medium)	TRAIL
TS (fmol/mg protein)	700±48	537±95[b]
DPD (fmol/mg protein/min)	5593±628	4234±311[b]
OPRT (pmol/mg protein/min)	10.7±1.2	20.7±3.5[b]
TP (pmol/mg protein/min)	104±22	116±28

TRAIL, TNF-related apoptosis-inducing ligand; TS, thymiddylate synthesis; DPD, dihydropyrimidine dehydrogenase; OPRT, oratate phosphoribosyltransferase; TP, thymidine phosphorylase; S.D., standard deviation.

[a] Caki-1 cells were treated with medium or TRAIL (100 ng/ml) for 24 h. The medium was aspirated and Caki-1 cells were washed three times with Roswell Park Memorial Institute (RPMI) medium. The expressions of the enzymes were measured as described in Materials and methods. The results are expressed as the mean±S.D. of three different experiments.

[b] $P<0.05$ versus control.

4. Discussion

This study demonstrated that treatment of established and freshly isolated RCC cells with a combination of TRAIL and 5-FU resulted in the potentiation of cytotoxicity and apoptosis and reversed their resistance. Comparable results have been obtained by another investigator [22]. Synergy was achieved with subtoxic concentrations of 5-FU. This may be of clinical relevance since high concentrations of 5-FU are toxic *in vivo*. Furthermore, the concentrations of 5-FU that sensitised RCC cells to TRAIL-mediated apoptosis *in vitro* are within the range clinically achievable in patients with RCC. Since TRAIL is not toxic to most normal tissues both *in vitro* and *in vivo* [7,8], combination treatment with TRAIL and 5-FU may be considered a promising strategy to eliminate RCC cells without harming normal tissues.

Several possible mechanisms of resistance to TRAIL-mediated apoptosis have been reported such as low expression of TRAIL receptors, DR4 and DR5, and the enhanced expression of antagonistic TRAIL receptors, DcR1 and DcR2 [23,24]. The existence of multiple receptors for TRAIL suggests an unexpected complexity in the regulation of signalling by this cytokine. Anti-apoptotic molecules such as bcl-2 and bcl-xL may be potential resistant factors for TRAIL-mediated apoptosis [25,26]. Since TRAIL induces apoptosis in cancer cells in a caspase-dependent fashion, the resistance to TRAIL-mediated apoptosis might be dependent on the level of expression of the caspases [27,28]. FLIP was shown to bind to caspase 8 and prevent activation of the downstream events leading to apoptosis including TRAIL-mediated apoptosis [29,30].

The mechanisms responsible for 5-FU resistance in cancer cells are multifactorial. Most of the administered 5-FU is degraded through a catabolic pathway with DPD [31,32]. The efficacy of 5-FU is related to the plasma level of this agent, which is inversely related to the level of DPD activity [33,34]. Thus, high DPD activity is correlated with 5-FU resistance. 5-FU itself is inactive and requires its intracellular conversion to FdUMP. FdUMP exerts its cytotoxic activity through the formation of a ternary complex with TS and 5,10-methylene-tetrahydrofolate, resulting in the inhibition of TS and blockade of the DNA synthetic process [35,36]. Previous studies performed on several cancers have demonstrated that there is a close relationship between the overexpression of TS and the resistance to 5-FU [37,38]. Defective membrane transport, reduced activity of 5-FU-anabolising enzymes including OPRT and TP, low FdUMP level and reduced TS affinity to FdUMP have also been proposed as mechanisms for resistance to 5-FU.

To enhance TRAIL-mediated apoptosis, there are two major pathways. One is the suppression of anti-apoptotic molecules and the other is the upregulation of pro-apoptotic molecules. Bcl-2 and bcl-xL, which are the major inhibitors of the mitochondrial apoptotic pathway, are regulated by anticancer agents [39,40]. Paclitaxel reduces the activity of bcl-2 by inducing the phosphorylation of bcl-2 [26,41]. However, our data demonstrated that 5-FU had no effect on the expression of bcl-2. Bax is a pro-apoptotic molecule that counters the apoptotic repressor activity of bcl-2 [42]. A previous study and our data have both shown that 5-FU enhances the expression of bax [43]. Treatment with 5-FU also induces p53 expression. Since p53 upregulates bax expression [44,45], the enhanced bax expression might be due, in part, to the enhanced expression of p53 following 5-FU treatment.

Although the upregulation of bax expression by 5-FU is suggestive as a possible mechanism for the increase in TRAIL-mediated apoptosis, the precise mechanisms are not fully understood. Treatment of Caki-1 cells with 5-FU induced the expression of p53, which induces apoptosis. Genotoxic drugs and cisplatin enhance the expression of a pro-apoptotic TRAIL receptor, DR5 [46,47]. However, preliminary experiments demonstrate that 5-FU had no effect on the expression of TRAIL receptors on Caki-1 cells. Further studies are required to elucidate the effect of 5-FU on the various signalling molecules involved in the TRAIL apoptotic pathways.

The current data have shown that TRAIL enhances the intracellular accumulation of 5-FU and FdUMP partly by modification of the expression of enzymes involved in the metabolisms of 5-FU. However, the effect is modest. The mechanisms by which TRAIL enhances the 5-FU-mediated cytotoxicity are not clarified in this study and await further investigations.

TRAIL, as well as Fas ligand, may play an important role in T cell-mediated and natural killer cell-mediated cytotoxicity and apoptosis against cancer cells [48,49]. This study has demonstrated that treatment with TRAIL in combination with 5-FU resulted in a significant potentiation of cytotoxicity and apoptosis against RCC cells. In addition, preliminary experiments have demonstrated that treatment of freshly isolated RCC cells with 5-FU enhanced their susceptibility to lysis by autologous lymphocytes. These findings suggest that the enhanced TRAIL-mediated apoptosis following 5-FU treatment might be one of the mechanisms responsible for the enhanced susceptibility of 5-FU-treated RCC cells to cytotoxic lymphocytes, and that a combination of 5-FU and immunotherapy might be an alternative approach in the treatment of 5-FU/immunotherapy-resistant RCC.

The overall response rate of patients with RCC to current anticancer immunotherapeutic and chemotherapeutic agents including 5-FU has gradually improved. However, immunotherapy/chemotherapy-resistance and the recurrence of RCC remain major problems and more effective therapies are necessary for these patients. This study shows that combined treatment with TRAIL and 5-FU resulted in a synergistic cytotoxicity and apoptosis against both established and freshly separated RCC cells. Although *in vivo* studies are needed, these findings suggest that treatment with a combination of TRAIL and 5-FU might be useful in patients with immunotherapy/5-FU-resistant RCC as a new form of therapy with more selective cytotoxicity and less toxicity.

Acknowledgement

This work was supported in part by Grant-in-Aids from the Japanese Ministry of Education, Science and Culture (No. 12470336).

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