Partial Enhancement of Hyperthermia-induced Apoptosis by Increasing Enzymatic Activity of Thymidine Phosphorylase in Human Gastric Carcinoma Cell Lines

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ABSTRACT

We examined the effects of hyperthermia and/or 5'-deoxy-5-fluorouridine (5'-DFUR) on the induction of apoptosis of three human gastric cancer cell lines, MKN-1 (carrying mutated p53), TMK-1 (mutated) and MKN-74 (wild-type), by comparison with the expression of thymidine phosphorylase (dThdPase) which converts 5'-DFUR to 5-fluorouracil (5-FU). The expression level of dThdPase was higher in TMK-1, lower in MKN-74, and absent in MKN-1. The enzymatic activity was up-regulated by hyperthermia (44°C for 30 min) in the former two, but not in MKN-1. Hyperthermia or 5'-DFUR gradually induced apoptosis in MKN-74 and TMK-1, the value of the apoptotic index (AI: percentage of apoptotic cells) at 48 h was higher by treatment with hyperthermia than by 5'-DFUR. Sequential treatment with hyperthermia and 5'-DFUR markedly enhanced induction of apoptosis in both MKN-74 and TMK-1, with values of AI at 48 h being 31.4% and 54.8%, respectively. Flow cytometric analysis revealed increased sub G1 fraction in both MKN-74 and TMK-1, and cell arrest in G2/M in MKN-1, which was resistant to the apoptosis induced by the hyperthermia and/or 5'-DFUR. These results indicated that the sequential treatment with hyperthermia and 5'-DFUR enhanced the induction of apoptosis, which might partially be correlated with dThdPase expression.

Thymidine phosphorylase (dThdPase), a member of the pyrimidine nucleoside phosphorylase (PyNPase) family, catalyzes the reversible phospholysis of thymidine, and converts 5'-deoxy-5-fluorouridine (5'-DFUR) to 5-fluorouracil (5-FU) (1, 8, 22, 27). dThdPase is identical to platelet-derived endothelial cell growth factor (PD-ECGF), which has potent angiogenic activity *in vivo* and *in vitro* (4, 7, 33). It has been demonstrated that the expression level is higher in tumor tissue than in corresponding normal tissue in a variety of human tumors (17, 20, 23, 26, 28-

32). Higher levels of expression of dThdPase are well known to be correlated with poorer prognosis in patients with gastric, pancreatic and colonic carcinomas. For example, Maeda *et al.* examined 120 gastric carcinomas and found that higher dThdPase expression was significantly correlated with increases in intratumoral microvessel density and hepatic metastasis (17).

In turn, gastric carcinoma is one of the most frequent malignancies in Japan and other countries. Although the incidence is becoming lower, the disease is still responsible for a significant number of deaths *per annum* in Japan. Surgical treatment is undoubtedly the first choice for treatment of gastric cancer patients. Hyperthermia is well known to induce apoptosis in various cancer

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cells *in vivo* and *in vitro* (5, 10, 16). Hyperthermic peritoneal perfusion (HPP) combined with anticancer therapy has been also conducted in cases with advanced gastric carcinoma in an attempt to reduce the tumor size and improve prognosis. Kaibara *et al.* analyzed patients with gastric cancer showing subserosa infiltration, and reported 10-year survival rate in patients receiving HPP was better than that in the other patients without HPP, although this was not statistically significant (9, 11). Thus, the applicability of HPP seems to be limited for treatment of advanced gastric carcinoma.

Recently, we confirmed that human gastric carcinoma cell lines underwent apoptosis following short-term hyperthermia, in contrast to the necrosis induced by long-term hyperthermia (3). Hyperthermia-induced apoptosis of gastric cancer cells occurred in both p53 gene-dependent and -independent manners, accompanied by the expression of heat shock protein 70 (3). In this study, we examined the role of dThdPase in hyperthermia-induced apoptosis. Our results provided useful information regarding the relationship between hyperthermia and dThdPase expression or its enzymatic activity in the induction of apoptosis of human gastric carcinoma.

MATERIALS AND METHODS

Cell Lines

We examined three cell lines derived from human gastric cancer, MKN-1, MKN-74 and TMK-1 (6, 21, 24). MKN-1 was established from an adenosquamous cell carcinoma carrying a mutant p53 gene. MKN-74 is a well-differentiated tubular adenocarcinoma carrying the wild-type p53 gene. TMK-1 is a poorly differentiated adenocarcinoma carrying a mutant p53 gene. All the cell lines were cultured in RPMI 1640 (Cosmo Bio, Tokyo) containing 10% fetal bovine serum, 2 mM glutamine and 100 U/mL penicillin in a humidified 5% CO₂ incubator at 37°C. Cells were passaged twice weekly and routinely examined for *mycoplasma* contamination.

Western Blotting for Endogenous dThdPase

Before or after heat treatment at 44°C for 30 min, the cells were trypsinized, washed in phosphate-buffered saline (PBS) and solubilized in lysis buffer (50 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.1% NP-40, 5 mM NaF, 1 mM PMSF, 1 ng/mL

leupeptin, 10 ng/mL soybean trypsin inhibitor, 1 ng/mL aprotinin, 10 ng/mL n-tosyl-l-phenylalanyl chloromethyl ketone) for 60 min on ice. Lysates were centrifuged at 2,500 g for 5 min. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Lab., Richmond, CA) using bovine serum albumin as the standard. Aliquots of 30 µg of protein were resolved by electrophoresis on 12% polyacrylamide gels, electrotransferred to polyvinylidene difluoride filters (Millipore, Bedford, MA), and blotted with mouse monoclonal antibody to dThdPase (1:500; obtained from Nippon Roche Research Center, Kanagawa, Japan). Blots were developed with peroxidase-labeled anti-mouse IgG (1:1000; MBL Co., Nagoya, Japan) using enhanced chemiluminescence (ECL Detection System; Amersham, Bucks, UK).

dThdPase Enzyme Assay

Cultured cells were collected and sonicated in 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl₂ and 50 µM potassium phosphate. This solution was then centrifuged at 105,000 g for 90 min. The supernatant was dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.4) and 1 mM β-mercaptoethanol and was used as a source of crude dThdPase. The protein concentration was determined by the method of Lowry *et al.* (15). All procedures were performed at 4°C. The reaction mixture (120 µL) for the enzyme-activity assay contained 183 mM potassium phosphate (pH 7.4), 10 mM 5'-DFUR, and the crude enzyme from human cells. The reaction was performed at 37°C for 60 min and was then terminated by the addition of 360 µL methanol. After removal of the precipitate by centrifugation, an aliquot of the reaction mixture (100 µL) was added to 20 µM 5-chlorouracil as an internal standard and then applied to the high-performance liquid chromatography (HPLC) column (ERC-ODS-1171). The solvent system used was 50 mM sodium phosphate buffer (pH 6.8) containing 5 mM 1-decane sulfonic acid: methanol (85:15, v/v). The amount of 5-FU produced was measured with a UV monitor (280 nm).

Hyperthermia and 5'-DFUR Treatment

Stock 5'-DFUR, obtained from Nippon Roche

Research Center (Kanagawa, Japan), was dissolved in 50% dimethyl sulfoxide (DMSO) to a concentration of 10 mM and diluted with medium to the desired experimental concentrations.

Approximately 1×10^5 cells were seeded in 50 mL flasks and incubated for 24 h at 37°C. The medium was replaced with fresh medium heated to 44°C (Thermalrobo; iuchi Co., Japan) and the cells were maintained at 44°C for 30 min in a water bath. 5'-DFUR was added directly to the culture medium immediately after heat stress. Then, cells were transferred to a 37°C incubator.

Cell Viability and Apoptotic Index (AI)

After treatment, the cells were incubated at 37°C for 24, 48 and 72 h, then trypsinized and harvested. Cell viability was counted by trypan blue staining. Cultured cells were stained with 1 mg/mL Hoechst 33258 for 15 min and analyzed by fluorescence microscopy (EFD-3; Nikon, Tokyo, Japan) with excitation at 360 nm to detect apoptosis. AI was defined as $AI = \text{apoptotic cells} / 200 \text{ cells} \times 100$.

DNA Fragmentation

The treated cells (1×10^7) were trypsinized, washed twice with PBS, and suspended in lysis buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.5% SDS and 0.5 mg/mL proteinase K). After incubation overnight at 37°C, DNA was extracted with phenol/chloroform and precipitated with isopropanol according to standard protocols. Finally, after RNase treatment, DNA was dissolved in Tris-EDTA (TE) buffer. Aliquots of 10 μ g of DNA were subjected to electrophoresis on 2% agarose gels, incubated in 2 μ g/mL ethidium bromide and visualized under ultraviolet (UV) illumination.

Flow Cytometric Analysis

The treated cells were fixed with 70% ethanol for more than 4 h at 4°C. The ethanol was removed and cells were solubilized in citrate-phosphate buffer for 30 min at room temperature. Next, the cells were added to PBS and 10 mg/mL RNase (DNase free), and incubated for 30 min at 37°C. Finally, the cells were resuspended in PBS and 1 mg/mL propidium iodide (PI) for 30 min in the dark, then the nuclear DNA content was analyzed with a flow cytometer (FCS-1; Japan Spectros-

copic Co., Tokyo, Japan), and 1×10^4 cells were examined in each sample.

RESULTS

First, we analyzed the expression of dThdPase and its enzymatic activity. Western blotting analysis revealed a higher level of expression of dThdPase in TMK-1, and a lower level in MKN-74 (Fig. 1). No expression was detected in MKN-1 (Fig. 1). Enzymatic activity of endogenous dThdPase before and after heat treatment was 107.2 unit/mg and 130.4 unit/mg in TMK-1, and 0.6 unit/mg and 1.4 unit/mg in MKN-74, respectively (Table 1). Although the heat treatment enhanced enzymatic activity, there was no statistical significance between the value before and after treatment in the both TMK-1 and MKN-74. No enzymatic activity was observed in MKN-1.

Next, we examined the effects of 5'-DFUR treatment (10 mM), and/or hyperthermia (44°C for 30 min) on the induction of apoptosis in the three cell lines. Hoechst 33258 staining confirmed a gradual increase in number of apoptotic cells (Fig. 2A, B). DNA ladder formation was detected on agarose gels in TMK-1 and MKN-74 (Fig. 2C), but not MKN-1. At 48 h after treatment, AI was higher with hyperthermia than with 5'-DFUR treatment in MKN-1 and TMK-1, but not MKN-1 which did not undergo apoptosis (Fig. 3). The induction of apoptosis was markedly enhanced by hyperthermia followed by 5'-DFUR treatment in all the three lines (Fig. 3). AI with

Table 1 Enzymatic activity of endogeneous dThdPase^a

Cell lines	Heat treatment(unit/mg)	
	Before	After
MKN-1	N.D.	N.D.
MKN-74	0.6 $\pm$ 0.0	1.4 $\pm$ 0.2
TMK-1	107.2 $\pm$ 74.7	130.4 $\pm$ 90.7

^adThdPase enzyme assay is determined as described in materials and methods.

Fig. 1 Expression of endogenous dThdPase determined by Western blotting before (37°C; A) or after (44°C; B) treatment.

Fig. 2 Morphological analysis of untreated TMK-1 cells (A, $\times 400$) or at 48 h after combined treatment (B, $\times 400$). Cells were stained with Hoechst33258. Cells with fragmented nuclei corresponding to apoptotic cells are noted. (C) Detection of DNA ladder formation on agarose gel electrophoresis in untreated MKN-74 cells (Lane 2) or at 48 h after combined treatment (Lane 3). A 123-bp DNA ladder molecular weight standard was run in Lane 1.

the 5'-DFUR treatment, hyperthermia and sequential treatment with both was 3.9%, 13.7% and 54.8% in TMK-1, and 7.3%, 17.8% and 31.4% in MKN-74, and 1.1%, 2.5% and 4.1% in MKN-1. Thus, TMK-1 showed the strongest response among the three cell lines in the induction of apoptosis by sequential treatment with hyperthermia and 5'-DFUR.

Fig. 4 shows cell viability and AI after sequential treatment with hyperthermia and 5'-DFUR in the three cell lines. Cell viability gradually decreased reaching approximately 20% in TMK-1, 60% in MKN-74, and 70% in MKN-1. AI gradually increased in TMK-1, in which approximately

65% of the cells showed apoptosis at 72 h after treatment. AI became slightly lower at 72 h than at 48 h in MKN-74, indicating that a few

Fig. 3 Combined treatment, treatment of 5'-DFUR alone or hyperthermia alone-induced apoptotic indices at 48 h after treatment in human gastric cancer cell lines. Apoptotic index was determined as described in Materials and Methods. Data are average values of three independent experiments.

Fig. 4 Combined treatment-induced survival alteration (A) and -induced apoptotic indices (B) in human gastric cancer cell lines. Cells were treated at 44°C for 30 min, 1 mM 5'-DFUR was added and harvested after 24, 48 or 72 h. Alterations in survival and apoptotic index were determined as described in Materials and Methods. Data are average values of three independent experiments.

apoptotic cells with fragmented nuclei might have been completely destroyed. Sequential treatment was less effective in MKN-1, in which flow cytometric analysis demonstrated a decrease in G1 fraction and increases in S and G2/M fractions at 24 and 48 hours after treatment, followed by a decrease in the G2/M fraction (Fig. 5). On the other hand, the sub G1 fraction, corresponding to apoptotic cells, increased from 24 h after treatment in TMK-1 (Fig. 5).

DISCUSSION

We induced apoptosis with 5'-DFUR, hyperthermia and sequential treatment with both in three human gastric carcinoma cell lines. The susceptibility of these cell lines to the treatment was obviously variable. Apoptosis of TMK-1 and MKN-74 was confirmed not only by morphological analysis, but also by detection of DNA ladder formation on agarose gels. Following combination treatment, however, MKN-1 showed cell cycle arrest, but not obvious apoptosis. This might be partially explained by a

total absence of dThdPase expression in MKN-1, while the reason for the hyperthermia-resistance of the cells remains unclear.

Paradoxically, repeated experiments always revealed lower mean AI at 48 h than at 72 h with sequential treatment in MKN-74. This might have been due to complete destruction of the cells, resulting in nuclear dust which is readily diffusible and could not be counted as apoptotic cells by Hoechst staining. Therefore, it is feasible that mean AI might be higher than the value demonstrated in this study.

AI was rather higher in MKN-74 than in TMK-1 following treatment with 5'-DFUR or hyperthermia. Combination treatment, however, obviously enhanced apoptosis in TMK-1. This might partially be explained by the far higher expression of dThdPase and its enzymatic activity in TMK-1 than in MKN-74. In fact, heat treatment up-regulated dThdPase enzymatic activity in both cell lines. These findings suggested the clinical efficacy of hyperthermia as a therapeutic tool for at least some forms of gastric carcinoma. In other words, the effect of hyperthermia is not

Fig. 5 Flow cytometric analysis in MKN-1 and TMK-1 cells. After combined treatment, the cells were incubated at 37°C for 24, 48 or 72 h and harvested. Flow cytometric analysis was performed as described in Materials and Methods.

necessarily constant and not to be expected in all gastric carcinomas.

In contrast to the sequential treatment, 5'-DFUR induced apoptosis more effectively in MKN-74 than in TMK-1, despite the considerably lower expression levels of dThdPase and also lower enzymatic activity in the former. The differences in susceptibility to 5'-DFUR may have been due to the direct cytotoxic effect of 5'-DFUR and chemosensitivity of the cell lines, which might depend on genetic alterations, especially in p53 gene status. We found no previous reports concerning the direct effects of 5'-DFUR for cultured cancer cell lines. Although dThdPase converts 5'-DFUR to 5-FU which exerts cytotoxic effects, our study did not exclude the possibility that 5'-DFUR also acts as a cytotoxic agent.

There is now considerable evidence to suggest that chemosensitivity is affected by genetic alterations, especially p53 gene status (13, 14). In fact, cancer cells carrying mutated (mt) p53 are known to be more resistant to 5-FU than cells with wild-type (wt) p53. One of the roles of wt p53 is to sense DNA damage and either initiate repair or trigger the apoptotic pathway when there is a sufficient level of damage to the cells. We also demonstrated that 5-FU induced apoptosis in human gastric carcinoma cell lines, MKN-45 and MKN-74 carrying wild-type p53 gene, but not in MKN-28 carrying a mutated p53 gene or KATO-III lacking the gene. Thus, higher susceptibility to the combined treatment might be affected by p53 gene status. Enhancement of apoptosis in MKN-74 might occur predominantly via a p53 gene-dependent pathway.

Lenz *et al.* (12) reported that colorectal cancers with wt p53 had significantly lower levels of thymidylate synthase (TS), the only *de novo* source of thymidine in cells, compared to cancer with mt p53. They suggested a regulatory relationship between p53 and TS. In fact, it has been shown that TS protein binds to p53 mRNA, supporting a possible regulatory connection (2). This is comparable, in part, with the lower level of expression of dThdPase observed in MKN-74 carrying wt p53 than in TMK-1 carrying mt p53. The apoptosis-resistant nature of MKN-1, as described above, might be partially explained by mt p53. Recently, it was demonstrated that transcriptional activity of mt p53 was dependent on the site of the mutated codon (18, 25). Mutations have been confirmed at codons 143 and 147 in MKN-1, and 173 in TMK-1. This might reflect

the different responses to the treatment in the induction of apoptosis.

Miyadera *et al.* (19) recently found a potent inhibitor of dThdPase, TPI, which has been shown to suppress proliferation of the xenotransplanted TMK-1 into nude-mice. Valuable information on the mechanism of the hyperthermia-induced apoptosis might be obtained by analyzing the cell lines treated with TPI, which awaits further investigation.

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