Synergistic growth suppression induced in esophageal squamous cell carcinoma cells by combined treatment with docetaxel and heavy carbon-ion beam irradiation

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Abstract. Heavy carbon-ion beam therapy has revealed several potential advantages over X-rays. Heavy-ion therapy has been applied for various solid tumors including esophageal squamous cell carcinoma (SCC). Although the local control rate of carbon ion radiotherapy for esophageal cancer has revealed better rates than that of conventional radiotherapy, some patients have shown resistance to the treatment. No study has evaluated whether anti-cancer drugs can enhance the anti-tumor effect of heavy carbon-ion beam irradiation. Therefore, we evaluated the efficacy of docetaxel, fluorouracil, cisplatinum, doxorubicin and gemcitabine to enhance the anti-tumor effects of heavy carbon-ion beam irradiation on human esophageal SCC cells in both *in vitro* and *in vivo* experiments. Fluorouracil, cisplatinum, doxorubicin and gemcitabine showed only additive anti-tumor effects. On the other hand, growth suppression was significantly potentiated by the combined treatment with heavy carbon-ion beam and docetaxel as compared to that treated with either agent alone. These data suggest that heavy carbon-ion beam irradiation combined with docetaxel may be a potentially useful therapeutic strategy for locally advanced esophageal SCC.

Introduction

Esophageal cancer is a highly malignant disease, in which progression of disease is observed in most patients at initial presentation (1). Neoadjuvant cytoreduction treatments are frequently used for the purpose of tumor downstaging, increasing the resection rate, and possibly improving survival (2). Although the combined treatment of radiation and anti-cancer drugs is a widely used therapeutic strategy, no satisfactory treatment regimen has yet been devised due to severe side effects and the acquisition of resistance (3-5). A new effective combined treatment strategy is a necessity for improving the therapeutic effects.

The biological effects of high linear energy transfer (LET) radiotherapy with heavy-ion are more pronounced than low-LET radiation, such as X-rays or gamma rays (6). High LET radiations are suitable for local control of tumors because of its high relative biological effectiveness, and reduced oxygen enhancement ratio. Another advantage of heavy carbon-ion beam irradiation is its superior dose distribution (7). The sharp Bragg peak of the heavy carbon-ion beam enables the localization of energy to the target volume. The chief biological effects of heavy carbon-ion beam irradiation are thought to be mediated by DNA damage. The extent of cell death after charged-particle irradiation may depend on irreparable DNA double-strand breaks (8,9). Therefore, phase I/II studies of heavy carbon-ion beam irradiation have been undertaken for the treatment of esophageal squamous cell carcinoma (SCC) and have produced encouraging results (10,11). Although the local control rate of heavy-ion radiation was better than X-rays alone, it was not better than chemoradiation therapy. Therefore, a suitable treatment strategy is needed to enhance anti-tumor effects of heavy-ion radiation.

Several studies have indicated that synergistic suppressive effects have been obtained with a combination treatment of anti-cancer drugs with X-ray radiation on various cancer cells including those of head and neck, lung, and colorectal cancers (12-14). However, there are no studies regarding the combination treatment of heavy carbon radiotherapy and anti-cancer drugs. Cisplatinum, fluorouracil and docetaxel are key anti-cancer drugs for treatment of esophageal carcinoma. Thus, the purpose of this study was to investigate the effects of these drugs in combination treatment with heavy carbon-ion beam irradiation against human esophageal SCC cells *in vitro* and *in vivo*.

Materials and methods

Human esophageal squamous cell carcinoma cell line (TE-2) and culture. The human esophageal squamous cell carcinoma cell line (TE-2) was obtained from the Japan Cell Research

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Key words: heavy carbon-ion beam, docetaxel, esophageal cancer, squamous cell carcinoma

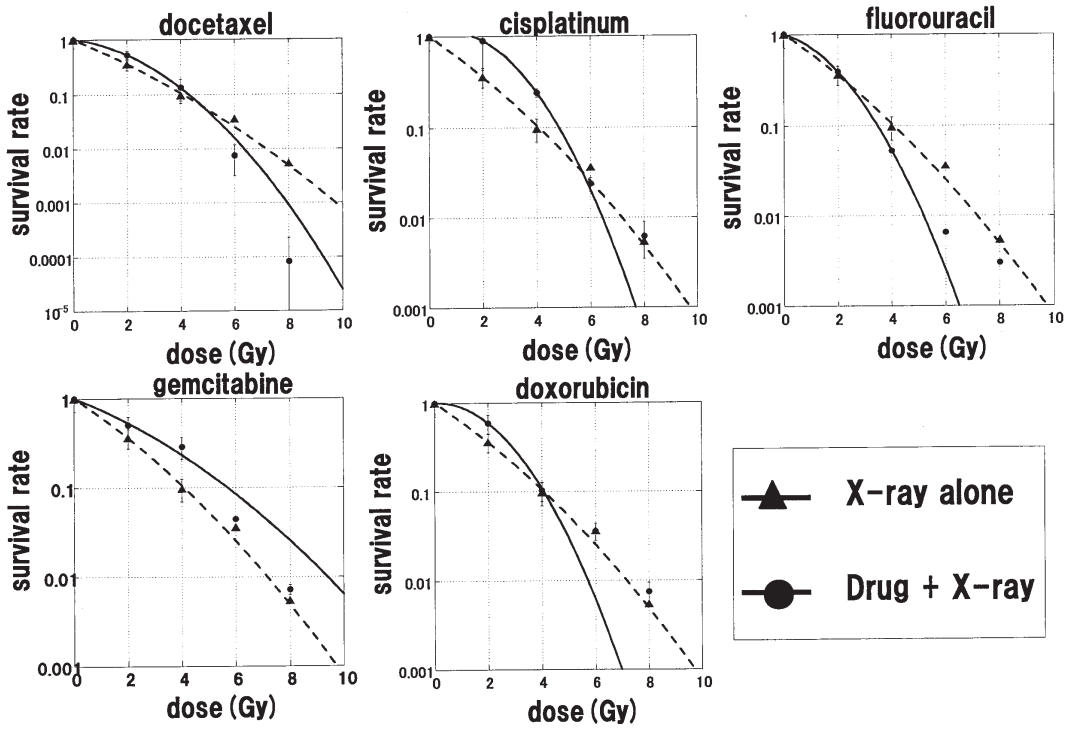

Figure 1. *In vitro* growth suppression of human esophageal squamous cell carcinoma cells irradiated with X-ray combined with a 48-h drug exposure (docetaxel, cisplatinum, fluorouracil, gemcitabine, doxorubicin). The survival curves here, and in Figures 2-4 were normalized by dividing by the surviving fraction treated with drug only.

Bank (15). This cell line was established from a poorly differentiated human esophageal squamous cell carcinoma containing the wild-type p53 gene. All tumor cell lines were maintained as monolayer cultures in Dulbecco's modified Eagle medium (DMEM)/F-12 (DMEM:F-12=1:1) (Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum, 0.1% L-glutamine, and 0.05% penicillin-streptomycin. The media and sera were purchased from Sigma Chemical Co. (St. Louis, MO).

Anti-cancer drugs. Docetaxel was purchased from Aventis Pharma Ltd. (Tokyo). Cisplatinum was purchased from Nipponkayaku Ltd. (Tokyo). Fluorouracil and doxorubicin were purchased from Kyouwahakkou Ltd. (Tokyo). Gemcitabine was purchased from Japan Eli Lilly Ltd. (Kobe). Cells were maintained in 25-cm^2 plastic flasks and were incubated in a 5% CO_2 incubator at 37˚C. Cells were treated with various concentrations of each drug for 48 h. Median effective dose (IC_{50}) of 48-h exposure for each drug was investigated by the colony forming assay (16). In brief, after drug exposure, the cells were removed from the culture by trypsinization in 0.05% trypsin in a 1 mM ethylenediamine tetraacetic acid (EDTA) solution. Cells (200) were plated onto 60-mm-diameter plastic dishes for colony formation assay. Colonies were fixed and stained with a 0.2% crystal violet solution in 20% methanol after a 14-day incubation. Colonies with >50 cells were counted as a survival colony. The survival fractions were calculated as the ratio of survival colonies per number of plated cells.

X-ray and heavy carbon-ion beam irradiation. X-ray radiation was given before, after, or in the midst of administration of drug at a dose rate of 1.04 Gy/min *in vitro* experiments. X-ray irradiation was carried out at room temperature with a Shimadzu (Tokyo) generator operated under 200 kVp and 20 mA, with 0.5 mm Al and 0.5 mm Cu filters.

The cells and tumors were irradiated with carbon-ions that were accelerated ≤290 MeV/n by the HIMAC (Heavy-ion Medical Accelerator in Chiba) synchrotron at the National Institute of Radiological Science. The irradiation system and biophysical characteristics of heavy carbon-ion beam irradiation have been described (17,18). Briefly, the initial energy of the heavy carbon-ion beam was 290 MeV/n. We used high-LET beams obtained by using polymethyl methacrylate absorbers with various thickness to change energy of the beam (19). The LET values in all *in vitro* experiments were calculated to be 70 keV/μm. Heavy carbon-ion beam radiation was given before or after exposure to each drug. The LET values in all *in vivo* experiments at tumor sites were calculated to be 50 keV/μm. Heavy carbon-ion beam irradiation, carried out at room temperature, was given 24 h before or after administration of the drug. The dose rate of the beam was ~3 Gy/min.

In vitro experiments. Cells were maintained in 25-cm^2 plastic flasks and were incubated in a 5% CO_2 incubator at 37˚C. Cells were treated with drug for 48 h. Because the timing of exposure to anti-cancer drugs is critical in establishing a clinical protocol, irradiation was carried out before, after, or during drug exposure. After irradiation or drug exposure, the cells were removed from the culture by trypsinization in 0.05% trypsin in a 1 mM EDTA solution. Cells (200) were plated onto 60-mm-diameter plastic dishes as controls in the colony

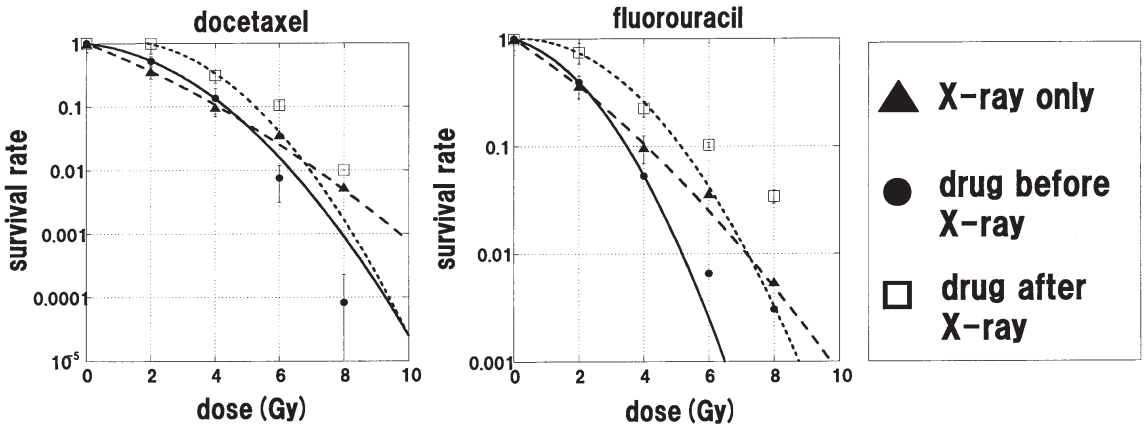

Figure 2. *In vitro* growth suppression of human esophageal squamous cell carcinoma cells irradiated with X-ray combined with a 48-h docetaxel and fluorouracil exposure (before or after irradiation).

formation assay. For single treatment cells, 400-30,000 cells were plated. For combination treatment, $1x10^3$ to $6x10^4$ cells were plated. Colonies were fixed and stained with a 0.2% crystal violet solution in 20% methanol after 14 days of incubation. Colonies with >50 cells were counted as a survival colony. The survival fractions were calculated as the ratio of survival colonies per number of plated cells. All experiments were carried out in triplicate (19). Cell survival rates were compared to the number of survival colonies without irradiation.

Cell cycle analysis. Cells were treated with various concentrations of docetaxel for 12, 24, and 48 h. Single cell suspensions were stained with propidium iodide for cell cycle analysis (20). Flow cytometry was performed on a FACS Calibur (Becton Dickinson, San Jose, CA). G0/G1 doublet discrimination was done by measuring the pulse width relative to the pulse area. A gate was set around single cells in the dot plots showing pulse width relative to pulse area. Each analysis included at least 20,000 events. Modfit (Verity Software House, Topsham, ME) was used for cell cycle analysis.

Evaluation of effects on tumor growth. Balb/c nu/nu athymic male mice (6-8 weeks old, Clea, Japan) were used. The animal experiments were performed at the Laboratory Animal Center of the National Institute of Radiological Science. The guidelines from the National Institute of Radiological Sciences Safety and Health Regulations for Handling Experimental Animals (2001) were followed. Cells ($5x10^6$) in 0.2 ml were injected subcutaneously into nude mice. Two weeks after inoculation (indicating as day 0) in the right thigh, the tumor size reached ~250-400 mm^3. The animals were randomized on day 0 of treatment to control and treated groups (5 mice each/group) to adjust mean tumor volume of each group. Docetaxel was injected with a 27-gauge hypodermic needle on day 1. The tumor-bearing mice received intraperitoneal injections of docetaxel at a dose of 20 mg/kg. This dose of docetaxel is roughly equivalent to the clinically used human dose of 70 mg/m^2. On day 2, 24 h after injection, the tumor was irradiated with a single dose of 5 Gy. Mice were anesthetized by intraperitoneal injections of pentabarbiturate prior to irradiation. The tumor size was measured with calipers 3 times a week over a period of 5 weeks. The tumor volume was calculated according to the (length x width^2)/2 and presented as the mean (± standard deviation) mm^3.

Results

IC_{50} of anti-cancer drugs for TE-2 cell line. IC_{50} values of the TE-2 cells were determined by the colony forming assay against each of the five anti-cancer drugs. The median effective dose (IC_{50}) was: docetaxel (70 nM), cisplatinum (670 nM), fluorouracil (4600 nM), gemcitabine (2.5 nM), and doxorubicin (26 nM).

In vitro cytotoxic effects of drugs and X-ray irradiation. The *in vitro* survival curves of TE-2 cells after a 48-h exposure to each of the five drugs followed by X-ray irradiation of 0-8 Gy are shown in Fig. 1. The survival curves were normalized by dividing by the surviving fraction treated only with drug. Docetaxel, fluorouracil and doxorubicin showed a synergistic growth suppression effect on cell survival at irradiation doses of 6 and 8 Gy. On the other hand, this synergistic effect was not seen with cisplatinum and gemcitabine.

With respect to the time when the cells were exposed to the drug, cell survival curves were compared for docetaxel and fluorouracil exposure before and after irradiation (Fig. 2). Synergistic growth suppression was shown with docetaxel exposure both before and after irradiation. For fluorouracil, however, this effect was observed only with cells treated before irradiation.

In vitro cytotoxic effects of drugs and heavy carbon-ion beam irradiation. The *in vitro* survival curves of TE-2 cells treated for 48 h with each of the five drugs followed by heavy carbon-ion beam irradiation of 0-5 Gy are shown in Fig. 3. The survival curves are normalized by dividing by the surviving fraction treated only with drug. Docetaxel plus irradiation showed a synergistic effect on cell survival at irradiation doses of 4 and 5 Gy. The other four drugs plus irradiation showed only additive effects to radiation alone.

To study the timing of docetaxel exposure, TE-2 cell survival curves were compared between those exposed to

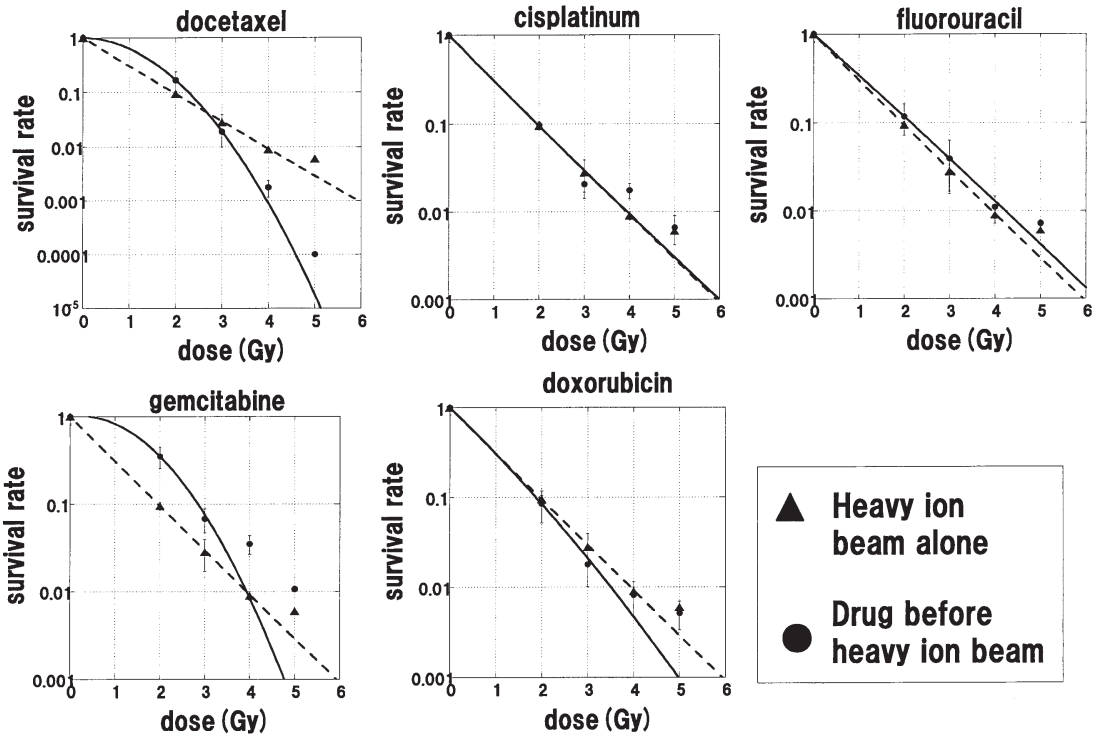

Figure 3. *In vitro* growth suppression of human esophageal squamous cell carcinoma cells irradiated with heavy carbon-ion beam combined with a 48-h drug exposure (docetaxel, cisplatinum, fluorouracil, gemcitabine, doxorubicin).

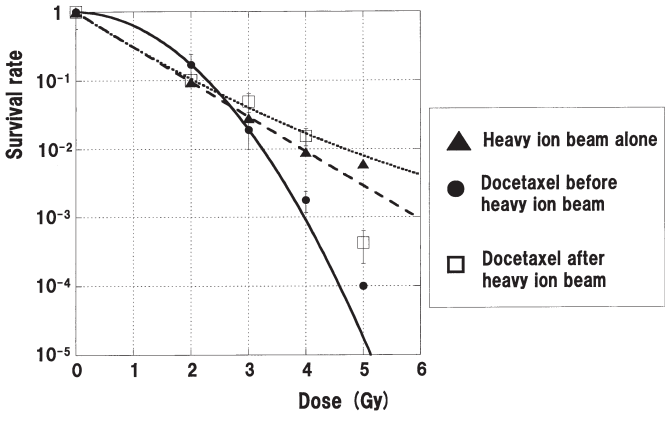

Figure 4. *In vitro* growth suppression of human esophageal squamous cell carcinoma cells irradiated with heavy carbon-ion beam combined with a 48-h docetaxel exposure (before or after irradiation).

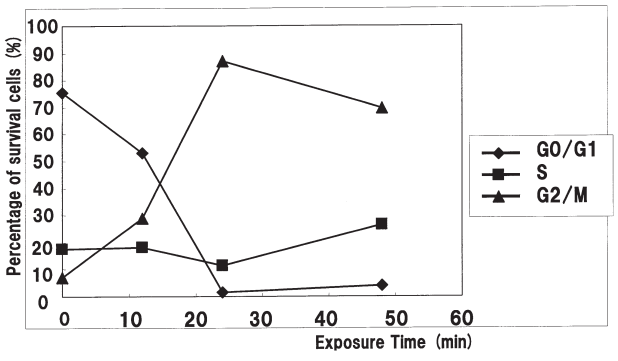

Figure 5. Alterations in the percentage of cells in G0/G1 phase (◆), S phase (■), G2/M phase (▲) after docetaxel treatment.

docetaxel before and after heavy carbon-ion beam irradiation (Fig. 4). Docetaxel exposure after irradiation showed only an additive effect, whereas docetaxel exposure before irradiation showed a synergistic suppressive effect on cell survival at irradiation doses of 4 and 5 Gy.

Cell cycle analysis. Fig. 5 shows that the number of cells in G2/M phase significantly increased at 12 h after docetaxel exposure. The percentage of cells in G2/M phase ranged from 87% at 24 h to 70% at 48 h after docetaxel exposure. Increasing exposure time resulted in a simultaneous increase in a G2/M.

In vivo combination treatment with heavy carbon-ion beam irradiation and docetaxel administration. Athymic nude mice were inoculated with TE-2 cells and were randomly divided into 4 groups as follows: i) control group, ii) docetaxel only injection group, iii) heavy carbon-ion beam irradiation only group, iv) docetaxel injection before heavy carbon-ion beam irradiation group. Tumor growth suppression was observed in all of the three treated groups. However, statistically significant tumor growth suppression was demonstrated only for the combined docetaxel plus heavy carbon-ion beam radiation group (see asterisks in Fig. 6).

Discussion

Although heavy carbon-ion beam irradiation has been shown to obtain good local responses for esophageal SCC with minor

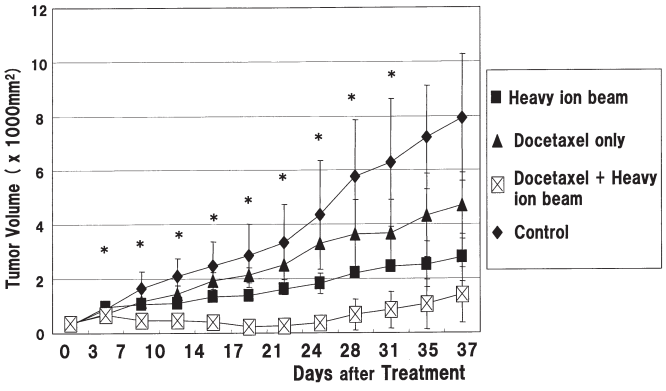

Figure 6. The effect of heavy carbon-ion beam 5 Gy irradiation combined with docetaxel (33 mg/kg i.p.) on *in vivo* tumor growth of esophageal squamous cell carcinoma. Values represent the mean and standard deviation of 5 mice. P-values were determined by the Mann Whitney U test.

bone marrow suppression, damage to the adjacent normal mucosa has been observed because of the cytotoxicity of high LET (21). Since a number of patients have shown resistance to treatment, combining treatment with drugs may be in order to enhance the anti-tumor effects of heavy carbon-ion beam irradiation. Combination treatment may also make it possible to reduce the total irradiation dose in order to minimize cytotoxicity to adjacent organs. In the present study, the administration of anti-cancer drugs combined with heavy carbon-ion beam irradiation efficiently enhanced the anti-tumor effect on human esophageal SCC both *in vitro* and *in vivo* experiments with nude mice.

Because heavy carbon-ion beam irradiation has a significantly higher LET beam than X-ray, cells irradiated by heavy carbon-ion beam showed less variation in cell-cycle-related radio sensitivity and decreased repair of radiation-induced DNA injury (22). Our results showed that docetaxel had a synergistic effect with heavy carbon-ion beam irradiation on esophageal SCC. This suggested that sensitivity to heavy carbon-ion beam irradiation was also partly dependent on cell-cycle accumulation in the G2/M phase. Several authors have suggested that the mechanism of radio sensitization by taxanes is the accumulation of cells in G2/M phase, the most radio-sensitive phase of the cell cycle (23-25). Docetaxel plus heavy carbon-ion beam irradiation given at the peak of the G2/M phase block induced a synergistic effect. This was clearly shown by the cell survival curves which were significantly reduced from the radiation only group.

In vivo experiments with nude mice showed again that docetaxel had a synergistic growth suppression effect when combined with heavy carbon-ion beam on esophageal SCC. However, Suzuki *et al* reported that docetaxel plus X-ray had only an additive effect in an *in vivo* model using SCCIIV, a skin squamous cell carcinoma (26). This difference in the radio sensitizing effect of docetaxel on squamous cell carcinoma may be attributed to differences in sensitivity to docetaxel between TE-2 and SCCIIV. In other words, the different results may be attributed to differences in the accumulation rates of G2/M phase cells after exposure to docetaxel. TE-2 cells (>80%) were accumulated in the G2/M phase aftera 24-h exposure to docetaxel. This ratio was relatively higher than that of SCCIIV. Several authors reported that cisplatinum plus X-ray had a synergistic effect in a human hepatoma cell line (27). Only a slight additive effect for cisplatinum was found in our experiments because the *in vitro* survival curves were similar between the combination group and the radiation alone group. These differences may also be attributed to differences in the cell lines.

Because the mechanism of radio sensitization by docetaxel was suggested in various studies to be due to the accumulation of cells in the G2/M phase, it was necessary for the exposure of cells to docetaxel to be given before radiation treatment (23-25). This was confirmed by the results reported herein. In this study, five major anti-cancer drugs were selected that are usually used for treatment of adenocarcinoma and squamous cell carcinoma of esophagus in the clinical setting. In *in vitro* experiments, drugs were used at the median effective dose (IC_{50}) because it was suspected that extremely high or low doses of drugs were not appropriate to evaluate additive or synergistic effects in combination treatment with radiation. In *in vivo* experiments, docetaxel was administered to mice by intraperitoneal (i.p.) injections. Some authors reported that plasma concentrations of docetaxel after intravenous (i.v.) injections were higher than after i.p. injections (28). Others reported that there were no significant differences in the plasma levels of docetaxel between the i.v. and i.p. groups (29,30). Therefore, both methods were considered appropriate for the preclinical model.

Heavy carbon-ion beam in itself is an effective therapeutic modality for esophageal SCC. In this study, it has been shown that docetaxel treatment synergistically enhanced anti-tumor effects of heavy carbon-ion beam irradiation on a human esophageal SCC cell line. Although the most severe adverse effect of docetaxel is bone marrow suppression (31), heavy carbon-ion beam irradiation may be appropriate for combined treatment because it has shown less toxicity for bone marrow than X-ray irradiation (11).

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