Effective chemoradiotherapy protocol with 5-fluorouracil for cervical squamous cell carcinoma *in vitro*

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Summary

Purpose of investigation: 5-Fluorouracil (5FU) is frequently used in concurrent chemoradiotherapy for patients with advanced cervical cancer, although its optimal chemoradiotherapy protocol has not yet been established. In search of an optimal chemoradiotherapy protocol, some *in vitro* experiments were carried out.

Methods: The radiosensitive human cervical squamous cell carcinoma cell line ME180 was examined to investigate the effects of 5FU on radiosensitivity and the effects of irradiation on 5FU-sensitivity.

Results: 5FU dose-dependently enhanced cellular radiosensitivity at therapeutic concentrations. Although high doses of γ-ray irradiation significantly reduced the 5FU-sensitivity, a low dose of irradiation at therapeutic doses (< 2.5 Gy) had no effect on 5FU-sensitivity of the irradiated cells. Cells pretreated with 5FU eight hours before irradiation showed significantly higher 5FU-sensitivity than cells concurrently treated with 5FU and irradiation. In contrast, cells treated with 5FU eight hours after irradiation showed significantly lower 5FU-sensitivity than cells concurrently treated with 5FU and irradiation. Moreover, all four post-irradiation surviving subclones obtained from repeatedly irradiated ME180 cells showed significantly lower 5FU-sensitivity than the non-irradiated parent cells.

Conclusion: 5FU acts as a radiosensitizer for cervical squamous cell carcinoma and 5FU-sensitivity is reduced in irradiated cells. Therefore, 5FU administration immediately before irradiation may be a more effective treatment than concurrent chemoradiotherapy or post-irradiation chemotherapy with 5FU.

Key words: 5-fluorouracil; Chemoradiotherapy; Cervical cancer; Squamous cell carcinoma; Radiosensitivity; Radiosensitizer.

Introduction

Radiotherapy is the most commonly used therapy for patients with locally advanced cervical squamous cell carcinoma (SCC), since cervical SCC cells are usually highly radiosensitive. However, tolerable irradiation doses for humans are limited and large cancer tissue masses cannot be completely killed by standard radiotherapy. Moreover, radiotherapy is never effective for lesions outside the irradiated areas. Therefore, in order to kill distant cancer cells outside the irradiated fields, enhance the radiosensitivity of the cancer cells during radiotherapy, and kill the surviving cancer cells after non-radical radiotherapy, concurrent chemoradiotherapy has sometimes been applied to locally advanced cervical cancer patients. Regarding chemoradiotherapy for advanced cervical cancer patients, concurrent chemoradiotherapy with 5-fluorouracil (5FU) and/or cisplatin has frequently been applied to patients and is reported to show better survival ratios than treatment with radiotherapy alone [1-3]. However, few investigations into the effective use of anticancer drugs with radiotherapy have been reported. A recent study reported that cervical cancer patients pretreated with bleomycin, vincristine, mitomycin and cisplatin (BOMP) chemotherapy prior to radiotherapy demonstrated a lower survival ratio than patients treated with radiotherapy alone [4]. These results led to the hypothesis that BOMP chemotherapy before radiotherapy reduces the radiosensitivity of cervical cancer cells.

5FU is one of the frequently used anticancer drugs in concurrent chemoradiotherapy, especially for patients with cervical SCC and head and neck SCC [1-3, 5]. Recently, oral administration of 5FU-derivatives was found to produce a better survival ratio in cervical cancer patients [6]. In the present study, we investigated the optimal combination treatment and mechanisms of chemoradiotherapy with 5FU using a radiosensitive cervical squamous cell carcinoma cell line.

Materials and Methods

Cell line and cell culture

The human cervical SCC cell line ME180 [7], which is radiosensitive [8], was obtained from the Japan Collection of Research Bioresources (JCRB) Cell Bank (Tokyo, Japan), and cultured in OPTI-MEM (GIBCO-BRL, Gaithersburg, MD, USA) containing 5% fetal calf serum (FCS; EQUITECH BIO Inc., Ingram, TX, USA), 100 U/ml penicillin (GIBCO-BRL) and 100 μg/ml streptomycin (GIBCO-BRL). 5FU was a kind gift from Kyowa-Hakko Co. Ltd (Tokyo, Japan).

Cell viability assays

Cell proliferation was assayed using a non-RI colorimetric assay kit (XTT; Boehringer-Mannheim, Mannheim, Germany). The growth-inhibitory effects of radiation and 5FU on the cells

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were assayed as follows. Cells in the log phase were detached with a mixture of 0.25% trypsin and 1 mM EDTA (GIBCO-BRL), and then cultured overnight in 96-well culture plates (5,000 cells/well). On day 2, the cells were irradiated with various doses of γ-rays using an irradiator (MBR 1520A; Hitachi-Medico, Tokyo, Japan). On day 4, the viable cells were counted using the XTT kit. In separate experiments, cells were treated with various concentrations of 5FU in order to examine the modulatory effects of 5FU on the cell death induced by irradiation. The cells were then irradiated with various doses of γ-rays, followed by a 2-day culture. Finally, the relative viable cell numbers (%) were calculated with the aid of the XTT kit. All the experiments were performed two or three times to verify the results. Data are shown as the mean ± SD and comparative data (n = 6) were analyzed by ANOVA.

Establishment of surviving subclones after repeated irradiations

Post-irradiation surviving subclones were established as follows. ME180 parent cells were cultured in five 96-well culture plates (10,000 cells/well) and irradiated with γ-rays (10 Gy) once a week for four weeks. In a preliminary experiment, more than 90% of the ME180 cells were killed after a single γ-ray irradiation at 10 Gy. Surviving cells were collected from each of four wells with viable cancer cell colonies and re-cultured at a lower cell density (0.1-20 cells/well) in a limiting dilution study. The cloning efficiencies of the limiting dilution cultures were < 10% (3.7-9.1%). Finally, four months after the first irradiation, four monoclonal subclones (Clones 1-4) that survived the irradiations were established.

Results

Initially, the effects of 5FU on the radiosensitivity of ME180 cells were examined. As shown in Figure 1, 5FU dose-dependently enhanced the cellular radiosensitivity of ME180 cells, and this was especially apparent in the cells irradiated with a low dose of γ-rays (2.5 Gy).

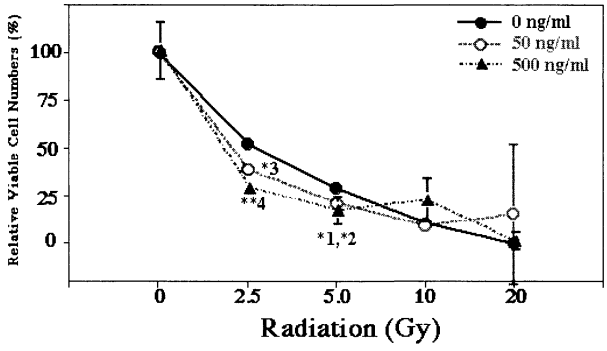

Figure 1. — Effect of 5FU on the radiosensitivity of ME180 cells.
Following the addition of a small amount of 5FU, ME180 cells were irradiated with various doses of γ-rays within 20 minutes. The final 5FU concentrations in the culture media were 0, 50 and 500 ng/ml. The solid line with closed circles shows the control radiosensitivity curve of cells cultured without 5FU. The dotted lines with open circles and closed triangles show cells treated with 5FU. 5FU dose-dependently enhanced the radiosensitivity of the cells. At each dose, the cell viabilities were compared between the cells with and without 5FU treatment. *1-*3: $p < 0.05$; **4: $p < 0.01$.

Next, the effects of irradiation on the cellular 5FU-sensitivity were investigated. As shown in Figure 2, irradiation significantly reduced the 5FU-sensitivity in a dose-dependent manner. However, when the cells were irradiated with a low dose of γ-rays (2.5 Gy), there was no difference between the 5FU-sensitivity of the non-irradiated and irradiated cells.

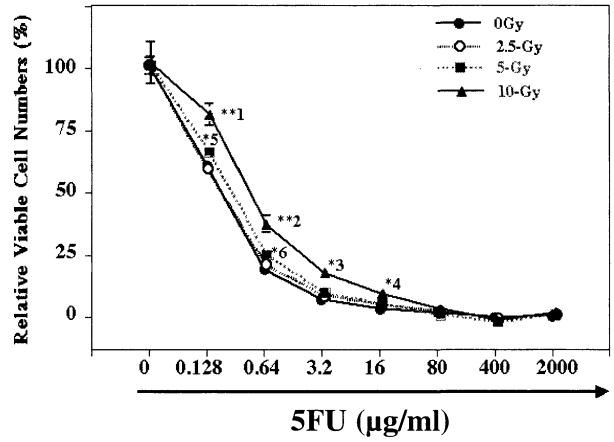

Figure 2. — Effects of irradiation on the 5FU-sensitivity of ME180 cells.
Following the addition of various concentrations of 5FU, ME180 cells were irradiated with various doses of γ-rays within 20 minutes. The solid line with closed circles shows the control 5FU-sensitivity curve of cells cultured without irradiation. A low dose of γ-rays (2.5-Gy) had no effect on the 5FU sensitivity curve (dotted line with open circles) of ME180 cells, while high doses of γ-rays (5-Gy and 10-Gy) significantly reduced the 5FU-sensitivity (dotted line with closed squares and solid line with closed triangles, respectively). The cell viabilities (%) were compared between the cells with and without irradiation. *3-*6: $p < 0.05$; **1-**2: $p < 0.01$.

Next, to determine whether the 5FU-sensitivity of the cells can be altered before or after irradiation, the 5FU-sensitivities were compared among three groups of irradiated cells. The 5FU-sensitivity of cells treated with 5FU eight hours before irradiation was significantly higher than that of cells concurrently treated with 5FU and irradiation (Figure 3A). In contrast, the 5FU-sensitivity of cells treated with 5FU eight hours after irradiation was significantly lower than that of cells concurrently treated with 5FU and irradiation (Figure 3B).

Finally, in order to determine whether post-irradiated cancer cells maintained a higher 5FU-sensitivity following irradiation, we established four post-irradiation surviving subclones and examined their 5FU-sensitivities. All four subclones demonstrated significantly lower 5FU-sensitivities than the parent ME180 cells (Figure 4), suggesting that post-irradiation cellular responses may reduce the 5FU-sensitivity.

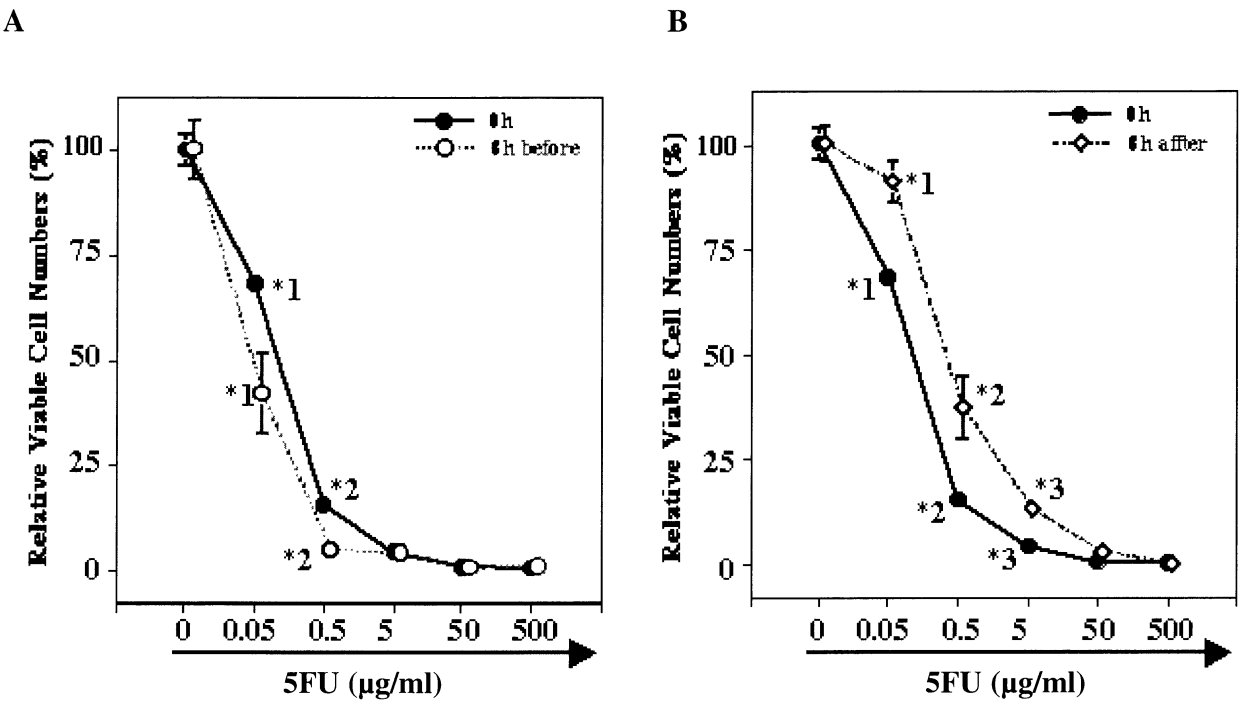

Figure 3. — Differential effects of 5FU-treatment before and after irradiation on the 5FU-sensitivity of ME180 cells.
A. The solid line with closed circles shows the control 5FU-sensitivity curve of cells irradiated with a single dose of γ-rays (2.5 Gy) immediately after 5FU addition to cells. The dotted line with open circles shows the 5FU-sensitivity curve of cells irradiated eight hours after 5FU treatment. The 5FU-sensitivity of cells treated with 5FU eight hours before irradiation was significantly higher than that of cells concurrently treated with 5FU and irradiation. *1, *2: $p < 0.05$.
B. The solid line with closed circles shows the control 5FU-sensitivity curve of cells irradiated with a single dose of γ-rays (2.5 Gy) immediately after 5FU addition to cells. The dotted line with open circles shows the 5FU-sensitivity curve of cells irradiated eight hours before 5FU treatment. The 5FU-sensitivity of cells treated with 5FU eight hours after irradiation was significantly lower than that of cells concurrently treated with 5FU and irradiation. *1, *2, *3: $p < 0.05$.

Discussion

Standard radiotherapy of cervical cancers is frequently non-radical for locally advanced cancers with huge primary tumors, wide invasion to the pelvic walls and lymph nodes, or possible distant micrometastases. Therefore, concurrent chemoradiotherapy is sometimes applied to patients with unresectable locally advanced cervical cancer, although the most effective combination of anticancer drugs and radiation has not yet been investigated in detail. Continuous drip-infusions of 5FU have been widely used in standard chemotherapy and chemoradiotherapy protocols for advanced cervical cancer patients. However, when and how 5FU should be administered to cancer patients for maximum effectiveness during chemoradiotherapy has not yet been clarified. The present study using radiosensitive human cervical SCC cells has clarified optimal conditions for combined chemoradiotherapy with 5FU.

5FU dose-dependently enhanced the radiosensitivity of cancer cells at therapeutic concentrations, suggesting that it acts as a radiosensitizer. Although high doses of irradiation reduced the cellular 5FU-sensitivity in a dose-dependent manner, a low dose of irradiation had no effect on the 5FU-sensitivity. Therefore, therapeutic doses of irradiation, which are usually < 2.5 Gy, may not reduce the 5FU-sensitivity of cancer cells. Moreover, cancer cells that survive after irradiation may have a lower 5FU-sensitivity. The 5FU-sensitivity of cells treated with 5FU eight hours before irradiation was significantly higher than that of cells concurrently treated with 5FU and irradiation. In contrast, cells treated with 5FU eight hours after irradiation showed a significantly lower 5FU-sensitivity than cells concurrently treated with 5FU and irradiation. From these results, we suggest that 5FU should be administered to patients with cervical cancer before irradiation. Tabata *et al.* [4] reported that BOMP chemotherapy before radiotherapy for cervical cancer patients produced a significantly lower survival ratio than treatment with radiotherapy alone, suggesting that the radiosensitivity of cancer cells could be reduced by neoadjuvant BOMP chemotherapy. The report indicates that chemoradiotherapy for cervical cancers may be a less effective therapy than radiotherapy alone if the chemotherapeutic drugs are inadequately administered. The results of the present study suggest that pretreatment with 5FU enhances the radiosensitivity of cancer cells accompanied by increased 5FU-sensitivity. Moreover, it is highly possible that post-irradiation cellular responses

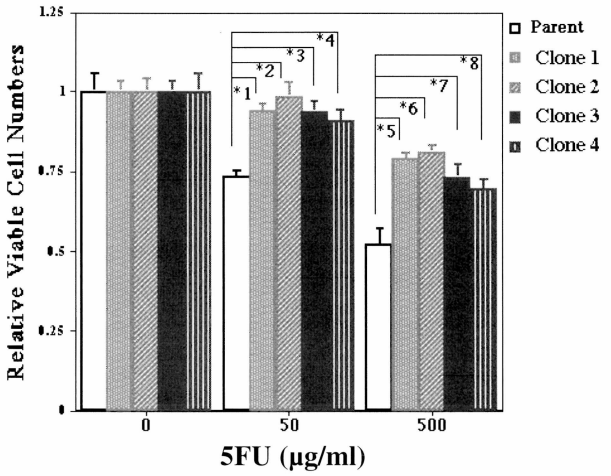

Figure 4. — 5FU-sensitivities of post-irradiation surviving subclones.
The 5FU-sensitivities of four subclones (Clones 1-4) established from cells that survived irradiation were compared with the 5FU-sensitivity of non-irradiated ME180 cells. The mean viable cell numbers without 5FU-treatment were set as 1 (100%). The relative viable cell numbers of cells treated with 50 or 500 ng/ml of 5FU were compared between the parent cells and the post-irradiation surviving subclones. All four post-irradiation surviving subclones (dotted, closed or lined bars) showed significantly lower sensitivities to 5FU than the parent cells (open bars). *1-*8: $p < 0.05$.

reduce 5FU-sensitivity. Therefore, 5FU administration immediately before irradiation may be a better treatment than concurrent chemoradiotherapy or post-irradiation chemotherapy with 5FU.

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