# Effects of mitomycin C on radiation-induced cell death in human cervical squamous cell carcinomas

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**Summary**

In order to find an effective protocol for chemoradiotherapy with mitomycin C (MMC) for advanced cervical cancer patients, effects of both MMC and irradiation on chemoradiosensitivity were examined using the radiosensitive human cervical squamous cell carcinoma cell line ME180. MMC and low doses of irradiation did not affect radiosensitivity of the cells. A high dose of γ-ray irradiation (10 Gy) significantly reduced MMC sensitivity of the cells. All of the four post-irradiation surviving subclones that were established from repetitively irradiated ME180 cells, demonstrated significantly higher MMC sensitivity than that of the non-irradiated parent cells. However, there was no difference in MMC sensitivity among three groups of irradiated cells; (1) cells treated with MMC eight hours before irradiation, (2) cells concurrently treated with MMC and irradiation and (3) cells treated with MMC eight hours after irradiation. These results indicate that MMC injections after completion of radiotherapy may be a better therapy than concurrent chemoradiotherapy with MMC.

*Key words:* Mitomycin C; Chemoradiotherapy; Cervical cancer.

## Introduction

The most common therapy for patients with locally advanced cervical squamous cell carcinoma (SCC) is radiotherapy, because cervical SCC cells usually have a high radiosensitivity. Tolerable irradiation doses for humans are limited and large cancer tissue masses cannot be completely killed by standard radiotherapy. Moreover, radiotherapy is never effective on the lesions outside of the irradiated areas. Therefore, in order to kill distant cancer cells outside of the irradiated fields, to enhance the radiosensitivity of the cancer cells during radiotherapy, and to kill the surviving cancer cells after non-radical radiotherapy, concurrent chemoradiotherapy has sometimes been applied to locally advanced cervical cancer patients.

With regard to chemoradiotherapy for advanced cervical cancer patients, concurrent chemoradiotherapy with cisplatin (CDDP), bleomycin (BLM), 5-fluorouracil (5-Fu) or mitomycin C (MMC) have been frequently applied to patients and are reported to show better survival ratios in treated patients than in patients treated with radiotherapy alone [1-8]. However, few investigations of the effective use of anticancer drugs with radiotherapy have been reported. A recent report has shown that cervical cancer patients pretreated with combined chemotherapy including BLM, vincristine, MMC and CDDP (BOMP) before radiotherapy demonstrated a lower survival ratio than patients treated with radiotherapy alone [9]. These results led to the hypothesis that BOMP chemotherapy before radiotherapy reduces radiosensitivity of the cervical cancer cells.

MMC is one of the anticancer drugs that has been frequently used in concurrent chemoradiotherapy, especially for patients with cervical SCC and head and neck SCC [6-7, 9-15]. In the present study, we investigated the optimal combination treatment and mechanisms of chemoradiotherapy with MMC using a radiosensitive cervical squamous cell carcinoma cell line.

## Materials and Methods

### *Cell line and cell culture*

Human cervical SCC cell line ME180 [16], which has wild-type p53 genes and is radiosensitive, was used in the study. The ME180 cell line was obtained from the Japan Resources of Cell Bank (JRCB, Tokyo, Japan). All the cells used in this study were cultured in OPTI-MEM (GIBCO-BRL, Gaithersburg, MD, USA)/5% fetal calf serum (FCS) (EQUITECH BIO Inc., Ingram, TX, USA)/penicillin (100 U/ml) (GIBCO-BRL)/streptomycin (100 μ/ml) (GIBCO-BRL). The MMC used in this study was a gift from Kyowa-Hakko Co. Ltd (Tokyo, Japan).

### *Cell viability assay*

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

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*Establishment of the surviving subclones from repetitive irradiation*

The subclones were established as follows. ME180 parent cells were cultured in five 96-well culture plates (10,000 cells/well) and 10-Gy of γ-ray was irradiated once a week to the plates four times, which were cultured for approximately four weeks. In a preliminary experiment, more than 90% of ME180 cells were killed after a single irradiation of 10-Gy γ-ray. Cells were collected from each of the four wells with viable cancer cell colonies and re-cultured with a lower cell density (0.1-20 cells/well) by a limiting dilution study. Cloning efficiencies of the limiting dilution cultures were under 10% (3.7%-9.1%). Finally, four months after the first irradiation, four monoclonal subclones that survived post-irradiation were established.

## Results

First, the effect of MMC on the radiosensitivity of ME180 cells was examined. MMC did not have any significant effect on radiosensitivity curves of the ME180 cells (Figure 1). Secondly, the effect of irradiation on MMC sensitivity of ME180 cells was examined. A low dose of irradiation (2.5 Gy and 5 Gy) did not have any effect on MMC sensitivity of the cells, although a higher dose (10 Gy) of irradiation significantly reduced MMC sensitivity of the cells (Figure 2). Thirdly, in order to evaluate MMC sensitivity in the post-irradiation surviving cancer cells, we established four subclones that survived post-irradiation according to the methods described above and examined their MMC sensitivity. All the established four subclones demonstrated significantly higher MMC sensitivity than the parent ME180 cells (Figure 3), suggesting the possibility that MMC sensitivity of the cells could be enhanced after irradiation. Finally, to determine if MMC sensitivity of the cells may be enhanced immediately after irradiation, the MMC sensitivity was compared among the three groups of irradiated cells.

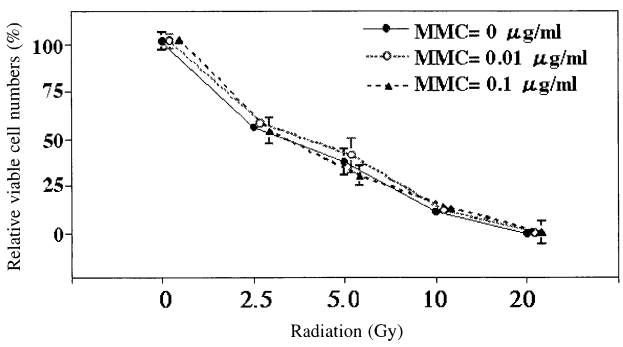


Figure 1. — *Effect of MMC on radiosensitivity of ME180 cells.* A small amount of MMC solution was added to ME180 cells, which were then irradiated with various doses of γ-ray within 20 minutes. Final MMC concentrations in the culture media were 0, 0.01, and 0.1 μg/ml. The solid line with closed circles shows the control radiosensitivity curves of the cells cultured without MMC. The dotted lines with open circles or closed triangles show cells treated with MMC. There was no significant change in the three radiosensitivity curves.

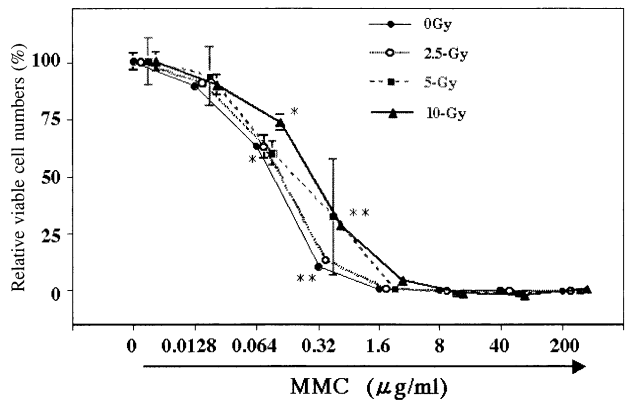


Figure 2. — *Effects of irradiation on MMC sensitivity of ME180 cells.*
After various concentrations of MMC were added to the ME180 cells, they were irradiated within 20 minutes with various doses of γ-ray. The solid line with closed circles shows the control MMC sensitivity curve of cells cultured without irradiation. Low doses of γ-ray irradiation did not affect any MMC sensitivity curves (dotted lines) of the ME180 cells, while 10-Gy of γ-ray irradiation significantly reduced the MMC sensitivity (solid line with closed triangles) (*$p < 0.05$, **$p < 0.05$).

There was no difference in MMC sensitivity between the cells treated with MMC eight hours before irradiation and the cells concurrently treated with MMC and irradiation (Figure 4a). There was also no difference in MMC sensitivity between the cells treated with MMC eight hours after irradiation and the cells concurrently treated with MMC and irradiation (Figure 4b).

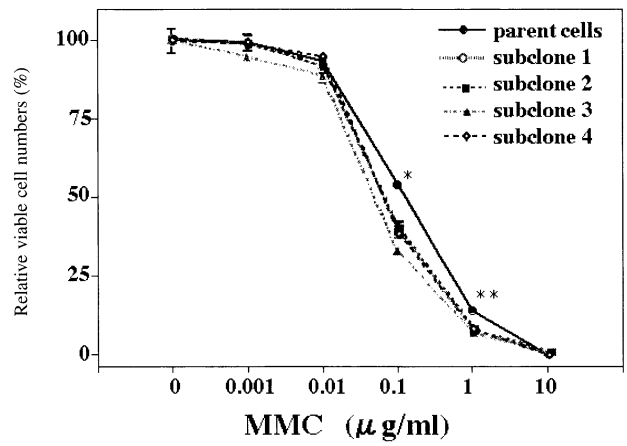


Figure 3. — *MMC sensitivities of post-irradiation surviving cells.* MMC sensitivities of the four subclones established from cells that survived irradiation were compared with the MMC sensitivity of non-irradiated ME180 cells. The solid line with closed circles shows the control MMC sensitivity curve of the ME180 parent cells. All of the four subclones that survived post-irradiation (dotted lines) demonstrated a significantly higher sensitivity to MMC than that of the parent cells (*$p < 0.05$, **$p < 0.05$).

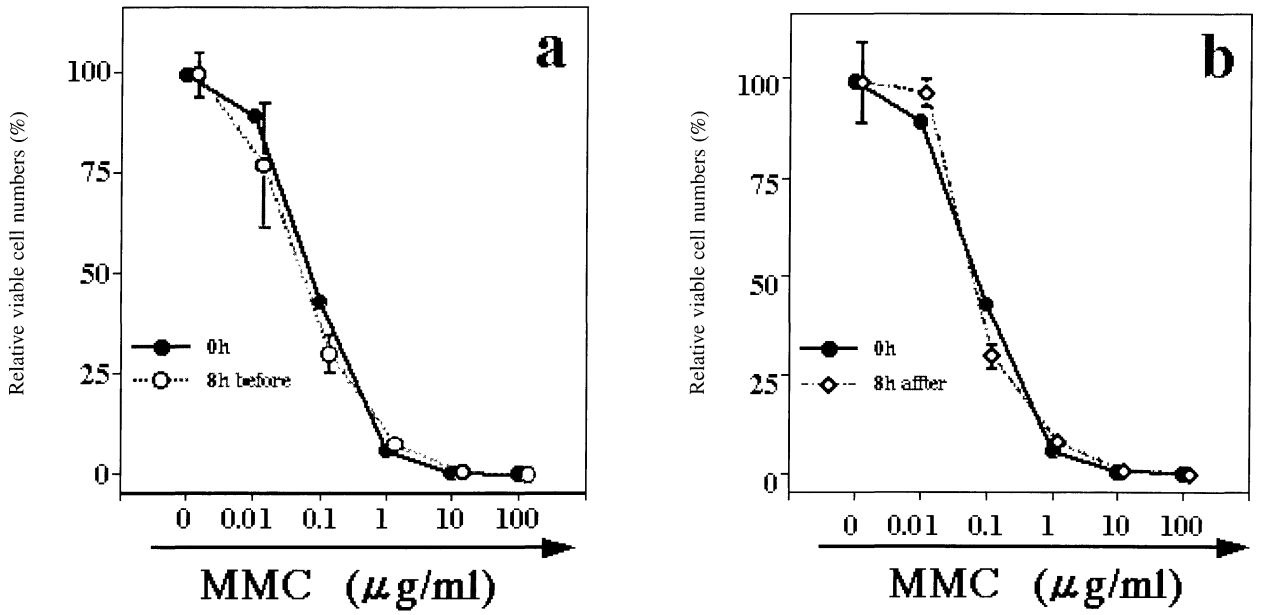


Figure 4. — *Effects of stimulation between MMC treatment and γ-ray irradiation on MMC sensitivity of ME180 cells.*
a) The solid line with closed circles shows the control MMC sensitivity curve of the cells that were irradiated with a single dose of 2.5 Gy immediately after MMC was added to cells. The dotted line with open circles shows the MMC sensitivity curve of the cells irradiated 8 hours after MMC treatment. No significant effect of irradiation 8 hours after MMC treatment on MMC sensitivity of the cells was found.
b) The solid line with closed circles shows the control MMC sensitivity curve of the cells that were irradiated with a single dose of 2.5 Gy immediately after MMC was added to cells. The dotted line with open circles shows the MMC sensitivity curve of the cells irradiated 8 hours before MMC treatment. No significant effect of irradiation 8 hours before MMC treatment on MMC sensitivity of the cells was found.

## Discussion

Standard radiotherapy of cervical cancers is frequently non-radical for locally advanced cervix cancers with huge primary tumors, wide invasions to pelvic walls and lymph nodes, or possible distant micrometastases. Therefore, concurrent chemoradiotherapy has sometimes been applied to patients with unresectable locally advanced cervical cancer, although an effective combination with anticancer drugs and radiation has not been investigated in detail. MMC injections have been widely used in standard chemotherapy and chemoradiotherapy for advanced cervical cancer patients [6, 7, 17]. However, when and how MMC should be effectively injected into the cancer patients during radiotherapy has not been clarified yet. The present study using radiosensitive human cervical SCC cells has shown optimal conditions in the case of combined chemoradiotherapy with MMC. Although MMC does not affect any radiosensitivity of the cancer cells, a high dose of irradiation may reduce MMC sensitivity. Moreover, cancer cells that survived after irradiation may have a higher MMC sensitivity, even four months after the first irradiation. However, a post-irradiation increase in MMC sensitivity could not be found within eight hours after irradiation. These results suggest that MMC should be administered to patients with cervical cancer more than eight hours after irradiation. Tabata et al. reported that BOMP chemotherapy with MMC before radiotherapy for cervix cancer patients demonstrated a significantly lower survival ratio than that of patients with radiotherapy alone [9], suggesting that radiosensitivity of the cancer cells could be reduced by the neoadjuvant BOMP chemotherapy. These results caution that chemoradiotherapy for cervix cancers may be a worse therapy than radiotherapy alone in cases of inadequate administration of the chemotherapeutic drugs. In conclusion, MMC injections after completion of radiotherapy may be a better therapy than the concurrent chemoradiotherapy with MMC. Moreover, the present investigative procedure could be applied to investigate an improved combination with radiotherapy and another anticancer drug for cervical cancer patients.

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