Methylxanthine sensitization of human colon cancer cells to 186Re-labeled monoclonal antibody

Methylxanthine Sensitization of Human Colon Cancer Cells to ^{186}Re-Labeled Monoclonal Antibody

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Tumor cells lacking the functional p53 suppressor gene may arrest at the G2 phase of the cell cycle after exposure to ionizing radiation, resulting in increased radioresistance. Methylxanthines (MTXs), such as pentoxifylline (PTX) or caffeine (CAF), can inhibit the G2-phase checkpoint arrest of damaged cells and thus radiosensitize them. However, the effect of MTX in cells irradiated with low-dose-rate β-emission is not well understood. **Methods:** A clonogenic assay was performed with LS180 human colon cancer cells lacking the functional p53 suppressor gene. Cells were irradiated with increasing concentrations of ^{186}Re-mercaptoacetyltriglycine (^{186}Re-MAG3)-labeled A7 monoclonal antibody against colorectal cancer (0–925 kBq/mL) at 37°C in 5% CO_2 for 24 h in the presence or absence of PTX (0–2 mmol/L) or CAF (0–5 mmol/L). The enhancement ratio (ER) with MTX was calculated as a ratio of 50% cell-killing concentration of ^{186}Re-MAG3-A7 in control cells to that in cells treated with PTX or CAF. The cell cycle distribution was analyzed with a flow cytometer. **Results:** The concentration of 50% cell kill was 474 kBq/mL ^{186}Re-MAG3-A7. Both PTX and CAF dose dependently enhanced the cytotoxicity of ^{186}Re-MAG3-A7: ERs of 0.5 mmol/L PTX, 2 mmol/L PTX, 1 mmol/L CAF, and 5 mmol/L CAF were 1.50, 2.18, 1.54, and 2.63, respectively. Flow cytometry showed that the percentage nonirradiated cells in the G2/M phase of the cell cycle was 11.3% ± 1.66%. On the other hand, cells exposed to ^{186}Re-MAG3-A7 accumulated in the G2/M phase of the cell cycle (40.2% ± 1.46%), which was inhibited by the presence of 1 mmol/L PTX (19.8% ± 8.12%) or 2 mmol/L CAF (26.9% ± 6.21%). **Conclusion:** Cellular modulation of the cell cycle with PTX and CAF radiosensitized LS180 colon cancer cells exposed to ^{186}Re radiation.

Key Words: radiosensitization; methylxanthine; ^{186}Re; β-irradiation; cell cycle

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Internal radiation therapy (IRT) using tumor-specific radiopharmaceuticals, such as radiolabeled monoclonal antibodies (mAbs), ^{131}I-metaiodobenzylguanidine, and ^{90}Y-antisomatostatin receptor ligands, has been examined for the management of patients with malignant tumors. The response rate of patients with refractory malignant lymphoma to radioimmunotherapy is high (*1*,*2*). However, results in other types of tumors are less favorable, and therefore the sensitization of tumor cells to radiation with radionuclides should be beneficial in enhancing the efficacy of IRT.

Cells with DNA damage induced by high-dose-rate radiation are known to arrest at the G1 or G2 phase of the cell cycle and repair the damage, resulting in augmentation of their radioresistance (*3*). G1-phase arrest is mediated by the p53 suppressor gene function (*4*,*5*), and the G2-phase arrest is also affected by the p53 status to some extent (*6*,*7*). However, G2-phase arrest can occur in the absence of functional p53, as observed in many types of cancer cells (*8*). Substantial evidence indicates that inhibition of the G2 arrest with methylxanthines (MTXs), such as pentoxifylline (PTX) and caffeine (CAF), increases p53-independent apoptosis after radiation (*9–15*). Inhibition of the G2 checkpoint arrest also increases the death of cells with defective apoptotic pathways through the induction of giant cells, which would result from multiple rounds of DNA synthesis in the absence of mitosis (*9*,*10*).

Low-dose-rate radiation may also induce the checkpoint arrest of irradiated cells (*16*,*17*). However, most knowledge about the radiosensitization effect of MTX is concerned with high-dose-rate external-beam radiation, and little is known about the effect of low-dose-rate radiation with radionuclides emitting β-particles. Anderson et al. (*16*) showed the radiosensitization effect of CAF in Chinese hamster ovary cells irradiated with ^{131}I, and Macklis et al. (*17*) showed it in EL-4 lymphoma cells exposed to ^{90}Y. However, Macklis et al. indicated that Raji lymphoma cells were not sensitized by CAF and that the radiosensitization effect of CAF was not related to the cell cycle. Therefore,

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the cell cycle effect in cells exposed to low-dose-rate radiation seems specific to the cell line. It is generally accepted that the resistance of colorectal carcinomas to ionizing radiation is associated with p53 mutation, and the inhibition of G2 arrest with CAF reportedly increased the death of colorectal cancer cells exposed to high-dose-rate γ-radiation (*10*). The reported response rate of colon carcinomas to radioimmunotherapy was not high (*18*); therefore, the effect of MTX on colon cancer cells exposed to β-radiation is worth investigating.

^{186}Re has abundant intermediate-energy β-emission (71% of 1.07 MeV and 21% of 0.94 MeV). Its γ-emission of 137 keV (9%) is suitable for external detection with gamma cameras and produces a less nonspecific radiation dose than does the high-energy γ-emission of ^{131}I (364 keV), which is the most widely used nuclide in IRT. In this study, we examined the effect of PTX and CAF on the survival of human colon cancer cells lacking functional p53 protein after exposure to a ^{186}Re-labeled anti-colorectal cancer mAb in vitro.

MATERIALS AND METHODS

Cell Culture and Radiolabeling

LS180 human colon cancer cells lacking functional p53 protein (American Type Culture Collection, Rockville, MD) were maintained as a monolayer in a medium (Dulbecco's modified Eagle medium; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere, harvested with 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid, and inoculated into six-well culture plates. ^{186}Re-perrhenate ($^{186}ReO^{4-}$) was produced by the ^{185}Re(n,γ) reaction (Japan Atomic Energy Research Institute, Tokaimura, Japan), chelated with *S*-benzoylmercaptoacetyltriglycine (*S*-benzoyl-MAG3) (a gift from Dr. Yasushi Arano, Kyoto University), and conjugated to A7 (an IgG1 anti-colorectal carcinoma antibody) (*19*). Briefly, the mixture of $^{186}ReO^{4-}$, $SnCl_2$, and *S*-benzoyl-MAG3 at the mol/L ratios of 2.3:1 for *S*-benzoyl-MAG3:Re and 8.0:1 for Sn^{2+}:Re was heated under a N_2 stream, resulting in ^{186}Re-MAG3, which was conjugated to A7 after esterification with 2,3,5,6-tetrafluorophenol (TFP) (Nacalai Tesque, Kyoto, Japan). ^{186}Re-MAG3-A7 was purified with a PD-10 column (Pharmacia LKB Biotechnology, Uppsala, Sweden), and its immunoreactivity was assessed using LS180 cells with an antigen excess.

Clonogenic Assay

LS180 cells were incubated in six-well culture plates with 0–925 kBq/mL ^{186}Re-A7 in 2 mL medium for 24 h. PTX (Sigma, St. Louis, MO) and CAF (Sigma) were added simultaneously to the wells at concentrations of 0–5 mmol/L and 0–2 mmol/L, respectively. The cells were washed with phosphate-buffered saline (PBS) twice and maintained in fresh medium for 10 d. Cell survival was determined by counting colonies fixed with 80% methanol and stained with crystal violet. Colonies containing ≥50 cells were considered as survivors. The surviving fraction was corrected by plating efficiency at each concentration of PTX and CAF. A triplicate assay was performed in all experiments. The data were fit to an exponential function to obtain a radioactivity concentration, producing a surviving fraction of 0.5 (i.e., 50% cell kill). An enhancement ratio (ER) at each concentration of PTX or CAF was calculated as the ratio of 50% killing concentration in control cells to that in cells treated with PTX or CAF.

Cell Cycle Analysis

The distribution of cells in the phases of the cell cycle was analyzed with a flow cytometer (Cytoron Absolute; Ortho Diagnostic Systems, Raritan, NJ). Briefly, control cells and cells irradiated with 463 kBq/mL ^{186}Re-MAG3-A7 in the presence or absence of 1 mmol/L PTX or 2 mmol/L CAF were suspended at 2×10^6 cells/250 μL PBS, fixed with 250 μL 100% ethanol, and stored at 4°C. The cells were washed and resuspended with 250 μL ribonuclease A (500 U/mL; Sigma). They were then stained with 250 μL propidium iodide (50 μg/mL; Sigma). The DNA content was analyzed with a flow cytometer, and the cellular distribution in the cell cycle was determined. The results were analyzed by an unpaired *t* test. In the analyses, the level of significance was set at 5%.

RESULTS

The incorporation of ^{186}Re into *S*-benzoyl-MAG3 was 98%. The radiochemical yield of ^{186}Re-MAG3-TFP production was 89%, and 50% of ^{186}Re-MAG3-TFP was conjugated to A7 mAb. The specific activity of purified ^{186}Re-MAG3-A7 was 90 MBq/mg, and its immunoreactivity was 63%.

Figure 1 and Table 1 summarize the effect of PTX and CAF on the survival of LS180 colon cancer cells irradiated with ^{186}Re-MAG3-A7. The data of surviving curves were well fit to an exponential function, showing $R^2 > 0.96$ for all assay conditions. The concentration of 50% cell kill was

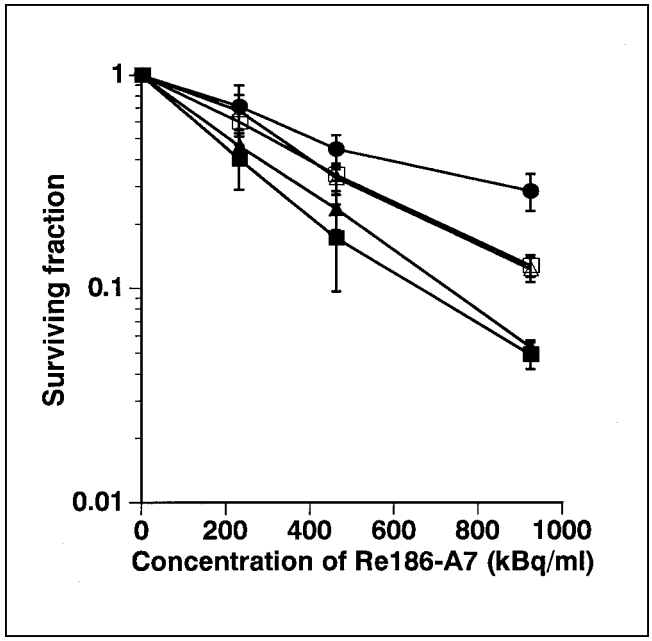

FIGURE 1. Surviving fraction of LS180 colon cancer cells exposed to ^{186}Re-MAG3-A7 anti-colorectal mAb in presence of PTX or CAF. ●, Control cells; △, PTX (0.5 mmol/L); ▲, PTX (2 mmol/L); □, CAF (1 mmol/L); ■, CAF (5 mmol/L). Values are mean ± SD of triplicate assays.

TABLE 1
Radiosensitization Effect of MTXs

Treatment	50% kill dose* (kBq/mL)	ER†
Control group‡	474	—
PTX, 0.5 mmol/L	316	1.50
PTX, 2 mmol/L	217	2.18
CAF, 1 mmol/L	307	1.54
CAF, 5 mmol/L	180	2.64

*Concentration of radiocompound producing survival fraction of 0.5.
†Expressed as 50% kill dose of control/50% kill dose with PTX or CAF.
‡Irradiated without PTX or CAF.

474 kBq/mL, and both PTX and CAF dose dependently enhanced the cytotoxicity of ^{186}Re-MAG3-A7.

Flow cytometry revealed that the cells exposed to ^{186}Re-MAG3-A7 accumulated in the G2/M phase of the cell cycle; 40.2% of the irradiated cells were found in the G2/M phase compared with 11.3% of the control cells ($P < 0.0001$) (Fig. 2). The presence of PTX or CAF inhibited the G2/M-phase accumulation of irradiated cells ($P < 0.01$).

DISCUSSION

Once DNA damage is induced by ionizing radiation or cytotoxic drugs, some of the damaged cells die through the apoptotic pathway. Others survive by repairing the damage during transient arrest at cell cycle checkpoints in the G1 or G2 phase. G1-phase arrest is mediated by the p53 suppressor gene function (*4*,*5*), and many tumor cells fail to arrest at the G1 phase because of lack of functional p53. However, G2-phase arrest can occur in the absence of functional p53, as observed in many types of cells (*8*), and a correlation between G2-phase arrest and radioresistance has been shown (*20*,*21*). The inhibition of the G2 arrest with MTXs, such as PTX and CAF, increases p53-independent apoptosis after radiation (*9*–*15*), and the mechanism underlying the inhibitory effect of CAF on the G2-phase arrest was recently shown to be the inhibition of the Chk1 inhibitory function on the phosphatase Cdc25c (*22*).

Although the positive effect of MTX in cells exposed to high-dose-rate external-beam radiation has been well documented, only a few reports on low-dose-rate β-irradiation are available, and the effect of cell cycle modulation on β-irradiation is not fully understood. In this study, we presented evidence of the radiosensitizing effect of PTX and CAF in colon cancer cells exposed to ^{186}Re radiation and found a possible correlation between radiosensitization

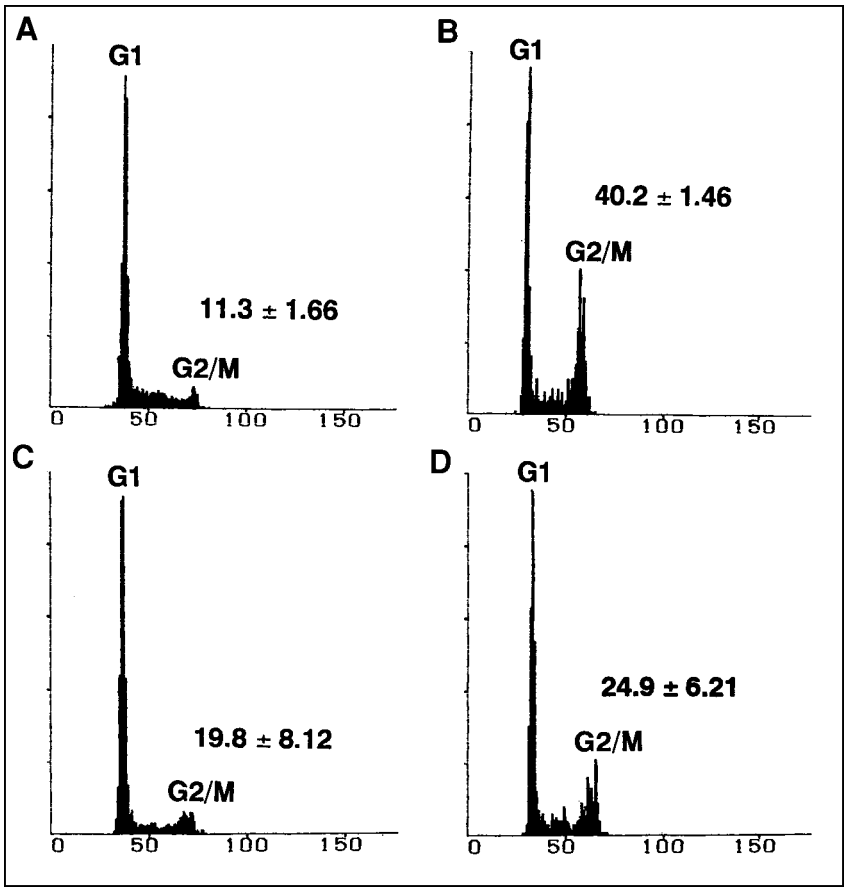

FIGURE 2. Representative histograms of flow cytometry of LS180 colon cancer cells. (A) Nonirradiated cells. (B) Cells irradiated with ^{186}Re-MAG3-A7 (463 kBq/mL for 24 h). (C) Cells irradiated in presence of PTX (1 mmol/L). (D) Cells irradiated in presence of CAF (2 mmol/L). Mean ± SD ($n = 3$) of percentage cells in G2/M phase of cell cycle is indicated.

and inhibition of the G2-phase arrest of irradiated cells. Anderson et al. (*16*) similarly reported a radiosensitization effect of CAF in Chinese hamster ovary cells irradiated with ^{131}I.

In contrast to these findings, the effectiveness of low-dose-rate irradiation is usually explained by the G2-phase accumulation of cells, because cells are radiosensitive in the G2 phase (*23*). Ning and Knox (*24*) recently reported the higher survival rate of CAF-treated HL60 leukemia cells exposed to low-dose-rate γ-radiation with a ^{137}Cs source. They concluded that the CAF-induced inhibition of the G2 arrest decreased the proportion of cells in the radiosensitive G2 phase, resulting in a higher survival rate of CAF-treated cells. On the other hand, the findings of Macklis et al. (*17*) are inconsistent with this hypothesis. They found that EL-4 lymphoma cells, showing less G2 accumulation than Raji lymphoma cells, were much more sensitive to low-dose-rate β-radiation of ^{90}Y than Raji cells, whereas these two cell lines showed equal sensitivity to high-dose-rate γ-radiation. Deweese et al. (*25*) indicated that cell cycle modulation was not a dominant determinant in five prostate cancer cell lines exposed to low-dose-rate γ-radiation. These inconsistent reports suggest that the effect of cell cycle modulation on the cytotoxicity of low-dose-rate radiation is likely specific to the cell line (*26*).

Radiation-induced apoptosis plays an important role in the responses of cells to irradiation, and MTX increases the p53-independent apoptosis of cells after irradiation (*9*,*10*). The expression level of Bcl-2, an anti-apoptotic oncogene, may therefore correlate with the induction of apoptosis by MTX (*22*). Other genes, such as p21, bax, RAF1, and p34, may also be involved (*27–31*). Investigation with many cell lines will be required to achieve a better understanding of the effect of MTX in cells exposed to low-dose-rate radiation with radiopharmaceuticals.

A limitation for the in vivo use of MTX is the difficulty in achieving the concentration required for radiosensitization through cell cycle modulation when administered systemically (*16*). To obtain a sufficiently high concentration, Tsuchiya et al. (*32*,*33*) intra-arterially infused CAF in patients with bone and soft-tissue sarcomas at a dose of 1.5 g/m^2 for 3 consecutive days in combination with chemotherapeutic agents. They found a much better response rate with this protocol than with conventional chemotherapy, suggesting the possible applicability of MTX to enhance the IRT effect. In addition, for the treatment of patients with intraperitoneal dissemination, we may administer these agents intraperitoneally, and it may be possible to obtain the lesional concentrations required for the radiosensitization effect of these agents in vivo. However, because IRT is a systemic therapy and patients with early systemic metastatic diseases would be the most suitable candidates for IRT (*34*), systemic administration of MTX would be needed. Therefore, the development of novel MTX derivatives of higher potential or other types of agents acting as cell cycle modulators would be a priority.

To enhance the efficacy of IRT, improved targeting of radiopharmaceuticals to tumors would be beneficial (*35*). It has been shown that PTX may improve tumor perfusion and oxygenation in vivo (*36*,*37*). Honess et al. (*36*) showed a 50%–70% increase of tumor perfusion by the administration of PTX in a nontoxic dose range in an animal model, and Song et al. (*37*) also showed an enhanced antitumor effect of x-irradiation with increased tumor oxygenation in a model administered with a similar nontoxic dose of PTX. These reports indicate that this kind of agent would play a significant role in enhancing IRT efficacy by increasing tumor accumulation of radiopharmaceuticals and improving tumor oxygenation status.

CONCLUSION

PTX and CAF inhibited the G2-phase arrest of LS180 human colon cancer cells exposed to ^{186}Re radiation and decreased their survival. These results encourage further investigation of the cell cycle checkpoint modulation of tumor cells for the enhancement of IRT.

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