# Application of Apoptogenic Pretreatment to Enhance Anti-tumor Immunity of Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)-secreting CT26 Tumor Cells

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**ABSTRACT**

**Background:** As an attempt to develop a strategy to improve the protective immune response to GM-CSF-secreting CT26 (GM-CSF/CT26) tumor vaccine, we have investigated whether the apoptogenic treatment of GM-CSF/CT26 prior to vaccination enhances the induction of anti-tumor immune response in mouse model. **Methods:** A carcinogen-induced mouse colorectal tumor, CT26 was transfected with GM-CSF gene using a retroviral vector to generate GM-CSF-secreting CT26 (CT26/GM-CSF). The CT26/GM-CSF was treated with ɣ-irradiation or mitomycin C to induce apoptosis and vaccinated into BALB/c mice. After 7 days, the mice were injected with a lethal dose of challenge live CT26 cells to examine the protective effect of tumor vaccination *in vivo*. **Results:** Although both apoptotic and necrotic CT26/GM-CSF vaccines were able to enhance anti-tumor immune response, apoptotic CT26/GM-CSF induced by pretreatment with ɣ-irradiation (50,000 rads) was the most potent in generating the anti-tumor immunity, and thus 100% of mice vaccinated with the apoptotic cells remained tumor free for more than 60 days after tumor challenge. **Conclusion:** Apoptogenic pretreatment of GM-CSF-secreting CT26 tumor vaccine by ɣ-irradiation (50,000 rads) resulted in a significant enhancement in inducing the protective anti-tumor immunity. A rapid induction of apoptosis of CT26/GM-CSF tumor vaccine at the vaccine site might be critical for the enhancement in anti-tumor immune response to tumor vaccine. **(Immune Network 2005;5(2):110-116)**

**Key Words:** CT26 tumor, GM-CSF, apoptotic pretreatment, cancer vaccine, anti-tumor immune response

## Introduction

Cancer is a complicate and life-threatening disease to human and becomes the second-ranking cause of death led only by heart disease in the world. Current treatment modalities to human cancer include the surgical resection, chemotherapy, radiation therapy, and immunotherapy. Since cancer cells can be viewed as altered self-cells from an immunological perspective, recent information from basic studies of cellular and molecular immunology and advanced recombinant DNA technology have continually developed several types of cancer immunotherapy to augment or supplement the immune response to cancer cells. Immunotherapy with cancer vaccine is expected to be a potential therapeutic approach to the treatment of various human cancers. The immunological killing of tumor cells generally depends on tumor-specific cytotoxic T cell response and thus causes no side effects of damaging normal cells. Although the influence of cytokine in enhancement of anti-tumor immunity of tumor vaccine was initially examined with interleukin-2 (1), a subsequent comparison study evaluating tumor lines transduced with multiple cytokines genes showed that granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transduced tumor cells induced the most potent, specific, and long-lasting antitumor immunity in murine models (2). The efficacy of GM-CSF-secreting tumor vaccines has been observed in several human tumors including renal cell carcinoma (RCC) (3), melanoma (4), prostate cancer (5), pancreatic cancer (6), and breast cancer (7). The therapeutic activity of

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GM-CSF is thought to be due to the paracrine action of the cytokine on differentiation and proliferation of dendritic cells at the vaccine site (2-6). In addition, the GM-CSF-secreting vaccines elicit multiple immune responses exerted by various immune cells including Th1/Th2, B cells, macrophages, and eosinophils (3).

Apoptosis, a programmed cell death, plays an important role in the development and homeostasis of multicellular organisms mainly through eliminating unwanted or damaged cells. As the initial trigger provoking apoptotitc cell death, numerous physiological and nonphysiological signals such as TCR engagement (8), tumor necrosis factor (9), Fas ligation (10), oxidative stress (11), growth factor withdrawal (12), heat shock (13), irradiation (14), and chemotherapeutic agents (15) have been implicated. In relation to induction or suppression of an immune response to tumor vaccines, the role of apoptosis has also been described. Some groups have shown that uptake of apoptotic cells by dendritic cells results in T-cell tolerance (16), whereas others have demonstrated that antigen produced by apoptotic cells increased the immunogenicity of the antigen (17,18). In the present study, we have investigated whether the apoptogenic pretreatment of GM-CSF-secreting CT-26 tumor vaccine enhances the induction of antitumor immune response *in vivo*. In addition, the optimal condition of γ-irradiation for the apoptogenic pretreatment of GM-CSF-secreting CT-26 tumor was investigated. The results show that γ-radiation-mediated apoptogenic pretreatment result in a significant enhancement in the protective anti-tumor immunity of the GM-CSF-secreting CT26 tumor vaccine.

## Materials and Methods

*Tumor cell lines.* CT26 is chemically induced colonic epithelial tumor induced by N-nitrogen-N-methylurethane in BALB/c mice. CT26/GM-CSF and CT26/B7 were generated by transfection of GM-CSF gene and co-stimulatory molecule B7 gene into CT26, respectively. All tumor cells were cultured in RPMI 1640 containing 10% FBS, penicillin (100 units/μl), streptomycin (100μg/ml), 2 mM L-Glutamine (GIBCO), and 5 mM HEPES (pH 7.0), and grown at 37°C in 5% $CO_2$. The RPMI 1640 medium was referred to as the complete medium.

*Irradiation or mitomycin-treatment of tumor cells.* Tumor cells were trypsinized and washed three times with 1X HBSS. The cells ($5\times10^6$/ml) were γ-irradiated with 5,000, 25,000, or 50,000 rads (1 rad=0.01 Gy) using $Cs^{137}$ source. The irradiated cells were washed once with 1X HBSS, and then injected subcutaneously for vaccination or kept in culture for further analysis of viability and capability of secreting GM-CSF. For treatment with mitomycin C, the cells ($5\times10^6$/ml) were treated with mitomycin C at a concentration of 100μg/ml at 37°C for 1 hr. The treated cells were washed three times with 1X HBSS, and then injected subcutaneously for vaccination or kept in culture for further analysis of viability and capability to secret GM-CSF.

*Induction of necrosis of tumor cells.* For induction of necrosis, the tumor cells ($5\times10^6$/ml) underwent 3 freeze/thaw cycles using a 37°C water bath and liquid nitrogen. The complete necrosis was confirmed by trypan blue staining before vaccination.

*Flow cytometry analysis of apoptosis.* Apoptosis of the tumor cells that were γ-irradiated or treated with mitomycin C was assayed using ApoAlert™ Annexin V Apoptosis Kit (Clontech, USA) according to the manufacturer's instructions. Briefly, the cells ($5\times10^5$ cells) were washed with 1X Binding buffer, and resuspended in 200μl of 1X Binding buffer, and then incubated with 5μl of Annexin V antibody for 5 min before analysis using Beckton Dickins FACSan.

*Assay of apoptotic DNA fragmentation.* Apoptotic DNA fragmentation induced in tumor cells following $^{137}$Cs-irradiation or mitomycin C-treatment was determined as previously described (19). Briefly, the cells were harvested at indicated time points by centrifugation and then treated with a lysis buffer (0.8% sodium lauryl sarcosine, 20 mM EDTA, 100 mM Tris, pH 8.0) for 30 min on ice. After centrifugation at 16,000 g, the supernatant was collected and brought to 1% SDS, and treated for 2 hr at 50°C with RNase A and subsequently with proteinase K for 2 hr at 37°C. The DNA fragments were precipitated with 2.5 volumes of ethanol in the presence of 5 M ammonium acetate and visualized following electrophoresis on a 1.5% agarose gel.

*Vaccination.* Male BALB/c mice (8～10 weeks old) were used. The tumor cells following γ-irradiation or mitomycin C-treatment were washed and resuspended in 1X HBSS buffer at a concentration of $5\times10^6$/ml. The vaccine cells ($5\times10^5$) were injected subcutaneously into the right hind flank, whereas the challenge live tumor cells ($1\times10^5$) were injected subcutaneously into left hind flank at 7 days after the vaccination. All cells were injected in 0.1 ml using a 1 ml syringe with 27-gauge needle. All mice were kept under specific pathogen-free conditions. Mice were checked for the presence of tumors every other day for tumor growth. For individual vaccination groups 10 mice were employed and the experiment was repeated three times.

## Results

*Production of GM-CSF by CT-26 or GM-CSF gene-transduced CT-26.* For generation of GM-CSF-secreting

CT26 (CT26/GM-CSF) or co-stimulatory B7-expressing CT26 (CT26/B7), CT26 cells were transfected with GM-CSF gene or B7 gene using a retroviral vector plasmid. Sequentially, the production level of GM-CSF was compared among CT26/GM-CSF, CT26/B7, and CT26. Individual cells ($5\times10^4$) were cultivated in a 96-well plate for 24 hr. The plate was centrifuged at 1,200 rpm for 5 min, and the GM-CSF in the supernatant was analyzed by mouse GM-CSF Quantikine ELISA plate (R&D systems, Minneapolis, MN, USA). As shown in Fig. 1, the CT26 cells appeared to produce 685 pg/ml and the CT26 transfected with B7 gene (CT26/B7) 690 pg/ml, whereas GM-CSF gene-transduced CT26 (CT26/GM-CSF) produced 4,322 pg/ml. These results indicated that the transfection of GM-CSF gene into CT26 resulted in 6.3 times enhancement in the secretion level of GM-CSF secretion as compared to the secretion level of CT26 wild type.

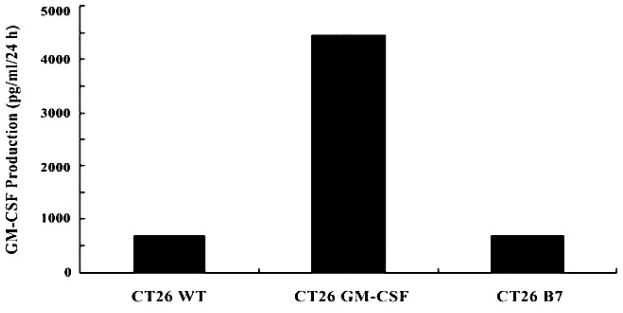


**Figure 1.** GM-CSF production of CT26 wild type, GM-CSF gene-transduced CT26, and B7 gene-transduced CT26 cells. Equal numbers of cells ($5\times10^4$ cells) were incubated in a 96-well plate at 37°C for 24 hr. The concentration of GM-CSF in the supernatants was measured by enzyme-linked immunosorbent assay using GM-CSF Quantikine ELISA plate from R&D Systems (Minneapolis, MN, USA).

*Induction of apoptotic cell death by γ-irradiation or mitomycin C-treatment in GM-CSF-transduced CT26*. To investigate the effect of apoptogenic pretreatment on the anti-tumor immunity induced by GM-CSF-secreting tumor vaccine, apoptosis of GM-CSF-transduced CT26 was induced prior to injection. Since tumor cell vaccines are generally γ-irradiated before vaccination to avoid proliferation *in vivo* (20), we also employed γ-irradiation as well as mitomycin C treatment as the source for induction of apoptosis. After the GM-CSF-transduced CT26 cells were γ-irradiated with 5,000, 25,000, or 50,000 rads using $Cs^{137}$ source, the cells were cultivated in the complete medium and apoptotic DNA fragmentation assay was done to verify and quantitate induction of apoptosis after 24, 48, and 72 hr of cultivation. As shown in Fig. 2, γ-irradiation-induced apoptotic DNA fragmentation of GM-CSF gene-transduced CT26 was detected at 24 hr and increased in a time- and irradiation dosage-dependent manner. Although mitomycin C-treatment failed to induce an easily detectable level of the apoptotic DNA fragmentation of GM-CSF gene-transduced CT26 at 24 hr, it was able to induce a significant level of apoptotic DNA fragmentation after 48 and 72 hr, indicating that the apoptosis induced by γ-irradiation was more rapid than that induced by mitomycin C treatment. On the other hand, the chromosomal DNA purified from GM-CSF gene-transduced CT26 following the freeze/thaw cycle did not show the apoptotic DNA frag-

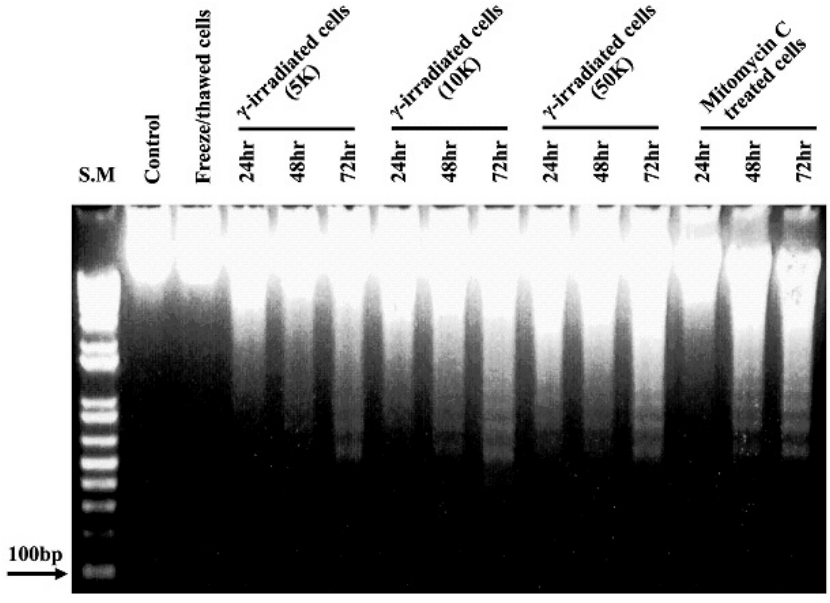


**Figure 2.** Kinetic analysis of γ-irradiation- or mitomycin C-induced apoptotic DNA fragmentation in GM-CSF gene-transduced CT26 cells. The cells were of γ-irradiated with indicated dosages or treated with mitomycin C. The apoptotic DNA fragmentation was analyzed using Triton X-100 lysis method, and visualized on 1.2% agarose gel electrophoresis.

mentation, demonstrating that the freeze/thaw cycle resulted in cell dying mainly from necrosis as described previously (21).

In order to confirm and quantitate the apoptotic cells after the apoptogenic treatment by γ-irradiation or mitomycin C, the cells were immunostained for annexin V and phosphatidylserine, and analyzed with a flow cytometry. As shown in Fig. 3, while 26% of the mitomycin C-treated tumor cells were stained positive for annexin V 24 hr after the treatment and in vitro culture, 50% of the cells appeared to be annexin V-positive after 72 hr, indicating that about half of the cells treated with mitomycin C underwent apoptotic cell death after 72 hr of in vitro cultivation. After γ-irradiation with 5,000 rads, 57% of the cells were stained positive for annexin V at 24 hr and >80% of the cells became apoptotic. These results indicated that γ-irradiation was able to induce apoptosis of the GM-CSF gene transduced CT26 cells more rapidly than was mitomycin C treatment. Under these conditions, the effect of apoptogenic treatments on the capability of the cells to secret GM-CSF was investigated using the culture supernatant and mouse GM-CSF ELISA plate. The results showed that the secretion level of GM-CSF by CT26/GM-CSF appeared to increase in early period following γ-irradiation, but was reduced in proportion to decline in the cell viability (data not shown).

*Enhancement of protective anti-tumor immunity following apoptogenic pretreatment of tumor vaccine CT26/GM-CSF cells prior to vaccination.* To investigate the effect of apoptogenic pretreatment of CT26/GM-CSF vaccine on the potency of protective immune response to the vaccine *in vivo*, apoptotic CT26/GM-CSF vaccine cells induced by γ-irradiation or mitomycin C treatment were injected subcutaneously into the right hind flank of mice, and 7 days later a lethal dose ($1\times10^5$) of challenge live tumor cells ($1\times10^5$) were injected subcutaneously into left hind flank. As shown in Fig. 4, all control mice, which were injected with PBS instead of tumor vaccine, developed tumor within 10 days after injection of the lethal dose of challenge tumor. In contrast, all mice vaccinated with apoptotic tumor cells induced by pretreatment with γ-irradi-

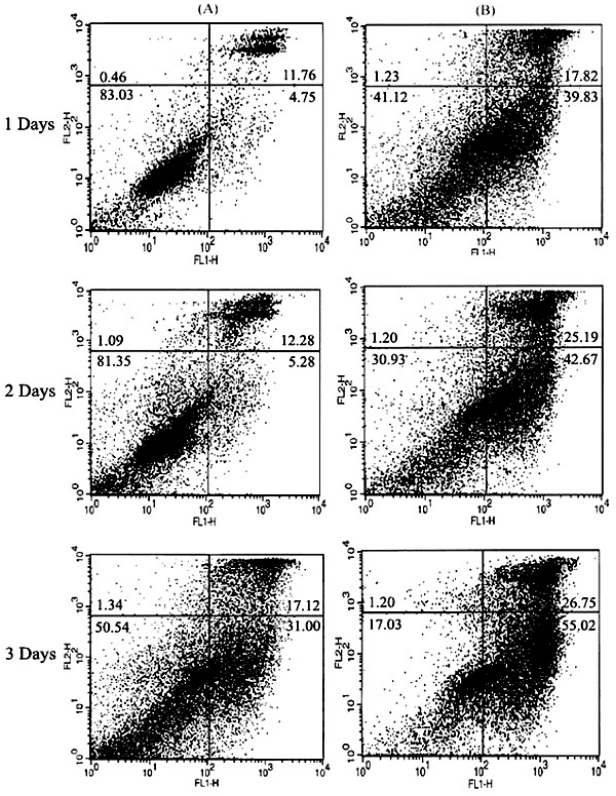


**Figure 3.** Induction of apoptosis by mitomycin C (A) or γ-irradiation (B). Flow cytometry analysis by annexin V-FITC/PI staining methods. GM-CSF gene-transduced CT26 cells were treated with 100μg/ml of mitomycin at 37°C for 1 hr or irradiated with 50,000 rads, and then the cells were kept in culture at 37°C for the indicated time periods. The cells were stained using ApoAlert™ Annexin V Apoptosis Kit (Clontech, USA) for Annexin V/PI staining as described in Materials and Methods. The annexin $V^+/PI^-$ cells were considered as the cells in an early stage of apoptosis, and the double positive cells were in the late phase of apoptosis, wherease the single PI-positive cells were necrotic cells. Numbers refer to the percentage of cells encountered in each quadrant.

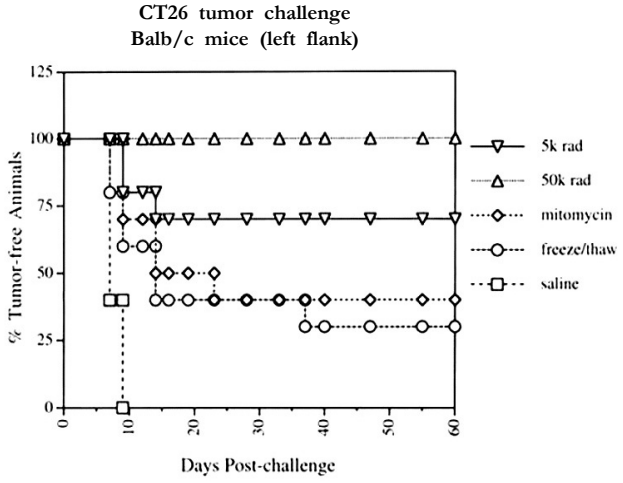


**Figure 4.** Protection against tumor development after vaccination with apoptotic CT26/GM-CSF or necrotic CT26/GM-CSF cells. Male BALB/c mice were subcutaneously vaccinated into the right hind flank with equivalent numbers of the vaccine cells ($5\times10^5$ cells) pretreated with γ-irradiation at 5,000 rads (▽), γ- irradiation at 50,000 rads (△), mitomycin C (◇), freeze/thaw cycle (○), and for true control the mice were injected with saline (□). After 7 days, the vaccinated mice were challenged with a lethal dose of live CT26 tumor cells ($5\times10^5$) in the left hind flank. The mice were monitored every other day for tumor growth and were scored positive when tumors were palpable.

ation at a dosage of 50,000 rads remained tumor free for 60 days. Under the same conditions, 65% of mice vaccinated with apoptotic tumor induced by γ-irradiation with 5,000 rads remained tumor free. When the mice vaccinated with CT26/GM-CSF cells pretreated with mitomycin to induce apoptosis were injected with a lethal dose of challenge tumor, 35% of mice remained tumor free for 60 days. On the other hand, only 25% of mice vaccinated with necrotic CT26/GM-CSF cells remained tumor free for 60 days after tumor challenge. These results indicated that apoptotic CT26/GM-CSF cells induced more potent protective immune response to tumor vaccine than did necrotic counterpart, and that although both pretreatment of CT26/GM-CSF vaccine cells with γ-irradiation and with mitomycin C were able to induce apoptosis, γ-irradiation was better method than mitomycin C for the apoptogenic pretreatment of the tumor vaccine to generate the protective immune response to tumor vaccine. Since pretreatment with γ-irradiation induced apoptosis of CT26/GM-CSF vaccine cells more rapidly than did mitomycin C treatment, and since the induction speed of apoptosis of the tumor vaccine by γ-irradiation appeared to increase in proportional to the irradiation dosage, these results suggest that the rapid induction of apoptosis of CT26/GM-CSF tumor vaccine resulting from apoptogenic pretreatment might be the most critical for generating enhancement in anti-tumor immune response to tumor vaccine.

## Discussion

Recent advances in tumor immunology, which aims to study of antigens on tumor cells and the immune response to these antigens, have revealed various tumor antigens that are unique to tumor cells. Tumor antigens can be shown to induce both humoral and cell-mediated immune responses that result in the destruction of the tumor cells; however, the cell-mediated immunity generally appears to play the major role in the anti-tumor immune response. In addition, a number of tumors have been shown to induce tumor-specific cytotoxic T lymphocytes (CTLs) that recognize tumor antigens presented by class I MHC molecule on the tumor cells. Over the past few years, several laboratories have tried to devise the strategies for the development of vaccine against melanoma and other tumors (22,23). Since antigen presentation is a critical step in inducing the protective immune response to any immunization including cancer vaccination, and since dendritic cells are the most potent professional antigen-presenting cells capable of initiating T cell-dependent immune responses, the dendritic cells have been employed in strategy of tumor vaccination in order to enhance immunization against tumor antigens via manipulating the fashion of antigen presentation (24). In this context, autologous and allogeneic GM-CSF secreting tumor cell vaccines are recently used for various types of cancer immunotherapy (3,5-7). The GM-CSF-expressing tumor cell vaccines are known to be very efficient at inducing tumor-specific immune response in mice and in preliminary clinical trials, because GM-CSF is a potent stimulator of dendritic cells and furthermore a local production of GM-CSF induces differentiation of bone-marrow derived progenitors into activated dendritic cells, which take up the antigen from the vaccination site and process it to stimulate T cells (25). In these processes, dendritic cells can take up antigens in the form of exosomes from either living cells or phagocytosed tumor cells dying from necrosis or apoptosis.

Since aliquots of tumor cell vaccines harvested from a large scale cultivation are routinely stored in the frozen condition until they are required for vaccination, and since the frozen cells are thawed and pretreated with γ-irradiation prior to vaccination, the tumor vaccines generally include necrotic as well as apoptotic cells resulting from thawing and pretreatment steps in the clinical practice. Thus, in the present study we have investigated the effect of apoptogenic pretreatment of GM-CSF-expressing CT26 on the induction of anti-tumor immune responses *in vivo*. The GM-CSF-expressing CT26 (CT26/GM-CSF) was generated by transfection of GM-CSF gene into CT26 that is previously known to be murine colonic epithelial tumor induced by a chemical mutagen N-nitrogen-N-methylurethane (26). The CT26/GM-CSF was able to secret GM-CSF about 6 times more than CT26 wild type. Two different methods such as γ-irradiation and mitomycine C treatment were used for the apoptogenic pretreatment of CT26/GM-CSF vaccine cells. When apoptotic DNA fragmentation assay as well as flow cytometric analysis using annexin V staining was performed to verify and quantitate the apoptosis of CT26/GM-CSF vaccine cells following the pretreatment, γ-irradiation at the dosage ranging from 5,000 to 50,000 rads appeared to be more apoptogenic as compared to mitomycin C treatment (100μg/ml). In addition, apoptotic cell death of CT26/GM-CSF following γ-irradiation was induced in an irradiation- dosage dependent manner. Flow cytometric analysis using annexin V staining revealed that only 26% of the tumor cells were stained for annexin V at 24 hr and 50% of the tumor cells were annexin V-positive at 72 hr after mitomycin C treatment, whereas 57% of the tumor cells were annexin V-positive at 24 hr and $>80\%$ of the tumor cells were annexin V-positive at 72 hr after γ-irradiation with 50,000 rads. These results demon-

strate that γ-irradiation induced apoptotic cell death of CT26/GM-CSF cells more rapidly and strongly than mitomycin C treatment. Under these apoptogenic pretreatment conditions, the vaccination effectiveness in generating the protective immune response to tumor challenge was also investigated *in vivo*. The results showed that γ-irradiation with 50,000 rads was the best apoptogenic pretreatment among the conditions tested, and 100% of mice vaccinated with CT26/GM-CSF pretreated with γ-irradiation (50,000 rads) were remained tumor free for 60 days following injection of a lethal dose of challenge live tumor cells. Although the CT26/GM-CSF pretreated with mitomycin C was also able to undergo apoptosis *in vitro*, its potency to induce anti-tumor immunity *in vivo* was not so significant as compared to γ-irradiated counterparts, but slightly better than necrotic CT26/GM-CSF prepared by the freeze/thaw cycle. Previously, it was shown that all mice vaccinated with necrotic CT26 wild-type cells developed tumor in 20 days after tumor challenge, whereas 60% of mice remained tumor-free after vaccination with apoptotic cells, indicating that apoptotic tumor vaccine induced more potent protective immune response to tumors than necrotic tumor vaccine (18). Under the same conditions, an immunohistochemical analysis on the tissue sections of vaccine sites showed that there were more dendritic- as well as T-cell responses after injection of apoptotic cell vaccine than necrotic cell vaccine, suggesting that dendritic cells at vaccine sites might internalize tumor-specific antigens from the site and activate $CD4^+$ and $CD8^+$ T cells much more efficiently when apoptotic rather than necrotic cells were injected. The current results also demonstrate that apoptotic tumor vaccine secreting GM-CSF is more potent than its necrotic counterpart in inducing anti-tumor immunity against tumor challenge. Consequently, these previous and current results indicate that apoptotic tumor cells possess better efficacy as the vaccine than necrotic counterpart, regardless of ectopic overexpression of GM-CSF in the tumor vaccine. It is noteworthy that all mice vaccinated with necrotic tumor CT26 wild type cells prepared by freeze/thaw cycle were previously reported to develop tumors in 20 days after tumor challenge (18). However, in our experiments, 25% of mice vaccinated with necrotic CT26/GM-CSF cells prepared by freeze/thaw cycle appeared to remain tumor free for 60 days after tumor challenge, suggesting an influence of GM-CSF in enhancement of anti-tumor immunity raised against necrotic tumor vaccine. Since pretreatment with γ-irradiation (50,000 rads) could induce the most rapid and significant apoptosis of CT26/GM-CSF cells, the current results indicate that the rapid induction of apoptosis of CT26/GM-CSF tumor vaccine needs to occur at the vaccination site where secreted GM-CSF from the vaccine cells might recruit and activate dendritic cell functions in relation to antigen presentation.

In summary, these results demonstrated that apoptogenic pretreatment of GM-CSF-secreting CT-26 tumor vaccine prior to injection was able to enhance the induction of anti-tumor immune response *in vivo*. The γ-irradiation with 50,000 rads was the best apoptogenic pretreatment among the conditions tested, and 100% of mice vaccinated with CT26/GM-CSF pretreated with γ-irradiation (50,000 rads) were remained tumor free for 60 days following injection of a lethal dose of challenge live tumor cells. These results also suggest that a rapid induction of apoptosis of CT26/GM-CSF tumor vaccine needs to occur at the vaccine site in order to enhance the protective immune response to tumor challenge.

## Footnotes

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