Effect of Rice Cell-Derived Human Granulocyte-Macrophage Colony-Stimulating Factor on 5-Fluorouracil-Induced Mucositis in Hamsters

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an important regulator of the maturation and function of cells in the granulocyte and macrophage lineages, and also plays a significant role in wound healing. In a previous study, we expressed human GM-CSF in rice cells (rice cell-derived human GM-CSF; rhGM-CSF). The purpose of the present study was to evaluate its effect on wound healing in oral mucositis. Oral mucositis was induced in Syrian hamster cheek pouches by 5-fluorouracil treatment and mechanical scratching. Ulcerated areas were treated from days 3 to 14 with an application of 200 µL saline, or of the same volume of a solution containing 0.04, 0.2, or 1 µg/mL rhGM-CSF. Treatment of hamsters with rhGM-CSF reduced the ulcerated areas of the oral mucosa, compared with the control. In the healing process, the mucositis tissue layer of the rhGM-CSF-treated group showed significantly decreased myeloperoxidase activity and increased numbers of proliferating cell nuclear antigen (PCNA)-positive cells. Treatment with rhGM-CSF also affected expression of inflammatory cytokines in the ulcerative mucosal tissue. These results demonstrate the efficacy of plant-produced rhGM-CSF in wound healing and have significant implications for the development of rhGM-CSF as a therapeutic agent for ulcerative oral mucositis.

Key words granulocyte-macrophage colony-stimulating factor; mucositis; wound healing; hamster buccal pouch; plant-based expression system

Oral and gastrointestinal mucositis is a troublesome adverse effect that develops in patients receiving radiotherapy or chemotherapy. Especially, ulcerative mucositis is a painful inflammation for patients receiving chemotherapy, and is estimated to affect 40% of all chemotherapy patients, 75% of patients exposed to high dose chemotherapy with bone marrow transplantation or peripheral blood stem cell support, and more than 90% of patients irradiated for head and neck cancer. Mucositis results from the ability of these treatments to nonspecifically target rapidly proliferating cells of the basal epithelial layer. In most cases, mucositis causes pain and limits oral nutrition intake. In severe cases, mucositis affects the success of anti-cancer therapy due to the reduced tolerance of patients to chemotherapy. It can also affect the patient’s quality of life as well as the cost of medical treatment. The onset of oral mucositis usually occurs within 5 to 7 d of administration of chemotherapy, and occurs in 5 stages, termed the initiation stage, primary damage response stage, signal amplification stage, ulceration stage and the healing stage. The course of mucositis is stimulated by many pro-inflammatory cytokines such as tumor necrosis factor-a (TNF-a) and interleukin 6 (IL-6). There are some of the chemotherapeutic agents that can induce mucositis are anthracyclines, taxanes, alkylating agents, antimitabolites, and vanca alkald.

Several agents have been reported to prevent oral mucositis, including l-glutamine, amifostine, benzydamine, N-acetylcysteine, keratinocyte growth factor, and cytokines such as IL-11 and transforming growth factor-beta 3 (TGF-β3). Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates proliferation of cells of the granulocyte and macrophage cell lineages and also plays an important role in wound healing by stimulating keratinocyte and fibroblast growth. GM-CSF has been shown to have significant effects on healing of cuts, burns, leg ulcers, and skin grafts. Some reports have suggested that GM-CSF exerts a wound healing effect in part by enhancing cellular regeneration. GM-CSF is known to stimulate regeneration of fibroblast and epithelial cells in oral mucositis, and stimulates proliferation of the oral mucosal cells by enhancing IL-1 transcription and translation. IL-1 has a protective effect on oral mucosa against radiation by increasing mucosal cell proliferation.

In this study, we used rice cell-derived recombinant human GM-CSF (rhGM-CSF) to treat oral mucositis. There are some benefits to developing medicinal agents from plant cells. 1) The medium for maintaining plant cells is cheaper than that of animal cells. 2) Plant culture medium contains fewer protein ingredients, making it easier to separate and purify the target proteins from the medium. Plants are the promising expression system for the production of human recombinant proteins because of low operating costs and less potential contamination with human pathogens. Thus, there are several advantages to expressing hGM-CSF in plant cells.

Plant-based expression systems for recombinant proteins have begun to receive acceptance for pharmaceuticals and other commercial markets. Plant-derived protein products provide safer, more cost-effective alternatives to traditional platforms using microbial cultures, cultured animal cells and, more recently, transgenic animals. The first potentially therapeutic proteins expressed in plants were human serum albumin expressed in tobacco and potato leaves and suspension cells, and a monoclonal antibody that was expressed in tobacco leaves. The plant-derived therapeutic antibodies are already in clinical trials, and, given the economic benefits. So plant systems could be used to produce pharmacologically significant and commercially valuable proteins. This is known as

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molecular farming.\textsuperscript{20,21)}

GM-CSF also regulates several aspects of the maturation of leukocytes, macrophages, and dendritic cells in the dermis and submucosa,\textsuperscript{22)} and also influences migration of epithelial cells. In wound healing, GM-CSF facilitates migration at the wound margins as a consequence of signals from mesenchymal cells and the extracellular matrix (ECM), which stimulates proliferation, migration and differentiation.\textsuperscript{23)} Eventually, these processes culminate in wound healing. Human GM-CSF has potential N-glycosylation sites (Asn27, Asn37) and several O-glycosylation sites.\textsuperscript{23–25)} It is known that degrees of glycosylation differ according to cell type and that in vivo stability increases with N-glycan content.\textsuperscript{26)} The glycosylation ratio of rhGM-CSF was higher than that of yeast-derived recombinant human GM-CSF (yrhGM-CSF).\textsuperscript{27)} N-Glycosylation of hGM-CSF has a significant role in continuing its half-life in the blood stream. The plasma rhGM-CSF level was better maintained than that of yrhGM-CSF. Hence rhGM-CSF was more effective than yrhGM-CSF in recruiting leukocyte.\textsuperscript{27)}

The present study was performed to evaluate the effect of plant-derived rhGM-CSF on wound healing in a hamster oral mucositis model.

**MATERIALS AND METHODS**

**Experimental Animals** Six-week-old male Syrian hamsters (100–110 g in weight) from SLC (Hamamatsu, Japan) were used in this study. The animals were acclimated for 1 week prior to the experiments. Throughout the experimental period, the hamsters were maintained in a clean laboratory animal room with controlled environmental conditions of 22±2°C, 50±5% relative humidity, and a 12h:12h light:dark cycle. The animals had free access to sterile water and commercial feed. All procedures and animal treatments were performed in the clean laboratory animal room according to the guidelines of laboratory animal experimentation of Chung-Ang University. This work was approved by the appropriate Chung-Ang University Ethical Committees related to the use of laboratory animals.

**Induction of Experimental Mucositis and Treatment with rhGM-CSF** Induction of ulcerative mucositis in the hamster buccal pouch was based on a previously reported method.\textsuperscript{28)} The animals were given an intramuscular injection of 60 mg/kg 5-fluorouracil (5-FU) on days 0 and 2. The 5-FU treatment was performed as a series of injections of 10 mg/kg 5-FU every 20 min, due to unacceptable mortality when administered as a single dose.\textsuperscript{29)} The buccal pouch mucosa was irradiated by superficial scratching using of the 23- and a 26-gauge needle.\textsuperscript{28)}

Scratching was performed by dragging the tip several times across the everted cheek pouch. Between days 3 and 14, the animals were topically administered 200–110 g in weight from SLC (Hamamatsu, Japan) were used in this study. The animals were acclimated for 1 week prior to the experiments. Throughout the experimental period, the hamsters were maintained in a clean laboratory animal room with controlled environmental conditions of 22±2°C, 50±5% relative humidity, and a 12h:12h light:dark cycle. The animals had free access to sterile water and commercial feed. All procedures and animal treatments were performed in the clean laboratory animal room according to the guidelines of laboratory animal experimentation of Chung-Ang University. This work was approved by the appropriate Chung-Ang University Ethical Committees related to the use of laboratory animals.

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Scratching was performed by dragging the tip several times across the everted cheek pouch. Between days 3 and 14, the animals were topically administered 200 µL of 0.04, 0.2, or 1 µg/mL rhGM-CSF (Hanson Biotech, Ltd., Daejeon, Korea) to the ulcerated mucosa, once a day. Control groups were treated with the same volume of sterile phosphate-buffered saline (PBS). Each treatment and control group initially consisted of 11 animals. Before administration of rhGM-CSF, animals were anesthetized with Zoletile 50 (Virbac) 0.4 mL/kg and Rompum® (BAYER) 0.1 mL/kg by intramuscular injection.

**Macroscopic Analysis and Gross Evaluation of Mucositis** The ulcerated mucosa was evaluated daily between days 3 and 8, and was photographed on each day. Mucositis was assessed and graded on a score of 0–5\textsuperscript{30)}: 0, normal epithelium; 1, erythema and congestion; 2, severe erythema and congestion with mucosal erosion in aspects of mucosa; 3, severe erythema and congestion, ulcers in one or more sites, whole area of ulcers or abscesses over 25% of the mucosal epithelium; 4, severe erythema and congestion, whole area of ulcers or abscesses over about half of the mucosal epithelium; and 5, extensive ulceration and abscesses, diffuse hemorrhagic areas.

**Myeloperoxidase Activity Assay** Measurement of myeloperoxidase (MPO) activity was based on a method previously reported, with some modifications.\textsuperscript{30)} Areas of ulcerative buccal tissue were resected from each animal on days 5 and 8. The tissue was weighed and homogenized in MPO assay buffer using a Mikkola homogenizer, and the homogenate was then centrifuged at 4°C for 15 min (10000×\textit{g}). The pellet was resuspended in hypotonic lysis buffer (900 µL of 0.2% (w/v) NaCl) for 30 s followed by the addition of an equal volume of a solution containing 1.6% (w/v) NaCl and 5% (w/v) glucose. The suspension was then centrifuged at 4°C for 5 min (2000×\textit{g}). The pellet was resuspended in a buffer solution (MPO assay buffer containing 0.5% (w/v) hexadecyl trimethyl ammonium bromide), sonicated, and centrifuged at 4°C for 20 min (12000×\textit{g}). The supernatant was assayed for MPO activity using 3,3′,5,5′-tetramethylbenzidine (18.4 mm) and H$_2$O$_2$ (0.017%). MPO activity was measured by absorbance at 630 nm, and the results are expressed as activity in mU/ (mL·mg) tissue.

**Proliferating Cell Nuclear Antigen Immunohistochemistry** The resected buccal tissues were fixed in 10% formaldehyde, paraaffin-embedded, sectioned, and mounted. Sections were deparaffinized in 2 changes of xylene and rehydrated in graded ethanol. After washing in PBS (3×10 min), the sections were incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. The slides were incubated in protein blocking agent (Immunotech, France) for 5 min, and then incubated with primary anti-proliferating cell nuclear antigen (PCNA) antibody (1:2000 dilution, Sigma-Aldrich Inc., U.K.) overnight at 4°C in a humidified chamber. The slides were washed in PBS (3×10 min) and incubated with biotinylated secondary antibody (Immunotech, France) for 5 min. Streptavidin peroxidase reagent (Immunotech, France) was then added for 10 min. Finally, the sections were developed with 3,3′-diaminobenzidine (DAB) (Immunotech, France) for 5–10 min. Developed sections were lightly counterstained with Mayer’s hematoxylin (Dako, Denmark), dehydrated in a graded series of ethanol, cleared in xylene, cover-slipped, and mounted with Canada Balsam (Sigma-Aldrich Inc., U.K.). The results are expressed as the ratio of PCNA-positive cells to the total number of cells counted. Cell counting was performed on 5 randomly chosen microscope fields at a magnification of ×200. Positively immunostained signals were detected by color thresholding on the brown color of DAB peroxidation, while total cells were counted by thresholding on the brown color plus the blue color (hematoxylin counterstain).\textsuperscript{31)}

**RNA Isolation and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis** Ulcerative mucosal tissues were resected on days 5 and 8 and stored at −80°C until required. RNA was extracted
using Trizol reagent (Invitrogen, CA, U.S.A.). For real-time qPCR, 0.1 µg of total RNA was reverse transcribed using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed in triplicate using the MyiQ Single-Color Real-Time PCR Detection System and an iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.). Relative expression was normalized to β-actin levels. The primer sequences used were: β-actin, forward (5′-CAC AGC CGA GAG GGA AAT-3′) and reverse (5′-CCA GGG AAG AGG ATG-3′); TGF-β, forward (5′-CTG GGC TGG ATT-3′) and reverse (5′-GGT GTG GTT GGT TGT AGA GG-3′); TNF-α, forward (5′-CTATG CCTCAG CTT-3′) and reverse (5′-GGTAGG TCTCCTCAGTG-3′); IL-1β, forward (5′-GGT GTG GTG TCTCAG TG-3′) and reverse (5′-AGG TCTCAG GTG CTC-3′). The thermal cycling conditions were pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 s, annealing at 56°C for 15 s, and extension at 72°C for 20 s.

**Data Analysis** All values are represented as mean±standard deviation (S.D.) of the indicated number of experiments. Statistical significance was calculated by Student’s t-test and p values below 0.05 were considered significant.

**RESULTS**

**Macroscopic Analysis and Gross Evaluation of Mucositis**

The combination of 5-FU administration followed by mechanical trauma to the oral mucosal tissue resulted in extensive oral mucositis in the hamsters. The significant lesions exhibited severe damage to the epithelium, underlying connective tissue, and muscle bundles. After the second mechanical irritation (on day 2), all animal groups showed severely ulcerated mucosal tissue (Fig. 1), with high mucositis scores (Fig. 2). However, after the third administration of rhGM-CSF (on day 5), it was evident that the healing rate was faster in animals that had been treated with rhGM-CSF than in the control group (Fig. 2). Especially, the rhGM-CSF-treated groups showed a clearly decreased mucositis score at day 4 (one day after the first treatment). The mucositis scores also showed that the healing effect of rhGM-CSF was dose-dependent.

**Effect of rhGM-CSF on Myeloperoxidase Activity in Oral Mucosal Tissue**

The ulcerative buccal tissue was resected on days 5 and 8. MPO activity was measured by absorbance at 630nm and expressed as mU/mL/mg of sample (n=4). **p<0.01 compared with control.**

![Fig. 1. Gross Evaluation of Ulcerative Mucositis](image1)

![Fig. 2. Histopathological Evaluation of Ulcerative Mucositis](image2)

![Fig. 3. Myeloperoxidase Activity in Buccal Tissue](image3)
MPO activity was found to be higher in the ulcerated tissue regions than in the unaffected regions. The MPO activity was high in the ulcerative mucosal tissue after the second irritation, and this was gradually reduced by administration of rhGM-CSF. The rhGM-CSF-treated animals showed a significant and dose-dependent decrease of MPO activity compared with the control group (Fig. 3). Although the animals in the group treated with the lowest dose of rhGM-CSF (0.04 µg/mL) did not show evidence of efficacy, the group treated with 1 µg/mL rhGM-CSF showed a significant decrease in MPO activity compared to the control group, on days 5 and 8.

**Proliferating Cell Nuclear Antigen Immunohistochemistry**

The purpose of this study was to investigate the effect of locally applied rhGM-CSF on cellular processes like proliferation and differentiation. PCNA is a specific immunohistochemical marker that is used to identify proliferating cells in tissue sections.

Many PCNA-positive cells were observed in the basal layer of the ulcerative mucosal tissue of rhGM-CSF-treated groups, on both day 5 and day 8 (Fig. 4). On day 5, there were more PCNA-positive cells in the rhGM-CSF-treated animals than in the control group. The PCNA-positive cell number in the rhGM-CSF group decreased between days 5 and 8, whereas it increased in the control group (Fig. 5). This result suggests that the therapeutic effects of rhGM-CSF were greater early in the healing process (on day 5) than at the later stage (on day 8).

**Quantitation of Pro-inflammatory Cytokine Expression**

We next used quantitative real-time RT-PCR to examine the expression of the pro-inflammatory cytokines IL-1β and TNF-α in the ulcerative mucosal tissue. On days 5 and 8, the mRNA levels of TNF-α and IL-1β were reduced in the rhGM-CSF groups compared to the control group (Fig. 6), with a dose-dependent effect being apparent on day 8.

**Quantitation of Transforming Growth Factor-β (TGF-β) Expression**

TGF-β is important for efficient wound healing.
because it stimulates fibroblast proliferation, myofibroblast differentiation, and angiogenesis. On day 5, the buccal tissues from animals in the rhGM-CSF-treated groups showed a dose-dependent increase in TGF-β mRNA levels compared with control animals. However, by day 8 the TGF-β mRNA levels were similar in all groups (Fig. 7). These data suggest that TGF-β participates in the early phase of the wound healing process but is likely not involved at later phases.

**DISCUSSION**

The management of ulcerative mucositis is essential in raising the quality of life in chemotherapy patients as well as the success of non-surgical treatments for cancer. Oral mucositis is considered a common debilitating complication of chemotherapy. The primary symptoms of oral mucositis are erosive and ulcerative lesions of the oral mucosa. The aim of treatment for oral mucositis is generally relieving symptoms and adjust possible infections. Continued research of effective treatments for oral mucositis is needed.

N-Linked carbohydrate moieties of hGM-CSF play a significant role in the biological specific activity in vitro and the adsorption rate from hypodermis and the clearance rate from bloodstream in vivo. It was found that rice cell derived hGM-CSF has higher glycosylation ratio and its ratio influence a pharmaceutical potency of rhGM-CSF.

This study examined the healing effect of rhGM-CSF on 5-FU-induced oral mucositis. The severity and dimension of mucositis were reduced rapidly in the rhGM-CSF-treated groups from days 5 to 8, as measured by both gross and immunohistochemical evaluations. In addition, the rhGM-CSF-treated groups showed a significant decrease in MPO activity compared with the control, PBS-treated group.

Cyclin/PCNA (36 kDa) is an auxiliary protein of DNA polymerase δ that is essential for DNA replication during the

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**Fig. 5. PCNA-Positive Cells in Buccal Tissue**

The number of PCNA-positive cells was counted in microscopic fields of tissue sections at ×200 magnification. Positive immunostaining signals were detected by color thresholding on the brown color of DAB peroxidation, while total cells were counted by thresholding on the brown color plus blue color (hematoxylin counterstaining). Day 5 (n=4) and day 8 (n=5). *p<0.05 compared with control.

**Fig. 6. Pro-inflammatory Cytokine Expression in Buccal Tissue**

TNF-α and IL-1β mRNA expression was quantified using real-time RT-PCR. Relative quantification was performed using the \(2^{-ΔΔC_t}\) method. (A) Day 5 (n=4) and (B) day 8 (n=5). *p<0.05 compared with control. \(C_t\), cycle threshold; \(ΔC_t\), cytokine–Ct, β-actin; \(ΔΔC_t\), sample–Ct, control.
S-phase of the cell cycle. In many normal tissues, PCNA-positive cells are limited to the proliferative compartment. Thus, PCNA immunohistology is a valuable tool for defining proliferative activity in diagnostic pathology.\(^{40}\) In this study, treatment with rhGM-CSF enhanced cell proliferation, as evidenced by the increase in PCNA-positive cells. The number of PCNA-positive cells in the rhGM-CSF-treated groups was higher than that of control group on day 5, suggesting that rhGM-CSF induces cell proliferation during the early steps of wound healing.

There are some reports linking cytokine levels to excisional wound healing. In excisional wound healing experiments, IL-1\(\beta\), TNF-\(\alpha\), and TGF-\(\beta\) were significant factors contributing to wound healing.\(^{6,32,33}\) TNF-\(\alpha\) and IL-1\(\beta\) are generally considered to be pro-inflammatory cytokines. Strong induction of IL-1 and TNF-\(\alpha\) expression is observed after irradiation of mucosal tissue, and this is maintained until the mid-phase of wound healing.\(^{36}\) Inhibition of TNF-\(\alpha\) signaling was found to accelerate excisional wound healing. Consistent with this, TNF-receptor p55 knockout mice exhibit increased angiogenesis, collagen production, and epithelial wound closure. Moreover, the inflammatory activity in wounds in these animals was found to be markedly decreased compared with controls.\(^{37}\)

In contrast to TNF-\(\alpha\) and IL-1\(\beta\), we found that TGF-\(\beta\) mRNA levels were increased in buccal tissue from the rhGM-CSF-treated animals. TGF-\(\beta\) is known to play a significant beneficial role in inflammation, angiogenesis, re-epithelialization, and connective tissue regeneration during wound healing. This cytokine stimulates the accumulation and proliferation of fibroblasts, which is required for efficient wound repair.\(^{38,39}\)

The most commonly recognized function of GM-CSF is stimulation of stem cells to differentiate into granulocytes (neutrophils, eosinophils, and basophils) and monocytes. In this study we focused on its effects in wound healing. GM-CSF interacts with specific receptors on fibroblasts to promote their proliferation, and additionally activates macrophages to remove damaged cells.\(^{40}\) The healing action of GM-CSF originates from these effects.

In this study, we observed reduced TNF-\(\alpha\) and IL-1\(\beta\) mRNA expression and increased TGF-\(\beta\) mRNA expression in the rhGM-CSF groups in the early phase of wound healing (on day 5). These results indicates that GM-CSF therapy could regulate the expression of cytokines involved in wound healing, which then accelerate ulcer re-epithelialization and mucosal tissue regeneration. IL-1\(\beta\) mRNA expression was decreased, but not much significantly. Although therapeutic effects of rhGM-CSF were evident on day 4, it cannot rule out the possibilities that these changes in the cytokine may be due to the result of early healing. To more clearly elucidate whether or not this decrease is shown as a therapeutic effect of rhGM-CSF and obtain a direct mechanism-based data. Topical application of GM-CSF on ulcerative buccal mucosa is thus expected to accelerate healing by suppressing the inflammatory reaction and reestablishing the epithelium.

In conclusion, plant-produced rhGM-CSF treatment was shown to increase the wound healing rate in animals with induced oral mucositis, especially early in the healing process. These results have significant implications for the development of rhGM-CSF as a therapeutic agent for ulcerative oral mucositis and plants expression system as an alternative system for recombinant therapeutic protein.

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