Oral Administration of Recombinant Human Granulocyte Colony-stimulating Factor in the Management of Radiotherapy-induced Esophagitis

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ABSTRACT

Radiation-induced esophagitis often results in treatment interruption, which may severely affect the probability of control of the local disease in patients undergoing chest radiotherapy (RT). No effective regimen that would reduce the incidence and severity of this complication has been identified up to now. Although acceleration of oral mucosal healing using topical recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) has been reported, the mechanism of such an interaction remains obscure. Effective topical application of rhGM-CSF for the treatment of radiation-induced esophagitis has never been reported in the past. In pharmacological studies, we observed that glycerol exerts a remarkable stabilizing effect on rhGM-CSF immunoreactivity. After studying the kinetics of esophageal emptying with nuclear imaging, we proposed a rhGM-CSF regimen that could be applied for topical treatment of esophagitis during RT. The regimen was applied for 5 consecutive days in a cohort of 36 patients undergoing chest RT, immediately after the documentation of grade 3 esophagitis. RT was not interrupted. Mucosal biopsies were performed endoscopically and examined immunohistochemically. Regression of dysphagia to grade 0/1 was observed in 19 of 36 (52%) patients, whereas grade 2 dysphagia persisted in 12 of 36 (33%) patients. Progression of dysphagia was seen in 5 of 36 (14%) patients. Recurrence of severe esophagitis within 5–8 days after rhGM-CSF therapy was observed in 7 of 31 (22%) patients with initial response to rhGM-CSF. Four of these patients presented significant improvement of symptomatology after additional rhGM-CSF medication. In immunohistochemical studies, active intraepithelial neovascularization and thymidine phosphorylase and vascular endothelial growth factor overexpression were observed in the damaged epithelium, which was not accompanied by macrophage or neutrophil infiltration. We conclude that rhGM-CSF topical therapy (p.o. administration) exerts a significant therapeutic effect against RT-induced esophagitis. The rhGM-CSF mucosal healing effect is probably due to its direct angiogenic activity and/or to the potentiation of the activity of other angiogenic factors released by the damaged epithelium.

INTRODUCTION

The importance of overall RT duration in the outcome of RT is well established for a variety of human carcinomas (1, 2). Radiation-induced esophagitis is the most important early side effect during RT of chest tumors (3), which often leads to treatment interruption for several days. Recent combinations of novel agents produce a high esophageal toxicity rate (4, 5). Therefore, the elimination of esophagitis, which is a principal cause for radiation treatment delay, may significantly improve the results of RT as well as preserve the patients’ quality of life during treatment.

Several supportive therapies, such as chlorexidine, benzodamamine, antibiotics, or even anti-ulcer agents have been used in the past, and the efficacy of such treatments is strongly questioned (6–9). s.c. use of granulocyte colony-stimulating factors and GM-CSFs, widely used in clinical practice to prevent and treat chemotherapy-induced neutropenia, has been shown to accelerate the healing of radiation- and chemotherapy-induced oral mucositis (10–13). It seems that local application of the growth factors may also have a significant effect (14–16). Although the mechanism by which GM-CSF promotes the mucosa healing remains unknown, it is postulated that it involves a direct effect on the mucosa in conjunction with an indirect activity via neutrophil or macrophage activation (12).

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3 The abbreviations used are: RT, radiotherapy; GM-CSF, granulocyte macrophage colony-stimulating factor; rhGM-CSF, recombinant human GM-CSF; VEGF, vascular endothelial growth factor; PEG, polyethylene glycol; MoAb, monoclonal antibody.
The role of p.o. administration of GM-CSF in the prevention of the acute esophageal toxicity of RT has never been studied. Although GM-CSF can easily be applied locally in the oral cavity, it is unknown whether the amount of the cytokine that remains on the esophageal walls after swallowing is clinically effective. Indeed, previous attempts to treat radiation-induced esophagitis with rhGM-CSF diluted in water failed to confirm a substantial benefit (data not shown). In the present Phase I and II study, we studied the kinetics of esophageal emptying and tried to identify which is the best solvent for oral administration of rhGM-CSF. A rhGM-CSF oral regimen was established thereafter and tested in a Phase II study. Moreover, we immunohistochemically examined biopsies from normal esophagus and irradiated esophagus to identify the possible mechanisms underlying the GM-CSF healing effect.

MATERIALS AND METHODS
Pharmacological Studies. Studies on rhGM-CSF mouthwashes use a concentration of 10 μg/ml in water three times a day. For esophageal application, repeated swallowing of a small quantity of the solution is indispensable to assure the prolonged presence of the growth factor on the esophageal walls. Esophageal motility may rapidly wash out the growth factor. The stability of the factor within the solvent may also be important because maintenance of high concentrations of the active factor for several hours by repeated p.o. intake is essential. Moreover, we postulated that a solvent with high viscosity could be useful because it would increase the contact time of the solution with the walls of the upper digestive tract.

rhGM-CSF is a 127-amino acid glycoprotein, and as such, it is assumed to be easily hydrolyzed. Glycerol is a high viscosity solvent that has been unsuccessfully used in the past for the treatment of radiation-induced esophagitis (17). We studied the effect of glycerol and of PEG on the stabilization of the rhGM-CSF molecule in solution by quantitatively measuring rhGM-CSF in an ELISA. rhGM-CSF (Mielogen) was provided by Schering-Plow SA, and glycerol (99.5%), and PEG (M, 8000) was provided by Sigma.

rhGM-CSF (8 ng) dissolved in sterile double-distilled water was incubated in sterile Eppendorf tubes in a total volume of 50 μl in the absence or presence of a 25%, 50%, or 75% final concentration of glycerol or a 25% final concentration of PEG. The samples (in triplicate) were incubated at 37°C for 1, 2, 8, or 24 h. At each time point (including time 0), the relevant samples were removed and stored at −80°C. Samples were collected at all time points, diluted with sterile double-distilled water, and tested by ELISA using the standardized human GM-CSF ELISA kit Endogen. We standardized the commercially available kit for optimal and reproducible quantitative determination of immunologically reactive rhGM-CSF in the presence of even higher glycerol concentrations. As controls in these assays, we used rhGM-CSF hydrolyzed by incubation in the presence of trypsin at 37°C for 30 min or denatured by heating at 56°C for 30 min.

Esophageal Emptying Studies. The esophageal emptying of water and glycerol (75%) was tested in five patients with non-small cell lung cancer before the beginning of RT. Informed consent was obtained from all patients. 10 mCi of 99mTc-radiolabeled sulfur colloid was diluted in 6 ml of water or glycerol. The patient was told to swallow the water solution and immediately lie on the bed of a SPECT scintigraphic camera (General Electric). The patient was also told to swallow once or twice during the 5-min imaging time upon the doctor’s recommendation. One h later, after swallowing 500 ml of water to wash the esophageal walls, the test was repeated using the solution of glycerol. Ten frames of 30 s each were taken. Scintigraphic images were collected and analyzed by the computer program provided by the camera. The total activity of the radioactive solution that was retained in the pharynx, esophagus, and stomach was analyzed by drawing the regions of interest on the related areas.

Phase II Study. This study was approved by the local study review and ethics board. Informed consent was obtained from all patients. Using the solutions and the swallowing recommendations suggested by the pharmacology and nuclear studies, 36 patients with locally advanced stage IIIb non-small cell lung cancer undergoing radical RT were recruited into a Phase II study to assess the efficacy of rhGM-CSF in alleviating radiation-induced dysphagia. Table 1 shows patient and treatment characteristics.

RT treatment planning was based on recent chest computed tomography scans. Anteroposterior radiation portals encompassing the primary tumor and part of the mediastinum were used to deliver a daily dose of 1.8 Gy with a LINAC 6 MV (Philips). More than two-thirds of the esophagus length was included in these fields in all patients. The homolateral supraclavicular area was also included in patients with an upper lobe mass. One or two oblique fields directed to the bulky tumor area were used to increase the tumor dose/fraction to 2.4 Gy. This additional dose was given immediately after treatment of the two anteroposterior fields (concomitant boost technique), with no interfraction interval. Patients received a normalized total dose (calculated with time correction for α:β ratio = 10 Gy) of 60 Gy (2). The planned overall treatment time was 5 weeks. The radiation dose delivered to the spinal cord (α:β ratio = 2 Gy) was less than 44 Gy.

<table>
<thead>
<tr>
<th>Table 1 Characteristics of patients recruited in the Phase II study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of patients</strong></td>
</tr>
<tr>
<td><strong>Age [yrs; mean (range)]</strong></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
</tr>
<tr>
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</tr>
<tr>
<td>Adenocarcinoma</td>
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<tr>
<td>Undifferentiated</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
</tr>
<tr>
<td>Stage IIIb</td>
</tr>
<tr>
<td><strong>RT field dimensions</strong></td>
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<tr>
<td>Large field</td>
</tr>
<tr>
<td>Boost</td>
</tr>
<tr>
<td><strong>Previous chemotherapy</strong></td>
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<tr>
<td>None</td>
</tr>
<tr>
<td>Pretreated</td>
</tr>
<tr>
<td>Taxane-based chemotherapy</td>
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<tr>
<td>Platinum-based chemotherapy</td>
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<tr>
<td>Anthracycline-based chemotherapy</td>
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</tbody>
</table>
Table 2 Immunologically reactive rhGM-CSF (pg/ml) at different time points and in different solutions, as assessed by ELISA

<table>
<thead>
<tr>
<th>rhGM-CSF/Time</th>
<th>0 h</th>
<th>1 h</th>
<th>2 h</th>
<th>8 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhGM-CSF in water</td>
<td>1430</td>
<td>495 (−65%)</td>
<td>235 (−84%)</td>
<td>100 (−93%)</td>
<td>29 (−98%)</td>
</tr>
<tr>
<td>rhGM-CSF in 25% glycerol</td>
<td>1344</td>
<td>885 (−34%)</td>
<td>706 (−47%)</td>
<td>420 (−69%)</td>
<td>274 (−80%)</td>
</tr>
<tr>
<td>rhGM-CSF in 50% glycerol</td>
<td>1434</td>
<td>1031 (−28%)</td>
<td>859 (−40%)</td>
<td>542 (−62%)</td>
<td>329 (−77%)</td>
</tr>
<tr>
<td>rhGM-CSF in 75% glycerol</td>
<td>1467</td>
<td>1043 (−29%)</td>
<td>950 (−36%)</td>
<td>807 (−45%)</td>
<td>681 (−53%)</td>
</tr>
<tr>
<td>rhGM-CSF in 25% PEG</td>
<td>1390</td>
<td>966 (−30%)</td>
<td>880 (−37%)</td>
<td>512 (−63%)</td>
<td>201 (−86%)</td>
</tr>
</tbody>
</table>

RESULTS

Glycerol Increases rhGM-CSF Stabilization. Table 2 shows the percentage of immunologically reactive rhGM-CSF at different time points and in different solutions. Glycerol exerts a remarkable stabilizing effect on rhGM-CSF immunoreactivity. One hour after incubation at 37°C in the presence of 50–75% glycerol, 71% of the rhGM-CSF immunoreactivity is still detectable, whereas only 35% of immunoreactive rhGM-CSF was detected in the control sample in the absence of glycerol. A stabilizing action on rhGM-CSF comparable to that of glycerol was also produced by PEG at a final concentration of 25%.

rhGM-CSF Esophageal Emptying Rate. The rate of esophageal emptying of the radioactive material was found to be similar for both water and glycerol solutions. About half (45 ± 8%) of the total radioactivity was distributed in the oropharyngeal and hypopharyngeal region, 31 ± 8% of the total radioactivity was in the esophageal area, and 20 ± 6% of the total radioactivity was distributed in the esophageal area. Increasing the volume of the swallowed liquid resulted in an increase in gastric accumulation, whereas pharyngeal and esophageal retention remained unchanged (data not shown). Esophageal radioactivity remained stable throughout the 5-min observation period, and the only factor effecting an immediate decrease by approximately 20–30% was swallowing (Fig. 1).

Prescription of rhGM-CSF Oral Administration. Taking into account the data mentioned above, the rhGM-CSF solution should be given according to strict rules. The patient should prepare 800 μg of rhGM-CSF in 5 ml of water for injection and then dissolve this in 20 ml 95% glycerol. Six ml of the solution should then be taken p.o., and the patient should stay in a supine position for 10 min and try to avoid swallowing. During the next 30-min period, the patient should avoid intake of any food or fluid. The procedure stated above should be repeated four times within 4–5 h. When not in use, the rhGM-CSF solution should be kept at 4°C.

Biopsies and Immunohistochemistry. A total of 12 of 36 patients underwent an endoscopic evaluation of the esophagus before the beginning of RT and 2 days after the end of the 5-day rhGM-CSF treatment. Biopsies were performed in the normal areas (before RT or outside the radiation portals) and damaged areas (within the radiation portals) of the esophagus. Tissue samples were fixed in formalin and embedded in paraffin. Two-μm sections were placed on poly-L-lysine-coated slides. Samples were immunostained using the alkaline phosphatase/antialkaline phosphatase technique for CD31 endothelial cell antigen (DAKO), CD68 monocyte-specific antigen (DAKO), CD3 pan-lymphocyte antigen (DAKO), and CD15 granulocyte antigen (DAKO). Moreover, we examined the expression of two angiogenic factors: (a) thymidine phosphorylase (platelet-derived endothelial cell growth factor, P-GF.44C MoAb; Oxford, United Kingdom; Ref. 19); and (b) VEGF (VG1 MoAb; Oxford, United Kingdom; Ref. 20).

Phase II Study. A 5-day treatment with the rhGM-CSF in glycerol regimen was given to 36 patients undergoing chest RT immediately after the documentation of grade 3 dysphagia. This early radiation toxicity appeared in 10 of 36 patients during the third week, in 22 of 36 patients during the fourth week, and in all 36 patients during the fifth week. Glycerol was generally well tolerated, with most of patients complaining of its very sweet, nauseating, taste. Six of 36 patients complained of gastric pain and were supported with omeprazole (20 mg/day, p.o.).

Although RT was not interrupted, a significant, impressive relief was reported even within 24 h. Fig. 2A shows the rate of resolution of dysphagia during the 5-day therapy period. Dramatic regression of dysphagia to grade 0/1 was observed in 19 of 36 (52%) patients, whereas grade 2 dysphagia persisted in 12 of 36 (33%) patients. Progression of dysphagia to severe grade 4, requiring hospitalization and i.v. fluid administration, was seen in 5 of 36 (14%) patients. These patients were supported as
described previously, and there was no need to insert a naso-gastric feeding tube because all patients with grade 4 esophagitis regressed to grade 3 within 3 days after RT interruption. However, recurrence of grade 3/4 esophagitis within 5–8 days after rhGM-CSF therapy was observed in 7 of 31 (22%) patients with an initial response to rhGM-CSF, which points out the need for treatment continuation. Four of these patients presented significant regression of esophagitis after additional rhGM-CSF glycerol medication. The regression of esophagitis did not seem to depend on the time of its onset.

**Immunohistochemistry.** Biopsies from normal esophageal mucosa showed the expected papillary histological structure with an inner basal layer and an outer strata of differentiated cells. Epithelial cells were well orientated, and CD31 staining showed scarce vessels at regular distances (Fig. 3A). Thymidine phosphorylase and VEGF were weakly expressed or absent.

CD64, CD15, and CD3 did not reveal any inflammatory cell component.

Biopsies from irradiated areas showed a damaged epithelium with degenerative changes and a marked lack of organization (Fig. 3, B and C). Necrotic debris and occasional lymphocytic infiltration (CD3 or even CD31 staining) were also observed. CD31 staining revealed focal areas of intense intraepithelial neovascularization, with an apparent dilation of the stained capillaries (Fig. 3, B and C). A strong cytoplasmic and nuclear thymidine phosphorylase reactivity (Fig. 3D) as well as a strong VEGF cytoplasmic expression was observed in the regenerating epithelial cells. No monocytes positive for CD68 or thymidine phosphorylase or granulocytes positive for CD15 could be identified. All 12 biopsies were from patients that responded to treatment. All five patients with progressive esophagitis refused endoscopy.

**DISCUSSION**

There is plenty of evidence that GM-CSF is active in the prevention and restoration of the early mucosal damage induced by both chemotherapy and RT. Gabrilove et al. (10) reported a study in which the s.c. administration of granulocyte colony-stimulating factor in patients with urological cancer receiving methotrexate-based chemotherapy significantly reduced the incidence of mucositis. A reduction of mucosal toxicity obtained by systemic administration of rhGM-CSF has also been reported in patients receiving high-dose chemotherapy with or without total body irradiation before bone marrow transplantation (21–24). A protective effect of systemic administration of rhGM-CSF against radiation-induced mucositis has also been reported (25).

In a recent study by Chi et al. (12), advanced-stage head and neck carcinoma patients treated with chemotherapy were
randomized to receive s.c. rhGM-CSF for 10 days after the first or second cycle of chemotherapy. In this study, the protective effect of rhGM-CSF seemed to be irrelevant to the neutrophil counts, and a direct effect on the oral mucosa was suggested. Local application of rhGM-CSF may therefore be more effective by exposing epithelia (or other mucosa cell components) to high growth factor concentrations. In a study by Ibrahim and al-Mulhim (16), rhGM-CSF mouthwashes were used to treat neutropenia-independent oral mucositis. A water solution of 5 \( \mu \text{g/ml} \) was used four to six times daily immediately after mucositis documentation. A rapid decrease in the severity of mucositis was observed within 2 days in 29% of patients. Reynoso et al. (26) also reported a significant attenuation of mucositis in patients treated with chemotherapy and allogeneic bone marrow transplantation by the application of rhGM-CSF mouthwashes. However, in a randomized trial with increasing concentrations of rhGM-CSF, mouthwashes did not reveal any protective effect (15).

Esophageal toxicity is a major problem during RT for lung cancer (3). Combinations of RT with novel chemotherapeutic agents result in a significant increase of severe dysphagia, which usually becomes dose-limiting (4, 5). Protracted overall RT time results in reduced local disease control (2). Previous trials using rhGM-CSF diluted in water in patients with severe radiation esophagitis failed to show convincing evidence of therapeutic activity (data not shown). In a preclinical study, we observed that rhGM-CSF disintegrates rapidly in water solution. This observation may well explain the failure of the randomized study by Cartee et al. (15) to show a protective effect of rhGM-CSF against oral mucositis. No more than 16% of the active drug remains within 2 h at 37°C. It is therefore important that studies using rhGM-CSF mouthwashes pay special attention to the instructions for the preparation and use of the solution. We found a significant stabilizing effect of glycerol on rhGM-CSF immunoreactivity, which is of importance if the prepared solution is to be used multiple times within the same day. Despite the high viscosity of glycerol, scintigraphic tests revealed that the permanence of the drug on the pharyngeal and esophageal walls depends mainly on the solution volume swallowed and the number of swallowing movements. About 20% of the 6-ml radioactive liquid was distributed in the esophagus. It was therefore suggested that repeated oral administration of small quantities, the patient in a supine position, and limiting swallowing to a minimum (at least for the following 10 min) would result in a 30–40-min exposure of the esophageal mucosa to a high concentration of rhGM-CSF.

In a Phase II trial, we used 800 \( \mu \text{g} \) of rhGM-CSF in 75% glycerol solution (25 ml), which was divided in four equal doses and consumed p.o., strictly following the instructions, within 4–5 h (once a day) by 36 patients with grade 3 radiation-induced esophagitis. An impressive reversal of esophagitis, even during RT continuation, was noted. These dramatic effects should be attributed to the stabilizing effect of glycerol on rhGM-CSF as well as the strict ritual for its administration, which was suggested by the previously conducted nuclear studies of esophageal emptying. Although the study was not randomized, its
design allows reliable conclusions on GM-CSF efficacy. Grade 3 radiation esophagitis is always expected to progress to grade 4 unless RT is interrupted. Although a 1-week treatment interruption is usually enough for the restoration of esophagitis, sometimes a longer interval may be necessary (4). In the present study, chest RT continued despite the appearance of grade 3 esophagitis. Instead of progression of its severity, a rapid regression of esophagitis was noted in 85% of cases (50% regressed to grade 0/1, and 33% regressed to grade 2). Recurrent esophagitis occurred in only 22% of responders, and retreatment was effective in more than half of the cases. The reason why 15% of cases did not respond to therapy or why 22% of responders recurred during RT continuation may be that different radiosensitivities of endothelial or epithelial cells exist among individuals. A wide range of lymphocyte and fibroblast sensitivity to radiation among individuals has been confirmed (27, 28), and recent studies suggest that a genetic susceptibility to DNA damage may exist in the general population (29). Impaired ability to repair DNA may account for the failure of GM-CSF to accelerate restoration of the mucosal damage in a subset of patients.

The mechanism of rhGM-CSF activity in the repair of RT-damaged mucosa remains unknown, although there is evidence that the peripheral blood leukocyte status does not interfere with the process (13). The immunohistochemical studies performed in irradiated esophageal mucosa show an activation of angiogenic factor expression by epithelial cells and a prominent, patchy, irregular, intraepithelial formation of new vessels. In a recent study, Bussolino et al. (30) showed that rhGM-CSF exerts a direct activity on endothelial cell proliferation and migration and also potentiates the angiogenic activity of other angiogenic factors. A direct activation of Janus-activated kinase 2 (JAK2) on endothelial cells after GM-CSF binding to surface receptors has been reported recently (31). The irregular angiogenesis and overexpression of thymidine phosphorylase and VEGF by the irradiated esophageal epithelium observed in our study may show that neovascularization is important in the restoration of a normal epithelium lining during and after RT. The exogenous addition of rhGM-CSF would increase the stimulatory effect of other angiogenic factors produced by the epithelial component, which would accelerate endothelial cell migration, proliferation, and new vessel formation. Inflammatory cells such as macrophages and neutrophils were absent, whereas the presence of lymphocytes was frequently noted. We reported previously that lymphocytic tumor infiltration, together with endothelial cell migration, was frequently observed in tumor areas with thymidine phosphorylase expression (32). Thymidine phosphorylase and lymphocyte interplay may also be important in the normal tissue healing processes. A direct stimulatory effect of rhGM-CSF on epithelial cells to enhance the production of angiogenic factors or even a direct role on epithelial cell growth (33) cannot be excluded. In future studies, a comparative evaluation of angiogenesis in responders versus nonresponders would allow a more reliable conclusion on the role of angiogenesis in the healing of irradiated esophageal mucosa.

We conclude that exposure of esophageal mucosa to high, clinically effective concentrations of rhGM-CSF after oral administration of GM-CSF in glycerol solution during RT for chest tumors is feasible. A significant therapeutic effect against radiation-induced esophagitis has been verified in the present study. We also provide evidence that the mucosa-healing effect of rhGM-CSF may be linked to activation of intraepithelial angiogenic pathways. rhGM-CSF may directly enhance endothelial cell migration and proliferation and may potentiate the activity of other angiogenic factors released by the irradiated epithelium.

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