DIFFERENTIAL EFFECT TRIGGERED BY A HEPARAN MIMETIC OF THE RGTA FAMILY PREVENTING ORAL MUCOSITIS WITHOUT TUMOR PROTECTION

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Purpose: Oral mucositis is a common side effect induced by radio/chemotherapy in patients with head and neck cancer. Although it dramatically impairs patient quality of life, no efficient and safe therapeutic solution is available today. Therefore, we investigated the protective efficacy of a new heparan mimetic biopolymer, RGTA-OTR4131, used alone or in combination with amifostine, for oral mucositis and simultaneously evaluated its effect on tumor growth in vitro and in vivo.

Methods and Materials: A single dose of 16.5 Gy was selectively delivered to the snout of mice, and the effects of OTR4131 or amifostine-OTR4131 were analyzed by macroscopic scoring and histology. The effect of OTR4131 administration on tumor growth was then investigated in vitro and in xenograft models using two cell lines (HEP-2 and HT-29).

Results: Amifostine and OTR4131 significantly decreased the severity and duration of lip mucosal reactions. However, amifostine has to be administered before irradiation, whereas the most impressive protection was obtained when OTR4131 was injected 24 h after irradiation. In addition, OTR4131 was well tolerated, and the combination of amifostine and OTR4131 further enhanced mucosal protection. At the tumor level, OTR4131 did not modify HEP-2 cell line clonogenic survival in vitro or protect xenografted tumor cells from radiotherapy. Of interest, high doses of OTR4131 significantly decreased clonogenic survival of HT-29 cells.

Conclusions: RGTA-OTR4131 is a well-tolerated, natural agent that effectively reduces radio-induced mucositis without affecting tumor sensitivity to irradiation. This suggests a possible transfer into the clinic for patients’ benefit. © 2009 Elsevier Inc.

Heparan mimetic, Dextran derivative, RGTA, Radiation-induced mucositis, Radioprotector.

INTRODUCTION

Oral mucositis (OM) is a common acute toxicity associated with radiation therapy and various chemotherapy regimens of head and neck cancers. These anticancer treatments cause erythematous and erosive and ulcerative mucositis, leading to impaired nutrition, enhanced risk of infection, and rapid deterioration of patient quality of life. OM can also result in delayed treatment and dose reductions, which in turn may affect the probability of local tumor control.

Although pathogenesis of acute mucositis is a complex process, the pathogenic paths are now well described and rely on four interdependent phases: (1) an initial inflammatory/vascular phase in which paracrine mediators including pro-inflammatory cytokines; (2) an epithelial phase that results from direct impact of radiation and chemotherapy on dividing cells and from indirect damages triggered by paracrine factors; (3) a mixed ulcerative/bacteriological phase due to epithelial depletion, which promotes colonization of...
abnormal oral flora; and finally (4) a healing phase involving granulation tissue formation and epithelial renewal. This later phase may lead to permanent or transient structural and functional recovery that may or may not be followed by delayed fibrotic reactivation (1).

Current therapies for acute OM are mostly for symptom control, and recent analysis of results obtained by 11 clinical trials lead to the recommendation of amifostine use as prophylactic treatment to prevent acute OM occurrence (2). Amifostine’s efficacy is mostly based on its free-radical scavenger properties and provides significant tissue protection when injected intravenously before irradiation (2). Its use is limited by side effects including hypotension and skin reactions (3–6). Recently, palifermin, an FGF7-based therapy, has indeed proved its efficacy in preventing OM in preclinical models and patients (7). This growth factor–based therapy raises specific issues regarding secondary tumor promotion. We investigated an alternative wound-healing-prone approach using innovative engineered biopolymers termed RGTAs (ReGeneraTing Agents). RGTAs have been developed during the past decade and are now being developed for clinical applications within a biotechnology company (SAS). RGTAs mimic the protecting and potentiating properties of heparin sulfates toward heparin binding growth factors (HBGF). They enhance repair of bone (8), skin (9), muscle (10), and digestive tissues including the gums (11, 12), and aid in treatment of 5-FU-induced mucositis (13). The mechanism of action involves protection of endogenous HBGF and correction of collagen abnormalities by modulation of FGF-2 and TGF-β1 pathways (9, 14–21).

We investigated the relevance of a specific RGTA derivative, RGTA-OTR4131, for the treatment of OM in head and neck cancer patients undergoing radiotherapy. Therefore, we used a simplified preclinical model of radiation-induced OM using C57B6 mice and large single fraction of irradiation known to induce very severe mucositis and investigated various sequences of RGTA-OTR4131 administration. In a first intent, amifostine was used as a reference to be compared with RGTA-OTR4131, and we found similar radioprotective effect of both agents. Interestingly, an almost complete protection of the oral mucosa was found when amifostine and OTR4131 were combined, suggesting an additive effect of the two compounds. Finally, the radioprotective action of OTR4131 on tumor was addressed in vitro and in vivo using head and neck cancer cell line and showed no tumor protection, suggesting clinical interest.

MATERIALS AND METHODS

Reagents

Heparan mimetic: The heparan mimetic RGTA-OTR4131 is a synthetic derivative of dextran T40 composed of about 200 glucosidic units linked through 1–6 bonds. The polymeric structure of OTR4131, characterized by the average substitution degree (SD) of carboxymethyl (CM), sulfate (S), and acetate (Ac) groups per glucosidic unit, is defined as follows: $SD_{CM} = 0.5, SD_{S} = 1.1, SD_{Ac} = 0.2$ (Fig. 1). This structural characterization was confirmed by prod-
Effect of RGTA in tumor growth

Animals and diet: Female Balb/c nude mice 6–8 weeks old (Janvier CERT, Le Genest) were used. During the experimental period, 5–10 animals were housed per plastic cage and the temperature (22 ± 2°C), lighting (12 h), and humidity (60% ± 10%) in the animal room were under control. Animals were used in compliance with the European Union recommendations on laboratory animal care. The experimental basal diet used in this study was solid food ad libitum (R.03, F91360, U.A.R., Villemoisson, France) and water.

Tumor cell lines: HEP-2, a human pharynx carcinoma cell line, and HT-29, a human colorectal carcinoma cell line, were maintained in MEM medium (Eurobio, Les Ulis, France) supplemented with 10% fetal calf serum, 0.1% L-glutamine, and 0.2% penicillin-streptomycin at 37°C in a 5% CO₂ humidified atmosphere.

Tumor xenografts: HEP-2 and HT-29 tumor xenografts were obtained by subcutaneous injection of 3 × 10⁶ cells in the right flank of nude Balb/c mice. When the tumor size reached a mean of 5 mm, animals were randomly allocated to groups of 8–9 mice treated with (a) IR alone; (b) IR and an IP injection of RGTA-OTR4131 (1 mg/kg or 40 mg/kg in 100 μL saline) 24 h after IR; (c) amifostine (200 mg/kg) 10 min before IR; (d) IR with the amifostine-OTR4131 combination; (e) an IP administration of OTR4131 (1 mg/kg) without IR; (f) no treatment (controls; Fig. 3). IR consisted of a 15-Gy dose delivered to the tumor with a 225-kV X-ray radiation source with a 0.5 mm Cu filter. IR was locally confined to the tumor with the rest of the body lead shielded, mice were restrained by plastic supports without use of anesthesia. Tumor response was defined as a relative change in tumor volume compared with the volume at the beginning of therapy. The tumor volume was estimated from two dimensional tumor measurements by the following formula: tumor volume = length (mm) × width² (mm⁴)/2 (24). Tumor volume was measured twice weekly.

Microvessel density assessment: Once mice were sacrificed, tumors were removed, fixed (Finefix, Milestone), and stained with hematoxylin and eosin and immunostained with smooth muscle actin for perivascular pericytes. The quantification of stained vessels on immunostained slides was automatically achieved with PixCyt, a software designed by the GRECAN (Centre François Baclesse, Caen, France). This system combines a dedicated slide scanner (Nikon 8000) and computer-assisted image analysis.

Clonogenic assays: HEP-2 and HT-29 cells were seeded in triplicate in 25-cm² flasks. A single dose of 0–6 Gy of IR was delivered by a 137Cs source 4 h after cells were seeded. For each dose delivered, flasks were subdivided into two groups with or without adding OTR4131 (10, 100, 300, or 500 μg/mL) 2 h after irradiation. After 12 days, colonies were fixed and stained with crystal violet. Only colony compounds exhibiting at least 50 cells were counted, and plating efficiency (colonies counted/cells seeded) was calculated. Surviving fraction was expressed as the following ratio: plating efficiency treated / plating efficiency control.

Statistical analysis: The effects of treatments on the mouse lip mucosa were assessed using mixed models analyzing the Parkins score at D(x). Similarly, the effects of treatments on tumor volume were assessed using mixed models analyzing the tumor volume at
D(x) and taking into account the initial tumor volume. This model allowed us to analyze repeated measurements even when there was a difference in the number of measurements per mouse. The analysis of the leukocyte infiltration was also performed by mixed model at D9 and D19, taking into account the number of measurements per mouse.

The percentage of cells growing without OTR4131 and of cells growing with each dose of OTR4131, which led to a colony compared by logistic regression. These comparisons were performed in irradiated and unirradiated cells.

RESULTS

**RGTA-OTR4131 administration prevents acute radiation-induced mucositis**

Monitoring of OM in mice (n = 8–10) was performed using the macroscopic Parkins scoring system (Table 1), which defines severe OM with a score >2. Exposure to single large dose of IR induced a biphasic response with two types of damage, the first and most severe occurring 9–11 days after IR, and the second, milder type occurring 15–19 days after irradiation. Several sequences of OTR4131 were investigated, and treatments given 3 or 24 h after IR were found the most efficient because only the first lesion type remained but was greatly attenuated with maximum scores equal to 1.5 and 1.2, respectively. The difference between the RGTA-OTR4131 (1 mg/kg)-treated and IR groups was statistically significant when RGTA-OTR4131 was administered 3 h after IR (p = 0.01) and highly significant (p = 0.0009) when OTR4131 was administered 24 h after IR (Fig. 4a). Amifostine treatment administered before IR displayed similar radioprotective effect, and no statistically significant difference in the lip mucosal reactions was observed between amifostine and OTR4131 groups (3 h, p = 0.30; 24 h, p = 0.90; Fig. 4a). Interestingly, the combined administration of amifostine 10 min before IR with OTR4131 24 h after IR yielded a striking protective effect and almost completely abrogated macroscopic damages induced by 16.5-Gy irradiation on the lip mucosa (p = 0.0003; Fig. 4b).

**RGTA-OTR4131 administration prevents development of radiation-induced structural damages in the mucosa**

Histopathological analysis was performed within the time course of the experiment. Figure 5a showed regular histology in nonirradiated tissues: smooth and thin keratinized epithelium with a rectilinear basement membrane was observed. Submucosal and muscle bundle layers were well organized with normal connective tissue composed of scanty cells and few blood vessels. Nine days after IR (Fig. 5b), marked tissue damage was apparent with edema of the submucosa and muscle. In patchy areas, epithelium exhibited an amorphous appearance consistent with cell necrosis. Muscle layers were dystrophic, with some muscle bundles also showing zones of necrosis. Epidermal and hair follicle depletion after IR was accompanied by an increase of collagen classically defined as granulation tissue. Administration of RGTA-OTR4131 and amifostine improved tissue structure,
Fig. 5. (a) Normal labial mucosa 100×, hematoxylin and eosin and Masson’s staining (b) 9 days after irradiation (IR) and (c) 19 days after IR. Histological analysis was performed on labial mucosa, as described in Methods and Materials. Five experimental groups were studied: a control (normal skin) group, an irradiation group, a group treated 10 min before IR with amifostine (200 mg/kg, intraperitoneal [IP’]), an IR group treated 24 h after with OTR4131 (1 mg/kg, IP) and an irradiated group treated with both amifostine (200 mg/kg, IP) and with OTR4131 (1 mg/kg, IP) 24 h after IR.
mucosal thickness, and collagen deposition were reduced. Interestingly, thin epithelial layer with papillae and hair follicles was maintained in the RGTA-OTR4131-treated group; however, vascular dilatation persisted. In mice treated with the combination of OTR4131 and amifostine, the improvement was further enhanced.

Nineteen days after irradiation (Fig. 5c), epithelial barrier integrity was restored, but severe epithelial hypertrophy was observed. In addition, significant collagen deposition consistent with prefibrotic remodeling was observed within the submucosa. Amifostine treatment did not improve mucosal or submucosal structure. However, treatment with OTR4131 and a combination of OTR4131 and amifostine improved tissue recovery, with a histological structure similar to that observed in normal lips. Epidermis was thin, and collagen deposition was reduced.

Quantification of acute inflammation was performed by counting the number of infiltrating leukocytes. The number of inflammatory cells infiltrating mucosa and submucosa 9 days after IR was 3.2-fold higher compared with that found in normal mucosa. Nine and 19 days after IR, leukocyte infiltration was significantly reduced by OTR4131 treatment when administered 24 h after IR \( (p < 0.0001) \) and restored to the level measured in the normal mucosa in groups treated with the combination of OTR4131 and amifostine \( (p < 0.0001) \) between IR and IR+OTR4131+amifostine; \( p = 0.13 \) at Day 9 and \( p = 0.77 \) at Day 19 between control without IR and IR+OTR4131+amifostine). Amifostine also decreased leukocyte infiltration in a statistically significant manner \( (p < 0.0001) \) at Day 9 and \( p = 0.001 \) at Day 19; Fig.6).

OTR4131 does not affect radiation sensitivity or protect tumor from irradiation

Intrinsic radiosensitivity assessed in vitro did not show any significant influence of 10 \( \mu \)g/mL OTR4131 on HEP-2 cell clonogenic survival \( (p = 0.06) \) (Fig. 7a). However, high doses of OTR4131 (100, 300, 500 \( \mu \)g/mL) significantly decreased clonogenic survival of HT-29 cells \( (p < 0.0001) \). In addition, in this cell type (HT-29), 300 and 500 \( \mu \)g/mL OTR4131 decreased surviving fraction by 25% \( (p < 0.0001) \); Fig. 7b).

RGTA-OTR4131 (1 mg/kg IP) does not affect tumor growth delay of HEP-2 cells engrafted into nude mice (Fig. 8a). The effect of low and high doses of OTR4131, combined or not with amifostine was investigated using two different cell lines, HEP-2 and HT-29, xenografted.

**Fig. 7.** (a) Clonogenic assays for HEP-2 cells \( (p = ns) \). (b) Clonogenic assays for HT-29 cells. In unirradiated cells, there is a significant decrease in clonogenic survival in the presence of 100, 300, and 500 \( \mu \)g/mL OTR4131 \( (p < 0.0001) \). In cells irradiated with 2 Gy, there is a significant inhibition of clonogenic survival in the presence of 300 and 500 \( \mu \)g/mL OTR4131 \( (p < 0.0001) \), but not in the presence of 100 \( \mu \)g/mL \( (p = ns) \).
None of the treatments affected tumor growth delay, and none induced any kind of toxicity in mice (Fig. 8b).

Histopathological analysis (Fig. 8d and 8e) of the intratumoral vascularization was performed using smooth muscle actin staining in HT-29 xenograft. Interestingly, amifostine-OTR4131 combination seemed to decrease the number of intratumoral vessels but remained statistically nonsignificant (Table 2).

**DISCUSSION**

Conventional treatment by radiotherapy for head and neck cancer consist of high doses of IR that inevitably lead to development of severe acute side effects. In addition, new radiotherapy regimens such as accelerated radiation therapy (26) and the combination of radiotherapy with new targeted drugs such as erbitux (27, 28) improve therapeutic index but also increase acute toxicities, including severe mucositis, which may lead to tissue necrosis. Therefore, new strategies that could prevent occurrence of these severe side effects without interfering with anticancer treatment are highly anticipated.

We investigated the potential protective action of a new compound derived from the RGTAs family on severe radiation-induced OM. RGTAs-related compounds are known as potent anti-inflammatory and pro-healing agents (8–13, 22), and here we show for the first time that RGTA-OTR4131 protects mouse lip mucosa from severe acute radiation injury, remarkably decreasing the severity and duration of mucositis. The experimental model of OM used in these experiments was induced by local IR using a single X-ray fraction of

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<th>Table 2. Microvascular density: Percentage of vascular stained areas vs. cancer cell compartment</th>
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<td><strong>Percentage of vascular stained areas versus cancer cell compartment (%)</strong></td>
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<tr>
<td>IR 15 Gy</td>
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<td>IR+RGTA 1 mg/kg</td>
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*Abbreviation: IR = irradiation.*
RGTA protects radiation-induced mucositis ● M. Mangoni et al.

16.5 Gy. Previous dose escalation experiments led us to define 16.5 Gy as the optimal single fraction dose usable to induce severe lip reaction without causing death. This irradiation regimen has been extensively used in our laboratory (29, 30) and others (23). One could argue that a single fraction of 16.5 Gy is irrelevant to the clinical situation, but this model displays various advantages: first, it is less stressful to mice (animals are immobilized in plastic supports during irradiation); second, it induces severe acute mucositis, which is the worst situation with regard to therapeutic intervention. We reasoned that obtaining a therapeutic gain with RGTA-OTR4131 in this extreme condition would be a good indicator of efficacy in the less damaging settings induced by fractionated irradiation. Further experiments are currently ongoing to evaluate the protective effect of OTR4131 after fractionated irradiation to determine the efficient sequence of administration. The mechanisms involved in OTR4131 protective action at the normal tissue level remain to be investigated, but we can speculate on its well-established anti-inflammatory properties. RGTA are also known to enhance tissue repair (17), neutralize metalloproteases responsible for tissue destruction (14–16), and neutralize collagen synthesis and deposition (18).

In this study, the most efficient timing of administration in mice was a single IP injection delivered 24 h after IR. It provided a protection similar to that obtained with amifostine, which is protective only when given before IR. More interesting, the combination of amifostine and OTR4131 provided an almost complete protection at the normal tissue level without any tumor protection. The isoeffect equation D/D1 = d1 + (α/β) / d2 + (α/β), where d1 and d2 are dose for fraction of two different schedules and D1 and D2 total doses (31), suggests that 16.5 Gy can be expected to induce similar oral damages than a total dose of 30 Gy given with a fraction size of 3 Gy (32) or 30 Gy with a fraction size of 6 Gy (33). This means that OTR4131 should be efficient when administered after completion of fractionation regimen. This postradiation efficacy is highly relevant to clinical practice because it would avoid interference with the anticancer action of radiotherapy. However, considering the marked radioprotective activity in normal tissues, we directly investigated whether OTR4131 had an effect on tumor growth after IR. Therefore, we used one cell line derived from head and neck carcinoma (HEP-2) and checked the relevance of the findings with HT-29 (colon carcinoma). Clonogenic survival assays performed in vitro showed no modulation of HEP-2 and HT-29 radiation sensitivity by OTR4131. This result was confirmed in vivo in HEP-2 and HT-29 xenograft mouse models in which OTR4131 did not display any tumor-protecting property whether or not combined with IR. Interestingly, high doses of OTR4131 inhibited HT-29 tumour cell growth in vitro. This later result suggests that OTR4131 might trigger an organ-specific antitumor effect that is worth further preclinical investigation. In addition, the absence of tumor protection found in vivo encourages us to study OTR4131 more extensively for clinical application in the treatment of radiation-induced mucositis.

In conclusion, our study demonstrates that OTR4131 exhibits marked protective activity against radiation-induced mucositis in mice without tumor protection. The combination of OTR4131 and amifostine completely abrogates radiation-induced mucositis, as confirmed by histopathological analyses. We are continuing investigations to elucidate the selective activity of these drugs on healthy tissues.

REFERENCES