An Engineered Biopolymer Prevents Mucositis Induced by 5-Fluorouracil in Hamsters

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Oral mucositis is a common, treatment-limiting, and costly side effect of cancer treatments whose biological underpinnings remain poorly understood. In this study, mucositis induced in hamsters by 5-fluorouracil (5-FU) was observed after cheek-pouch scarifications, with and without administration of RGTA (RG1503), a polymer engineered to mimic the protective effects of heparan sulfate. RG1503 had no effects on 5-FU-induced decreases in body weight, blood cell counts, or cheek-pouch and jejunum epithelium proliferation rates, suggesting absence of interference with the cytotoxic effects of 5-FU. Extensive mucositis occurred in all of the untreated animals, and consisted of severe damage to cheek pouch tissues (epithelium, underlying connective tissue, and muscle bundles). Only half of the RG1503-treated animals had mucositis, over a mean area 70% smaller than in the untreated animals. Basement membranes were almost completely destroyed in the untreated group but was preserved in the RG1503 group. RG1503 blunted or abolished the following 5-FU-induced effects: increases in matrix metalloproteinase (MMP)-2, MMP-9, and plasmin, and decreases in tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. These data indicate that mucositis lesions are related to massive release of proteolytic enzymes and are improved by RG1503 treatment, this effect being ascribable in part to restoration of the MMP-TIMP balance. RG1503 given with cancer treatment might protect patients from mucositis. (*Am J Pathol* 2004, 164:739–746)

Mucositis is a common and debilitating side effect of radiation therapy and chemotherapy for cancer.¹ The lesions develop in the oral and gastrointestinal tract mucosae, most notably at nonkeratinized sites. Mucositis of the oral cavity affects the cheeks, lips, soft palate, ventral

surface of the tongue, and mouth floor. The ulcerative lesions are painful, restrict food intake, and often act as a portal of entry for indigenous oral flora.² Mucositis is a source of morbidity, adversely affects quality of life, and incurs substantial economic cost. The risk of mucositis is influenced by the diagnosis, patient age, pre-existing oral health, and type and frequency of treatment administration.³

The cytotoxic agents used to treat cancer impair the proliferation of basal epithelial cells in the oral mucosa. The result is an atrophic epithelium that is susceptible to spontaneous or traumatic ulceration.⁴ Other factors, including changes in the endothelium and extracellular matrix, may contribute to the pathogenic process.⁵ The biological events underlying the development of mucositis are poorly understood. In particular, the molecular mechanisms responsible for tissue damage have not been investigated. To date, no treatments capable of reliably preventing or treating mucositis are available. Of the many agents that have been used in an attempt to improve mucositis, many are merely symptomatic.⁶ GM-CSF and rhIL-11, which modulate inflammation, protect connective tissue, and inhibit apoptosis, have been recently tested.⁵

Using a hamster model, we evaluated the effects of a RGTA (regenerating agent), namely RG1503, on mucositis. RGTAs are polymers engineered to mimic some of the protective effects of heparan sulfate and used to enhance tissue repair and regeneration. These compounds are potent anti-inflammatory agents. *In vivo*, RGTAs modify inflammation kinetics,⁷ and *in vitro* they directly inhibit the activities of plasmin, cathepsin G, and neutrophil elastase,^{8–10} which may contribute to the genesis of mucosal epithelial damage. RGTAs protect heparin-binding growth factors against proteolytic degradation and enhance their bioavailability,^{11,12} allowing them to stimulate tissue repair, as shown in several *in vivo* models.^{7,13–15} These properties prompted us to investigate whether RGTA administration prevented chemotherapy-induced mucositis by protecting against tissue damage and/or stimulating tissue repair.

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Materials and Methods

RGTA Synthesis

The RGTA used in this study was a carboxymethyl sulfate dextran (CMDS-RGTA, code no. RG1503) synthesized from a native T40 dextran (molecular weight, 40 kd; Pharmacia Fine Chemicals, Uppsala, Sweden). Briefly, sequential substitutions with carboxymethyl groups were performed on any of the three hydroxyl groups of the dextran glucose residues, and benzamide and/or O-sulfonate (= sulfate) groups were added. The purified derivatized dextran was ultrafiltered, and its chemical composition was determined by microanalysis and spectrophotometry.⁹

Experimental Procedures

Forty-five male golden Syrian hamsters (Centre d'Elevage Dépré, St. Doulchard, France) weighing 100 to 120 g each were used in compliance with European Union recommendations on laboratory animal care. They received food and water *ad libitum*. One group (15 animals) received no treatment and served as the control group. Mucositis was induced in the other animals as follows: on days 0, 5, and 10, the animals received an intraperitoneal injection of 5-fluorouracil (5-FU) (60 mg/kg; Sigma Chemical Co., St. Louis, MO), and on days 1, 2, and 3, both cheek pouches of each animal were everted under gaseous anesthesia (Forene; Abbot, St. Remy, France), three parallel 1-cm scarifications were made using a dental burr rotating at low speed. On days 4 and 12, saline was injected intramuscularly in the left hind leg of 15 hamsters (sham-treated group). The remaining 15 hamsters were treated in the same conditions with RG1503 (1.5 mg/kg in saline) (RG1503-treated group).

On day 15, 2 hours before sacrifice, each animal received an intraperitoneal injection of bromodeoxyuridine (BrdU, 60 mg/kg; Sigma). The animals were killed after anesthesia with an 8% chloral hydrate solution (Prolabo, Fontenay, France) injected intraperitoneally. After exsanguination by cardiac puncture, the cheek pouches were everted and standardized photographs were taken under a Tessovar Photomacrographic Zoom System (Zeiss, Oberkochen, Germany) for visual evaluation of the lesions. The photographs were used to assess the presence and mean area of mucositis lesions.

Tissue Sampling

The right and left cheek pouches were gently dissected out. The left cheek pouch was halved. One half was transferred to Hanks' balanced salt solution complemented with 1.5 mmol/L Ca^{2+} (Life Technologies, Stafford, TX) and was incubated at 37°C. The medium was sampled after 48 hours and frozen (−20°C) until use. The other half of the pouch was stored in RNAlater (Ambion, Austin, TX) at −20°C for evaluation of mRNA expression.

The right cheek pouches were fixed in 40% ethanol at 4°C. The samples were dehydrated by increasingly concentrated alcohol solutions, embedded in methyl methacrylate (Merck, Darmstadt, Germany), and polymerized at −20°C. They were then processed for sectioning in a Polycut E microtome (Leica, Wetzlar, Germany). Serial 4- μm sections were cut. A segment of jejunum, an intestinal site characterized by a high proliferation index, was sampled and processed in the same way as the right cheek pouch.

Histological Study and Morphometry

The sections were either stained with toluidine blue (pH 3.8) and Masson's trichrome for morphological analysis or processed for immunohistochemistry of selected markers.

To reveal BrdU-positive cells, sections were treated with 0.1% trypsin (20 minutes at 37°C, Sigma) and then 2 mol/L HCl (30 minutes) to denature the DNA. After incubation (20 minutes at 37°C) with normal horse serum (Vector, Burlingame, CA), the sections were exposed first to monoclonal anti-BrdU antibody (1:1000, 2 hours at 37°C; Sigma) and then to the secondary biotinylated antibody [mouse anti-horse immunoglobulin (Ig) G, 1:200, 90 minutes at room temperature; Vector]. After treatment with 3% hydrogen peroxide (10 minutes) and avidin-biotin peroxidase complex (ABC Vectastain kit, Vector) for 60 minutes, 3,3'-diaminobenzidine tetrahydrochloride (Sigma) was used as the chromogen. Phosphate-buffered saline (PBS) (0.1 mol/L) was used for washing between incubations. Negative controls were prepared by omitting the primary antibody, replacing the primary antibody with rabbit nonimmune serum at the same dilution, or using an irrelevant secondary antibody (goat anti-rabbit Ig G instead of mouse anti-horse IgG).

Laminin was revealed using a rabbit polyclonal anti-rat antibody (1:25; Sigma) cross-reacting with hamster antigen. Mouse monoclonal antibodies directed against proforms and active forms of matrix metalloproteinase (MMP)-2 and MMP-9 (1:100), type IV collagen (1:100), and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 (1:50; France-Biochem, Meudon, France) were also used. The sections were treated with 1% glycine (Sigma) for 30 minutes at room temperature. After incubation with the primary antibody for 2 hours at room temperature, the sections were incubated with the secondary biotinylated antibody (goat anti-rabbit IgG for laminin and horse anti-mouse IgG for the other molecules, 1:200, 90 minutes; Vector). Subsequent processing and evaluation of immune reaction specificity were as described above.

Morphology was assessed at $\times 260$ or $\times 420$, using a semiautomatic image analyzer. Mucositis severity was assessed by measuring cheek pouch tissue thickness (in μm , 60 measurements per section). In six random fields per section of cheek pouch and jejunum, the BrdU-positive cell count was determined; this count was expressed as the percentage of epithelial cells and served as an indicator of cell proliferation.

Zymography Evaluation of Gelatinases A (MMP-2) and B (MMP-9)

MMP-2 and MMP-9 were identified in the supernatant of Hanks' media (Life Technologies), using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis impregnated with 1 mg/ml of gelatin I (60 V for 10 minutes and then 140 V for 45 minutes). The gels were washed twice for 30 minutes in 2.5% Triton X-100 (Bio-Rad, Hercules, CA) after migration to renature and activate the proteins. They were incubated 20 hours at 37°C in 100 mmol/L Tris-HCl, 5 mmol/L CaCl₂, 0.005% Brij-35 (Bio-Rad), 0.001% NaN₃, pH 8.8. They were stained with 0.25% G250 Coomassie blue (Bio-Rad), and cleared in 40% methanol and 10% acetic acid. Proteases in supernatant are correlated with proteases in tissue.¹⁶ To assign the lysis bands to an MMP class, ethylenediaminetetraacetic acid (30 mmol/L) or 4-2-aminoethylbenzene sulfonyl, fluoride hydrochloride (Pefabloc, 2 mmol/L), both MMP inhibitors, and the serine protease inhibitor phenylmethyl sulfonyl fluoride (1 mmol) were added to the incubation buffer (all inhibiting agents from Sigma). The standard was supernatant from cultured HT1080 fibrosarcoma cells, an established cell line constitutively expressing MMP-2 (72 kd) and MMP-9 (92 kd).

The zymograms were recorded with a video camera. The average area (Ar, in pixels) of lysis bands was measured semiautomatically by following the contour. The black and white images generated by the camera were converted to images in 256 shades of gray and analyzed using mathematical morphology software. Complete digestion of the substrate corresponded to gray level 255 and absence of hydrolysis to gray level 0. Enzyme activity evaluated comparatively with that of the HT1080 cell line was expressed in gelatinase activity units (GAU = [Ar_{gl}]/[Ar_{HT1080-gl_{HT1080}], where gl is the gray level), and gelatinase activity per mg of tissue was expressed as arbitrary units (AU = GAU/tissue weight).}

Western Blotting Evaluation of MMP-2, MMP-9, TIMP-1, and TIMP-2

Ten percent sodium dodecyl sulfate-polyacrylamide gel electrophoreses were run under Laemmli conditions. Total proteins (20 µg per lane) from Hanks' supernatants were electrotransferred onto a nitrocellulose membrane (Bio-Rad) (400 mA for 1 hour). Nonspecific binding sites were blocked for 1 hour [0.1% PBS + 0.05% Tween 20 (TPBS) and 5% skim milk powder]. The membranes were incubated with primary mouse monoclonal MMP-2, MMP-9, TIMP-1, or TIMP-2 antibodies¹⁷ (all 1:500; France-Biochem) in TPBS 1% skim milk powder (2 hours at room temperature) and with the secondary antibody (goat anti-mouse, 1:1000, 2 hours). At all steps, the membranes were rinsed in TPBS 1% skim milk powder. Peroxidase activity was detected by chemiluminescence (Western Blot Chemiluminescence Reagent Plus; NEN, Boston, MA). Controls were prepared by omitting the primary antibody.

Figure 1. Changes in body weight during the 15-day experimental period. The healthy controls slightly gained weight, while all of the 5-FU-treated hamsters rapidly lose body weight. The RG1503 treatment had no significant influence on this consequence of 5-FU administration. The animals had an average loss of 23% of their initial weight on day 15. *, $P < 0.05$ versus the corresponding controls.

Determination of Plasmin and uPA

Plasmin activity in the supernatant was measured using the S-2251-based assay (Chromogenix, Mölndal, Sweden) on 96-well microtiter plates incubated at 37°C for 1 hour. Plasmin generation was detected by measuring *p*-nitroaniline release from the substrate at 410 nm using a microplate reader. Control experiments performed without substrate showed that the samples contained no intrinsic enzyme activity. Urokinase plasminogen activator (uPA) activities were measured using the S-2444-based assay (Chromogenix) as above. Activities were corrected for tissue weight (in mg).

Statistics

Data were compared by nonparametric tests (Kruskal-Wallis test followed, if significant, by group comparisons with the Mann-Whitney *U*-test). Differences were considered significant when *P* values were < 0.05 . All data are given as means $\pm$ SEM.

Results

Animals in the two 5-FU-treated (sham- and RG1503-treated) groups lost weight as compared to the controls (-23% , $P < 0.004$ on day 15; Figure 1). The two 5-FU-treated groups did not significantly differ at any time point. No other differences in physical health or behavior were noted between the 5-FU-treated groups. Neither were any differences in absolute blood cell counts or leukocyte differential counts found between the two 5-FU-treated groups at sacrifice (data not shown).

Gross Evaluation of the Lesions

All sham-treated animals had mucositis in both cheek pouches, seen as bulbous lesions over a large area of inflamed mucosa. Among the RG1503-treated animals, only half (7 of 15) had mucositis (chi-square test, $P < 0.001$), which was far less extensive than in the sham-treated group, the mean area involved being 70% smaller ($P < 0.001$) (Figure 2).

Figure 2. Macroscopic views of mucositis on day 15 after induction. **a:** Sham-treated animal. A verrucose lesion overlays a large area of inflamed mucosa. **b:** RG1503-treated animal. The lesion is restricted to a small zone of inflamed mucosa. When lesions were present in the RG1503-treated animals, the mean mucositis area (in cm^2) was strongly reduced. *, $P < 0.001$ versus the sham-treated group. Scale bar, 0.5 cm.

Histological Study and Morphometry

Control cheek pouch tissues were thin, with a smooth keratinized epithelium, a rectilinear basement membrane, a submucosal layer, and a muscle bundle layer (Figure 3a). The submucosa was primarily fibrous, with a scanty cell population and a few blood vessels. Sham treatment induced marked tissue modifications with considerable thickening because of edema of the submucosa and muscle. The surface of the pouch had an irregular warty appearance. The basement membrane was rectilinear. In places, the epithelium exhibited an amorphous appearance consistent with cell necrosis (Figure 3b). Some muscle bundles showed zones of necrosis (Figure 3c). With RG1503 treatment, tissue thickness was reduced as compared to the sham-treated group but remained increased as compared to the con-

Figure 3. Mucositis-induced tissue disturbances. **a:** Epithelium from a control animal. The tissue is thin with a well-individualized keratin layer (small arrows). Toluidine blue staining. **b:** Localized amorphous change of the epithelium (arrows) involving the peripheral cell layers; this feature probably corresponds to cell necrosis. The epithelium is thick with enlarged intercellular spaces. Toluidine blue staining. **c:** Zone of muscle necrosis (arrow). Masson's trichrome. **d:** MMP-2 immunostaining showing presence of the enzyme at the periphery of muscle bundles (arrows) corresponding to zones of muscle necrosis. Scale bars, 20 μm .

Table 1. Changes in Cheek Pouch Thickness (in μm)*

	Cheek pouch thickness
Control group	254.46 $\pm$ 32.4
Sham-treated group	1163.03 $\pm$ 208.4 [†]
RG1503-treated group	419.65 $\pm$ 93.35 [‡]

* Mucositis strongly thickened the pouch tissue, while RGTA treatment also completely controlled tissue changes. Evaluation was made in histological sections (15 hamsters per group). $m \pm \text{SE}$.

[†] $P < 0.005$ from control group.

[‡] $P < 0.01$ from sham-treated group.

rol group (although not significantly). Surface irregularities and necrotic zones were visible.

Sham treatment increased cheek pouch thickness by 460% as compared to controls ($P < 0.0001$). The RG1503 treatment strongly reduced this parameter (-78% , $P < 0.001$ versus the sham-treated group) (Table 1). Sham treatment decreased the BrdU-positive cell count in both the pouch and jejunum epithelia (-80% , $P < 0.005$ versus controls). RG1503 treatment had no effect on this decrease (Figure 4). Except for the reduction in proliferating cells, no histological change was seen in the jejunum mucosa.

Immunostaining for laminin, a major component of basement membranes, was continuous and thick in the control animals. In the sham-treated group, the laminin layer was reduced to a few islets. Laminin labeling was absent around the muscle fibers, at the boundary with the underlying connective tissue. In contrast, laminin immunostaining in the RG1503-treated group was unchanged as compared to the controls (Figure 5). Type IV collagen immunostaining showed the same pattern as for laminin.

Immunostaining for MMP-2 was faint and scattered throughout the connective tissue in the control animals. A similar distribution pattern was seen in the sham-treated group, but the staining was more pronounced. Localized areas of dense MMP-2 immunostaining corresponded to zones of muscle necrosis seen in adjacent Masson's trichrome-stained sections (Figure 3d). In the RG1503-treated group, no immunostaining for MMP-2 was detected; MMP-2 was not expressed by the keratinocytes near the basement membrane or around the muscle bundles.

In the sham-treated group, MMP-9 immunostaining was present under the epithelial basement membrane, within the connective tissue, and around the muscle bun-

Figure 4. Proliferation rate in the two different epithelium (in percentage of total epithelial cells). The 5-FU treatment strongly reduced mitosis in the cheek pouch and the jejunum (the latter was chosen for further measures because its epithelium exhibited a higher renewal rate). The RG1503 treatment did not interfere with the anti-proliferative property of 5-FU. $P < 0.005$ versus the control group.

Figure 5. Immunostaining for laminin. **a:** Control group. The laminin layer was continuous and thick along the basement membrane of both the epithelium (arrows) and muscle bundle (arrowhead). **b:** In the sham-treated group, the laminin layer was almost completely destroyed with only some islets persisting along the basement membrane of the epithelium (arrows) and adjacent muscle bundles (arrowhead). **c:** RG1503-treated animal. The laminin layer was well preserved along the epithelium and around the muscle bundles. Scale bar, 50 μ m.

dles (Figure 6). In the RG1503-treated group, MMP-9 immunostaining was faint in the connective tissue and absent near the muscles. No MMP-9 immunostaining was seen in the control cheek pouch tissues.

TIMP-1 and TIMP-2 immunostaining was faint in the connective tissue and mucositis lesions from sham-treated animals. In the RG1503-treated group, TIMP-2 staining was strong in the connective tissue and around the muscle fibers but was absent along the epithelial basement membrane, and TIMP-1 was expressed within the connective tissue. No TIMP-1 or TIMP-2 immunostaining was found in the control animals.

Activities and Presence of Proteinases in Hanks' Medium

Plasmin activity was increased in the sham-treated group (+25%, $P = 0.01$ versus the control group) but remained at the control value in the RG1503-treated group (Figure 7). As compared to the controls, uPA activity was decreased in the sham-treated group (Figure 7) and in the RG1503-treated group (−40% and −42%, respectively; $P = 0.01$ versus controls for both comparisons).

ProMMP-2 (64 kd) and MMP-2 (58 kd) zymography activities were markedly increased in the sham-treated group (+233%, $P = 0.02$ and +181%, $P = 0.01$ versus the control group, respectively) (Figure 8). In the RG1503-treated group, in contrast, these activities were

similar to those in the controls. Neither proMMP-9 nor MMP-9 activity was detected in the controls. In the sham-treated group, proMMP-9 (102 kd) and MMP-9 (90 kd) were markedly increased ($P = 0.01$ and $P = 0.001$, versus the control group, respectively). In the RG1503-treated group, smaller increases were found (−50% for pro-MMP-9, $P < 0.05$; and −75% for MMP-9, $P = 0.03$, respectively, versus the sham-treated group). Adding ethylenediaminetetraacetic acid or Pefabloc to the incubation buffer abolished the gelatinolytic bands on the zymograms, whereas adding phenylmethyl sulfonyl fluoride had no effect.

Protein content was evaluated by Western blotting (Figure 9). Staining for proMMP-2 and MMP-2 was faint in the control group and strong in the sham-treated group. In the RG1503-treated group, staining was faint for proMMP-2 and virtually absent for MMP-2. TIMP-2, the naturally occurring MMP-2 inhibitor, was markedly reduced in the supernatants from sham-treated tissue samples as compared to controls, and this effect was partly countered by RG1503. ProMMP-9 protein was not detected in the supernatants from control animals. ProMMP-9 was increased in the sham-treated group, and again this effect was attenuated by RGTA treatment. MMP-9 was low in supernatants from the control and sham-treated groups and was undetectable in supernatants from the RG1503-treated group. TIMP-1, the naturally occurring MMP-9 inhibitor, was reduced in the

Figure 6. Immunostaining for MMP-9 (gelatinase B). **a:** In this mucositis sample the immunostaining was sparse in the connective tissue, suggesting a generalized reaction. **b:** A strong immunostaining underlined the basement membrane in this mucositis animal. Scale bar, 20 μ m.

Figure 7. Proteolytic activities in the cheek pouch supernatants as assessed by a spectroscopic technique. Plasmin activity behaves differently under RG1503 treatment. uPA activity decreased in all 5-FU-treated groups. *, $P < 0.01$ versus the control group. +, $P < 0.01$ versus the sham-treated group.

sham-treated group, and this effect was attenuated by RG1503.

Discussion

In this study we attempted to gain insight into the biological underpinnings of 5-FU-induced mucositis lesions by collecting quantitative data, of which none are available to date in the literature. At the same time, we obtained objective information on the potential of RG1503 as a therapeutic agent for mucositis induced by 5-FU therapy. We found that RG1503 treatment either prevented or attenuated mucositis.

Mucositis Induced by 5-FU

In this hamster model, mucositis became visible 5 days after 5-FU treatment and culminated on day 15,¹⁸ this was the reason why we decided to test RG1503 treatment at this moment to fully appreciate its possible preventive effect. Indeed, florid lesions were seen over an inflammatory mucosa, as well as marked thickening and irregularity of cheek pouch tissues. All pouch tissues were affected; in particular, the muscle bundles located deep in the submucosa were edematous and showed patchy necrosis. At this late stage, no inflammatory cell infiltrate was observed. As expected, 5-FU induced a marked

Figure 8. Gelatinolytic activities in the supernatants of cheek pouches cultured for 2 days in Hanks' medium. The sham-treated animals had a strong increase of the zymogen and active forms of MMP-2 and MMP-9. The RG1503 treatment lowered the gelatinolytic activities. AU (arbitrary units) = gelatinolytic activity units (GAU)/mg of cultured tissue (see the text for definition of GAU). *, $P < 0.01$; **, $P < 0.001$ versus the control group. +, $P < 0.01$; ++, $P < 0.001$ versus the sham-treated group.

Figure 9. Western blot for MMP-2, MMP-9, TIMP-1, and TIMP-2. Mucositis enhanced MMP-2 and MMP-9 and depressed TIMP-1 and TIMP-2. In contrast the RG1503 treatment normalized these molecules to controls. Only the complexed forms of the TIMPs were evidenced on the blots. **Line 1**, control group; **line 2**, sham-treated group; **line 3**, RG1503-treated group.

decrease in epithelial cell proliferation, rendering the pouch lining susceptible to mechanical trauma. Cell lysis also contributed to disorganize the epithelium, but apoptosis did not occur, at least at this stage (data not shown). Fragmentation of the laminin and type IV collagen layers was noted, indicating basement membrane destruction. The epithelium seemed detached from the submucosa, a mechanism that may contribute to epithelium stripping.

These structural alterations were found to reflect a metabolic imbalance. The basement membrane degradation strongly suggested release of proteolytic enzymes in the mucosa. Plasmin and MMPs hydrolyze various basement membrane components, including laminin^{19,20} and type IV collagen.^{10,21} In our study, plasmin activity in the supernatants was significantly increased, whereas uPA was decreased, suggesting that the uPA pool had not been replenished by new synthesis and/or that the enzyme had been destroyed by specific proteases. Plasmin and uPA activate various hydrolytic enzymes, including proMMP-2 and proMMP-9, via dose- and time-dependant mechanisms.^{22,23} Plasmin generated on the cell surface can also activate MMP-1 and MMP-3, which in turn activate the gelatinases.²² Activation of proMMP-9 can also be mediated by MMP-2.²⁴ Accordingly, we found that MMP-2 and MMP-9 activities were strongly increased in the mucositis lesions, as compared to the control tissues. Moreover, MMPs levels as assessed by Western blotting were elevated in the mucositis lesions, whereas their naturally occurring inhibitors TIMP-1 and TIMP-2 were markedly diminished. This imbalance in the TIMP/MMP ratio probably indicates a major role for MMPs in the genesis of mucositis lesions.

Because of its experimental design, this study does not provide information on the kinetics of mucositis onset

and development. In particular, data on leukocyte infiltration are lacking; this may be of interest because polymorphonuclear leukocytes and macrophages release significant amounts of proteolytic enzyme on activation.

Treating Mucositis with RG1503

RG1503 prevented mucositis in approximately half the animals and reduced its severity in the other half. From the reductions in area and thickness of the lesions, we estimated that the mean lesion volume decreased 15-fold with RG1503 treatment. The improvements in mucositis lesions were assessed using a range of criteria that accurately identified RG1503 targets.

Importantly, RG1503 had no influence on cell proliferation in the pouch or jejunum epithelia, indicating that this agent did not interfere with the effects of 5-FU treatment. Thus, RG1503 may protect the mucosa without reducing the effectiveness of chemotherapy. Additional support for this selective effect is the absence of differences in body weight and blood cell count changes between the two 5-FU-treated groups.

Although some epithelial necrosis and surface irregularities persisted with RG1503 therapy, tissue thickening and muscle necrosis were markedly reduced. These improvements occurred despite the decrease in epithelial cell proliferation detected as a decrease in BrdU-positive cell counts. The presence of MMP-9 along the basement membrane and its marked reduction after RG1503 treatment suggest a role for this enzyme in the basement membrane destruction associated with mucositis. Protection of the basement membrane was demonstrated by the preservation of laminin and type IV collagen immunostaining in the RG1503 group. Similarly, the improvement in muscle necrosis was associated with preservation of a laminin-positive basement membrane around the muscle bundles. Protection of basement membrane integrity by RG1503 has been reported also in a model of ischemic muscle healing.²⁵ We found in an earlier study that RG1503 enhanced type III collagen deposition within the epithelium basement membrane of periodontal pockets in hamsters.⁷ As laminin and type IV collagen are heparin-binding molecules,²⁶ RG1503 may also mimic the properties of heparan sulfate toward these molecules. Indeed, we speculate that RG1503 may bind to the multiple heparin-binding sites of various matrix glycoproteins, such as laminin,²⁰ fibronectin, rendered accessible during the course of inflammation after degradation by heparinase of the natural heparan sulfates, as well as interact with specific heparin-binding peptide sequences of other basement membrane proteins such as collagen IV.¹⁹ As RG1503 is not degraded by heparanase (data not shown), we expect that these interactions are stable and the subsequent protection of these molecules lasts during the healing process. Moreover, the matrix-bound RGTA may maintain or reconstitute an appropriate three-dimensional architecture allowing the binding and activity of heparin-binding growth factors.

The reductions in tissue lesions provided by RG1503 reflected stabilization of the enzymatic cascade induced

by mucositis. RG1503 depressed plasmin activity but had no effect on uPA activity, in keeping with *in vitro* experiments in which RG1503 inhibited plasmin activity by binding to regulatory noncompetitive enzyme sites without affecting uPA activity.⁹ Downstream, MMP-2 and MMP-9 amounts and activities were markedly reduced by RG1503 treatment, in accordance with previous results from animals with periodontal disease.⁷ Heparin affects the transcription of various MMPs, including MMP-2 and MMP-9,²⁷ and the heparin-mimetic RG1503 may have similar effects. RG1503 treatment also increased the amounts of TIMP-1 and TIMP-2, the naturally occurring inhibitors of these gelatinases. Western blot analyses showed only TIMP within complexes, suggesting that RG1503 may have prevented the dissociation of MMP-TIMP complexes. Thus, RG1503 seems to be a potent anti-inflammatory agent in this mucositis model. RG1503 treatment either prevented the development of mucositis or reduced the severity of the lesions, and one of the mechanisms underlying this effect was normalization of the MMP-TIMP balance.

Other mechanisms may underlie the RG1503 effect on mucositis. RGTAs protect heparin-binding growth factors, such as fibroblast growth factor-1 and -2 and transforming growth factor- β , from proteolytic degradation, and act as low-affinity receptors by increasing interactions with their specific receptors.^{11,12,28} This may have enhanced healing of the tissues jeopardized by the local and systemic injuries. Interestingly, transforming growth factor- β and keratinocyte growth factor (or fibroblast growth factor-11, also a heparin-binding growth factor) reduce mucositis severity in the same experimental model.^{29,30} RG1503 may have protected and enhanced the actions of these heparin-binding growth factors. Also, heparin inhibits lipopolysaccharide binding to blood cells, reducing the release of proinflammatory cytokines,³¹ such as interleukin-1, possibly a key cytokine in mucositis.³ This is of interest because oral gram-negative bacteria colonize the injured mucosa tissues, aggravating mucositis severity.³² As heparan sulfate mimetics, RGTAs may share similar properties with heparin. The conjunction of these biological properties may account for the strong effect of RG1503 on hamster mucositis. The choice of RG1503 as a potential drug for mucositis treatment is further justified by its low *in vitro* anti-coagulant activity compared to heparin (at least 10-fold less in time thrombin test) and the *in vivo* absence of anti-coagulant activity in plasma after intravenous injection of 50 mg/kg of RGTA (unpublished data).

Considering its remarkable effectiveness in this mucositis model, RG1503 may prove valuable in preventing or improving mucositis, a common side effect of therapy that adversely affects the health and quality of life of cancer patients.

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