
Effects of ceramide inhibition on experimental radiation-induced oral mucositis

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Objective. Oral mucositis (OM) is a common toxicity of ionizing radiation (IR), which is used as treatment for head and neck cancer. Ceramide-mediated apoptosis may contribute to the pathogenesis of mucositis. In response to IR or other cellular stresses, ceramide production occurs either by the hydrolytic action of sphingomyelinase (SMase) or de novo via ceramide synthase.

Study design. Male golden Syrian hamsters (10 per group) exposed to a single dose of 40 Gy ionizing radiation (day 0) were treated with subcutaneous 0.2 mL injections of either neutral SMase, acidic SMase, or ceramide synthase inhibitor (5 mmol/L glutathione, 5 μ mol/L desipramine, or 1 μ mol/L fumonisin B₁, respectively) from day -1 to day 16. A control group was treated with saline. Two blinded examiners assessed clinical OM development from day 6 to day 26. Two animals per group were killed on days 3, 10, and 16 for immunohistochemical detection of ceramide expression in both the epithelium and in the connective tissue.

Results. The group exposed to fumonisin B₁ exhibited a statistically significant reduction in mean daily weight gain, mean mucositis score, duration of mucositis, and expression of ceramide in the epithelium on day 3 as well as in the connective tissue on days 10 and 16 relative to control. Immunohistologic analysis also revealed significant differences in ceramide expression on days 3 and 16 for animals treated with glutathione in both the epithelial and connective tissue when compared to the control.

Conclusions. These results suggest that IR triggers early de novo ceramide production and that inhibition of this process attenuates OM on a clinical level.

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Oral mucositis (OM) is a common side effect of radiation used to treat cancers of the head and neck. It is often of such severity as to require analgesics and is a cause of hospitalization, breaks in therapy, and feeding-tube placement.¹

The underlying biological events contributing to the pathogenesis of mucositis are still being defined and multiple pathways leading to epithelial cell death seem likely.² One such pathway involves radiation-induced activation of transcription factors such as nuclear factor-

kappaB, and their subsequent upregulation of genes that control the production of deleterious proteins. Alternatively, it has been hypothesized that nontranscription-dependent mechanisms also contribute to mucosal injury. One such mechanism is ceramide-mediated apoptosis.

Ceramide is a membrane sphingolipid consisting of an N-acylated (14 to 26 carbons) sphingosine (18 carbons). In response to various cellular stressors, such as radiation, it stimulates a number of target enzymes such as cathepsin D, kinase suppressor of RAS (KSR), cRAF, serine/threonine protein phosphatases (PP1 and PP2A), and PKC- ζ .³ The end result for all these pathways is growth arrest or caspase-initiated protein disassembly and subsequent cell death.³

The extent to which ceramide-mediated apoptosis activated by radiation damages tissue remains unclear. At present, it appears that at single doses of IR that result in 1 log of cell kill in vitro, apoptosis accounts for less than 50%, and more often only 10% to 25%, of cell death.^{4,5} However, certain cell types may be inherently resistant or susceptible to ceramide-driven apoptosis in response to IR. In fact, it is the primary method of cell kill in endothelial, lymphoid, hematopoietic, gastrointestinal crypt stem and some types of tumor cells.^{4,6,7}

Preliminary reports of some of the elements of this study were presented at the 39th American Society of Clinical Oncology (ASCO) Annual Meeting, Chicago, Ill, May 2003.

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The conjecture that radiation triggers the production of ceramide is relatively uncontested. The exact method by which ceramide levels increase, however, is in great dispute. Investigators describe 2 major mechanisms of ceramide production: hydrolysis of sphingomyelin and de novo formation. The former method involves conversion of membrane sphingomyelin to ceramide through the action of sphingomyelinase (SMase). Acute increases in ceramide concentration occur within 1 to 2 minutes of stress induction in this scenario.⁸ Several types of SMase, identified based upon pH optima, cellular location, and cation dependence, appear to exist: acidic lysosomal SMase (A-SMase); neutral membrane-bound Mg-dependent SMase (N-SMase); neutral Mg-independent SMase present in the myelin sheath and leukemic cell cytosol; neutral nucleic Mg- and dithiothreitol-stimulated SMase; neutral SMase reported in chromatin and envelope of rat liver cell nuclei; alkaline SMase; and Zn-dependent SMase.⁹ Of these, A-SMase and N-SMase have been most studied for their roles in ceramide-mediated apoptosis.

Particular stimuli may initiate different SMase enzymes. Published data show that in response to Fas ligand, 1 α ,25-dihydroxyvitamin D3 γ -interferon, chemotherapeutic agents (Ara C, daunorubicin), retinoic acid, heat stress, hydrogen peroxide, ischemia/reperfusion, arachidonic acid, glutathione depletion, interleukin-1 (IL-1) α and β , IR, and especially tumor necrosis factor (TNF) α , increases in N-SMase activity occur.⁶⁻¹¹ A-SMase upregulation results from UV radiation and IR as well as TNF- α , IL-1 β , and Fas.^{6,12} It must be noted that conflicting reports exist on this matter. It is likely, however, that different cell types use dissimilar mechanisms to generate ceramide. In the SQ-20B human head and neck squamous cell carcinoma line, radiation incurs apoptosis by upregulation of N-SMase and A-SMase in addition to de novo synthesis of ceramide.⁹

Condensation of serine and palmitoyl CoA by serine palmitoyltransferase (SPT) initiates the de novo pathway and generates 3-ketosphinganine.^{6,13} This is reduced to sphinganine, which is then N-acetylated by ceramide synthase (CS) to dihydroceramide. Introduction of a trans-4,5 double bond by a desaturase leads to creation of ceramide. Unlike the acute peak (within 1 to 2 minutes) seen with the SMase mechanism, this pathway requires hours to occur after stimulation before detectable levels of ceramide are noted.^{3,4,14-16}

The particular importance of the ceramide pathway in radiation-induced mucosal barrier injury was hypothesized by Paris et al,¹⁷ who noted that radiation-induced endothelial damage precipitated intestinal mucositis. The present study was undertaken with 2 objectives: first, to ascertain that the ceramide pathway was of significance in the development of oral mucositis; and

second, to determine which enzymatic pathway was most involved. We studied 3 different ceramide inhibitors (glutathione, desipramine, and fumonisin B₁) which have site and function specificity.

Recent evidence has shown the antioxidant properties of glutathione to play an important role in oxidant toxicity and regulation of stress-mediated apoptosis. Glutathione, a neutral SMase inhibitor, reduces lipid hydroperoxides through their Se-independent glutathione peroxidase activity which can also detoxify lipid peroxidation end products such as 4-hydroxynonenal (4-HNE).¹⁸ Desipramine, an acidic sphingomyelinase inhibitor, was observed to halt TNF- α -induced ROS production at the same concentration used in this study.¹⁹ Fumonisin B₁ is a potent and naturally occurring mycotoxin produced by the fungus *Fusarium verticillioides*. Fumonisin B₁ has been shown to affect a variety of cell signaling proteins, including protein kinase C (PKC), a serine/threonine kinase involved in a number of signal transduction pathways that include cytokine induction, carcinogenesis, and apoptosis.²⁰

MATERIAL AND METHODS

Animals

Forty male golden Syrian hamsters (Charles River Laboratories, Wilmington, Mass) aged 5-6 weeks and weighing between 60 g and 90 g were used. Animals were individually numbered, housed in groups of 4 with wood chip bedding and fed and watered ad libitum. A 12-hour light/dark cycle was set. Animals were acclimated for a minimum of 48 hours prior to radiation to minimize the effects of stress due to shipping. All animals were weighed daily and prior to radiation and monitored daily for survival. All protocols were approved by the Standing Committee on Animal Use of the Harvard Medical Area.

Dosing

Animals were randomly divided into 4 groups of 10 animals each. Each group was assigned a different ceramide inhibitor dose. Initial dosing levels were determined by a review of the literature, followed by a pilot dose-ranging study in hamsters.¹⁸⁻²⁹ The optimum dose of each agent was used in this study. All animals were given 0.2 mL subcutaneous injections of saline or inhibitor once daily from day -1 to day 16. Animals in each group received saline, 5 mmol/L glutathione, 5 μ mol/L desipramine, or 1 μ mol/L fumonisin B₁.

Radiation

On day 0, hamsters were anesthetized immediately prior to radiation via an IP injection with a ketamine/xylazine cocktail: 16 mg ketamine and 0.8 mg xylazine per 100 g body weight. The left buccal cheek pouch was

everted using blunt forceps and stabilized to a gel pad. The remainder of the animal was covered with a total body lead shield, leaving the cheek pouch exposed, and placed beneath an x-ray radiation source (YXLON MGG 42). Subsequently, animals were irradiated with 40 Gy delivered to the targeted mucosa by a 250 kV potential (1 mA) source at a focal distance of 50 cm and hardened with a 0.35-mm Cu filtration system. This dose has consistently been shown to produce ulcerative mucositis.

Mucositis assessment

Animals were anesthetized by inhalation of isoflurane and the left buccal pouches were everted to assess the severity of mucositis. Two blinded examiners evaluated the course of mucositis every other day from day 6 to 26 using an established 6-point scoring system.²⁰

Immunohistochemical ceramide assessment

Two animals from each group were killed on days 3, 10, and 16 and the left cheek pouch was excised. Following fixation in 10% buffered formalin representative sections from each tissue sample were embedded in paraffin blocks. Five-micrometer sections were then mounted on slides for later immunohistochemical analysis. Samples were deparaffinized in xylene, rehydrated in ethanol, and blocked in endogenous peroxidase (3% H₂O₂ in methanol). Following a rinse in deionized water, samples were microwaved in citrate buffer for 30 minutes at 199°F and then placed in phosphate buffer solution (PBS). Tissues were blocked in goat serum and then treated with Avidin/Biotin (Vector Laboratories; Burlingame, Calif). Tissues were then incubated in a humid chamber with a 1:100 dilution of monoclonal anti-ceramide specific antibody (Sigma-Aldrich, St Louis, Mo) and amplified with a 1:200 dilution of biotinylated secondary antibody (Vector Laboratories; San Jose, Calif). ABC solution (Vector Laboratories) was then applied to the samples for 45 minutes. Samples were visualized with DAB (DAKO Corporation; Carpinteria, Calif) (10 mg 3,3'-diaminobenzide in 10 mL 0.05 mol/L Tris buffer, 3 drops H₂O₂) and counterstained with Harris hematoxylin. Positively stained cells were quantified for each inhibitor, including the control, by determining the mean number of cells from 10 randomly selected high-powered fields (40×) per tissue per animal in the epithelium and the connective tissue for days 3, 10, and 16 in blinded fashion by 2 observers.

Statistical analysis

The number of animals with a blinded mucositis score of greater than or equal to 3 in each drug treatment group was compared to the control group. Differences were analyzed on a cumulative basis. A 1-way ANOVA test was used to determine the significance of the area under

the curve (AUC) of the mean daily weight gain. Treatment success was defined as a statistically significant lower number of hamsters with this score in a drug treatment group versus control as determined by chi-squared analysis. A Mann-Whitney rank sum test was used to determine the significance in positive cell counts. Type I errors for these statistical tests were set at .05.

RESULTS

Survival

All 4 animal groups achieved 100% (n = 40) survival. No deaths occurred during the course of this study.

Weight change

All animals exhibited linear weight gain throughout the study. Animals in the control, glutathione, desipramine, and fumonisin B₁ groups gained roughly 54%, 48%, 51%, and 42%, respectively, of their starting weights while only the fumonisin group experienced statistically less weight gain than the control. Statistically significant differences in mean AUC weight gain were noted between control, glutathione, and desipramine groups ($P = .043$; Fig 1). The fumonisin B₁-treated animals, however, displayed a statistically significant lower weight gain than their counterparts, a difference of 12%.

OM scores

The overall mean of OM, as scored from day 6 to 26, for control, glutathione, desipramine, and fumonisin B₁ groups were 1.94, 1.75, 1.91, and 1.52 respectively. No statistically significant differences were detected between control, glutathione, and desipramine animals. On the other hand, animals treated with the ceramide synthase inhibitor fumonisin B₁ showed a 22% decrease in total mean score compared to control ($P = .002$; Fig 2).

Peak oral mucositis for all groups occurred from day 16 to day 18 (Fig 3). Peak OM scores did not statistically deviate from control in any group except for those animals treated with fumonisin B₁. On day 16, animals injected with fumonisin showed a 16% lower mean OM score compared to control animals (2.62 and 3.13, respectively). A 23% lower mean OM score for the fumonisin group was detected on day 18 (2.50 versus 3.25 in control).

Indeed, only animals treated with fumonisin B₁ had significantly fewer percent animal days with severe mucositis (as evidenced by a score of 3 or greater) ($P = .036$; Fig 4). Animals in the control, glutathione, and desipramine groups exhibited ulcers at the rates of 34%, 28%, and 33%, respectively, during the course of this study. Ulceration was detected only 17% of the time in hamsters treated with fumonisin B₁.

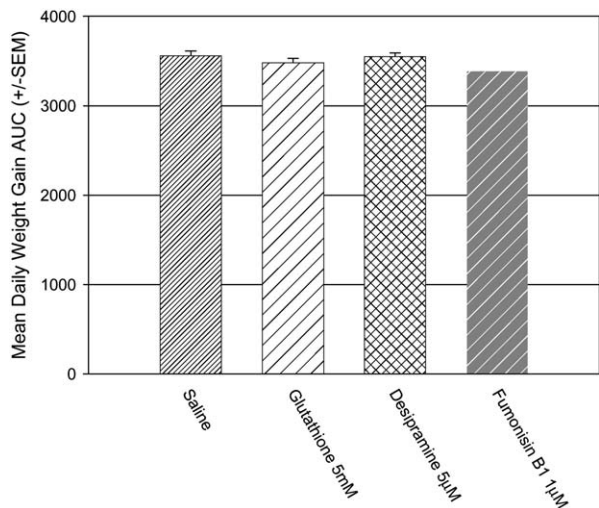


Fig 1. The One Way Anova test failed (ie, there are significant differences between groups; $P = .043$). T-tests indicated that there was a statistically significant difference between the control group and the Fumonisin B1 treated group.

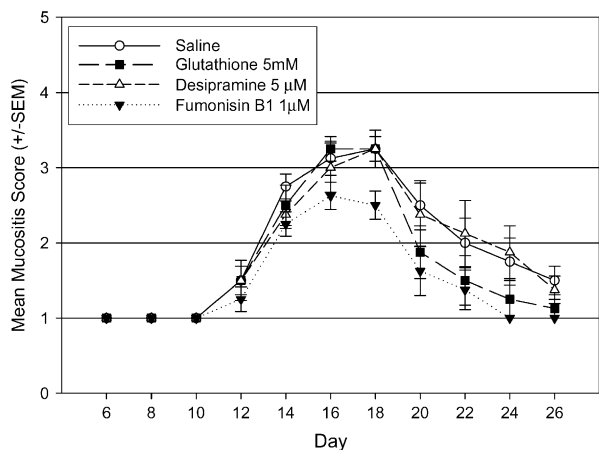


Fig 2. Mean OM scores of groups over course of the experiment. Note that mean OM scores of only animals treated with the ceramide synthase (Fumonisin B₁) inhibitor were significantly lower than those of control (day 14 to day 26).

Immunohistochemical ceramide assessment

To verify that our enzyme inhibitors blocked ceramide generation, left buccal cheek pouch samples were collected from 2 animals in each group to detect ceramide expression on days 3, 10, and 16 using indirect immunohistochemical techniques (Fig 5). In the epithelium of the oral mucosa, significant differences in positive cells relative to the control were observed on day 3 ($P = .004$) and day 16 ($P < .001$) for the glutathione group. Animals treated with fumonisin B₁ also exhibited statistically significant differences on day 3 ($P = .008$) and day 6 ($P = .014$) but also on day 10

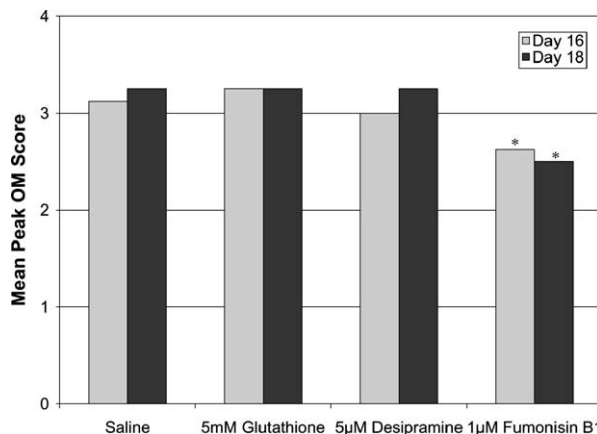


Fig 3. Mean peak OM scores from day 16 to day 18. Asterisk denotes mean peak OM score that is statistically different from that of control.

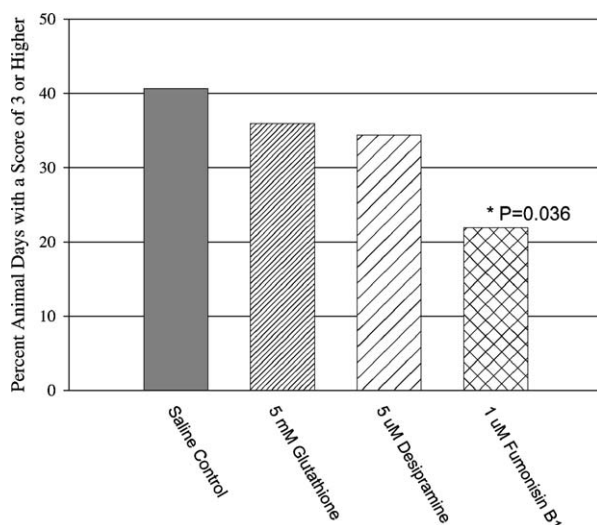


Fig 4. Animal-days with OM scores ≥ 3 . Total of 88 animal-days possible per group. Asterisk denotes that animal-days for 1 μM fumonisin B₁ group is statistically different from control by Chi-squared analysis; $P = .036$.

($P = .001$; Fig 6). Interestingly, the positive cellularity in the connective tissue statistically mirrored that in the epithelial only in the animals treated with glutathione relative to the control ($P < .01$ on day 3; $P < .01$ on day 10). Noteworthy, only on tissue samples on day 10 ($P < 0.005$) and day 16 ($P < .001$) were significant differences visually detected in tissues samples for animals that received fumonisin B₁ (Fig 7).

DISCUSSION

The results of this investigation suggest that inhibition of ceramide synthase is more effective than N-SMase or A-SMase in attenuating head and neck

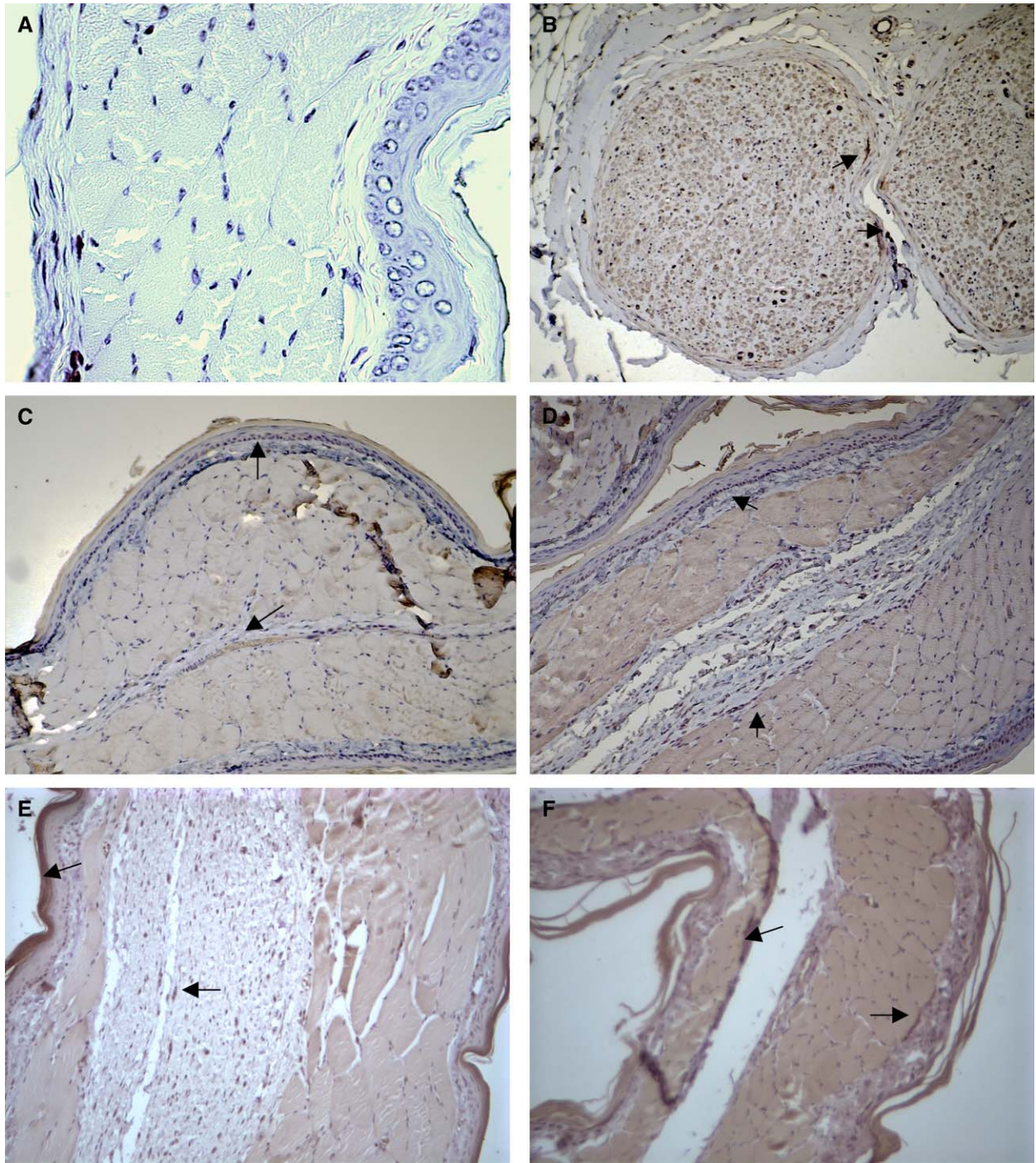


Fig 5. Immunohistochemical detection of ceramide (1:100) of oral mucosae receiving 40Gy x-radiation (day 0) observed on day 16 using 10X magnification. **A**, Negative control- hamster cheek pouch. **B**, Positive control-neural hamster tissue. **C**, Saline. **D**, 5µM Desipramine. **E**, 5mM Glutathione. **F**, 1µM Fumonisin B1.

(HN) IR-induced apoptosis and subsequently oral mucositis. Previous studies support this idea. Liao and colleagues reported that DNA damage from radiation signal posttranslational activation of CS is downregulated by ATM.¹⁴ EBV-immortalized B cell lines from

patients with ataxia telangiectasia (absent or non-functional ATM) showed increased CS, ceramide generation, and apoptosis. Stable transfection of wild type ATM cDNA reversed these events.¹⁴ Garzotto and associates described the abrogation of TPA- and

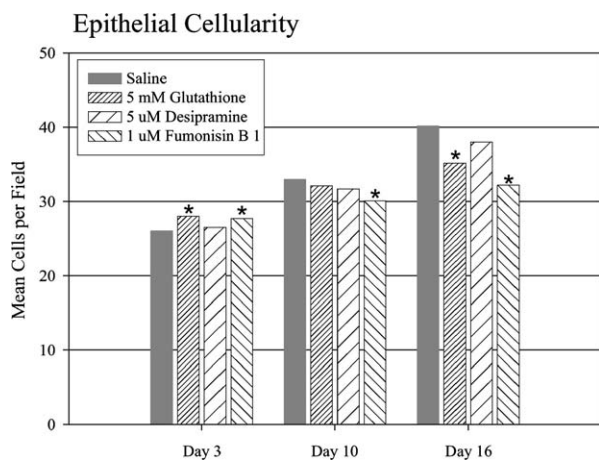


Fig 6. Mean positive cell counts per 40x field from hamster oral mucosa obtained at days 3, 10, and 16. Significance of differences in cell counts was determined using the Mann-Whitney rank sum test. Significant differences (noted by *; $P < .05$) seen on day 3 are significant increases relative to controls, while significant differences on days 10 and 16 are decreases relative to controls.

radiation-induced apoptosis in LNCaP prostate cancer cells after treatment with fumonisin B₁.³⁰ In addition, glutathione's antioxidative properties have shown that lipid peroxidation products can modulate mechanisms of stress-mediated signaling.¹⁸

Only the group of animals treated with fumonisin B₁ presented with a less severe (less ulceration) course of OM than the other groups relative to the control group. The general shape of the mucositis initiation, progression, and healing curve of the fumonisin B₁-treated animals, however, remains similar to that of the control group, which indicates that synthase inhibition does not affect the timing of OM progression. That is, animals in the fumonisin B₁ group did not have delayed initial upswing in OM scores (day 12) or early regression of the disease (days 20-26). In addition, no differences in OM score between groups existed from day 6 to 10. Course length and development remain unchanged, however in all groups, peak mucositis occurred between day 16 and 18. It appears then that de novo ceramide production contributes in a critical way to radiation stress several days after induction. This is supported by immunohistochemical detection illustrating variability in the expression of ceramide and then attenuation of ceramide on day 3 for the glutathione and fumonisin groups. We speculate that HN IR upregulates ceramide synthase around day 10 in the underlying connective tissue, at which the fumonisin B₁-treated animals began to deviate clinically from the other groups. Noteworthy, our results demonstrate that desipramine was not

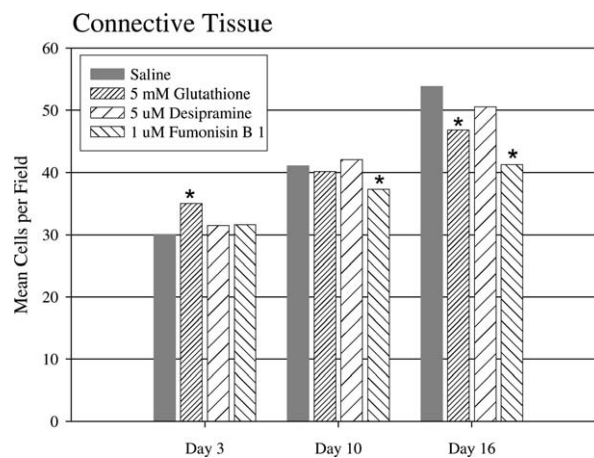


Fig 7. Mean positive cell counts per 40x field from hamster oral mucosa obtained at days 3, 10, and 16. Significance of differences in cell counts was determined using the Mann-Whitney rank sum test. Significant differences (noted by *; $P < .05$) seen on day 3 are significant increases relative to controls, while significant differences on days 10 and 16 are decreases relative to controls.

effective clinically in attenuating the production of ceramide through the inhibition of A-SMase.

It is interesting to note that animals treated with fumonisin B₁ endured a milder course of OM and presumably had less discomfort with mastication. However, only this group exhibited significantly less weight gain than the control. Fumonisin B₁ does not induce direct DNA damage and are not DNA reactive.³¹ Still, the adverse effects of fumonisin B₁ are well documented; it is a mycotoxin produced by *Fusaria* fungi that grow primarily on corn. As a result of CS inhibition, sphinganine accumulates in the cells. In particular, those of the liver and kidney are susceptible. Inhibitors of cyclin-dependent protein kinases (Cip1, Kip1, Kip2) are upregulated, which causes growth arrest.³² Programmed cell death ensues as well, most likely due to inhibition of PKC and disruption of the endothelial barrier.³² A number of experiments demonstrate that fumonisin B₁ is hepatotoxic, nephrotoxic, and carcinogenic in rodents.³⁰⁻³⁵ In all these studies, cancers were induced by at least 50 ppm (70 $\mu\text{mol/L/kg}$) of daily fumonisin B₁ after several months, if not years. An investigation by Voss et al showed that oral doses of 10 to 100 ppm (14 to 140 $\mu\text{mol/L/kg}$) of fumonisin B₁ given to female B6C3F mice over 28 days did not cause any significant decrease in body weight gain compared to control.³⁵ Our study gave no more than 12 ppm (17 $\mu\text{mol/L/kg}$) per animal daily. Perhaps fumonisin B₁ in lower concentrations can cause organ weight or total body mass changes or has yet-undescribed properties as an appetite suppressant.

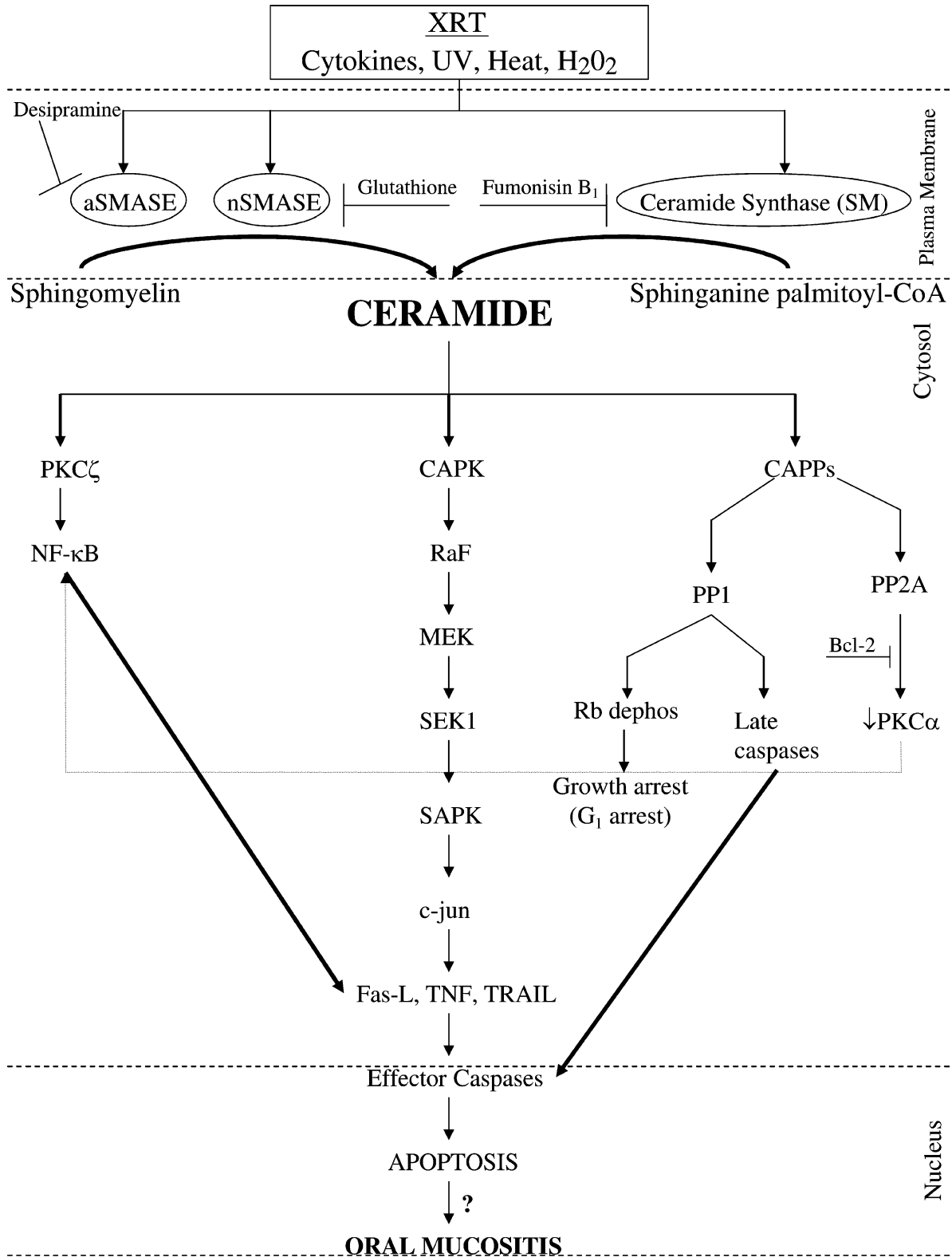


Fig 8. Schematic proposed mechanism illustrating the inhibition of the production of ceramide through the ceramide synthase and neutral and acidic sphingomyelin arms of this pathway.

Through its various effector mechanisms, ceramide plays a crucial role in cell homeostasis, communication and death in a number of organ systems (immune, endocrine, vascular, CNS).⁶ The study of this lipid is still in its nascent stages, but its link to stress-induced apoptosis is clear. The method by which this occurs is not. Our study finds that ceramide-mediated apoptosis has an obvious effect on HN IR-induced OM and implicates only de novo ceramide production via CS, but it is possible that N-SMase and A-SMase contribute to a degree (Fig 8). Indeed, modification of amount or function of other enzymes may attenuate ceramide. SPT is the first and rate-limiting enzyme in the de novo synthesis pathway; its inhibition leads to ceramide reduction.³ Conversely, sphingomyelin synthase, ceramidase, ceramide kinase, and glucosylceramide synthase all engage in the clearance of ceramide. Activation of these molecules may also prove to be antiapoptotic and clinically significant. Burek and colleagues studied Farber disease lymphocytes, which lack ceramidase.⁷ Equal to normal cells, these cells underwent apoptosis in response to IR, anticancer drugs, and staurosporine. When challenged with antiCD95, however, Farber lymphoid tissue showed a significant decrease in apoptosis. In studying these lipid mechanisms, the investigator must be aware that certain pathways may dominate or be vulnerable in certain cell types in response to particular stresses.

In future, we suggest in vivo use of various other ceramide inhibitors and dosing concentrations, immunohistochemical review of specific tissue types (endothelium in particular), and analysis of apoptotic markers and levels of enzyme. Further studies on cell death in oral mucositis subsequent to ionizing radiation are needed. If a ceramide inhibitor proves to be consistently successful in disease reduction, then it may be adapted for medical treatment.

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