Anti-ceramide antibody prevents the radiation gastrointestinal syndrome in mice

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Radiation gastrointestinal (GI) syndrome is a major lethal toxicity that may occur after a radiation/nuclear incident. Currently, there are no prophylactic countermeasures against radiation GI syndrome lethality for first responders, military personnel, or remediation workers entering a contaminated area. The pathophysiology of this syndrome requires depletion of stem cell clonogens (SCCs) within the crypts of Lieberkühn, which are a subset of cells necessary for postinjury regeneration of gut epithelium. Recent evidence indicates that SCC depletion is not exclusively a result of DNA damage but is critically coupled to ceramide-induced endothelial cell apoptosis within the mucosal microvascular network. Here we show that ceramide generated on the surface of endothelium coalesces to form ceramide-rich platforms that transmit an apoptotic signal. Moreover, we report the generation of 2A2, an anti-ceramide monoclonal antibody that binds to ceramide to prevent platform formation on the surface of irradiated endothelial cells of the murine GI tract. Consequently, we found that 2A2 protected against endothelial apoptosis in the small intestinal lamina propria and facilitated recovery of crypt SCCs, preventing the death of mice from radiation GI syndrome after high radiation doses. As such, we suggest that 2A2 represents a prototype of a new class of anti-ceramide therapeutics and an effective countermeasure against radiation GI syndrome mortality.

Introduction
Characterized clinically by anorexia, vomiting, diarrhea, dehydration, systemic infection, and, in extreme cases, septic shock and death, the radiation gastrointestinal (GI) syndrome involves destruction of crypt/villus units, loss of mucosal integrity, and infection by resident enterobacterial flora (1–3). While conventional radiobiology considers unrepaired or misrepaired DNA double-strand breaks in stem cell clonogens (SCCs) as autonomous lesions leading to irreversible tissue injury, our recent studies have challenged this paradigm, presenting genetic evidence that acute endothelial damage also plays a major role in GI tract injury (4–6). Within minutes of radiation exposure, endothelial acid sphingomyelinase (ASMase) is activated, catalyzing ceramide generation on the external plasma membrane of mouse and human endothelium to initiate apoptotic signaling (7, 8). Endothelium displays 20-fold more ASMase than other mammalian cells, almost exclusively in a secretory form, which makes them particularly vulnerable to ceramide-induced apoptosis (9, 10). Early evidence indicates that vascular compromise, consequent to endothelial cell apoptosis, impairs radiation-injured SCC DNA damage repair, resulting in SCC demise. In several mouse strains, endothelial apoptosis occurs between 8 and 15 Gy (4, 6), which encompasses doses that cause both sublethal (≤14 Gy) and lethal (≥15 Gy) GI tract injury (5), beginning at 1 hour and peaking at 4 to 6 hours after irradiation (4, 6, 11). Attenuation of intestinal endothelial apoptosis by genetic inactivation of ASMase-mediated ceramide generation enhances SCC survival, facilitating repair of crypt damage and rescue of animals from GI lethality (4, 6). These observations provide the basis for developing a neutralizing anti-ceramide monoclonal antibody as a potential radiation countermeasure.

Results and Discussion
Initial studies examined whether radiation-induced ceramide locally reorganizes endothelial plasma membranes to form ceramide-rich platforms (CRPs), sites of ceramide-mediated transmembrane signal transmission for diverse stresses in other mammalian cell types (7). These studies used bovine aortic endothelial cells (BAECs), as previous reports detail ionizing radiation activation of the ASMase apoptotic program in these cells (12–14). In this study, ionizing radiation (10 Gy) induced a rapid increase in BAEC ASMase enzymatic activity from a baseline of 171 ± 5 nmol/mg/h to a peak of 307 ± 24 nmol/mg/h 1.5 minutes after stimulation (P < 0.005 vs. unirradiated control; Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI59920DS1). Concomitantly, cellular ceramide increased from 157 ± 12 pmol/106 cells to 203 ± 10 pmol/106 cells (P < 0.01 vs. unirradiated control) within 1 minute of stimulation and persisted for over 2 minutes before decreasing toward baseline (Supplemental Figure 1B). Simultaneous increase of neutral sphingomyelinase or ceramide synthase activity was not detected (data not shown), confirming radiation-induced ceramide generation as ASMase mediated. At the same time, cell surface platforms enriched in ASMase and ceramide were observed by fluorescence microscopy (Figure 1A). Formation of CRPs, identified as ceramide clustered into cell surface macrodomains of 500 nm up to several microns (7), was detected as early as 30 seconds after irradiation, peaking at 1 minute with 32% ± 2% of the population

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exhibiting platforms ($P < 0.001$ vs. unstimulated control; Figure 1B). Platform formation was dose dependent at 1 minute, reaching a maximum at 11 Gy ($P < 0.001$), with an ED$_{50}$ of approximately 5 Gy (Figure 1C). This dose range is virtually identical to that published for induction of radiation-induced apoptosis in BAECs (15). Preincubation of BAECs with MID 15B4 (a commercially available anti-ceramide antibody), a strategy that neutralizes cell surface ceramide and blocks ceramide-induced coalescence in other cell types (16–19), inhibited radiation-induced formation of CRPs (Figure 1D). CRPs were observed in 43% ± 6% of BAECs pretreated with irrelevant IgM ($P < 0.01$ vs. nonirradiated control), whereas pretreatment with the anti-ceramide antibody MID 15B4 (1 μg/ml) reduced CRPs to 16% ± 2% of the population, which was not significantly different from the baseline 15% ± 3% in unirradiated cells ($P > 0.1$). Surface ceramide neutralization and CRP inhibition attenuated 10 Gy–induced apoptosis by 71% for up to 8 hours after stimulation (Figure 1E; $P < 0.001$). Similar anti-ceramide antibody inhibition of radiation-induced (5–20 Gy) CRP formation and apoptosis were observed in Jurkat T lymphocytes (Zhang and Kolesnick, unpublished observations).

Based on these observations, we generated a mouse monoclonal anti-ceramide IgM, termed 2A2, with specific affinity for ceramide compared with phosphorylcholine, diacylglycerol, dihydrosphingosine, sphingomyelin, or GM1, as determined by ELISA (Supplemental Figure 2). 2A2 inhibited CRP formation and ceramide-mediated apoptosis (data not shown) and dose dependently inhibited endothelial cell apoptosis in vivo. Intravenous 2A2 administration (1,000 μg/25 g mouse) to C57BL/6 mice 15 minutes prior to the LD$_{100}$ of 15 Gy whole-body irradiation (WBI) reduced peak endothelial apoptosis within the lamina propria microvasculature by 83%, from a mean of 9.2 ± 0.5 apoptotic endothelium per villus to 1.6 ± 0.3 apoptotic endothelium per villus (Figure 2A; $P < 0.05$). Thus, 2A2 phenocopies the genetic inhibition of radiation-induced intestinal endothelial apoptosis.
within the range of 8 to 15 Gy (Figure 2C), and animals administered purified 2A2 (0–1,000 μg) 15 minutes prior to 15 Gy WBI enhances crypt survival, quantified by the microcolony assay of Withers and Elkind (Figure 2, B and C). Data (mean ± SEM) are compiled from 3 experiments of 2 mice each, analyzing 10–20 intestinal circumferences per mouse.

Figure 2
2A2 antibody inhibits radiation-induced endothelial apoptosis and crypt lethality. (A) Purified 2A2 or irrelevant IgM control (1,000 μg) was injected intravenously into C57BL/6 mice 15 minutes prior to 15 Gy WBI, and proximal jejunum was harvested 4 hours thereafter. Endothelial apoptosis was identified by microscopic detection of TUNEL (brown) and CD34 (red) double-positive endothelium (indicated by arrows; original magnification, ×400). Data, compiled from 3 experiments using 3 mice each analyzing 200 intact villi per mouse, are presented as a histogram. Note that approximately half of the villi in wild-type mice display 10 or more apoptotic endothelial cells after irradiation, effectively abrogated by preventing CRP formation genetically (using aSMase–/– mice) or pharmacologically (2A2 prophylaxis). (B) 2A2 dose dependently protects small intestinal crypts. C57BL/6 mice were administered purified 2A2 (0–1,000 μg) 15 minutes prior to 15 Gy WBI. (C) Administration of 2A2 (1,000 μg) 15 minutes prior to 8 to 15 Gy WBI enhances crypt survival, quantified by the microcolony assay of Withers and Elkind. (B and C) Data (mean ± SEM) are compiled from 3 experiments of 2 mice each, analyzing 10–20 intestinal circumferences per mouse.
of 2A2 inhibition in Supplemental Figure 8). Previously published countermeasures are associated with clinically relevant toxicities or have displayed less efficacy when directly compared with 2A2. Basic fibroblast growth factor protects against radiation GI syndrome lethality (11) but is associated with serious hypotension (22, 23). The TLR5 agonist, CBLB502, is efficacious as a radioprotector of GI tract injury (24); however, reported data indicate that its effect is limited to lower radiation doses that are nonlethal to the GI tract (<13 Gy). Sphingosine-1-phosphate, while effective in protecting against radiation GI injury (25), was less effective than 2A2 in direct comparison (Supplemental Figure 9). Lastly, the angiopoietin derivative, COMP-Ang1 (26), was substantially less effective than 2A2 as a radioprotector in our laboratory (data not shown). 2A2 antibody thus complements other promising agents under development, including CBLB502 from Cleveland BioLabs (24), which has been demonstrated to be effective at preventing BM lethality. As such, 2A2 antibody fulfills a mandate of the United States Department of Health and Human Services Public Health Emergency Medical Countermeasures Enterprise, a national security measure designed to protect the American public from the deliberate use of weapons of mass destruction, consistent with the goals of the president’s Biodefense for the 21st Century and National Strategy for Medical Countermeasures against Weapons of Mass Destruction directives.

Methods

Antibody generation. BALB/c mice immunized by 3 intraperitoneal administrations of 2 × 10⁷ human Kaposi’s sarcoma (KS1767) cells at 1-week intervals were boosted 3 days prior to fusion. Harvested splenocytes were fused with mouse myeloma cells (P3X63Ag8.653; ATCC) at a 4:1 ratio using polyethylene glycol (MW 1,500 g/mol). Hybridomas were seeded at 1 × 10⁵ cells per well in RPMI 1640 selection medium plus 20% fetal bovine serum, 10% Hybridoma supplements (Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, and hypoxanthine-aminopterin-thymidine (Sigma-Aldrich). Supernatants were screened by FACS of KS1767 cells and ELISA using BSA-conjugated C₁₀₆-ceramide. Selected hybridomas were subcloned 4 times by limited dilution and screened by ELISA. 2A2 IgM was purified from ascites using mannann-binding protein beads.

Cell culture. BAECs were cultured in DMEM, switched at confluence to DMEM plus 2% normal calf serum for 1-week minimum (8), and preincubated for 18 hours before irradiation in DMEM plus 0.2% human albumin.

Ceramide quantification. Diacylglycerol kinase assay was used for ceramide quantification (27).

ASMase activity. ASMase activity was quantified by radioenzymatic assay using [³⁵C]-methylcholesteroliphingomycelin (Amersham) (28).

Detection of CRPs. Confluent BAECs were detached with PBS plus 0.1% collagenase, 0.02% EDTA, and 0.5% BSA at 37 °C for 5 minutes, gently dispersed to obtain a single-cell suspension, and resuspended at 0.5 × 10⁶ cells/ml DMEM plus 0.2% human albumin. After irradiation, cells were incubated at 37 °C and fixed with 2% paraformaldehyde for 15 minutes at 4 °C. Nonspecific sites were blocked with PBS containing 2% fetal bovine serum for 20 minutes, and cells were stained for surface ceramide using mouse monoclonal anti-ceramide antibody MID 15B4 IgM (1:50 dilution; Alexis Biochemicals) or for surface ASMase with rabbit polyclonal antiASMase AB 1598 (1:100 dilution) (16). Irrelevant mouse IgM or rabbit IgG served as isotype controls. After 3 washes with PBS plus 0.05% Tween-20, cells were stained for platforms with Texas Red–conjugated anti-mouse IgM or Cy3-conjugated anti-rabbit IgG (1:300 dilution, Roche), respectively, for 1 hour at 4 °C. Cells were mounted in fluorescent mounting medium (Dako). Fluorescence was detected using an Axiovert S-100 Zeiss microscope and SPOT digital camera. The percentage of cells containing platforms, i.e., those in which fluorescence condenses onto less than 25% of the cell surface, was determined in 150 to 250 cells/point.

Endothelial apoptosis. BEAC apoptosis was measured morphologically after Hoechst 33258 bis-benzimide staining (12). Small intestinal endothelial apoptosis was detected by double staining with TUNEL for apoptosis and a rat antibody against the endothelial cell surface marker CD31 (4).

Radiation source. Radiation was delivered to mice using a Shepherd Mark-I unit (model 68-SN643), operating at a Cs source at 2.12 Gy per minute. For events occurring during the first 10 minutes, the dose rate was 13.1 Gy per minute.

Cryopreservation survival assay. Cryopreservation survival assay was performed according to Withers and Elkind (11, 20).

Mouse survival after WBI and designation of autopsies findings. Actuarial mouse survival was calculated by the Kaplan-Meier method (29). Terminally sick animals displaying agonal breathing were sacrificed by hypercapnia/asphyxiation, and necropsy was performed to determine cause of death. Intestinal specimens, fixed in formaldehyde, were stained with hematoxylin (6). GI damage was diagnosed as cause of death when small intestines displayed denuded mucosa with nearly no villi or crypts or when mucosa displayed limited repair (6).
Statistics. Significance was calculated using 2-tailed Student’s t test and Mantel log-rank test. P < 0.05 was considered significant.

Study approval. Animal experimental protocols were approved by MSKCC Research Animal Resource Center.

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