Decreasing the Apoptotic Threshold of Tumor Cells through Protein Kinase C Inhibition and Sphingomyelinase Activation Increases Tumor Killing by Ionizing Radiation

Steven J. Chmura, Helena J. Mauceri, Sunil Advani, Ruth Heimann, Michael A. Beckett, Edwardine Nodzenski, Jose Quintans, Donald W. Kufe, and Ralph R. Weichselbaum

ABSTRACT

Approximately 30% of cancer deaths result from the failure to control local and regional tumors. The goal of radiotherapy is to maximize local and regional tumor cell killing while minimizing normal tissue destruction. Attempts to enhance radiation-mediated tumor cell killing using halogenated pyrimidines, antimitabolites, and other DNA-damaging agents or sensitizers of hypoxic tumor cells have met with only modest clinical success. In an unique strategy to modify tumor radiosensitivity, we used an inhibitor of the protein kinase C group A and B isoforms, chelerythrine chloride (chelerythrine), to enhance the killing effects of ionizing radiation (IR). Protein kinase C activity plays a central role in cellular proliferation, differentiation, and apoptosis. Chelerythrine increases sphingomyelinase activity and enhances IR-mediated cell killing through induction of apoptotic tumor cell death in a radioresistant tumor model both in vitro and in vivo. Although previous reports have suggested that IR-mediated apoptosis correlates with tumor volume reduction, we demonstrate for the first time that lowering the apoptotic threshold increases tumor cell killing in vivo.

INTRODUCTION

Approximately 500,000 individuals die of cancer in the United States yearly (1). Nearly 30% of these individuals will die due to the failure of cancer therapies to control local or regional disease. Historically, surgery was the major treatment for localized cancer (2). However, poor results in many large tumors and the morbidity of normal tissue resection have led to the use of additional cancer treatments (3). Initially, poor physical delivery and tumor targeting limited radiotherapy. Although recent advances in linear accelerator design, computerized treatment planning, and tumor visualization have optimized the physical delivery of IR, major biological obstacles to effective radiation therapy exist.

One major biological determinant of radiotherapy failures is tumor radioresistance (4—6). Exposure of mammalian cells to IR results in a loss of cellular reproductivity (mitotic death) by inducing DNA double strand breaks and lethal chromosomal aberrations (7). Morphological characteristics of IR-induced mitotic death include multinucleated giant cells, cell-cell fusions (7), and loss of membrane integrity, which is characteristic of necrotic cell death (8—10). Daughter cells with limited divisional potential can arise from lethally irradiated mother cells to form abortive colonies. In contrast to necrotic death, apoptosis induced by IR (8—10) results in activation of a genetic program initiated by cytoplasmic or nuclear events that culminates in cytoplasmic blebbing, chromatin condensation, and DNA fragmentation (11—13). The induction of apoptosis may occur immediately after irradiation (interphase death), after arrest in G2 (10), or following one or more cell divisions (14, 15). Thus, both mitotic and apoptotic cell death contribute to the killing effects of IR.

Strategies to sensitize radioresistant tumors have included the use of agents that damage DNA, interfere with DNA repair, or sensitize hypoxic tumor cells. These strategies have, however, met with limited clinical success (7, 16—18). Loss of the apoptotic response to IR has been linked to a radiation-resistant phenotype. Overexpression of antiapoptotic proteins or loss of pro-apoptotic function has been reported to alter radiosensitivity (19, 20). For example, loss of p53 function may be associated with radioresistance as a consequence of a diminished ability to undergo apoptosis in vitro (5, 21, 22). Acquired mutations in p53 are associated with both treatment resistance and tumor relapse (14, 23). Previous reports have demonstrated that the therapeutic responsiveness of tumors correlates with IR-induced apoptosis (5, 24). These findings have led to speculation that increasing the apoptotic response in radioresistant tumors may increase the therapeutic efficacy of IR. To date, however, no studies have demonstrated that altering the threshold of apoptosis in vivo to IR results in increased tumor killing.

The PKC family of serine/threonine kinases comprises at least 13 related isoforms (25) with differing sensitivities to calcium and lipid activators. The PKC family of kinases plays a central role in cell growth, differentiation, and apoptosis. PKC activation is functionally related to gene induction following exposure of mammalian cells to IR (26—29). Previous studies have demonstrated that PKC activation occurs following exposure of human tumor cell lines to IR and that protein kinase inhibitors may limit this response (30, 31). PKC activation limits the production of ceramide from the hydrolysis of sphingomyelin and rescues cells from IR-mediated apoptosis (32, 33). Inhibition of PKC and subsequent ceramide production alters the expression and function of antiapoptotic proteins (34, 35) and activates caspases (CED3/CPP32 proteases), which are required for IR-mediated apoptosis (36). These studies suggest that inhibition of PKC may represent a strategy to enhance IR-mediated cell death by apoptotic mechanisms in cells that are otherwise resistant to apoptosis.

We have used chelerythrine chloride (chelerythrine), a selective inhibitor of group A and B PKC isoforms (37), to test whether PKC inhibition increases the IR-mediated killing of p53-deficient and radioresistant human tumor cells. Chelerythrine was selected for these studies due to its demonstrated specificity for PKC isoforms, ease of delivery in vivo, and inhibition of PKC in an ATP-independent manner (37—39). These studies demonstrate that targeting and modulation of membrane and cytoplasmic events to enhance IR-mediated apoptosis and cell killing overcomes tumor cell radioresistance and may represent a unique therapeutic strategy.
MATERIALS AND METHODS

Drugs and Reagents. Chelerythrine, ATP, PBS, and PI were purchased from Sigma Chemical Co. (St. Louis, MO). Chelerythrine was dissolved in sterile water for the in vitro experiments or in PBS for intratumoral injections. [14C]Sphingomyelin (60 Ci/mmol), and [γ-32P]ATP were purchased from DuPont NEN. All solvents were high-performance liquid chromatography grade.

Cell Culture. The SQ-20B human head and neck squamous cell carcinoma line was grown in DMEM:F-12 (3:1) supplemented with 20% fetal bovine serum (Life Technologies, Inc., Grand Island, NY), 1% penicillin-streptomycin, and 1% hydrocortisone at 37°C in a humidified atmosphere containing 5% CO2.

Viability Assay via PI Exclusion. Cells (2.5 X 10^5) to 3.5 X 10^5) were cultured in 24-well tissue culture plates for all experiments. Cells were treated with various concentrations of chelerythrine, incubated for 30 min at 37°C, and then irradiated. Radiation for all samples was delivered using a 60Co irradiator (Gammacell 220, Atomic Energy of Canada) at a dose rate of 2.0 Gy/sec. At the indicated time points, cells were harvested, washed once, and resuspended in PBS containing 50 μl of 100 μg/ml PI. Cells were analyzed by flow cytometry (FACS) on a FACScan (Becton-Dickson) using Lysis II software.

DAPI Staining for Nuclear Visualization. Cells (5 X 10^5) were centrifuged at 1000 rpm and resuspended in approximately 100 μl of DAPI (Sigma: 1 μg/ml in PBS + 1% Triton X-100). One drop of this mixture was placed on a microslide with a coverslip. The cells were viewed by fluorescence microscopy using an Olympus BX-40 microscope with a X 16 or X40 fluorite objective, numerical aperture 0.75 (Leco l-UB527), and an UV filter cube (cx 330—385 nm, em 420 nm, wide band pass; Leco U-M536). Images were photographed using an Optronics cooled low-light video camera (Leco DEI-470TB) with a 2X coupler (Leco HR200-CMT). The image was saved to a digital file at 72 dpi.

Assay for Neutral and Acidic Sphingomyelinase Activity. The mixed micellar sphingomyelinase assay using 14C-labeled sphingomyelin was performed as described with minor modifications (40). Exponentially growing cells (2 X 10^5) were treated, collected, and lysed in 200 μl of buffer containing 20 mM HEPES, pH 7.4; 10 mM MgCl2; 2 mM EDTA; 5 mM DTT; 100 mM Na3VO4; 100 mM Na3MO4; 10 mM β-glycerophosphate; 750 μM ATP; 1 mM phenylmethylsulfonyl fluoride; 10μM leupeptin; 10 μM pepstatin; and 2% Triton X-100 for the neutral sphingomyelinase assay or 0.2% Triton X-100 for the acidic sphingomyelinase assay. After incubation for 5 min at 4°C, cells were lysed by treatment in a Dounce homogenizer, and nuclei were removed by low-speed centrifugation (800 X g) for 5 min at 4°C. The protein concentration was measured using the Bio-Rad protein assay system. Protein (50 μg) was incubated for 2 h at 37°C in a buffer (final volume, 30 μl) containing 20 mM HEPES, 1 mM MgCl2, and 0.9 μl of (N-methyl-14C) sphingomyelin for the neutral activity and in 20 mM sodium acetate (pH 5.0), 1 mM EDTA, and 0.1% Triton X-100 with the same amount of 14C-labeled sphingomyelin for the acidic activity. The reaction was run at this protein concentration. Phosphocholine was then extracted with 100 μl of chloroform, 100 μl of methanol, and 1 n HCl (100:100:1). Radioactive phosphocholine was measured by scintillation counting. Data are expressed as a percentage of control for both the acidic and neutral sphingomyelinases.

Growth of Human Tumor Xenografts. SQ-20B (1 X 10^6 to 5 X 10^5) tumor cells were injected into the right hind limbs of nude mice (Fredrick Cancer Research Institute). Xenografts were grown for 2—3 weeks to a mean tumor volume ± SE of either 811 ± 61 mm3 or 320 ± 38 mm3, and animals were sorted into treatment groups by tumor size and deviation. At day 0, initial tumor volume was determined by direct measurement with calipers. During treatment, tumor volumes were measured with calipers twice weekly and represented as a percentage of original tumor volume. Tumor volumes were calculated by the formula (a X b X c)/2, which was derived from that for an ellipsoid (d^3/6). Xenografts were injected intratumorally with 1 mg of chelerythrine per kg of body weight on days 0, 3, 7, and 10. Irradiated mice were immobilized in Lucite chambers, and the entire body was shielded with lead except for the tumor-bearing hind limb. Tumors with initial mean volumes ± SE of either 811 ± 61 mm3 (n = 102) or 320 ± 38 mm3 (n = 72) were injected with 1 mg of chelerythrine per kg of body weight on days 0, 3, 7, and 10 (LD50, 25 mg/kg IP). Radiation was delivered at 5 Gy/day on days 0, 1, 3, 4, 7, 8, 10, 11, 14, and 15 for a total of 50 Gy using a Maxitron generator (1.88 Gy/min). Tumors were treated with radiation alone, chelerythrine alone, buffer, or chelerythrine combined with radiation. Data were calculated as the percentage of original (day 0) tumor volume and graphed as fractional tumor volume ± SE. Significance was analyzed using the Mann-Whitney rank-sum test. Tumor cure was defined as regression to a volume of <10% of original size because, at this volume, scar tissue resembles residual tumor tissue.
PKC INHIBITION, SPHINGOMYELINASE, AND APOPTOSIS IN VIVO

**Fig. 2.** Chelerythrine enhances radiation-induced apoptosis and increases sphingomyelinase activity. A, the clonogenic assay was used to assess cell survival following 1 µM chelerythrine and increasing doses of IR. Cells were treated with 0 or 1.0 µM chelerythrine 30 min prior to irradiation. After 2 weeks, colonies were scored as viable if they were composed of >50 cells. *Data points,* mean of three individual experiments with duplicate or triplicate determinants; *bars,* SD. B, light microscopy of SQ-20B cells 36 h after treatment with 8 Gy (left) or 5 µM chelerythrine and 8 Gy (right). Increased phase brightness and rounding of cells is characteristic of apoptosis in this system. C, DAPI staining of nuclei reveals that the concomitant treatment of SQ-20B cells with 5 µM chelerythrine and 8 Gy results in nuclear characteristics consistent with apoptosis. ×500. D, chelerythrine enhances radiation-induced cell killing as measured by PI exclusion and FACS analysis 36 h after treatment. *Data points,* mean of three independent experiments; *bars,* SD.

**Histology of SQ-20B Xenografts.** Tumors were treated once with chelerythrine (2 mg/kg body weight) and 20 Gy of radiation or with 20 Gy of radiation alone, excised 6 h after treatment, and fixed in 10% neutral buffered formalin. Tumors were then trimmed and processed in a Tissue Tek II tissue processor. Tissues were embedded in paraffin, sectioned, and stained with H&E or with 100 µl of DAPI (Sigma D9542; 1 µg/ml in PBS + 1% Triton X-100), followed by examination for apoptosis and necrosis by light microscopy. Apoptotic bodies were defined as basophilic staining nuclei with condensed or fragmented appearance in tissue regions devoid of necrotic material. The percentage of apoptotic cells was derived from counting 1000 cells per tumor (10 high-power fields); the percentages were scored by an observer blinded to the treatment condition. A minimum of 4 tumors per treatment condition were evaluated.

**RESULTS**

**SQ-20B Tumor Cells Lack an Apoptotic Response to IR and Are Radioresistant.** We previously demonstrated that the SQ-20B cell line, which is derived from a human laryngeal carcinoma, is resistant to radiation (D₀ = 2.3 Gy) compared to other human cell lines (41, 42). Mutations within exon 5 of p53 in SQ-20B result in a
PKC INHIBITION, SPHINGOMYELINASE, AND APOPTOSIS IN VIVO

Fig. 3. A, neutral sphingomyelinase activity is enhanced following the concomitant treatment of cells with chelerythrine and IR. The mixed micellar assay for sphingomyelinase activity was used to quantitate increased neutral (pH 7.4) and acidic (pH 5.5) sphingomyelinase activity 5 min following treatment of cells with either chelerythrine, IR, or both and compared to unirradiated control cells. Columns, mean of three experiments; bars, SD. B, the addition of ceramide to irradiated cultures decreases clonogenic survival. Data points, mean of three individual experiments; bars, SD. •, control; □, +5 μM C2-ceramide. C, PI staining of SQ-20B cells treated with chelerythrine demonstrates that zVAD-fmk inhibits programmed cell death. zVAD-fmk (20 μM) was added 30 min prior to treatment with chelerythrine (CH). R1 defines the region of dead cells; a percentage is given in the upper right corner of the histogram.

lack of G1 arrest and no increase in p53 protein following IR exposure (42). To further study whether SQ-20B cells undergo apoptosis following IR alone, studies were conducted at 20 Gy to assess nuclear morphology. No detectable chromatin condensation or cytoplasmic blebbing was observed after 72 h (Fig. 1A). By contrast, SQ-20B cells treated with 10 μM chelerythrine exhibited chromatin condensation, cytoplasmic blebbing, and internucleosomal DNA by 12 h (Fig. 1B). These data demonstrate that although SQ-20B cells retain the capacity to undergo apoptosis, IR alone is insufficient to initiate the apoptotic response.

Chelerythrine Enhances IR-induced Apoptosis and Is Preceded by Activation of Neutral and Acidic Sphingomyelinases. Because SQ-20B cells retain the capacity to undergo apoptosis yet fail to initiate the apoptotic program following IR, we tested whether one-tenth the concentration of chelerythrine (1 μM) that induced apoptosis alone would increase IR-mediated cell killing. We assayed clonogenic survival following treatment with 1 μM chelerythrine alone, IR alone (1–7 Gy), or the combination of chelerythrine and IR (Fig. 2A). The combination of 1 μM chelerythrine and IR increased cell death, as reflected by abortive colony formation over the dose range of IR delivered (1–7 Gy), and resulted in reduction of clonogenic survival greater than the expected additive killing of either treatment alone. For example, 5 Gy in combination with 1 μM chelerythrine results in 80% more abortive colonies (9% survival) than would be expected with either chelerythrine (68% survival) and IR (23% survival) separately (15%). These data suggest that chelerythrine and IR interact and result in increased abortive colony formation and cell killing. It is interesting to note that although previous studies showed that PKC inhibitors shorten the G2-M phase in p53-deficient cells and increase cell killing by IR (43), 1 μM chelerythrine failed to shorten the G2-M phase in irradiated cells (data not shown).

We assayed the percentage of SQ-20B cells undergoing apoptosis...
PKC Inhibition, Sphingomyelinase, and Apoptosis in Vivo

Chelerythrine Induces Apoptosis through Activation of CED3/CPP32-like Proteases. Previous studies have indicated that IR-induced apoptosis is dependent on activation of CED3/CPP32 (caspases)-like proteases (36). To determine whether the induction of apoptosis by chelerythrine involves activation of caspases, we used the irreversible peptide inhibitor zVAD-fmk. The addition of zVAD-fmk blocked apoptosis induced by chelerythrine and the combination of chelerythrine and IR (Fig. 3C). These findings indicate that chelerythrine initiates apoptosis through activation of CPP32 and related caspases. The peptide inhibitor YVAD-cmk, which inhibits other cysteine proteases more closely related to interleukin-1 converting enzyme than to CPP32, failed to inhibit chelerythrine-induced apoptosis. These results, when taken together with those from the apoptotic and clonogenic assays, indicate that the marked increase in cell death within 36 h following the combined treatment of cells with chelerythrine and IR results, in part, from a decrease in the apoptotic threshold of SQ-20B cells to IR. These data are consistent with previous reports demonstrating that IR- and ceramide-mediated apoptosis is dependent on caspase activation (45–47).

Chelerythrine Enhances Apoptosis and Cell Killing by IR in Vivo. Previous studies have suggested that increasing the fraction of tumor cells undergoing apoptosis may enhance IR-mediated tumor cell killing (5, 24). To determine whether the increased apoptosis observed in vitro with the combined treatment of chelerythrine and IR in vivo occurs in vivo, we examined H&E tissue sections from tumors at 6 h after treatment. Tumor sections were removed following treatment with a single injection of chelerythrine alone (2 mg/kg), 20 Gy

Fig. 4. The combination of chelerythrine and IR induces apoptosis in vivo. Tumors were excised 7 days following treatment with either two doses of chelerythrine at 1 mg/kg (A), 20 Gy in 5 Gy fractions (B), a single dose of chelerythrine and 20 Gy (C), or two doses of chelerythrine and 20 Gy (D) and stained with DAPI as described in “Materials and Methods.” Arrows, examples of apoptotic nuclei. ×500.

by quantitating PI uptake, cell shrinkage by FACS analysis, and cell and nuclear morphology. Exposure of SQ-20B cells to 4–20 Gy induced little apoptosis compared to untreated controls at 36 h. The combination of 2.5 μM chelerythrine and 4–8 Gy induced morphological changes consistent with apoptosis (Fig. 2, B and C) at a greater frequency than observed with either IR or chelerythrine alone (Fig. 2D) as assayed by FACS analysis.

Previous work in our laboratory suggested that chelerythrine enhances IR-induced apoptosis, in part, through activation of sphingomyelinases (35). In addition, both IR and ceramide have been reported to induce apoptosis through activation of caspases (44–47). We therefore assayed neutral and acidic sphingomyelinase activity in irradiated SQ-20B cells using a mixed micellar assay. Whereas exposure of SQ-20B cells to 20 Gy increased neutral and acidic sphingomyelinase activity by approximately 25% within 5 min of treatment (Fig. 3A), consistent with previous findings in other cell lines (33–35, 48), 10 μM chelerythrine alone or in combination with 20 Gy increased neutral sphingomyelinase to 194% (P < 0.01) of untreated controls within 5 min of exposure to IR. Ceramide production was also increased by the combination of chelerythrine and IR (not shown). In addition, the addition of exogenous ceramide 30 min prior to irradiation enhanced IR-induced cell killing (Fig. 3B), suggesting that ceramide acts as a second messenger to enhance cell death in this system. These studies demonstrate that the combination of chelerythrine and IR increases sphingomyelinase activity before the appearance of apoptosis and that the addition of exogenous ceramide increases tumor cell killing.
alone, or a single injection of 2 mg/kg chelerythrine and 20 Gy. This time point was chosen to limit the necrotic regions within the tumors treated with both chelerythrine and IR that may skew the number of detectable apoptotic cells. H&E sections taken from four independent tumors in each treatment group were scored for apoptotic nuclei; 1000 cells were scored from four separate regions. In the tumors treated with IR alone or chelerythrine alone, 1.0 and 1.53% of cells were scored as apoptotic, respectively. By contrast, 3.0% of cells were apoptotic in tumors treated with chelerythrine and 20 Gy ($P < 0.001$, z test). Thus, the combination of chelerythrine and IR significantly increased the apoptotic fraction of tumor cells compared to tumors treated with either IR or chelerythrine alone.

To see the extent of apoptosis over time, tumor histology was examined 7 days after treatment with two injections of chelerythrine (1 mg/kg) and 20 Gy fractionated in 5-Gy doses on days 0, 1, 3, and 4. Whereas nearly 100% of representative histological tumor sections exhibited significant necrosis in the combined treatment group by day 7, DAPI staining of sections from regions without necrosis demonstrated numerous apoptotic nuclei (Fig. 4C). In contrast, tumors treated with 20 Gy of IR fractionated in 5-Gy intervals (Fig. 4B) or two doses of chelerythrine (Fig. 4A) showed few apoptotic nuclei as defined by chromatin margination and nuclear shrinkage and contained only modest increases in necrotic areas over untreated tumors (not shown). The histology studies demonstrate that exposure to chelerythrine and IR increases apoptosis in tumors within 6 h and that increased apoptosis is observed over the course of treatment.

We examined whether the observed increase in tumor cell apoptosis by chelerythrine following IR reflects enhanced tumor volume reduction in vivo. As shown in Table 1 and Fig. 5, chelerythrine and IR reduced 43% (18 of 42) of SQ-20B tumors in nude mice to less than 10% of their original volume at day 28 compared to 13% (6 of 44) and 12% (6 of 43) in IR and chelerythrine-only groups, respectively ($P < 0.001$, z test). Due to the rapid growth of mock-treated tumors and the necessity of sacrificing the control groups, data were analyzed to day 28. Because the induction of apoptosis is an ongoing and rapid process in a tumor, with individual cells taking under 60 min to complete the death program, a doubling in the number of apoptotic tumor may account for the observed rapid tumor size reduction and cures.

Treatment of tumors with IR induces areas of inflammation characterized by polymorphonuclear cells in the underlying epidermis and muscle tissue 7 days after treatment with 20 Gy fractionated at 5 Gy/day (not shown). The combined treatment of tumors with IR and chelerythrine did not enhance the acute inflammation in the normal tissue. In addition, chelerythrine treatment did not appear to hinder mobility or induce weight loss. Long-term studies, however, are needed to determine the effect on the therapeutic ratio of treatment of mice with chelerythrine and IR. These findings suggest that induction of apoptosis increases cell killing and tumor volume reduction following radiation treatment.

**Table 1.** Chelerythrine enhances tumor volume reduction by IR after 28 days

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>Number of tumors</th>
<th>% tumors &lt;10% original volume after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>0/38</td>
<td>0</td>
</tr>
<tr>
<td>50 Gy (5 Gy X 10 days)</td>
<td>6/44</td>
<td>13</td>
</tr>
<tr>
<td>Chelerythrine (1 mg/kg)</td>
<td>6/43</td>
<td>12</td>
</tr>
<tr>
<td>50 Gy and chelerythrine</td>
<td>18/42</td>
<td>43</td>
</tr>
</tbody>
</table>

* $P < 0.001$, z test.

Fig. 5. The combination of chelerythrine and IR increases tumor regression over IR alone. The SQ-20B xenograft system was used to assess the antitumor effects of chelerythrine and IR. Columns, the percentage of tumors that regressed to less than 10% of the original volume on days 0, 7, 21, and 28 after treatment.

**DISCUSSION**

Although previous reports have indicated that the radioscurability of tumors correlates with an apoptotic response to IR, we are the first to demonstrate that lowering the threshold at which tumor cells undergo apoptosis following IR contributes to enhanced tumor cell killing, regression, and cures. SQ-20B cells undergo little apoptosis when exposed to IR doses as high as 20 Gy in vitro or in vivo. By contrast, apoptosis was observed both in vitro and in vivo following exposure to IR and chelerythrine. The increase in mitotic death following the combined radiation and chelerythrine treatment may represent a nonapoptotic and therefore different mechanism of tumor cell killing in vivo.

Chelerythrine was selected for these studies due to its specificity for PKC group A and B isoforms relative to other kinase inhibitors, such as staurosporine and H7 (37). Whereas other selective PKC inhibitors, such as the bisindolylmaleimides (Ro31–8220), have been synthesized, chelerythrine inhibits PKC in an ATP-independent manner. ATP may reduce the relative efficacy of other inhibitors in vivo because the intracellular concentrations of ATP are much higher than those used to test the potency of kinase inhibitors in vitro (38). In addition, recent studies suggest that staurosporine and its derivatives may, paradoxically, activate certain PKC isoforms (49, 50). Chelerythrine is also soluble in PBS compared with staurosporine and calphostin C (39), which allows for more efficient delivery of the drug to the tumor.

The induction of apoptosis by the combination of chelerythrine and IR is preceded by activation of neutral sphingomyelinase. Sphingomyelinase activation and subsequent ceramide production have been found to precede IR-induced apoptosis in several cell types (33, 48, 51–54). Loss of ceramide production following IR treatment confers a radioresistant phenotype in cells derived from acid sphingomyelinase knockout mice and in tumor cells selected for a defect in neutral sphingomyelinase production (48, 55). In addition, chelerythrine (37) and calphostin C (56) induce apoptosis and ceramide production following the activation of a neutral sphingomyelinase (34, 35). Although the exact role of neutral and acidic sphingomyelinas...
IR-induced apoptosis has not been resolved (57), our data demonstrate the therapeutic potential of modifying the ceramide/sphingomyelinase signaling pathway in an in vivo tumor model. Our results also demonstrate that the increase in apoptosis observed following the combined treatment of cells with IR and chelerythrine is dependent on activation of the caspases. These data are consistent with recent reports demonstrating that IR and ceramide induce apoptosis through activation of CPP32 and other caspases (36). Our results suggest that chelerythrine may enhance IR-mediated apoptosis by targeting common components of the cell death pathway and thereby overcome resistance to IR-induced apoptosis in vitro and in vivo. Chelerythrine may represent a clinically useful PKC inhibitor to enhance the therapeutic index of IR, although more studies are required to assess the systemic effects of this chemotherapeutic agent. Chelerythrine decreases tumor cell survival and increases apoptosis at doses of X-rays typically used in a fractionated radiation therapy schedule (1–4 Gy). Because cell killing in a tumor receiving fractionated radiotherapy is magnified exponentially by the number of daily doses of IR delivered, the decrease in tumor cell survival following chelerythrine and IR treatments compared with IR alone results in a large increase in tumor cell killing at the end of treatment (58). The equal normal tissue toxicity observed between IR alone and the combined chelerythrine and IR treatment group suggests selective tumor cell killing. Whether selective tumor killing is due to the physical restriction of IR and chelerythrine to the tumor or whether it represents a biological specificity of PKC inhibition in malignant cells requires further investigation. In summary, inhibition of PKC represents a unique strategy for overcoming radioresistance through a decrease in the apoptotic threshold of tumor cells.

ACKNOWLEDGMENTS

We thank Ann Koons and the Flow Cytometry Facility for technical support.

REFERENCES


Decreasing the Apoptotic Threshold of Tumor Cells through Protein Kinase C Inhibition and Sphingomyelinase Activation Increases Tumor Killing by Ionizing Radiation

Steven J. Chmura, Helena J. Mauceri, Sunil Advani, et al.


Updated version  Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/57/19/4340](http://cancerres.aacrjournals.org/content/57/19/4340)

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.