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Source: Radiation Research, 190(3):262-270.
Published By: Radiation Research Society
https://doi.org/10.1667/RR15072.1
URL: http://www.bioone.org/doi/full/10.1667/RR15072.1

RADIATION RESEARCH **190**, 262–270 (2018)
0033-7587/18 $15.00

DOI: 10.1667/RR15072.1

Cell Line-Specific Direct Irradiation and Bystander Responses are Influenced by Fetal Bovine Serum Serotonin Concentrations

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Curtis, J. J., Seymour, C. B. and Mothersill, C. E. Cell Line-Specific Direct Irradiation and Bystander Responses are Influenced by Fetal Bovine Serum Serotonin Concentrations. *Radiat. Res.* 190, 262–270 (2018).

The radiation-induced bystander effect is mechanistically complex, involving many different signaling components. Serotonin, present in fetal bovine serum (FBS), has been implicated in the modulation of cellular responses to radiation. However, the role of this ubiquitous signaling molecule has yet to be elucidated with regard to cell line-specific radiation responses. In this study, cell survival was measured in HCT116 p53 wild-type (HCT116$^{+/+}$) and HaCaT cell cultures treated with media containing serotonin-depleted FBS and compared to our standard FBS-supplemented media, using clonogenic assays. We utilized an enzyme-linked immunosorbent assay to quantify the difference (4.3 $\pm$ 1.3 ng/ml) in serotonin concentrations among the media. Serotonin-depleted media significantly reduced survival in both nonirradiated cell lines. Furthermore, we sought to determine the effects to cells in this media exposed to direct irradiation as well as bystander media from irradiated cells. Cell survival was significantly increased when HCT116$^{+/+}$ cells were directly irradiated in serotonin-depleted media, while HaCaT cells showed no significant difference in survival between the media. Bystander investigations demonstrated that HCT116$^{+/+}$ cells were only able to generate a bystander effect when cultured in standard media conditions containing greater serotonin levels. Conversely, HaCaT cells were unaffected by the different media in terms of producing a bystander response, generating bystander effects irrespective of the media. Previous research linking serotonin receptors to the bystander effect, together with our results, indicate that receptor heterogeneity among cell types may underlie serotonin sensitivity in direct irradiation and bystander responses through serotonin receptor-mediated cell signaling cascades.

INTRODUCTION

After low-dose ionizing radiation exposure, a nontargeted pleiotropic biological cascade defined as radiation-induced bystander effect (RIBE) can arise in cells not directly traversed by a charged particle. The cellular responses associated with this event ultimately lead to cell death, which occurs as a result of nonirradiated cells receiving an unknown signal(s) from irradiated cells. Although this effect is commonly observed, the underlying mechanisms involved in bystander signaling remain complex and elusive. Many factors have been suggested to be important in mediating bystander effects, including photon emission (*1*), calcium signaling (*2*), oxidative metabolism, gap-junction intercellular communication, secreted soluble molecules (*3*) and many downstream signaling cascades (*2–4*). Presumably, all of these components are interconnected, however, the extent to which each potential aspect plays and their definitive roles in bystander signal production have yet to be fully elucidated. The challenge of ascertaining the bystander mechanism is augmented by variability in the production of bystander effects. Inconsistencies in the generation of effects have been linked to several small signaling molecules that can influence bystander responses considerably (*5*). In particular, 5-hydroxytryptamine (5-HT, serotonin) is repeatedly connected to the modulation of bystander signaling (*5–11*). Appreciable serotonin levels are found in fetal bovine serum (FBS), which is commonly used as a supplement in cell culture media preparations. The 5-HT concentrations in FBS samples can fluctuate substantially between suppliers and even among batches from the same supplier (*9*). Further exacerbating variability among serum samples, light exposure is known to cause depletion of FBS serotonin levels (*9*). This pronounced unpredictability in serum causes deviations in cell culture media serotonin concentrations that are sufficient to dictate the magnitude of both direct irradiation and bystander-induced cell death, wherein samples with increased 5-HT concentration are correlated to greater reductions in cell survival (*9*). It is evident that serotonin levels in FBS are a major cause for variability in bystander assays, paradoxically implicating serotonin-initi-

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ated cellular signaling cascades in the bystander mechanism.

Previously reported investigations into this phenomenon have revealed that cell culture media supplemented with serotonin can lead to increases in apoptosis frequency (*5*) that are concomitant with reductions in cell survival upon both direct irradiation (*5*) and media transfer from irradiated to nonirradiated cells (*5*, *8*, *10*). Moreover, it has been observed that 5-HT concentrations present in culture media are depleted after exposure of cells to radiation (*5*), indicative of serotonin binding to plasma membrane embedded 5-HT receptors. Studies have also utilized the 5-HT inhibitor, reserpine, for its ability to deplete monoamines. Reserpine abolishes bystander-induced cell death both *in vitro* in recipient cells receiving media from irradiated cells (*5, 7*) and in zebrafish (*7*). Yet, a discernable relationship between serotonin and RIBE does not appear to occur in all cell lines. Notably, it has been determined that the human keratinocyte HaCaT cell line is unresponsive to changes in cell culture media 5-HT concentrations when bystander effects are analyzed in these cells (*11*). It has been suggested that there may be interplay between serotonin-initiated signaling pathways and protein 53 (p53) activated pathways in the production of bystander signals (*8, 11*), however, this hypothesis is currently lacking tangible experimental backing. In addition, data on responses to direct irradiation in different media 5-HT concentrations in distinct cell lines are oddly absent from the literature. Most published studies exploring the effects of serotonin in terms of cellular irradiation responses have been performed exclusively on the HPV-G cell line (*5–7*, *9*, *10*) or have concentrated on cell line-specific bystander effects (*8*, *11*). Clearly, a large knowledge gap exists.

Serotonin is an extensively studied biogenic monoamine neurotransmitter signaling molecule synthesized from the essential amino acid tryptophan. Consistent with its physiological functions in the cardiovascular, central nervous and gastrointestinal systems in mammals, serotonin is found in high concentrations in platelets and serotonergic neurons, and is synthesized, stored and released by the enterochromaffin cells of the intestinal mucosa (*12*). The physiological responses of serotonin are mediated through seven receptor classes and 13 distinct receptor subtypes (*13*). Pathological evidence has accumulated linking serotonin to carcinogenesis, specifically, increases in serum levels of 5-HT in association with certain carcinomas, and expression of 5-HT receptors on several types of cancers (*12*). Serotonin has also been shown to be a mitogenic factor for a wide range of normal and tumor cells (*12*). While FBS serotonin levels may fluctuate, concentrations within the nanomolar range are sufficient to activate many 5-HT receptors (*14*), which encompass most cell culture media preparations. Nonetheless, in the context of cellular effects, even slight serotonin concentration fluctuations in culture media can govern the degree to which 5-HT receptors are activated and mediate various cellular responses. The receptor families of importance in bystander signaling are the 5-HT_2 and 5-HT_3 receptors, both capable of transiently elevating intracellular calcium levels (*15*). Accordingly, research into the selective 5-HT receptor antagonists has demonstrated that both 5-HT_3 receptor-specific (*5*, *6*) and 5-HT_2 receptor-specific (*6*) inhibitors can modulate bystander signaling. Additional support for the role of serotonin in RIBE was the observation that 5-HT_3 receptor expression increased in HPV-G cells after irradiation (*5*). Collectively, these data strongly suggest a role for 5-HT_3 receptors in bystander signaling.

To further investigate the significance of serotonin in the bystander mechanism, we assessed cell survival in the absence of irradiation, direct irradiation cell survival and bystander-induced cell death using serotonin-depleted media. These outcomes were examined in two distinct cell lines, HCT116 p53 wild-type ($HCT116^{+/+}$) and HaCaT, selected for their polar sensitivities to 5-HT when producing bystander signals. We suspected genotypic variability as the cause for heterogeneous bystander responses when distinct cell lines are cultured in varying media serotonin concentrations.

MATERIALS AND METHODS

Cell Culture

The human colon carcinoma cell line, $HCT116^{+/+}$ was selected for this study due to its previously demonstrated ability to generate and receive bystander signals (*4*). This cell line has also shown responsiveness and sensitivity to serotonin levels in cell culture media when bystander effects were measured (*8*). Cells were maintained in 75-cm^2 stock flasks (Falcon™, Durham, NC) containing Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco®/Life Technologies Inc., Grand Island, NY) supplemented with 10% FBS, 2 m*M* L-glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin sulphate. In addition, the nontransformed human keratinocyte HaCaT cell line was used in agreement with the literature, which characterized these cells as unresponsive to serum serotonin levels when producing bystander signals (*11*). HaCaT cells were cultured in stock flasks containing media of similar composition to $HCT116^{+/+}$ culture media; however, a 0.5-μg/ml hydrocortisone (Sigma-Aldrich®, St. Louis, MO) supplement was added. Both cell lines were incubated at 37°C, 95% relative humidity and 5% CO_2 until reaching 80–100% confluence, upon which cells were passaged. These adherent cells were detached with a 10-ml solution of 0.25% trypsin containing Dulbecco's phosphate buffered saline and 1 m*M* ethylenediaminetetraacetic acid dissociation reagent. After trypsinization, cells were neutralized with 10 ml of culture media and cell suspensions were subsequently pipetted into stock flasks containing 15 ml of fresh culture media. Cells were incubated after being passaged and received media renewals every 3–4 days. All reagents were obtained from Gibco/Life Technologies unless otherwise specified.

Enzyme-Linked Immunosorbent Assay to Quantify Serotonin Depletion

Following the approach by Mothersill *et al*. (*9*), FBS aliquots were directly exposed to 4 h of laboratory light to effectively deplete serum serotonin levels. A serotonin research enzyme-linked immunosorbent assay (ELISA) kit (Rocky Mountain Diagnostics Inc., Colorado Springs, CO) was used to quantify the FBS serotonin concentrations of both light-exposed FBS and FBS devoid from light exposure.

Previously, our group has utilized this experimental technique and specifically, this reagent in similar studies to quantify serotonin levels (*7*, *9*, *10*). Kit reagents and serum samples were stored at 4°C and allowed to reach room temperature prior to performing the ELISA. Standards and controls were pipetted at a volume of 10 µl into the wells of the acylation plate (pre-coated with acylation reagent) and were subsequently diluted to 100 µl with the kit-provided diluent (Tris buffer with 1% stabilizing agent) and deionized water. Serum samples were loaded into the wells of the acylation plate at volumes of 100 µl. Acylation buffer (25 µl) was added to all wells of the acylation plate and was incubated at room temperature on an orbital shaker (Thomas Scientific, Swedesboro, NJ) for 30 min at 600 rpm. The full volume of acylated standards, controls and samples were transferred to the microtiter plate (antigen pre-coated microwell plate) with 25 µl of serotonin antiserum. The microtiter plate was then incubated with adhesive foil for 16 h at 2–8°C. Well contents were discarded and the plate was washed three times with 300 µl of wash buffer and blotted dry each time. Next, 100 µl of enzyme conjugate was added to all wells and the plate was incubated at room temperature on a shaker (600 rpm) for 30 min. Plate washing was performed three more times as described. Substrate was added at a volume of 100 µl to the wells and incubated for 30 min at room temperature on a shaker. Finally, 100 µl of the stop solution was added to the microtiter plate and absorbance was read immediately at room temperature using an Infinite M200 Pro Plate Reader (Tecan, Männedorf, Switzerland) at a wavelength of 450 nm. A calibration curve was constructed by plotting absorbance readings of the standards as a function of each logarithmic standard concentration and the data were fit for a four-parameter nonlinear regression using the statistical software analysis package GraphPad Prism version 6 (LaJolla, CA). Thereafter, serotonin concentrations of the serum samples were obtained using the calibration curve and the measured absorbance readings of the samples, performed in quadruplicate. Light-exposed FBS samples and FBS devoid from light exposure were then used as supplements in cell culture media to generate serotonin-depleted media and standard media, respectively. The proceeding experiments were performed to evaluate cell survival among cells cultured in these distinct media. Special precautions were employed to avoid additional exposure to light during this procedure.

Clonogenic Assay and Direct Irradiation

Cell monolayers were detached with trypsin and resuspended in media to generate a single-cell suspension. Sample aliquots of this cell suspension were counted with a TC20 Automated Cell Counter (Bio-Rad® Laboratories, Mississauga, ON). Clonogenic cell densities of 500 cells per flask were seeded onto 25-cm^2 flasks (Falcon™, Durham, NC) in 5 ml of media and incubated for eight days. Cells were stained with 25% carbol fuchsin (RICCA Chemical Co.®, Arlington, TX) and counted according to the techniques described by Puck and Marcus (*16*), wherein macroscopic colonies equal to and over 50 cells satisfy criteria of reproductive cell survival. Clonogenic cell survival was first assessed in terms of survival without irradiation in both serotonin-depleted media and standard media for each cell line, in triplicate, for eight independent experiments. Cell survival in serotonin-depleted media was normalized to that of cells plated in standard media. Subsequently, clonogenic cell survival was evaluated in the different media conditions and in both cell lines receiving either a 0.3, 1.0 or 3.0 Gy dose. Irradiations were performed at room temperature 24 h after cell seeding, using a cesium-137 gamma radiation source. Flasks containing cells were positioned at a distance of 27 cm from the source, giving a dose rate of 0.26 Gy/min at the time of the experiments. Cells were irradiated for an amount of time required to achieve the aforementioned radiation doses. In both cell lines, the survival of each irradiated group was normalized to survival of nonirradiated cells for each respective media treatment. Survival values are presented as percentage cell survival for eight independent experiments, performed in triplicate.

Media Transfer and Clonogenic Assay for Bystander Activity

Procedural details for the methods of this section are adapted from previous work performed by our group. Detailed methods for bystander media transfer experiments can be found elsewhere (*17*). In brief, cells were detached with trypsin, resuspended in media and counted as described for the clonogenic assay. Donor and recipient 25-cm^2 flasks were plated in 5 ml of cell culture media containing 350,000 and 500 cells, respectively. Cell seeding of both donor and recipient flasks occurred 24 h prior to irradiation of donor flasks. At 1–2 h postirradiation, culture media from donor flasks was harvested and passed through a 0.2-µm Acrodisc® sterile filter (Pall Corp., Ville St. Laurent, Canada) to remove cells and debris from transferred media. This transferred media is commonly referred to as irradiated cell condition media (ICCM). Recipient cell media was discarded and replaced with ICCM from respective treatment group donor flasks. Control donor groups were sham irradiated and media was transferred to recipient cells, serving as a media transfer control level of survival. Experiments were performed to determine whether serotonin concentrations in donor and/or recipient flasks would modulate bystander signal production. Media transfer bystander assays were performed with three distinct arrays of bystander experimental setups: donor cells seeded in serotonin-depleted media and the ICCM transferred to recipient cells initially seeded in standard media (before media transfer); donor cells seeded in standard media and the ICCM transferred to recipient cells initially seeded in serotonin-depleted media; and donor cells seeded in standard media and the ICCM transferred to recipient cells initially seeded in standard media. Donor flasks received 0.3 and 1.0 Gy doses to generate the ICCM, which was then transferred to recipient flasks as described. Recipient cell survival-receiving ICCM were normalized to control recipient flasks undergoing media transfers; however, their respective donor cells were not irradiated. Cell colonies were stained and counted seven days after media transfer. Survival was measured for each distinct experimental array, performed in triplicate.

Statistical Analysis

Data are presented as mean ± standard error of mean (SEM) throughout this study. Statistical analyses were performed using GraphPad Prism version 6, in which significance was evaluated by comparing the survival of cells seeded in serotonin-depleted media and in standard media. In bystander assays, cell survival for irradiated groups were each independently compared to survival of the media transfer control group within each distinct experimental setup. Statistical significance was determined using a Student's t test and different levels of survival were considered to be significant at $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$.

RESULTS

Nonirradiated and Direct Irradiation Cell Survival in Serotonin-Depleted Media

Preceding cell survival examinations, FBS aliquots were measured for serotonin levels to quantify the magnitude of 5-HT depletion caused by exposure to 4 h of laboratory light. The outcome was a 4.3 ± 1.3 ng/ml decrease (or a 42% reduction) in cell culture media serotonin levels (Table 1). We observed a similar significant reduction in cell cloning efficiency in both HCT116$^{+/+}$ cells and HaCaT cells when serotonin-depleted media was used to seed the cells compared to standard media conditions in the absence of radiation exposure (Fig. 1). Serotonin-depleted media reduced cell survival by 13 ± 3% and 10 ± 2% of the

TABLE 1
Serum Serotonin Concentrations

Standard media (ng/ml)	5-HT-depleted media (ng/ml)
10.3 ± 0.3	6 ± 1

Note. Errors represent SEM, n = 4.

survival of cells cultured in standard media conditions for HCT116$^{+/+}$ and HaCaT cells, respectively.

Upon direct irradiation, the cell lines responded distinctly to the media preparation containing intentionally reduced serotonin levels. HCT116$^{+/+}$ cells exhibited a significantly greater degree of survival when plated in serotonin-depleted media at all radiation doses compared to cells cultured in standard media conditions (Fig. 2A). This effect was most significant when the cells received either a 0.3 or 1.0 Gy dose of radiation (Fig. 2A). In contrast, HaCaT cells displayed a similar level of survival when plated in either media (Fig. 2B). Regardless of the cell line and media used, clonogenic cell survival was radiation dose-dependent in all cases.

Bystander Activity of Cells Cultured in Serotonin-Depleted Media

Dissimilar results were observed when bystander activity was quantified in each cell line (Figs. 3 and 4). Significant reductions in cell survival occurred in HCT116$^{+/+}$ bystander cells, compared to sham-irradiated media transfer controls, only with the experimental setup in which both donor and recipient cells were seeded in standard cell culture media containing higher serotonin levels (Fig. 3C). When serotonin-depleted media was used for plating either the donor or recipient cells, clonogenic cell survival was not significantly reduced (Fig. 3A and B). Conversely, HaCaT cells exhibited significant reductions in clonogenic cell survival when bystander media from donor cells received 1.0 Gy was transferred to recipient cells for each experimental setup (Fig. 4). This showed that, irrespective of the 5-HT concentration of the media that the donor or recipient cells were cultured in, bystander media generated a significant reduction in HaCaT cell survival.

DISCUSSION

Bystander signaling appears to involve a complex interplay between several cellular components, leading to a multitude of signaling events that are supposedly interrelated. Of the many factors suggested to be connected to the cascade, our group has been especially interested in determining the role of serotonin in bystander signal production. Studies exploring the effects of 5-HT in bystander responses have largely been performed on the HPV-G cell line (*5–7*, *9–10*), however, diverse responses to serotonin have been shown in other cell lines (*8*, *11*). We have demonstrated cell line-specific survival pertaining to both direct irradiation and RIBEs when cells are cultured in media containing depleted serotonin levels.

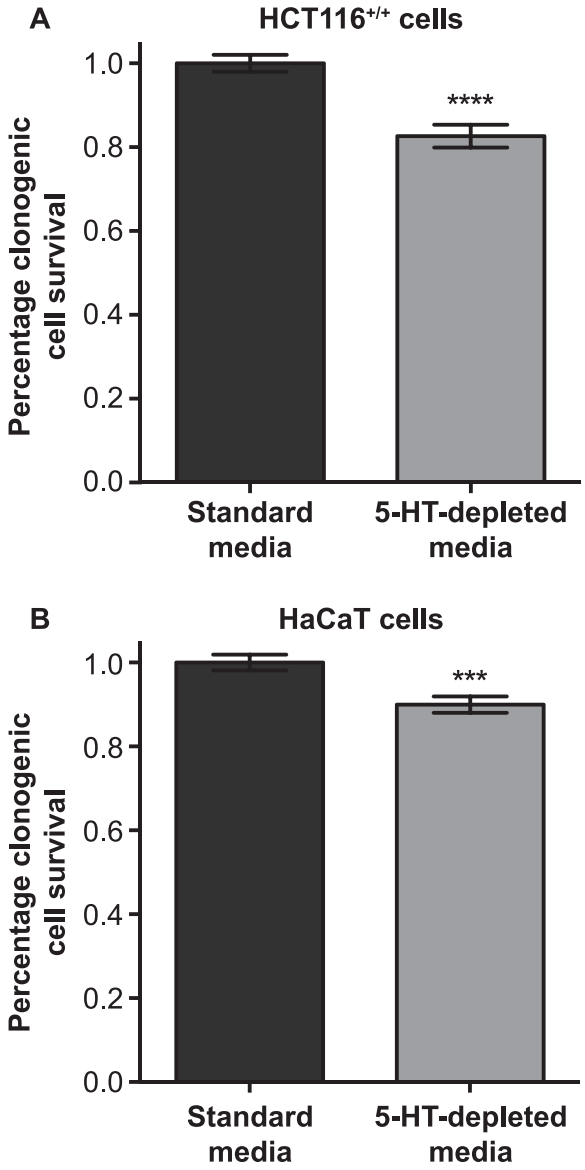

FIG. 1. Clonogenic cell survival in nonirradiated HCT116$^{+/+}$ cells (panel A) and HaCaT cells (panel B) seeded in cell culture media containing either serotonin-depleted FBS (serotonin-depleted media) or cell culture media containing FBS devoid of light exposure (standard media). Survival is presented as a percentage of control, normalized to the survival of cells in standard media conditions for each cell line. Error bars represent SEM for eight independent experiments performed in triplicate. Significance (****$P < 0.0001$ and ***$P < 0.001$) was determined using Student's t test to determine survival values of cells cultured in serotonin-depleted media to survival values of cells cultured in standard media.

As anticipated, 4 h of laboratory light exposure successfully depleted 5-HT concentrations in FBS samples that were then used as a supplement in cell culture media. This finding reiterates the importance of minimizing light exposure in cell culture work sensitive to serotonin. Initially evident when quantifying serum 5-HT levels was the seemingly low FBS serotonin concentration used in our

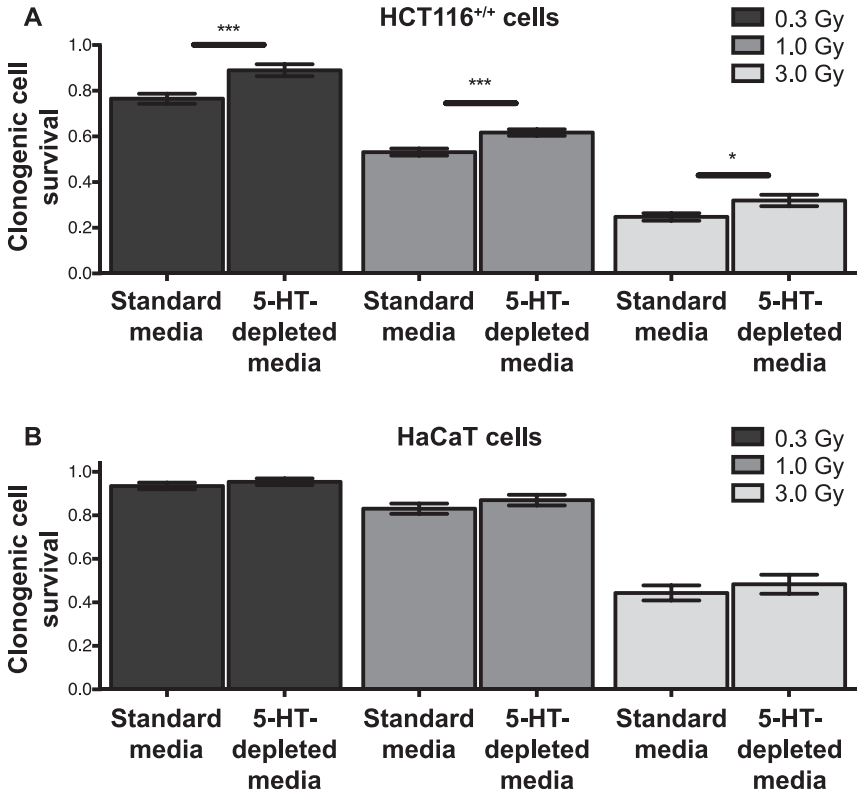

FIG. 2. Clonogenic cell survival after direct irradiation of HCT116$^{+/+}$ cells (panel A) and HaCaT cells (panel B) that received 0.3, 1.0 or 3.0 Gy. Survival values for standard media and serotonin-depleted media are both presented and are normalized to the survival of nonirradiated cells for each respective media. Error bars represent SEM for eight independent experiments performed in triplicate. Significance was determined using Student's *t* test to assess differences among media treatment groups within each radiation dose, where statistical significance was considered at $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$.

standard cell culture preparation even prior to light exposure. The concentration measured (10.3 ± 0.3 ng/ml) falls slightly below the range of concentrations presented by Mothersill *et al*. (*9*) in FBS samples measured across various manufacturers (13–89 ng/ml). However, this concentration is comparable and was subsequently proven to be adequate for bystander effect generation (Figs. 3C and 4C). In the HPV-G cell line, serum samples with 5-HT concentrations below 25 ng/ml were deemed to be insufficient to generate media transfer bystander effects (*9*). The supposed serum serotonin threshold concentration of 25 ng/ml for bystander signal production appears to be cell line-specific, as cell culture media containing serotonin levels below this value was sufficient to facilitate bystander effects.

Examination of cell survival in different media conditions prior to irradiation revealed that small nanomolar concentration differences in 5-HT were substantial enough to cause significant changes in plating efficiency (Fig. 1). Media containing depleted serotonin levels resulted in decreased cell survival in the absence of radiation in both cell lines. Inconsistent with this result, 5-HT-supplemented cell culture media has been shown to decrease cell survival (*5*). When gathering the results of this study, we realized this conflicting finding, wherein our cell lines exhibited increased survival with the media that contained higher serotonin concentration. Nonetheless, in the previously performed experiments, micromolar concentrations of 5-HT were added to culture media (*5*), rather than the nanomolar concentration difference utilized here. Collectively, these findings appear to indicate a biphasic relationship between serotonin concentration and cell survival, in which 5-HT has a toxic effect in the micromolar concentration range, and conversely, may be permitting cell survival when nanomolar concentrations are present in culture media. Pro-survival effects of serotonin have been shown in nonirradiated bystander media transfer control experiments with both HCT116$^{+/+}$ and HCT116$^{-/-}$ cells cultured in media supplemented with 100 ng/ml of 5-HT (*8*). These cells displayed lower levels of micronuclei induction compared to standard media conditions with lower 5-HT levels (*8*), suggesting that serotonin may actually promote cell survival at nanomolar concentrations. A similar biphasic relationship between serotonin and cell survival has been demonstrated in cultured epithelial thyroid cells where 5-HT concentrations below 5 μ*M* enhanced cell growth and survival, while concentrations exceeding 50 μ*M* activated cell apoptosis pathways (*18*). Serotonin has also been shown to increase

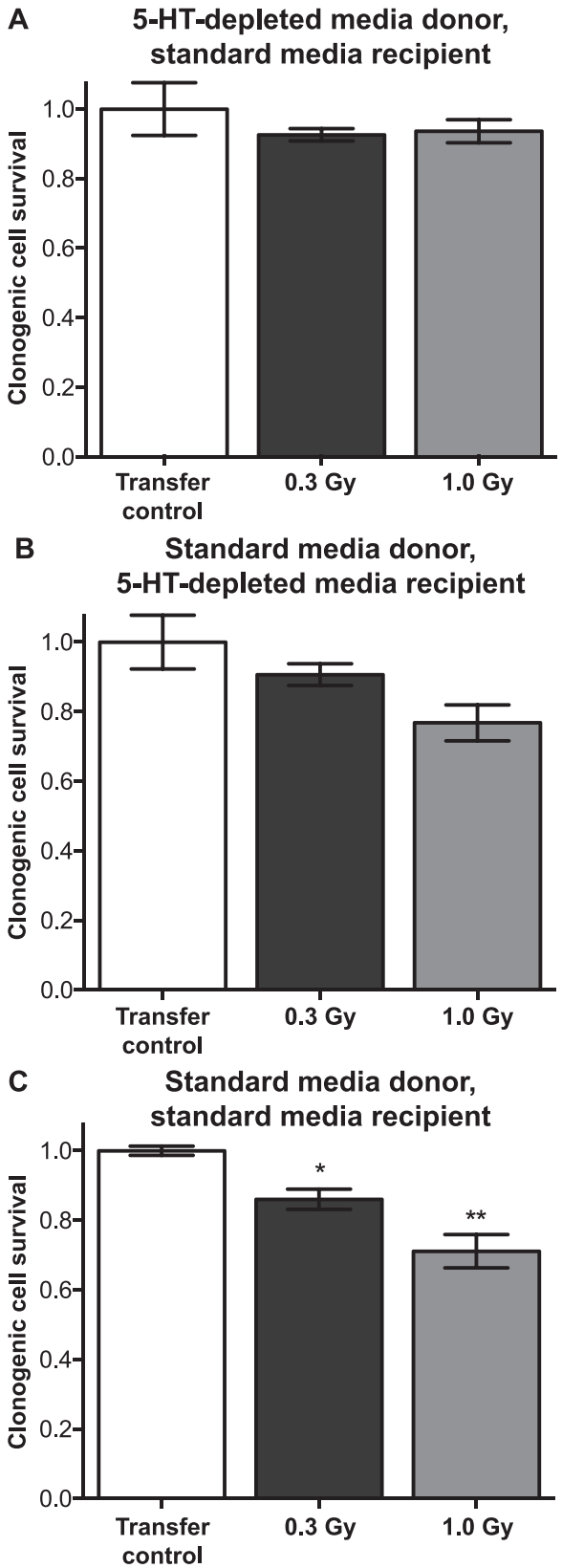

FIG. 3. Clonogenic cell survival of HCT116$^{+/+}$ bystander cultures after irradiation of donor cells in which: ICCM from donor cells cultured in 5-HT-depleted media was transferred to recipient cells initially (before media transfer) cultured in standard media conditions (panel A); ICCM from donor cells cultured in standard conditions was transferred to recipient cells initially cultured in 5-HT depleted media (panel B); and ICCM from donor cells cultured in standard conditions was transferred to recipient cells initially cultured in standard media (panel C). Survival is normalized to the media transfer control (0 Gy, sham irradiated) level of survival for each independent experimental setup (panels A–C). Values presented are mean with SEM error bars for each experiment performed in triplicate. Significance was determined using Student's *t* test (**$P < 0.01$; *$P < 0.05$) comparing media transfer control cell survival to cell survival of recipient cells receiving media transfer from irradiated cells.

cell survival (*19*) and stimulate growth in a wide range of both normal (*12*, *19*) and cancer (*12*) tissues. It has been postulated that when nanomolar concentrations of 5-HT are available, the effect of ionizing radiation is needed in addition to serotonin to trigger cell death cascades through intracellular calcium mobilization (*2, 5*). Furthermore, when excess serotonin is present in the media, it alone is sufficient to initiate calcium entry, leading to reductions in cell survival (*5*). Therefore, it is crucial that serotonin-sensitive assays are performed using consistent culture media conditions and in minimal lighting, since we have shown that even nanomolar concentration differences can significantly alter cell plating efficiency.

Direct irradiation has been shown to reduce HPV-G cell survival in correspondence with increasing culture media serotonin levels (*5*). Before this study, it was unknown if this effect was common across different cell lines and if nanomolar concentration differences in 5-HT are sufficient to reproduce this effect. We observed significant increases in HCT116$^{+/+}$ cell survival when cells were seeded in serotonin-depleted media and directly irradiated compared to standard media (Fig. 2A). Evidently, we assumed that some factor, or lack thereof, in the media was the cause for this discrepancy in survival. Previously published data by our group has shown that serotonin is depleted in cell culture media after irradiation of cells and expression of 5-HT_3 receptors increases with radiation exposure (*5*). These previously reported findings, with the data presented here, strongly suggest that the depleted serotonin levels within the light-exposed cell culture media were the cause for the significant increase in survival of these cells. When irradiated, these cells had reduced levels of 5-HT available for receptor binding, and serotonin receptor binding appears to be critical in initiating cell death cascades of directly irradiated cells by facilitating calcium entry into cells (*5*). These elevations of intracellular calcium cause an overload of calcium in the mitochondria leading to a transient loss of membrane potential, the production of reactive oxygen species and the activation of apoptosis pathways (*2*, *3*). Several others have also suggested that these elevations of cellular calcium levels in donor cells occurring after irradiation may be necessary for the release of a soluble bystander signal into the media, which can trigger these apoptosis cascades in recipient cells as if they were directly irradiated (*4*, *5*, *7*). Furthermore, radiation dose does not appear to alter the ability of HCT116$^{+/+}$ cells to respond to

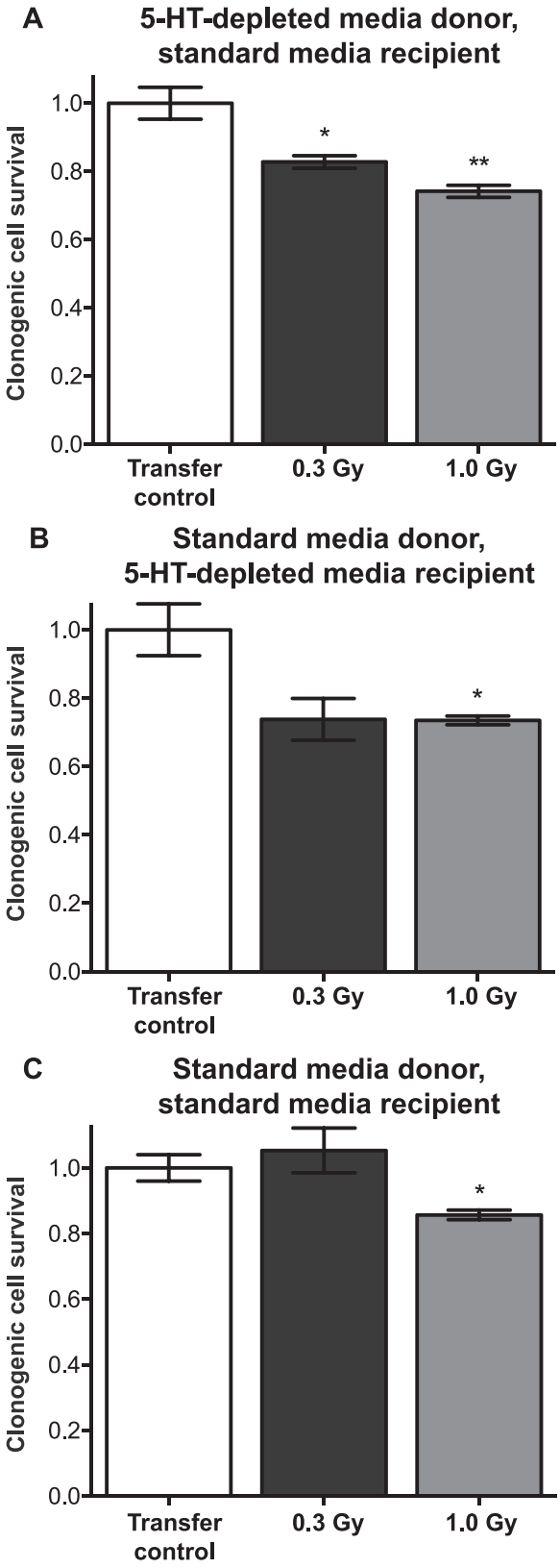

FIG. 4. Clonogenic cell survival of HaCaT bystander cultures after irradiation of donor cells in which: media from donor cells cultured in 5-HT depleted media was transferred to recipient cells initially cultured in standard media (panel A); media from donor cells cultured in standard conditions was transferred to recipient cells initially cultured in 5-HT depleted media (panel B); and media from donor cells cultured in standard media was transferred to recipient cells initially cultured in standard media (panel C). Survival is normalized to control (0 Gy, sham irradiated) cells (transfer control) for each independent experimental setup. Values presented are mean with SEM error bars for each experiment performed in triplicate. Significance values were determined using Student's *t* test comparing control cell survival to survival of irradiated media transfer groups for each experimental setup (**$P < 0.01$; *$P < 0.05$).

the serotonin in the media. At all radiation doses, we observed a significant difference in cell survival between media groups in these cells. Nevertheless, the most significant increases in survival occurred with the lower radiation doses of 0.3 and 1.0 Gy, suggesting the mechanism mediating serotonin-sensitive cell survival is saturable and, furthermore, susceptible to desensitization at a specific radiation dose, consistent with the assumption of 5-HT receptor-mediated cell death. In marked contrast, the survival of directly irradiated HaCaT cells was unaltered by culture media containing intentionally reduced serotonin levels (Fig. 2B). Past hypotheses for the differences among cell lines in their ability to respond to serotonin levels in media have concentrated on downstream signaling responses proposed to involve p53 pathways (*8*, *11*). The HaCaT cell line is known to contain a mutant p53 gene (*20*), however, these cells express a considerable amount of the protein and expression increases with radiation exposure (*21*). In comparison, the HCT116$^{+/+}$ cell line expresses much lower levels of the p53 protein that increase with radiation exposure (*21*), albeit, typical or wild-type protein function (*4*). The p53 pathway is compromised in many human tumors and could therefore lead to differential expression of bystander and direct irradiation effects (*4*). With previously collected data on effects of direct irradiation in correspondence with p53 function, the suggestion of p53 status dictating cell line-specific radiation responses to serotonin does not appear to coincide with the data presented. In experiments with HCT116$^{+/+}$ and HCT116$^{-/-}$ cells with wild-type and null p53 expression, respectively, there was no significant difference between responses of cells to direct irradiation (*4*), suggesting that p53 status does not play an appreciable role in responses to direct irradiation of these cells. Yet, when cell culture media preparations containing variable levels of 5-HT were used in this study, we observed an effect of serotonin in p53 wild-type cells (Fig. 2A) and not in a cell line with a mutant p53 gene (Fig. 2B). Although these diverse effects occur in different cell lines, p53 functionality has been well characterized in these cell lines (*4*, *20*, *21*), and the results indicate that p53 status may not be the ultimate cause for serotonin sensitivity when cells are directly irradiated. Regardless of the downstream signaling effects, responses to direct irradiation have been clearly linked to signaling cascades initiated by serotonin receptors, implicating differences in serotonin receptor expression as the fundamental cause for discrepancies in radiation responses among

cell lines. This hypothesis does not eliminate the role of p53 activated pathways, but rather suggests that signaling effects are initiated by serotonin receptor cascades that may lead to downstream interactions with p53 and other signaling pathways. Further investigation to validate this hypothesis is required. Nevertheless, direct irradiation cell survival appears to be sensitive to nanomolar concentrations of 5-HT, although, it is clear that not all cell lines are serotonin responsive.

Similar to responses to direct irradiation, bystander experiments showed variable responses among the two cell lines. In contrast to direct irradiation data, we assayed lower radiation doses, as it is well known that RIBE saturates with increasing radiation doses of approximately 1.0 Gy (*22*). Various types of media transfer experiments were performed to determine how nanomolar serotonin concentration fluctuations would influence bystander signal production in donor cells and bystander responses in recipient cultures. This revealed that HCT116$^{+/+}$ cells were only able to generate a bystander effect that resulted in a significant reduction in cell survival when standard media conditions were used for both the donor and recipient cells. It appears there may be a threshold concentration of serotonin that must be available in both donor and recipient cell culture media to permit bystander-induced cell death in HCT116$^{+/+}$ cells. If either donor or recipient cells were cultured in serotonin-depleted media, bystander signals could not be generated (Fig. 3A and B), indicating these experimental setups lacked necessary serotonin concentrations required to either initiate bystander production or facilitate bystander responses. These findings, together with direct irradiation data, establish marked serotonin dependence and nanomolar concentration sensitivity in the HCT116$^{+/+}$ cell line to produce radiation-induced cell death. In contrast, in HaCaT cells, bystander responses were generated when donor cells were exposed to 1.0 Gy and media was transferred to recipient cells, irrespective of the cell culture media conditions (Fig. 4). This suggests that, unlike HCT116$^{+/+}$ cells, HaCaT cells can generate bystander signal production even with low levels of serotonin available in the media. With a decrease in serotonin concentration in either donor or recipient media, bystander effects could still be generated. This replicates the findings observed by Lyng *et al.* (*11*) where bystander-induced cell death in these cells was not significantly altered by media containing variable serotonin concentrations. Diverse radiation effects are once again observed in the cell lines assayed, contributing to the suggestion that genotype differences are the cause for these effects. Differences in receptor status of the cell lines appear to be a likely explanation for bystander responses, analogous to the effects of direct irradiation. Presumably, as in donor cells, calcium-initiated cell death in recipient cultures requires serotonin to induce membrane calcium channel opening, increasing intracellular calcium levels. Consequently, serotonin sensitivity would then reflect the receptor profiles of cells, requiring more or less serotonin necessary in culture media to activate an appropriate number of receptors. The continuation of this work will comprehensively elaborate on the cell line-specific bystander effects to serotonin that we have demonstrated.

Intuitively, the genotype of a cell can strongly influence cellular radiation responses, especially bystander signaling. Genotypic variations among cell lines have been recognized as critically important in RIBE, substantially impacting both the production of bystander signals and the response to such signals (*4*, *23*). Etiologies of cellular radiation responses should expand to encompass genotypic variation among cells as a cause for cell culture media serotonin sensitivity. Differences in cell signaling effects activated in distinct cell lines may reflect receptor heterogeneity, potentially the underlying factor mediating a cell's ability to respond to media serotonin levels. While there are many factors at play in the bystander effect, and it is highly unlikely that one signaling pathway alone initiates the bystander response, serotonin and 5-HT receptor status are obviously involved in radiation-induced cell response. Additional work is necessary to categorically address receptor status in the cell lines assayed, however, this study has provided an avenue for exploring serotonin-mediated radiation response effects and a comprehensive investigation assessing the relationship between the results presented and full receptor status characterization will follow. The findings presented here have significance for determining the signaling mechanisms involved in the RIBE in different tissues.

In conclusion, we have confirmed a correlation between FBS serotonin concentration and cell death in directly irradiated cells and in bystander cell cultures receiving media from directly irradiated cells. These results strongly suggest that serotonin signaling is involved in direct irradiation cell death and bystander-induced cell death. However, this effect is not evident in all cell lines. Genotypic variation among different cell lines could be dictating these responses, resulting in heterogeneous cell signaling through distinct receptor populations in these different cells. While there remain unanswered questions, this study is the beginning of a thorough investigation into cell line-specific serotonin signaling events involved in RIBE, which will hopefully take us one step closer to determining the bystander mechanism.

ACKNOWLEDGMENTS

We recognize and give special thanks and appreciation to Dusan Vukmirovic for his overall support, guidance and advice during the preparation of this manuscript. Thank you to the honors biology and pharmacology program at McMaster University for the opportunity to conduct this research. Support for this research was provided by the Canada Research Chairs Program (to CEM, grant no. 950-221284), National CFIDS Foundation Inc. (to CEM), NSERC Collaborative Research and Development Grant (to CEM, grant no. RGPIN293153-12), CANDU Owners Group (to CBS, grant no. CRDPJ484381-15) and Bruce Power (to CEM).

Received: March 5, 2018; accepted: June 7, 2018; published online: July 2, 2018

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