Published in final edited form as:

Int J Radiat Biol. 2016 August ; 92(8): 427–433. doi:10.1080/09553002.2016.1186299.

Irradiated Human Endothelial Progenitor Cells Induce Bystander Killing in Human Non-Small Cell Lung and Pancreatic Cancer Cells

William T. Turchan[1], **Ronald H. Shapiro**[1], **Garrett V. Sevigny**[1], **Helen Chin-Sinex**[1], **Benjamin Pruden**[1], and **Marc S. Mendonca**[1,2,*]

[1]Department of Radiation Oncology, Radiation and Cancer Biology Laboratory, Indiana University School of Medicine, Indianapolis, IN 46202

[2]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202

Abstract

Purpose—To investigate whether irradiated human endothelial progenitor cells (hEPCs) could induce bystander killing in the A549 non-small cell lung cancer (NSCLC) cells and help explain the improved radiation-induced tumor cures observed in A549 tumor xenografts co-injected with hEPCs.

Materials and Methods—We investigated whether co-injection of CBM3 hEPCs with A549 NSCLC cells would alter tumor xenograft growth rate or tumor cure after a single dose of 0 or 5 Gy of X-rays. We then utilized dual chamber Transwell dishes, to test whether medium from irradiated CBM3 and CBM4 hEPCs would induce bystander cell killing in A549 cells, and as an additional control, in human pancreatic cancer MIA PaCa-2 cells. The CBM3 and CBM4 hEPCs were plated into the upper Transwell chamber and the A549 or MIA PaCa-2 cells were plated in the lower Transwell chamber. The top inserts with the CBM3 or CBM4 hEPCs cells were subsequently removed, irradiated, and then placed back into the Transwell dish for 3 h to allow for diffusion of any potential bystander factors from the irradiated hEPCs in the upper chamber through the permeable membrane to the unirradiated cancer cells in the lower chamber. After the 3 h incubation, the cancer cells were re-plated for clonogenic survival.

Results—We found that co-injection of CBM3 hEPCs with A549 NSCLC cells significantly increased the tumor growth rate compared to A549 cells alone, but paradoxically also increased A549 tumor cure after a single dose of 5 Gy of X-rays ($P < 0.05$). We hypothesized that irradiated hEPCs may be inducing bystander killing in the A549 NSCLC cells in tumor xenografts, thus improving tumor cure. Bystander studies clearly showed that exposure to the medium from irradiated CBM3 and CBM4 hEPCs induced significant bystander killing and decreased the surviving fraction of A549 and MIA PaCa-2 cells to 0.46 (46%) ± 0.22 and 0.74 ± 0.07 (74%) respectively ($P < 0.005$, $P < 0.0001$). In addition, antibody depletion studies demonstrated that the

[*]**Corresponding Author:** Marc S. Mendonca, Ph.D., Radiation and Cancer Biology Labs, 975 West Walnut St, IB-346, Indiana University School of Medicine, Indianapolis, IN 46202, Phone (317) 278-0404, Fax (317) 278-0405, mmendonc@iupui.edu.

DECLARATION OF INTEREST

The authors have no conflicts of interest to report.

bystander killing induced in both A549 and MIA PaCa-2 cells was mediated by the cytokines TNF-α and TGF-β ($P < 0.05$).

Conclusions—These data provide evidence that irradiated hEPCs can induce strong bystander killing in A549 and MIA PaCa-2 human cancer cells and that this bystander killing is mediated by the cytokines TNF-α and TGF-β.

Keywords

X-rays; CBM endothelial progenitor cells; bystander effects; A549 NSCLC cells; MIA PaCa-2 pancreatic cancer cells

INTRODUCTION

Over the past few decades, the long-term survival rates for many cancers have increased dramatically, primarily as a result of significant advances in diagnostic and therapeutic techniques (Siegel, Ma et al. 2014). Unfortunately, this has not been case for non-small cell lung cancer (NSCLC), which is frequently diagnosed at an advanced stage and has an overall 5-year survival rate of approximately 15% (DeSantis, Lin et al. 2014, Siegel, Ma et al. 2014). Similarly, increases in the long-term survival rate for pancreatic cancer have been quite marginal during this time period and as a result, the 5-year survival rate for pancreatic adenocarcinoma remains below 5% (DeSantis, Lin et al. 2014, Siegel, Ma et al. 2014). Although the reasons that improvements in diagnostic and therapeutic techniques have not translated into significant increases in long-term survival for these particular cancers are complex and multifold, it is reasonable to postulate that a major factor behind the lack of improvement in prognosis for these cancers is that both lung and pancreatic cancers are highly resistant to current cancer treatments (Gannon 1998, Tse, Dawson et al. 2008, El Maalouf, Le Tourneau et al. 2009, DeSantis, Lin et al. 2014, Siegel, Ma et al. 2014). Therefore, a better understanding of the core principles behind current cancer treatment methods, such as radiation therapy, may prove useful in the development of novel, more effective methods of treating lung and pancreatic cancer patients (Gannon 1998). An example of one such core principle, within the field of radiobiology, is the bystander effect.

The bystander effect is a non-targeted effect of ionizing radiation, in which cells that are directly irradiated can signal to unirradiated “bystander” cells, resulting in a response to radiation that is observed not only in the irradiated cells, but also in the bystander cells (Nagasawa and Little 1992, Deshpande, Goodwin et al. 1996, Mothersill and Seymour 1997, Azzam, de Toledo et al. 1998, Gannon 1998, Lorimore, Kadhim et al. 1998, Mothersill and Seymour 1998, Prise, Belyakov et al. 1998, Zhou, Randers-Pehrson et al. 2000, Belyakov, Malcolmson et al. 2001, Sawant, Randers-Pehrson et al. 2001, Goldberg and Lehnert 2002, Mothersill and Seymour 2002, Prise, Folkard et al. 2003, Maguire, Mothersill et al. 2005, Liu, Mothersill et al. 2006). A great deal of time and effort has been invested to investigate the specific factors and mechanisms that might mediate bystander-induced killing including: cell-cell communication via gap junctions, reactive oxygen species, and various cytokines such as TNF-α and TGF-β (Lehnert and Goodwin 1997, Narayanan, Goodwin et al. 1997, Azzam, de Toledo et al. 1998, Prise, Belyakov et al. 1998, Narayanan, LaRue et al. 1999, Iyer, Lehnert et al. 2000, Matsumoto, Hayashi et al. 2000, Seymour and Mothersill 2000,

Zhou, Randers-Pehrson et al. 2000, Belyakov, Malcolmson et al. 2001, Bishayee, Hill et al. 2001, Matsumoto, Hayashi et al. 2001, Mothersill and Seymour 2002, Azzam, de Toledo et al. 2003, Shao, Furusawa et al. 2003, Azzam, de Toledo et al. 2004, Edwards, Botchway et al. 2004, Moore, Marsden et al. 2005, Kadhim, Hill et al. 2006, Little 2006, Hamada, Matsumoto et al. 2007, Matsumoto, Hamada et al. 2007, Ryan, Smith et al. 2008, Burr, Robinson et al. 2010, Al-Mayah, Irons et al. 2012). However, there is evidence of significant heterogeneity within the bystander effect, as different cell types display not only differences in their ability to induce bystander effects, but also in their ability to respond to bystander signals (Mothersill and Seymour 1997, Burr, Robinson et al. 2010). Because of this heterogeneity, there is no guarantee that cells of one type can induce bystander killing in cells of another type, even if a bystander effect has been observed in both types of cells individually (Mothersill and Seymour 1997, Burr, Robinson et al. 2010). Furthermore, potential bystander signaling between stromal cells such as endothelial progenitor cells (EPCs) and cancers cells in tumors appears to be complex and requires further investigation. In this study, we tested whether subcutaneous co-injection of CBM3 human endothelial progenitor cells (hEPCs) with A549 NSCLC cells in nude mice would alter tumor xenograft growth and/or alter sensitivity to treatment with X-rays. We found that co-injection of CBM3 hEPCs with A549 cells compared to A549 cells alone significantly increased the tumor xenograft growth rate. However, after 5 Gy of X-rays, co-injection of CBM3 hEPCs with A549 cells compared to A549 cells alone significantly decreased tumor growth and increased tumor xenograft cure. We hypothesized and then investigated whether the increased X-ray-induced cell killing and tumor cure observed in the tumor xenografts co-injected with the CBM3 hEPCs and A549 cells could be due to cytotoxic bystander signaling between the irradiated normal stromal hEPCs and the A549 NSCLC cells.

MATERIALS AND METHODS

Cell lines

A549 (p53 wild type) NSCLC and MIA PaCa-2 (p53 mutant) pancreatic adenocarcinoma cell lines were obtained from Dr. John Turchi and Dr. Michele Yip-Schneider, Indiana University School of Medicine, respectively (Estabrook, Chin-Sinex et al. 2011). A549 was grown in Dulbecco's Modification of Eagle's Medium (DMEM), 1X (Mediatech, Herndon, VA, USA), 10% Fetal Bovine Serum (HyClone, Logan, UT, USA), and 1% penicillin/streptomycin (Mediatech). MIA PaCa-2 was grown in DMEM, 1X (Mediatech, Herndon, VA, USA), 10% Bovine Calf Serum (HyClone, Logan, UT, USA), and 1% penicillin/streptomycin (Mediatech). Stock cultures were plated with 7.5×10^5 cells on T-75 cell culture flasks (Corning, Corning, NY, USA) and were then grown to 70-100% confluency at 37° C, 5% CO_2, and 85% humidity. (These conditions were also used in all subsequently mentioned incubations, unless noted otherwise.) A549 population doubling time was 17.2 ± 2.4 h. MIA PaCa-2 population doubling time was 17.1 ± 2.5 h.

CBM3 and CBM4 hEPCs were derived from human umbilical cord blood and were obtained from Dr. David A. Ingram and Dr. Merv Yoder, Indiana University School of Medicine and were grown in endothelial cell growth medium-2 (EGM-2) (Lonza, Walkersville, MD, USA). Stock cultures were plated with 3.5×10^5 cells on T-75 cell culture flasks (Corning)

that were previously coated with type I rat-tail collagen (BD Biosciences, Bedford, MA, USA). (All subsequently mentioned plasticware used to grow hEPCs was coated with type I rat-tail collagen prior to plating the cells.) Cells were then grown to 70-100% confluency. CBM3 and CBM4 population doubling times were 17.6 ± 1.1 h and 17.8 ± 1.1 h, respectively.

X-ray machine dosimetry

Measurement of absolute dose on the Precision Model XRAD 320 X-ray unit and Faxitron Model CP160 unit used for these studies were performed according to AAPM Task Group 61 report, following the AAPM protocol for 40–300 kV x-ray beam dosimetry in radiotherapy and radiobiology, Med Phys, 28 (6), 2001. The dosimetry setup used for calibration included a PTW N30001 ion chamber and an electrometer calibrated by the University of Wisconsin Accredited Dosimetry Calibration Laboratory (UW-ADCL). The ion chamber was calibrated at energies UW120, 250M and Cs137. Dose measurements were performed for a standard setup in air and corrected for experimental setup.

A549 in vivo xenograft tumor growth studies in athymic nude mice

In vivo A549 NSCLC subcutaneous human xenografts with and without CBM3 hEPCs were established in female athymic nude mice (Harlan Laboratories, Indianapolis, IN, USA) by our standard procedures (Estabrook, Chin-Sinex et al. 2011). All experiments were conducted in accordance with university-approved IACUC protocols. Mice were injected in the flank on day 0 of the experiment with a cell preparation containing one of the following ratios of CBM3 cells to A549 cells (in millions of cells): 0:2, 1:2, 2:2, 4:2. Tumors were then allowed to grow for 1 week. On day 7 of the experiment, mice in the irradiated groups received a single 5-Gy dose of radiation using a X-RAD 320 Precision X-ray machine (Precision X-ray, North Branford, CT, USA)) operated at 250-kVp with the collimator set to produce a field size of 2.0 × 2.0 cm. Additional settings for the X-ray machine were as follows: 2.0 mm Al filter, d = 50.0 cm, and dose rate = 1.46 Gy/min. Tumor progression was monitored over 73 days, with weekly measurements to determine the oblong sphere volume of each tumor versus time and also the number of tumor sites that were successfully cleared/cured for each cell mixture and treatment group (Estabrook, Chin-Sinex et al. 2011).

In order to facilitate analysis, data was compiled into four different groups, as follows: A549 (mice injected with the 0:2, 1:2, or 2:2 CBM3:A549 cell preparations), R - A549 (mice injected with the 0:2 CBM3:A549 cell preparation and then irradiated on day 7 of the experiment), CBM3:A549 (mice injected with the 4:2 CBM3:A549 cell preparation), and R - CBM3:A549 (mice injected with the 4:2 CBM3:A549 cell preparation and then irradiated on day 7 of the experiment). Because data was not all collected simultaneously, nor by the same researcher on the same days after tumor implantation, it was necessary to use regression analysis to estimate tumor volume at a given time post-implantation in some instances and to normalize the volume data both with respect to oblong sphere volume and time. This data was then used to create a tumor growth curve and an independent, one-tailed, Student’s t-test was performed to determine the statistical significance of oblong sphere tumor volume at each day of measurement. In addition, the number of tumors cleared/cured

by radiation for each condition (R - A549 and R - CBM3: A549) was measured and then a one-tailed chi-squared test was used to determine statistical significance.

Assessment of bystander-induced killing by clonogenic survival assays

Unirradiated bystander target cells were plated in the bottom chamber of the 6-well cell culture plates (Corning), while cells to be irradiated were plated in upper Transwell permeable inserts, with a membrane pore-size of 0.4 μm, that fit into the 6-well cell culture plates (Corning). Plates or inserts with cancer cells were plated with 1.0×10^5 cells, while those with hEPCs were plated with 2.0×10^5 cells. After plating, cells were cultured for 24 h. Cells in the upper inserts were then irradiated using a 160-kVp Faxitron X-ray machine (Faxitron Bioptics, Tucson, AZ, USA). Settings for the X-ray machine were as follows: 0.5 mm Cu filter, d = 33.0 cm, and dose rate = 62.4 cGy/min. Following irradiation, the upper inserts were transferred to the lower wells containing the target cells (Fig. 1). After 3 h of incubation to allow for the potential production and diffusion of bystander signals between the upper and lower chambers, the upper inserts were removed and target cells in the bottom well were counted using a Z1 Coulter Counter (Beckman Coulter, Indianapolis, IN, USA). These cells were then plated on T-25 cell culture flasks (Corning). The flasks were plated with 300 cells and incubated for 10-12 days, while flasks in experiments with hEPCs as target cells were plated with 600 cells and incubated for 6-8 days. Three flasks were plated per condition, per experiment. Following incubation, colonies were stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and colonies with greater than 50 cells were scored as clonogenic survivors (Estabrook, Chin-Sinex et al. 2011, Mendonca, Chin-Sinex et al. 2011). In experiments where antibodies were used to titrate out the candidate cytokines TNF-α and TGF-β, the anti-TNF-α antibody (R&D Systems, Minneapolis, MN, USA) or the anti-TGF-β antibody (R&D Systems) was added to the irradiated medium in the upper irradiated well to achieve a concentration of 0.5 μg/mL or 7.0 μg/mL, respectively. Target cells in the lower chambers were then incubated for 3 h with the irradiated upper inserts containing the irradiated cells.

Clonogenic survival assays were performed with each of the following combinations of irradiated cells to target cells: CBM3 or CBM4 to A549 and CBM3 or CBM4 to MIA PaCa-2 cells. At least 3 trials of each experimental condition were performed. Since the bystander results were very similar for both CBM3 and CBM4 hEPCs, we only report the results for the CBM4 cells here. Data was graphed with respect to survival fraction and then an independent, one-tailed, Student’s t-test was performed to determine statistical significance.

Flow cytometry based propidium iodide and Annexin V-EGFP apoptosis assays

Medium transfer experiments were performed utilizing Transwell permeable inserts (Corning), as previously described, with CBM3 or CBM4 hEPCs as the irradiated cells and A549 or MIA PaCa-2 cells as the target cells. 5.0×10^5 target cells were then placed in a centrifuge tube (Corning) for each trial and centrifuged for 4 minutes at 4000 rpm using a clinical centrifuge (International Equipment Company, Needham Heights, MA, USA). Cells were then washed, resuspended and then centrifuged again using the aforementioned settings. Following the second centrifugation, the pelleted cells were resuspended in 0.5 mL

of 1X Binding Buffer (BioVision, Milpitas, CA, USA). 5.0 μL of Annexin V-EGFP (BioVision) and 5.0 μL of Propidium Iodide (PI) were then added to the cell suspensions, which were then incubated at room temperature and in the dark for 10 minutes. Flow cytometry was performed using a FACSCalibur cell analyzer with no APC (BD Biosciences) 3 h after the inserts containing the irradiated cells were removed from the target cells. Data was collected from the flow cytometer using CellQuest Pro (BD Biosciences). Flow cytometry was performed as described above, with the following combinations of irradiated cells to target cells: CBM4 to A549, MIA PaCa-2 to MIA PaCa-2, and CBM4 to MIA PaCa-2, with the irradiated cells receiving a 2-Gy dose of radiation. At least 3 trials of each experimental condition were performed.

Data obtained from flow cytometry apoptosis/necrosis analysis was used to create a dot plot in CellQuest Pro (BD Biosciences) with the Annexin V stain on the x-axis, and the PI stain, on the y-axis. The resulting plot was then divided into 4 quadrants which were labeled as follows: lower left, viable cells; lower right, early apoptotic cells; upper right, late apoptotic cells; upper left, necrotic cells. Flow cytometry data was then graphed with respect to the percentage of cells in each quadrant and then an independent, one-tailed, Student's t-test was performed to determine statistical significance of differences in the percentage of cells undergoing apoptosis after bystander treatment versus unirradiated controls (Estabrook, Chin-Sinex et al. 2011, Mendonca, Chin-Sinex et al. 2011).

RESULTS

Subcutaneous co-injection of CBM3 hEPCs with A549 cells in nude mice improved tumor xenograft growth but increased X-ray sensitivity

The addition of CBM3 hEPCs to A549 cells in a 4:2 ratio resulted in a significant increase in subcutaneous tumor xenograft growth in nude mice from days 38 to 73 after implantation compared to injection of the A549 cells alone (Fig. 2, *P <0.05, **P < 0.01). However, after 5 Gy of irradiation, tumor volume appeared to be consistently smaller in mice that were injected with a combination of A549 cells with CBM3 hEPCs versus mice that were injected with A549 cells alone. Linear regression analysis was performed to determine the rate of tumor volume growth per day after 5 Gy of X-rays. The analysis showed that in mice that received the injection of CBM3 hEPCs with A549 cells, tumors grew significantly more slowly following radiation (rate of tumor growth = −0.0278 mm^3/day), compared to mice that were injected with A549 cells alone (rate of tumor growth = 0.3415 mm^3/day) (*$P <$ 0.01). Moreover, after 5 Gy of irradiation 7 of the 8 mice that received the injection of CBM3 hEPCs with A549 cells had their tumors' cleared/cured versus only 3 of the 8 mice injected with A549 cells alone ($P < 0.05$).

Irradiated CBM4 hEPCs induced bystander killing in A549 NSCLC cells

Exposure to medium from CBM4 hEPCs that received a 2-Gy dose of radiation induced significant bystander killing in A549 cells, decreasing the mean survival fraction of these A549 cells to 0.46 ± 0.22 or 46 ± 22% ($P < 0.005$) (Fig. 3).

Irradiated CBM4 hEPCs induced bystander killing in MIA PaCa-2 pancreatic cancer cells

Exposure to medium from CBM4 hEPCs that received a 2-Gy dose of radiation induced significant bystander killing in MIA PaCa-2 cells, decreasing the mean survival fraction of these MIA PaCa-2 cells to 0.74 ± 0.07 or 74 ± 7 % ($P < 0.0001$) (Fig. 4).

Bystander killing induced by irradiated CBM4 hEPCs in A549 and MIA PaCa-2 cells is mediated by TNF-α and TGF-β

The addition of the antibodies against TNF-α and TGF-β to the upper chamber containing irradiated CBM4 hEPCs, completely inhibited the bystander killing in the A549 cells with survivals of 1.02 ± 0.44 ($P < 0.01$) and 0.98 ± 0.23 ($P < 0.05$), respectively (Fig. 3). Moreover, the addition of the antibodies against TNF-α and TGF-β to the upper chamber containing irradiated CBM4 hEPCs, also completely inhibited bystander killing in MIA PaCa-2 cells with survivals of 1.00 ± 0.04 ($P < 0.005$) and 0.97 ± 0.12 ($P < 0.05$), respectively (Fig. 4).

Bystander killing induced by irradiated CBM4 hEPCs involves the induction of apoptosis in MIA PaCa-2 cells but not in A549 cells

No increase in Annexin V positive apoptotic cells was evident in the A549 cells after a 3-hour exposure to the medium of irradiated CBM4 hEPCs; however, exposure of MIA PACA-2 cells to the irradiated hEPC medium did significantly increase the mean percentage of MIA PaCa-2 cells undergoing apoptosis from 12.53% ± 3.13% to 20.25% ± 2.90% ($P < 0.05$).

DISCUSSION

A great deal of evidence suggests that EPCs facilitate tumor growth by promoting vasculogenesis and angiogenesis (Lorimore, Coates et al. 2003, Ingram, Mead et al. 2004, Ingram, Caplice et al. 2005, Ingram, Mead et al. 2005, Broxmeyer, Srour et al. 2006, Ingram, Krier et al. 2007, Ding, Kumar et al. 2008, Sieveking, Buckle et al. 2008). The results of our A549 tumor xenograft growth studies in nude mice support this notion, as mice that were co-injected with CBM3 hEPCs and A549 cells in a 4:2 ratio developed significantly larger tumors than mice that were injected with the 0:2, 1:2, or 2:2 preparations (Fig. 2). However, surprisingly the addition of hEPCs to the A549 cells in a 4:2 ratio appeared to increase the subcutaneous tumor xenograft X-ray sensitivity and tumor cure after 5 Gy of X-rays (Fig. 2). We hypothesized that irradiated hEPCs may be inducing bystander killing in the A549 cancer cells in tumor xenografts, thus improving tumor cure. We utilized dual chamber Transwell dishes to test whether medium from irradiated CBM3 or CBM4 hEPCs would induce bystander cell killing in A549 NSCLC cells and as an additional control in another cancer cell type, namely MIA PaCa-2 pancreatic cancer cells.

We demonstrated that irradiated CBM3 and CBM4 hEPCs are able to induce significant bystander killing in both A549 and MIA PaCa-2 cells (Figs. 3 & 4). The reduction in mean survival fraction that was observed in MIA PaCa-2 cells following exposure to the medium of irradiated hEPCs was consistent with the approximately 25% reduction in survival fraction as a result of bystander killing that has been reported in the literature (Lyng,

Seymour et al. 2000, Gomez-Millan, Katz et al. 2012). Although the reduction in mean survival fraction seen in A549 cells following exposure to the medium of irradiated hEPCs was somewhat larger than expected, there is published evidence of heterogeneity with respect to the magnitude of bystander-mediated cell killing in different types of cells and our data would concur with this observation (Anzenberg, Chandiramani et al. 2008, Byeon, Choi et al. 2014). Interestingly, we only observed a significant increase in apoptosis in MIA PaCa-2 bystander cells, but not in A549 bystander cells following exposure to medium from irradiated CBM4 hEPCs, indicating that bystander killing can be mediated via apoptosis or other forms of cell death such as mitotic failure, which appears to be the case in A549 cells. Finally, bystander killing in both cancer cell lines appears to be mediated by the production of TNF-α and TGF-β by the irradiated CBM3 and CBM4 hEPCs, as bystander killing was significantly attenuated when antibodies against TNF-α and TGF-β were added to the medium of the irradiated hEPCs prior to incubation with the target cancer cells in the lower Transwell chamber. This observation supports the general consensus that both TNF-α and TGF-β play a significant role in the signaling necessary for the induction of bystander killing in the target cells (Lehnert and Goodwin 1997, Bishayee, Hill et al. 2001, Matsumoto, Hayashi et al. 2001, Moore, Marsden et al. 2005, Kadhim, Hill et al. 2006, Hamada, Matsumoto et al. 2007, Ryan, Smith et al. 2008). In the future, experiments need to be conducted to determine whether our observations can be generalized to other types of stromal cells and what range of cancer cell types are receptive to these cytotoxic bystander signals produced by irradiated stromal cells.

Our observation that irradiated normal hEPCs can induce bystander killing in cancer cells may give some insight into how cancer cells that do not directly receive lethal doses of radiation can still be killed by bystander signaling from the surrounding normal stromal cells that have been irradiated. Perhaps novel techniques in radiation oncology could be developed that maximize bystander cytotoxic signaling from the surrounding normal tissue, that could potentially result in better long-term success rates in treating patients with cancers that are currently rather resistant to radiation therapy (Widel, Przybyszewski et al. 2009). To put the potential for new treatment methods that better utilize bystander killing into perspective, it should be noted that the reduction in mean survival fraction in the cell lines studied resulting from exposure to the medium of hEPCs that received a 2-Gy dose of radiation was the equivalent of the reduction in survival fraction that would be expected following an approximately 1.1-Gy dose of direct radiation to the cancer cells. Thus, decreases in survival fraction as a result of bystander killing can potentially exceed 50% of the decreases in survival fraction accounted for by direct irradiation, making better utilization of the bystander effect a powerful potential paradigm in radiation therapy. Moreover, a better understanding of the mechanism that facilitates bystander killing could be used to develop novel combinations of chemotherapy and radiation therapy that maximize synergistic tumor killing. On the other hand, evidence that irradiated

cancer cells can induce a bystander effect in normal hEPCs is relevant in light of recent data that suggests that bystander signals may be responsible for increased genomic instability and increased risk of carcinogenesis in unirradiated normal tissues (Lewis, Mayhugh et al. 2001, McIlrath, Lorimore et al. 2003, Morgan 2011, Siegel, Ma et al. 2014). In order to reap the potential benefits of this information, future studies need to be conducted to determine how

bystander killing can be maximized in cancer cells, while minimizing the number of unirradiated normal cells that are affected by bystander signaling.

ACKNOWLEDGMENTS

The authors WTT, GVS, BP and MSM would like to acknowledge the NIH T35 training grant and internal support from the Department of Radiation Oncology, IUSM that helped fund the experiments conducted in this investigation.

REFERENCES

1. Al-Mayah AHJ, Irons SL, Pink RC, Carter DRF, Kadhim MA. Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation. Radiat Res. 2012; 177(5):539–545. [PubMed: 22612287]
2. Anzenberg V, Chandiramani S, Coderre JA. LET-dependent bystander effects caused by irradiation of human prostate carcinoma cells with X rays or alpha particles. Radiat Res. 2008; 170(4):467–476. [PubMed: 19024654]
3. Azzam EI, de Toledo SM, Gooding T, Little JB. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. Radiat Res. 1998; 150(5):497–504. [PubMed: 9806590]
4. Azzam EI, de Toledo SM, Little JB. Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. Oncogene. 2003; 22(45):7050–7057. [PubMed: 14557810]
5. Azzam EI, de Toledo SM, Little JB. Stress signaling from irradiated to non-irradiated cells. Curr Cancer Drug Targets. 2004; 4(1):53–64. [PubMed: 14965267]
6. Belyakov OV, Malcolmson AM, Folkard M, Prise KM, Michael BD. Direct evidence for a bystander effect of ionizing radiation in primary human fibroblasts. Br J Cancer. 2001; 84(5):674–679. [PubMed: 11237389]
7. Bishayee A, Hill HZ, Stein D, Rao DV, Howell RW. Free radical-initiated and gap junction-mediated bystander effect due to nonuniform distribution of incorporated radioactivity in a three-dimensional tissue culture model. Radiat Res. 2001; 155(2):335–344. [PubMed: 11175669]
8. Broxmeyer HE, Srour E, Orschell C, Ingram DA, Cooper S, Plett PA, Mead LE, Yoder MC. Cord blood stem and progenitor cells. Methods Enzymol. 2006; 419:439–473. [PubMed: 17141066]
9. Burr KL, Robinson JI, Rastogi S, Boylan MT, Coates PJ, Lorimore SA, Wright EG. Radiation-induced delayed bystander-type effects mediated by hemopoietic cells. Radiat Res. 2010; 173(6): 760–768. [PubMed: 20518655]
10. Byeon HJ, Choi SH, Choi JS, Kim TH, Lee ES, Lee KC, Youn YS. Apoptotic activity and antitumor efficacy of PEGylated TNF-related apoptosis-inducing ligand (TRAIL) in a Mia Paca-2 cell-xenografted mouse model. Biomed Pharmacother. 2014; 68(1):65–69. [PubMed: 24268811]
11. DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS, Jemal A. Cancer treatment and survivorship statistics, 2014. CA Cancer J Clin. 2014; 64(4):252–271. [PubMed: 24890451]
12. Deshpande A, Goodwin EH, Bailey SM, Marrone BL, Lehnert BE. Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extranuclear target. Radiat Res. 1996; 145(3):260–267. [PubMed: 8927692]
13. Ding, Y.-t.; Kumar, S.; Yu, D.-c. The role of endothelial progenitor cells in tumour vasculogenesis. Pathobiology. 2008; 75(5):265–273. [PubMed: 18931528]
14. Edwards GO, Botchway SW, Hirst G, Wharton CW, Chipman JK, Meldrum RA. Gap junction communication dynamics and bystander effects from ultrasoft X-rays. Br J Cancer. 2004; 90(7): 1450–1456. [PubMed: 15054470]
15. El Maalouf G, Le Tourneau C, Batty GN, Faivre S, Raymond E. Markers involved in resistance to cytotoxics and targeted therapeutics in pancreatic cancer. Cancer Treat Rev. 2009; 35(2):167–174. [PubMed: 19027240]
16. Estabrook NC, Chin-Sinex H, Borgmann AJ, Dhaemers RM, Shapiro RH, Gilley D, Huda N, Crooks P, Sweeney C, Mendonca MS. Inhibition of NF-kappaB and DNA double-strand break

repair by DMAPT sensitizes non-small-cell lung cancers to X-rays. Free Radic Biol Med. 2011; 51(12):2249–2258. [PubMed: 22019440]

17. Gannon F. Reaping the benefits of basic research. Nature. 1998; 392(6678):752–752. [PubMed: 9572128]
18. Goldberg Z, Lehnert BE. Radiation-induced effects in unirradiated cells: a review and implications in cancer. Int J Oncol. 2002; 21(2):337–349. [PubMed: 12118330]
19. Gomez-Millan J, Katz ISS, Farias V. d. A. Linares-Fernandez J-L, Lopez-Penalver J, Ortiz-Ferron G, Ruiz-Ruiz C, Oliver FJ, Ruiz de Almodovar JM. The importance of bystander effects in radiation therapy in melanoma skin-cancer cells and umbilical-cord stromal stem cells. Radiother Oncol. 2012; 102(3):450–458. [PubMed: 22169765]
20. Hamada N, Matsumoto H, Hara T, Kobayashi Y. Intercellular and intracellular signaling pathways mediating ionizing radiation-induced bystander effects. J Radiat Res. 2007; 48(2):87–95. [PubMed: 17327686]
21. Ingram DA, Caplice NM, Yoder MC. Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. Blood. 2005; 106(5):1525–1531. [PubMed: 15905185]
22. Ingram DA, Krier TR, Mead LE, McGuire C, Prater DN, Bhavsar J, Saadatzadeh MR, Bijangi-Vishehsaraei K, Li F, Yoder MC, Haneline LS. Clonogenic endothelial progenitor cells are sensitive to oxidative stress. Stem Cells. 2007; 25(2):297–304. [PubMed: 17023514]
23. Ingram DA, Mead LE, Moore DB, Woodard W, Fenoglio A, Yoder MC. Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. Blood. 2005; 105(7):2783–2786. [PubMed: 15585655]
24. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood. 2004; 104(9):2752–2760. [PubMed: 15226175]
25. Iyer R, Lehnert BE, Svensson R. Factors underlying the cell growth-related bystander responses to alpha particles. Cancer Res. 2000; 60(5):1290–1298. [PubMed: 10728689]
26. Kadhim MA, Hill MA, Moore SR. Genomic instability and the role of radiation quality. Radiat Prot Dosimetry. 2006; 122(1-4):221–227. [PubMed: 17164271]
27. Lehnert BE, Goodwin EH. A new mechanism for DNA alterations induced by alpha particles such as those emitted by radon and radon progeny. Environ Health Perspect. 1997; 105(Suppl 5):1095–1101. [PubMed: 9400706]
28. Lewis DA, Mayhugh BM, Qin Y, Trott K, Mendonca MS. Production of delayed death and neoplastic transformation in CGL1 cells by radiation-induced bystander effects. Radiat Res. 2001; 156(3):251–258. [PubMed: 11500134]
29. Little JB. Cellular radiation effects and the bystander response. Mutat Res. 2006; 597(1-2):113–118. [PubMed: 16413041]
30. Liu Z, Mothersill CE, McNeill FE, Lyng FM, Byun SH, Seymour CB, Prestwich WV. A dose threshold for a medium transfer bystander effect for a human skin cell line. Radiat Res. 2006; 166(1):19–23. Pt 1. [PubMed: 16808607]
31. Lorimore SA, Coates PJ, Wright EG. Radiation-induced genomic instability and bystander effects: inter-related nontargeted effects of exposure to ionizing radiation. Oncogene. 2003; 22(45):7058–7069. [PubMed: 14557811]
32. Lorimore SA, Kadhim MA, Pocock DA, Papworth D, Stevens DL, Goodhead DT, Wright EG. Chromosomal instability in the descendants of unirradiated surviving cells after alpha-particle irradiation. Proc Natl Acad Sci U S A. 1998; 95(10):5730–5733. [PubMed: 9576952]
33. Lyng FM, Seymour CB, Mothersill C. Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. Br J Cancer. 2000; 83(9): 1223–1230. [PubMed: 11027437]
34. Maguire P, Mothersill C, Seymour C, Lyng FM. Medium from irradiated cells induces dose-dependent mitochondrial changes and BCL2 responses in unirradiated human keratinocytes. Radiat Res. 2005; 163(4):384–390. [PubMed: 15799693]

35. Matsumoto H, Hamada N, Takahashi A, Kobayashi Y, Ohnishi T. Vanguards of paradigm shift in radiation biology: radiation-induced adaptive and bystander responses. J Radiat Res. 2007; 48(2): 97–9106. [PubMed: 17327685]
36. Matsumoto H, Hayashi S, Hatashita M, Ohnishi K, Shioura H, Ohtsubo T, Kitai R, Ohnishi T, Kano E. Induction of radioresistance by a nitric oxide-mediated bystander effect. Radiat Res. 2001; 155(3):387–396. [PubMed: 11182788]
37. Matsumoto H, Hayashi S, Hatashita M, Shioura H, Ohtsubo T, Kitai R, Ohnishi T, Yukawa O, Furusawa Y, Kano E. Induction of radioresistance to accelerated carbon-ion beams in recipient cells by nitric oxide excreted from irradiated donor cells of human glioblastoma. Int J Radiat Biol. 2000; 76(12):1649–1657. [PubMed: 11133047]
38. McIlrath J, Lorimore SA, Coates PJ, Wright EG. Radiation-induced genomic instability in immortalized haemopoietic stem cells. Int J Radiat Biol. 2003; 79(1):27–34. [PubMed: 12556328]
39. Mendonca MS, Chin-Sinex H, Dhaemers R, Mead LE, Yoder MC, Ingram DA. Differential mechanisms of x-ray-induced cell death in human endothelial progenitor cells isolated from cord blood and adults. Radiat Res. 2011; 176(2):208–216. [PubMed: 21663393]
40. Moore SR, Marsden S, Macdonald D, Mitchell S, Folkard M, Michael B, Goodhead DT, Prise KM, Kadhim MA. Genomic instability in human lymphocytes irradiated with individual charged particles: involvement of tumor necrosis factor alpha in irradiated cells but not bystander cells. Radiat Res. 2005; 163(2):183–190. [PubMed: 15658894]
41. Morgan WF. Radiation-induced genomic instability. Health Phys. 2011; 100(3):280–281. [PubMed: 21556289]
42. Mothersill C, Seymour C. Lethal mutations and genomic instability. Int J Radiat Biol. 1997; 71(6): 751–758. [PubMed: 9246188]
43. Mothersill C, Seymour C. Radiation-induced bystander effects, carcinogenesis and models. Oncogene. 2003; 22(45):7028–7033. [PubMed: 14557807]
44. Mothersill C, Seymour CB. Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. Radiat Res. 1998; 149(3):256–262. [PubMed: 9496888]
45. Mothersill C, Seymour CB. Bystander and delayed effects after fractionated radiation exposure. Radiat Res. 2002; 158(5):626–633. [PubMed: 12385640]
46. Nagasawa H, Little JB. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res. 1992; 52(22):6394–6396. [PubMed: 1423287]
47. Narayanan PK, Goodwin EH, Lehnert BE. Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. Cancer Res. 1997; 57(18):3963–3971. [PubMed: 9307280]
48. Narayanan PK, LaRue KE, Goodwin EH, Lehnert BE. Alpha particles induce the production of interleukin-8 by human cells. Radiat Res. 1999; 152(1):57–63. [PubMed: 10381841]
49. Prise KM, Belyakov OV, Folkard M, Michael BD. Studies of bystander effects in human fibroblasts using a charged particle microbeam. Int J Radiat Biol. 1998; 74(6):793–798. [PubMed: 9881726]
50. Prise KM, Folkard M, Michael BD. A review of the bystander effect and its implications for low-dose exposure. Radiat Prot Dosimetry. 2003; 104(4):347–355. [PubMed: 14579891]
51. Ryan LA, Smith RW, Seymour CB, Mothersill CE. Dilution of irradiated cell conditioned medium and the bystander effect. Radiat Res. 2008; 169(2):188–196. [PubMed: 18220470]
52. Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ, Hall EJ. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells in vitro can be initiated in the unirradiated neighbors of irradiated cells. Radiat Res. 2001; 155(3):397–401. [PubMed: 11182789]
53. Seymour CB, Mothersill C. Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve. Radiat Res. 2000; 153(5):508–511. Pt 1. [PubMed: 10790270]
54. Shao C, Furusawa Y, Aoki M, Ando K. Role of gap junctional intercellular communication in radiation-induced bystander effects in human fibroblasts. Radiat Res. 2003; 160(3):318–323. [PubMed: 12926990]

55. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014; 64(1):9–29. [PubMed: 24399786]

56. Sieveking DP, Buckle A, Celermajer DS, Ng MKC. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. J Am Coll Cardiol. 2008; 51(6):660–668. [PubMed: 18261686]

57. Tse RV, Dawson LA, Wei A, Moore M. Neoadjuvant treatment for pancreatic cancer--a review. Crit Rev Oncol Hematol. 2008; 65(3):263–274. [PubMed: 18063378]

58. Widel M, Przybyszewski W, Rzeszowska-Wolny J. [Radiation-induced bystander effect: the important part of ionizing radiation response. Potential clinical implications]. Postepy Hig Med Dosw (Online). 2009; 63:377–388. [PubMed: 19724078]

59. Zhou H, Ivanov VN, Gillespie J, Geard CR, Amundson SA, Brenner DJ, Yu Z, Lieberman HB, Hei TK. Mechanism of radiation-induced bystander effect: role of the cyclooxygenase-2 signaling pathway. Proc Natl Acad Sci U S A. 2005; 102(41):14641–14646. [PubMed: 16203985]

60. Zhou H, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ, Hei TK. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. Proc Natl Acad Sci U S A. 2000; 97(5): 2099–2104. [PubMed: 10681418]

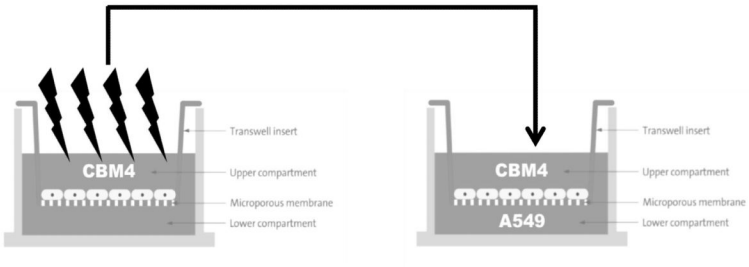

Figure 1.

Diagram of experimental setup using Transwell culture dishes with irradiated CBM4 human endothelial progenitor cells plated in upper Transwell permeable insert and the target cancer cells (either A549 or MIA PaCa-2, shown here as “A549”) plated in the lower compartment. Illustrations that were used to create this diagram were obtained from the Corning website.

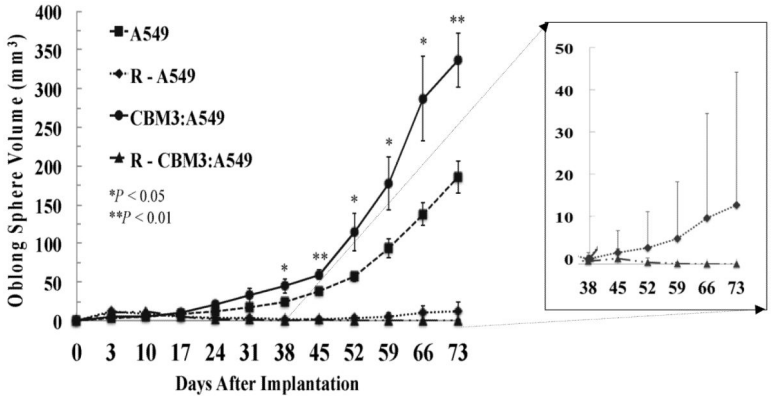

Figure 2.

Athymic nude mice were injected with either a combination of CBM3 human endothelial progenitor cells (hEPCs) with A549 cells in a 0:2, 1:2, or 2:2 ratio (A549 and R - A549) or a combination of CBM3 hEPCs with A549 cells in a 4:2 ratio (CBM3:A549 and R - CBM3:A549) on Day 0. Mice that received radiation (R - A549 and R - CBM3:A549) were irradiated on Day 7. On each day of measurement after the first 5 weeks of the experiment, tumor volume was significantly larger in mice that were injected with a combination of CBM3 hEPCs with A549 cells in a 4:2 ratio than in mice in the A549 cohort (0:2, 1:2, and 2:2 cell preparations) (*$P < 0.05$, **$P < 0.01$). However, after 5 Gy of irradiation, tumor volume appeared to be consistently smaller in mice that were injected with a combination of CBM3 hEPCs with A549 cells in a 4:2 ratio versus mice that were injected with A549 cells alone. Linear regression was performed to determine the rate of tumor volume growth per day and showed that mice that received the injection of CBM3 hEPCs with A549 cells was significantly lower (rate of tumor growth = −0.0278 mm^3/day) compared to mice that were injected with A549 cells alone (rate of tumor growth = 0.3415 mm^3/day) (*$P < 0.01$).

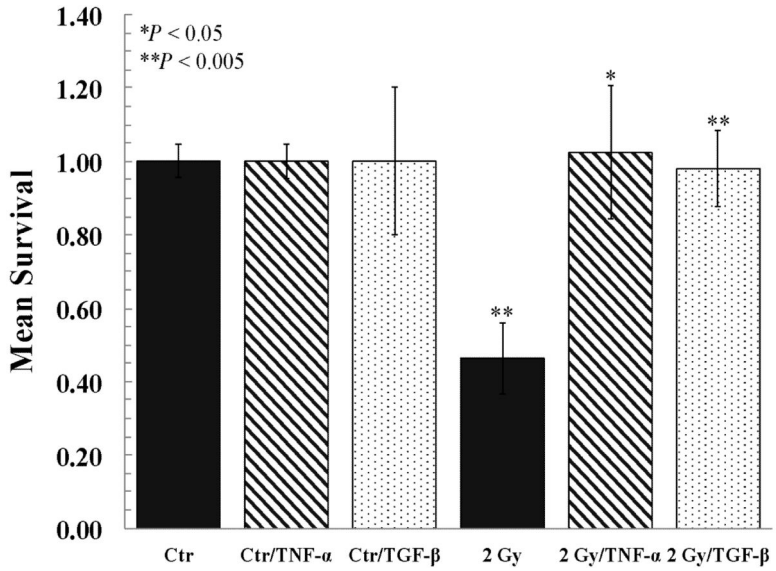

Figure 3.

A549 cells that were exposed to the medium of CBM4 human endothelial progenitor (hEPCs) cells that received a 2-Gy dose of radiation had a significantly lower mean survival fraction compared to A549 cells that were exposed to the medium of unirradiated CBM4 hEPCs (*$P < 0.005$). The addition of antibodies against TNF-α and TGF-β to the media of irradiated CBM4 hEPCs significantly increased the mean survival fraction of A549 cells exposed to these media compared to A549 cells that were exposed to the medium of irradiated CBM4 hEPCs that were not treated with antibodies (*$P < 0.01$, **$P < 0.005$).

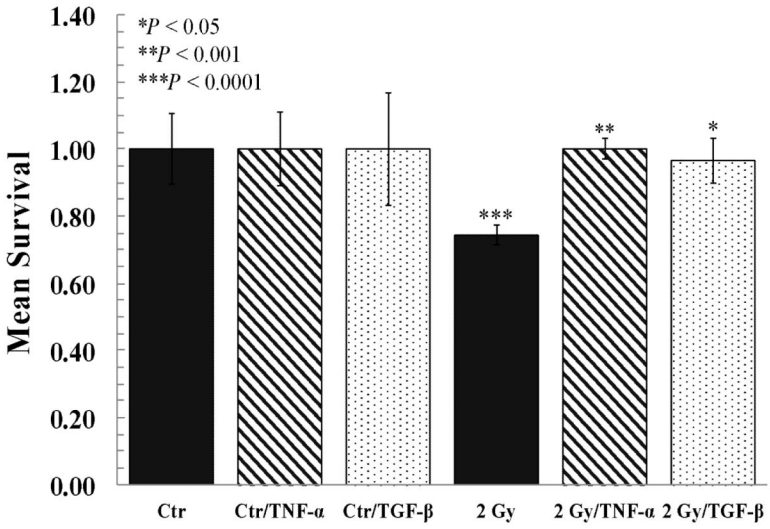

Figure 4.

MIA PaCa-2 cells that were exposed to the medium of CBM4 human endothelial progenitor cells (hEPCs) that received a 2-Gy dose of radiation had a significantly lower mean survival fraction compared to MIA PaCa-2 cells that were exposed to the medium of unirradiated CBM4 hEPCs (*$P < 0.0001$). The addition of antibodies against TNF-α and TGF-β to the media of irradiated CBM4 hEPCs significantly increased the mean survival fraction of MIA PaCa-2 cells exposed to these media compared to MIA PaCa-2 cells that were exposed to the medium of irradiated CBM4 hEPCs that were not treated with antibodies (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$).