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Medium from Irradiated Cells Induces Dose-Dependent Mitochondrial Changes and BCL2 Responses in Unirradiated Human Keratinocytes

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Exposure of unirradiated human keratinocytes to irradiated cell conditioned medium (ICCM) is known to cause a cascade of events that leads to reproductive death and apoptosis. This study investigates the effect of ICCM on clonogenic survival, mitochondrial mass and BCL2 expression in unirradiated keratinocytes. Exposure to 5 mGy, 0.5 Gy and 5 Gy ICCM resulted in a significant decrease in clonogenic survival. Human keratinocytes incubated with ICCM containing an antioxidant, *N*-acetylcysteine, showed no significant decrease in clonogenic survival. HPV-G cells incubated with ICCM containing a caspase 9 inhibitor showed no significant decrease in clonogenic survival when the ICCM dose was ≤ 0.5 Gy. A significant increase in mitochondrial mass per cell was observed after exposure to 5 mGy and 0.5 Gy ICCM. A change in the distribution of the mitochondria from a diffuse cytoplasmic distribution to a more densely concentrated perinuclear distribution was also observed at these doses. No significant increase in mitochondrial mass or change in distribution of the mitochondria was found for 5 Gy ICCM. Low BCL2 expression was observed in HPV-G cells exposed to 5 mGy or 0.5 Gy ICCM, whereas a large significant increase in BCL2 expression was observed in cells exposed to 5 Gy ICCM. This study has shown that low-dose irradiation can cause cells to produce medium-borne signals that can cause mitochondrial changes and the induction of BCL2 expression in unirradiated HPV-G cells. The dose dependence of the mitochondrial changes and BCL2 expression suggests that the mechanisms may be aimed at control of response to radiation at the population level through signaling pathways. © 2005 by

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ated cells that can reasonably be assumed to have occurred as a result of exposure of other cells to radiation. A number of methods exist for the study of this effect. Experimental approaches include microbeam irradiation using high- or low-LET radiation (2), irradiation with low fluences of α particles (3), and the transfer of culture medium from irradiated cells to unirradiated cells (1, 4). The bystander signal molecule(s) is as yet uncharacterized but has been reported to cause sister chromatid exchanges (3), chromosomal aberrations (5), changes in protein expression (6), micronucleus induction (2), initiation of apoptosis (7), and genomic instability (8). The bystander effect appears to be the dominant effect of radiation in the low-dose region, and it saturates at very low doses (9, 10). Clonogenic assays have shown that there is no significant difference between the level of effect of 0.5 Gy and 5 Gy irradiated cell conditioned medium (ICCM) (11). Lyng *et al.* (7) observed that exposure to ICCM causes a cascade of events that leads to apoptosis. Mitochondria, highly organized double membrane structures, are pivotal organelles in the cascade to cell death by apoptosis as shown by Newmeyer *et al.* (12). Disruption of this double membrane through a loss/gain of permeability induces a complex but regulated cascade of events leading to cell death. The traditional view of the role of the mitochondria during apoptosis is where the mitochondria are morphologically preserved (13). However, recent evidence shows changes in the structure of the mitochondria during apoptosis (14). The changing morphology of the mitochondria is characterized by hyperdensity of the matrix and reduction in mitochondrial size (15). These morphological changes along with an increase in production of ROS have been linked to an increase in mitochondrial mass per cell (16). There is now growing evidence indicating that exposure to apoptosis-inducing chemicals can induce mitochondrial proliferation resulting in an increase in mitochondrial mass per cell. Numerous chemicals, including herbimycin A (15), H₂O₂ (17) and genistein (17), have been shown to induce abnormal proliferation of mitochondria, which leads to an increase in mitochondrial mass. Mancini *et al.* (15) showed that herbimycin-treated Colo-205 cells had increased mitochondrial mass without an increase in net mitochondrial transmembrane potential. Limoli *et al.* (16) showed that there was a 15% increase in mitochondrial

INTRODUCTION

The bystander effect has been defined by Mothersill and Seymour (1) to be the detection of a response in unirradiated

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mass in a chromosomally unstable cell line after direct irradiation.

BCL2 has been identified as a key gene in the regulation of apoptosis (18) and is reported to be present in cells 30–60 min after irradiation (19). Harney *et al.* (20) reported that uroepithelial cultures showing the greatest reduction in cell numbers after irradiation had lower *BCL2* levels than those showing moderate or no reduction in cell numbers.

In the present study, human keratinocytes were exposed to 5 mGy, 0.5 Gy and 5 Gy ICCM. Clonogenic survival, mitochondrial mass and *BCL2* expression were investigated at each dose. An inhibitor of reactive oxygen species (ROS), *N*-acetylcysteine (NAC), was used to determine the role of ROS. An inhibitor of caspase 9 was used to investigate whether apoptosis at each dose was dependent on caspase.

MATERIALS AND METHODS

Cell Culture

Human keratinocytes immortalized with the HPV virus (HPV-G cells) were originally obtained as a kind gift from Dr. J. Di Paolo, NIH, Bethesda, MD (21). HPV-G cells were cultured in Dulbecco's MEM:F12 (1:1) medium (Sigma, Dorset, UK) containing 10% fetal calf serum (Gibco, Irvine, UK), 1000 IU 1% penicillin-streptomycin solution (Gibco), 2 mM L-glutamine (Gibco), and 1 µg/ml hydrocortisone (Sigma). Cells were maintained in an incubator at 37°C with 95% humidity and 5% CO₂. Subculture was routinely performed when cells were 80–100% confluent, using a 1:1 solution of 0.25% trypsin and 1 mM versene at 37°C.

Irradiation

Cells were irradiated in T-25 flasks (NUNC, Uden, Denmark) containing 5 ml of culture medium at room temperature using a cobalt-60 teletherapy source at St. Luke's Hospital, Dublin, at a flask-to-source distance of 80 cm. The dose rate during the experiment was approximately 1.8 Gy/min. TLDs were used to confirm that the appropriate dose was delivered. Once irradiated, the cells were returned to the incubator immediately.

Harvesting of ICCM

Donor flasks containing approximately 2×10^5 cells were irradiated 6 h after plating. Controls were sham-irradiated. Medium from irradiated and unirradiated cells was poured off donor flasks 1 h after irradiation and filtered through a 0.22-µm filter to ensure that no cells or other debris were still present in the medium. The filtrates were stored at -80°C in aliquots and thawed only once when required.

Measurement of Mitochondrial Mass per Cell

Mitochondrial mass was measured using a fluorescent dye, MitoTracker Green FM (Molecular Probes, Leiden). MitoTracker Green accumulates in the mitochondrial matrix where it covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues. An increase in green fluorescence clearly identifies an increase in mitochondrial mass (22). HPV-G cells were seeded at high concentrations on glass cover slips (diameter 24 mm) and incubated in normal culture medium for 3 h until the cells had attached. This medium was then removed. The cells were incubated at 37°C for 18 h with ICCM from cells exposed to 0 Gy, 5 Gy, 0.5 Gy and 5 mGy. The ICCM was then removed and the cells were washed in Mg²⁺/Ca²⁺ buffer (130 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM MgCl₂ and 1 mM CaCl₂). Cells were loaded

with MitoTracker Green FM (150 nM) made up in Mg²⁺/Ca²⁺ buffer for 20 min at 37°C and subsequently washed twice in buffer. The cover slips were then inserted into in-house designed petri dishes, which allowed imaging through the glass cover slip in the base of the dish. Using a Zeiss LSM 510 confocal microscope, MitoTracker Green FM was excited at 488 nm and fluorescence emission was recorded at 525 nm. Three fields of view were recorded for each dish and repeated as three independent experiments. The intensity of green fluorescence was measured using Zeiss LSM software. The mean fluorescence was divided by the number of cells in the field of view. An increase in fluorescence intensity indicated an increase in mitochondrial mass per cell (22). Control fluorescence was set to 100%, and all data were normalized to the control.

Distribution of Mitochondria

MitoTracker Green FM was used to stain the mitochondria in HPV-G cells as described above. Z-stacks were recorded using the Zeiss LSM 510 confocal microscope to confirm the location of mitochondria in the cell. The location of the mitochondria in the cell was scored either as being located diffusely in the cytoplasm or as being in a perinuclear location around the nucleus. Five hundred cells were scored for each of three independent runs. The slides were coded and scored blind. The percentage cells with either cytoplasmic distribution or perinuclear distribution was calculated.

Clonogenic Assay

Four hundred HPV-G cells were plated in T-25 flasks (NUNC, Denmark) in 4 ml of medium for determination of survival using the clonogenic assay technique of Puck and Marcus (23). These cells were called the recipient cells. These recipient cells received 3 ml of ICCM. Control recipient cells were set up and received medium harvested from sham-irradiated cells. Cells were then left for 7 days in an incubator at 37°C. The cells were then stained with Carbol Fuschin (BDH, Poole, UK) and colonies were counted.

Immunocytochemistry

Immunocytochemical analysis was performed using the streptavidin peroxidase method for cell culture using the Vectastain ABC kits. After exposure to ICCM, cells were washed twice in PBS to remove any debris and then fixed in 10% buffered formalin. The primary antibody, mouse monoclonal Bcl2 (Dako, Denmark), was applied for 1 h (1:50 dilution). Biotinylated anti-mouse reagent was then added to the cells for 30 min followed by streptavidin peroxidase for a further 30 min, with a wash in PBS in between each step. The chromagen, 0.02% DAB (Sigma), was then added for 10 min in darkness and washed off in distilled water. Cells were then counterstained with Harris hematoxylin and mounted with glycerol. A negative control, where no primary antibody was added, was included in each experimental run. Positive staining was determined by brown staining in the cytoplasm. Numbers of cells positive for *BCL2* were scored in random areas on each of three replicate cultures and expressed as the percentage of the total cells counted (over 500 cells were counted per culture).

Inhibition of Reactive Oxygen Species

HPV-G cells were incubated with 3 ml of ICCM containing 0.5 mM *N*-acetylcysteine (NAC) (Sigma) and assayed for changes in mitochondrial mass and clonogenic survival as described above.

Caspase 9 Inhibitor/Mch6 Inhibitor, Z-LEHD-FMK

Clonogenic recipient HPV-G cells were incubated with ICCM containing 1×10^{-3} IU caspase 9 inhibitor (Gentaur, Brussels) per milliliter of cell culture medium, as recommended by the supplier for the duration of the clonogenic assay. The caspase 9 inhibitor used was a Mch6 inhibitor, Z-LEHD-FMK. Clonogenic survival was determined as described above.

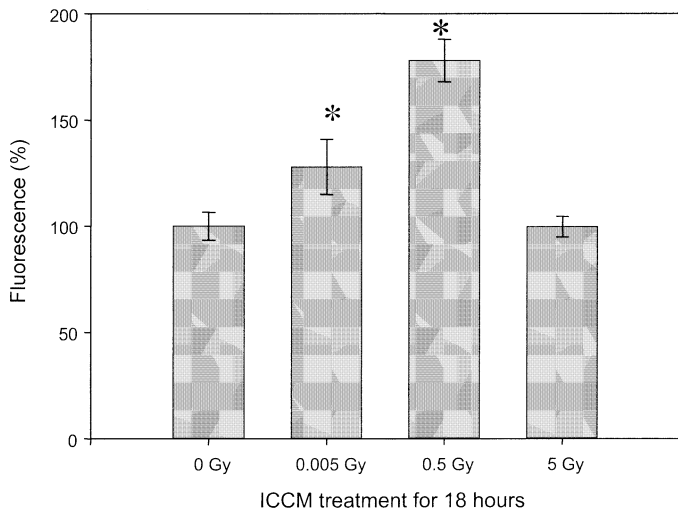


FIG. 1. Percentage of fluorescence levels of MitoTracker FM in HPV-G cells after 18 h exposure to ICCM. * $P < 0.05$.

Statistics

Data are reported as means \pm standard errors. Experiments were performed in triplicate. Significance was determined by a Student's unpaired t test, and the differences were considered significant if $P \leq 0.05$.

RESULTS

After 18 h of treatment of HPV-G cells with ICCM derived from cells exposed to 5 mGy and 0.5 Gy, there was an increase in fluorescence per cell as indicated by MitoTracker Green FM. This indicates an increase in mitochondrial mass per cell (Figs. 1, 2). However, no increase in fluorescence compared to the control was observed after 18 h of exposure to 5 Gy ICCM (Figs. 1, 2).

HPV-G cells exposed to 0 Gy ICCM displayed a diffuse cytoplasmic distribution of the mitochondria. After 18 h of exposure to 5 mGy and 0.5 Gy ICCM, a change in distribution of the mitochondria to a more densely concentrated perinuclear distribution was observed (Figs. 3, 4). This change in distribution of mitochondria from a cytoplasmic distribution to a perinuclear distribution was not observed when HPV-G cells were exposed to 5 Gy ICCM (Fig. 3).

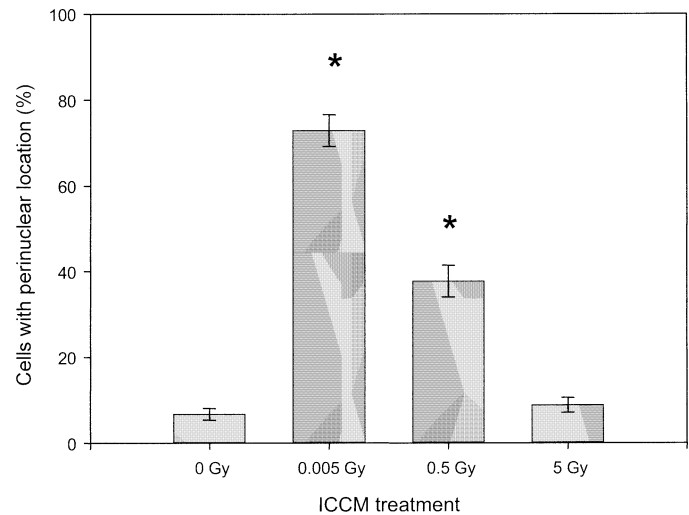


FIG. 3. Percentage of cells with a perinuclear mitochondrial distribution after 18 h exposure to ICCM. * $P < 0.05$.

To assess if reactive oxygen species play a part in the observed increase in mitochondrial mass per cell, ICCM containing an antioxidant, NAC, was added to unirradiated cells. No significant increase in mitochondrial mass per cell was observed in the cells incubated with ICCM containing NAC (Fig. 5).

After 18 h exposure of HPV-G cells to 5 mGy, 0.5 Gy and 5 Gy ICCM immunocytochemical staining for anti-apoptotic BCL2 was carried out to examine whether up-regulation of anti-apoptotic pathways was observed. There was a slight increase in BCL2 expression in cells exposed to 5 mGy ICCM and a small but not significant increase in BCL2 expression for cells exposed to 0.5 Gy ICCM (Fig. 6). However, for cells exposed to 5 Gy ICCM, there was a large significant increase in BCL2 expression (Fig. 6).

After recipient cells were treated with ICCM derived from cells exposed to 5 mGy and 5 Gy, a significant decrease in clonogenic survival was observed (Fig. 7).

The caspase 9 inhibitor, Z-LEHD-FMK, prevented the reduction in clonogenic survival caused by 5 mGy ICCM but not that caused by 5 Gy ICCM. NAC prevented the

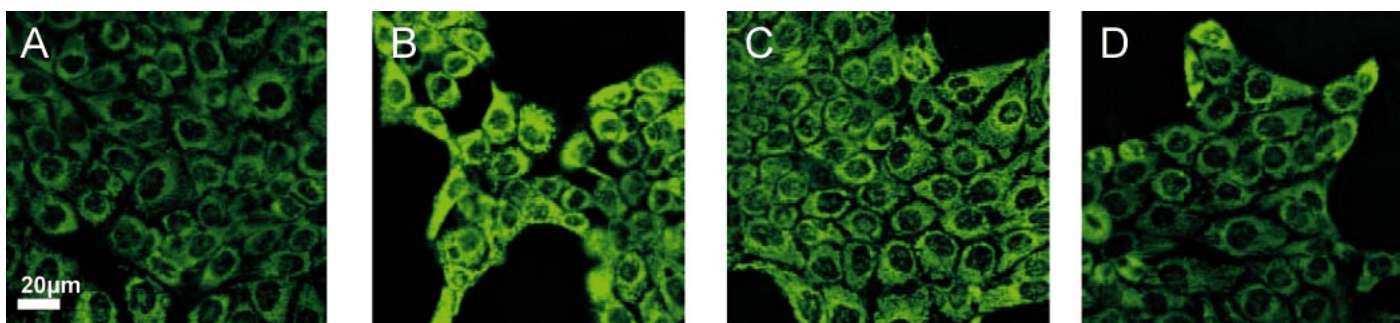


FIG. 2. Fluorescence confocal images showing mitochondrial mass in HPV-G cells as indicated by MitoTracker Green FM. HPV-G cells were exposed to (panel A) 0 Gy ICCM (control), (panel B) 5 mGy ICCM, (panel C) 0.5 Gy ICCM, and (panel D) 5 Gy ICCM. All exposures were for 18 h.

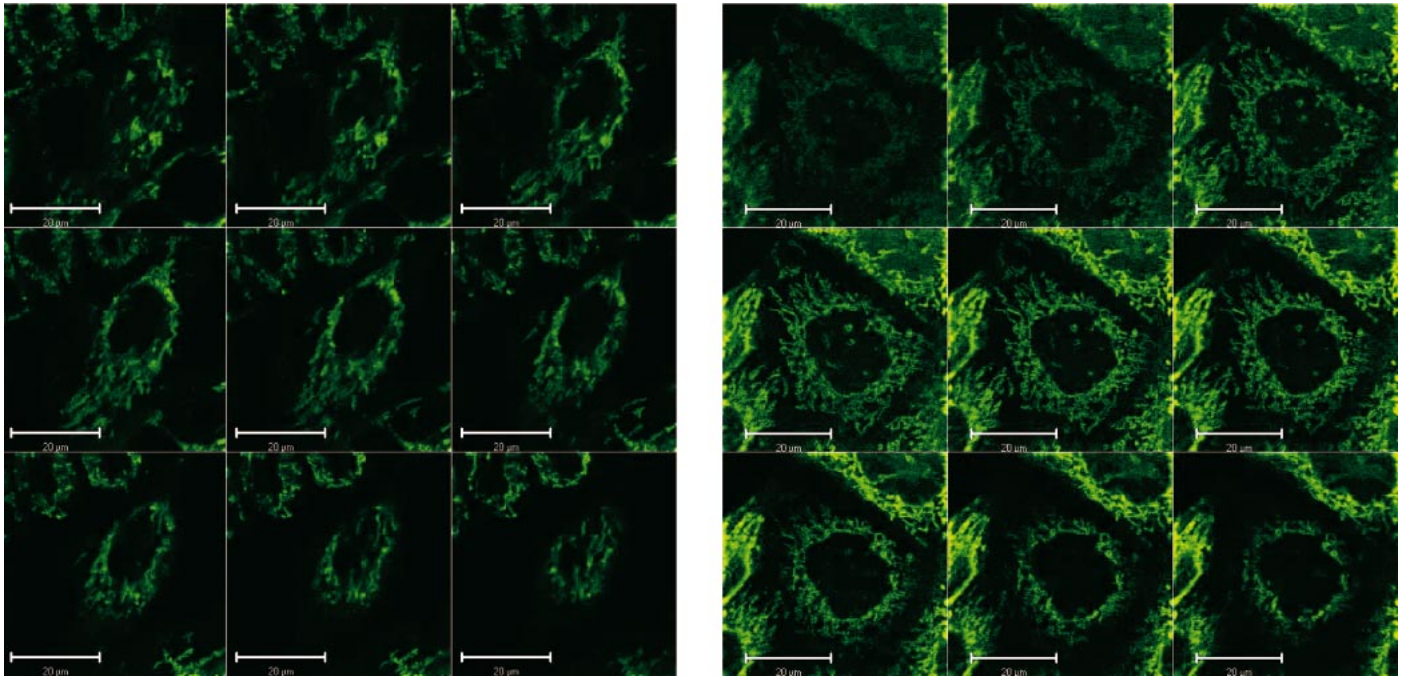


FIG. 4. Z-stack of HPV-G cell stained with MitoTracker Green FM. Panel A: Diffuse location of mitochondria in the cytoplasm of cells treated with 0 Gy ICCM. Panel B: Perinuclear location of mitochondria in HPV-G cells treated with 5 mGy ICCM for 18 h. $Z = 0.2 \mu\text{m}$.

reduction in clonogenic survival at both ICCM doses (Fig. 8).

DISCUSSION

The results show that exposure of HPV-G cells to ICCM containing the currently unknown medium-borne signal causes changes in mitochondrial mass, distribution of mitochondria, and induction of BCL2 expression.

Exposure to ICCM ≤ 0.5 Gy for 18 h caused a significant increase in mitochondrial mass per cell, whereas exposure to 5 Gy ICCM caused no significant increase in mitochon-

drial mass per cell. The mechanism of increase in mitochondrial mass remains unknown. Mancini *et al.* (15) have suggested that mitochondrial dysfunction could be specially programmed or targeted with a redox-sensitive factor governing mitochondrial proliferation. Reports in the literature suggest that a decrease in mitochondrial function may trigger a compensatory response in cells that induces mitochondrial biogenesis (24).

Spodnik *et al.* (25) have reported that α taxol causes proliferation of the mitochondria, with a simultaneous increase in the intracellular levels of α -tubulin. Nacodazole

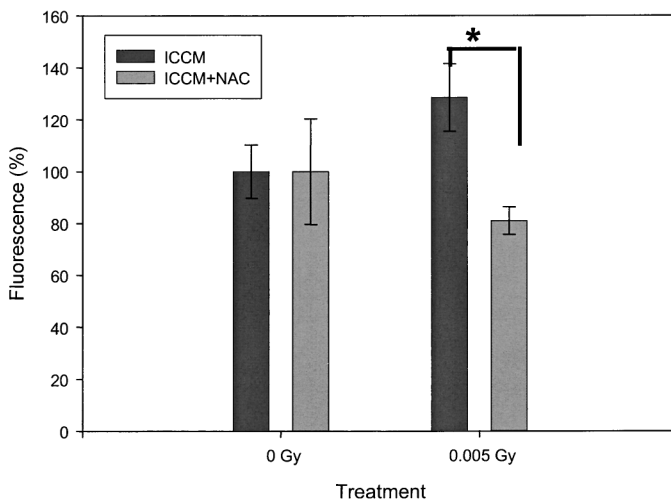


FIG. 5. Percentage of fluorescence of MitoTracker Green in HPV-G cells after 18 h exposure to ICCM plus 0.5 mM NAC. $*P < 0.05$.

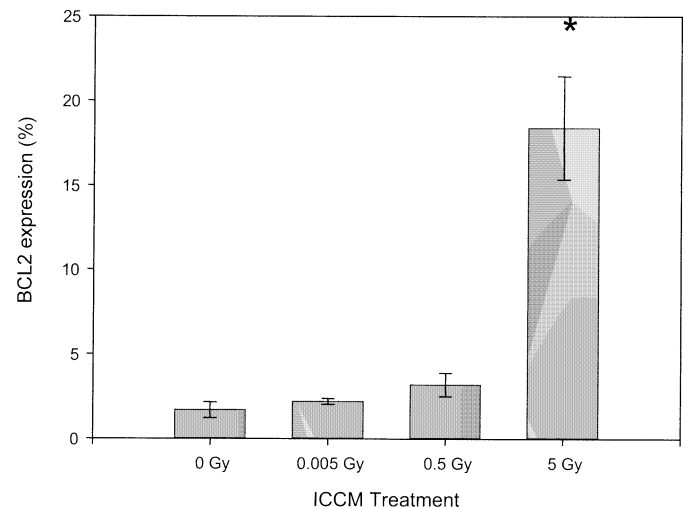


FIG. 6. Expression of BCL2 in HPV-G cells 18 h after exposure to ICCM. 500 cells per sample per independent run were scored. $*P < 0.002$.

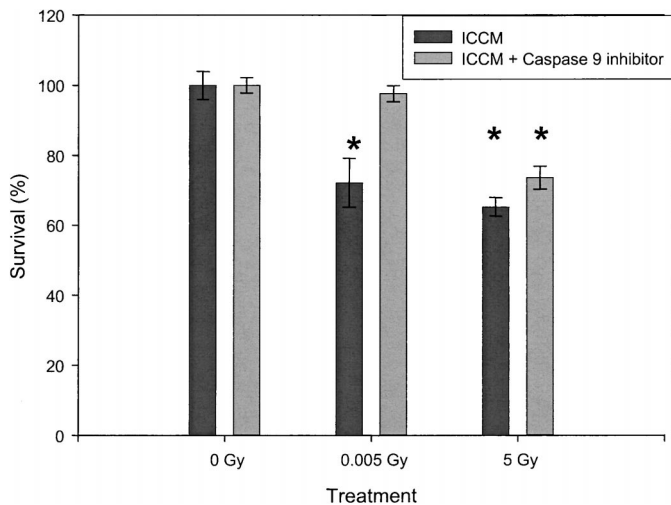


FIG. 7. Clonogenic survival of HPV-G cells 7 days after exposure to ICCM and ICCM plus caspase 9 inhibitor. * $P < 0.05$.

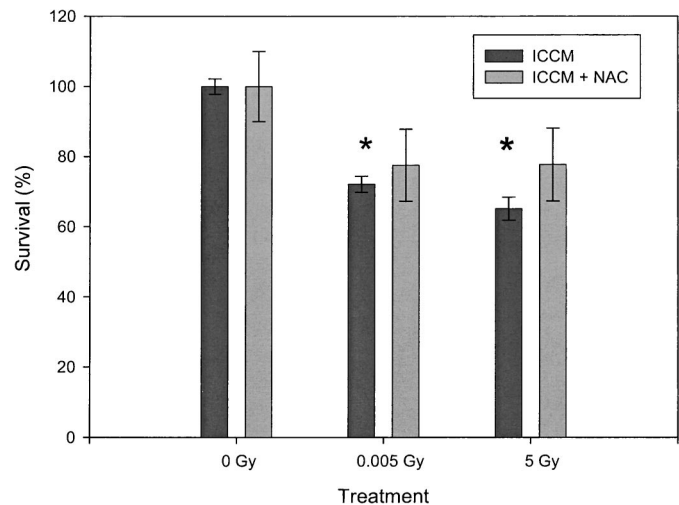


FIG. 8. Clonogenic survival of HPV-G cells 7 days after exposure to ICCM and ICCM plus NAC. * $P < 0.05$.

or colchicines, which depolymerize microtubules, prevent mitochondrial proliferation, with nocodazole also preventing proliferation of mitochondria caused by exposure to herbimycin A (25).

ROS have been linked to an increase in mitochondrial mass (16), and ROS are known to play an important role in the perpetuation of the bystander effect (26, 27). It was observed that the addition of 0.5 mM NAC to 5 mGy ICCM prevented the increased mitochondrial mass per cell. NAC, a thiol-containing antioxidant, has been shown to protect cells against damage associated with oxidative stress, although the exact ROS involved is currently unknown (31). It has been shown to eliminate arsenite-induced apoptosis (32). Mancini *et al.* (15) reported that the observed increase in mitochondrial proliferation in Colo-205 cells treated with herbimycin A was inhibited by NAC.

A change in the distribution of the mitochondria was observed in unirradiated HPV-G cells exposed to ≤ 0.5 Gy ICCM. This change in distribution was observed to occur only when there was an increase in mitochondrial mass per cell. The change in distribution could be related to the increased activity in the nucleus resulting from the need to induce protein synthesis to deal with the oxidative stress. Mitochondrial aerobic metabolism is important fuel for this process. Mothersill *et al.* (28) showed that energy metabolism was involved in the expression of bystander effects. Miranda *et al.* (29) have shown that mitochondrial DNA depletion also results in a perinuclear distribution pattern. Wakabayashi *et al.* (30) reported that when microtubules are destabilized, mitochondria lose their normal spatial distribution in the cell and cluster around the nucleus.

As with all toxic exposures to any cell, it is the initial response to the dose that determines whether the cell dies or lives and what pathway of cell death it takes. From the BCL2 immunocytochemistry, it has been shown that exposure to 5 Gy ICCM resulted in a greatly increased expression of BCL2. Exposure to 0.5 Gy ICCM resulted in a

small but not significant increase in BCL2 expression. Low BCL2 expression was observed after exposure to 5 mGy ICCM. Previous work by this group, using irradiated bladder epithelium, showed that BCL2 appears to be induced at doses around 1 Gy but not at lower doses (20, 33). Therefore, there may be a damage level or dose level threshold that needs to be exceeded for BCL2 to be induced.

When BCL2 expression was high (above the proposed BCL2 threshold level of 1 Gy), there were no observed changes in mitochondrial mass or distribution but when BCL2 expression was low (below the proposed BCL2 threshold level of 1 Gy), an increase in mitochondrial mass and change in distribution was observed.

To further understand these observed mitochondrial changes above and below the proposed BCL2 threshold, clonogenic studies were carried out with ICCM containing either a caspase 9 inhibitor, Z-LEHD-FMK, or NAC.

NAC was observed to prevent cell death caused by exposure to both 5 mGy and 5 Gy ICCM. This result concurs with those of other studies implicating ROS in the perpetuation of the bystander effect (26, 27).

The caspase 9 inhibitor Z-LEHD-FMK was found to block cell death caused by exposure to 5 mGy ICCM but not 5 Gy ICCM. Lyng *et al.* have observed that exposure to 5 Gy ICCM induces apoptosis (7). Currently there is a growing literature discussing caspase-independent and -dependent pathways (34). If the cell has access to caspases, it will undergo apoptosis in a caspase-dependent manner. However, if caspase activity is eliminated by inhibitors, such as IAPs or BCL2, cells can still die, displaying apoptotic morphology, in a caspase-independent manner. We have observed that for exposure to 5 Gy ICCM, there is a large significant increase in BCL2 expression. Increased BCL2 expression prevents caspase activation, possibly by complexing with CED4-like adaptor proteins and preventing their oligomerization (35). The present study suggests that apoptosis caused by exposure to ≤ 0.5 Gy ICCM (no

significant BCL2 expression) occurs through a caspase-dependent pathway, whereas exposure to 5 Gy ICCM (significant BCL2 expression) occurs through a caspase-independent pathway.

In conclusion, it appears that low-dose irradiation can produce medium-borne signals that can cause mitochondrial changes and induction of BCL2 in unirradiated HPV-G cells. The dose dependence of the mitochondrial and BCL2 responses suggests that the mechanisms may be aimed at control of response to the radiation dose at the population level.

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