

Exosomes Are Involved in Mediating Radiation Induced Bystander Signaling in Human Keratinocyte Cells

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There is much evidence supporting the existence of bystander effects in cells that were never exposed to radiation. Directly irradiated cells and bystander cells can communicate with each other using gap junctional intercellular communication or by releasing soluble factors into the surrounding medium. Exosomes and microvesicles are also known to mediate communication between cells. The main aim of this study is to establish whether exosomes and microvesicles are involved in radiation induced bystander signaling. Human keratinocytes, HaCaT cells, were irradiated (0.005, 0.05 and 0.5 Gy) using γ rays produced from a cobalt 60 teletherapy unit. After irradiation, the cells were incubated for 1 h and the irradiated cell conditioned medium (ICCM) was harvested. Exosomes were isolated from the ICCM using ultracentrifugation. Exosomes were characterized using light scattering analysis (LSA) and scanning transmission electron microscopy (STEM). Cytotoxicity and reactive oxygen species assays and real time calcium imaging were performed either with ICCM from which exosomes and microvesicles were removed or with the exosome fraction resuspended in cell culture media. The characterization data showed a particle size distribution indicative of both exosomes (30–100 nm) and microvesicles (>100 nm) and the light scattering analysis showed increased concentration of both exosomes and microvesicles with increasing dose. Western blotting confirmed the presence of an exosomal protein marker, TSG 101. Treatment of unirradiated cells with ICCM in which exosomes and microvesicles were removed resulted in abrogation of ICCM induced effects such as reduction in viability, calcium influx and production of reactive oxygen species. Addition of exosomes to fresh media produced similar effects to complete ICCM. These results suggest a role for exosomes and microvesicles in radiation induced bystander signaling.

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INTRODUCTION

The radiation induced bystander effect can be defined as a biological effect in cells after receiving signals from directly irradiated cells (1–7). Bystander signals may be transferred to surrounding cells either by gap junctional intercellular communication or by the production of soluble extracellular factors released from irradiated cells. Soluble signaling factors such as reactive oxygen species (ROS) (8–12), nitric oxide (NO) (12–14), secondary messengers like calcium (4, 8–10), cytokines such as interleukins (15–17), TGF β (18, 19), TNF α and TRAIL (20, 21) have been found to play a major role in radiation-induced bystander effects. A new mechanism for communication by soluble factors could be by intercellular communication by exosomes. Cells may release microvesicles (100–1,000 nm) by pinching off from the plasma membrane or endocytic secretions called exosomes (50–100 nm) (22). These vesicles can contain mRNA, micro RNA (miRNA) and proteins that can be functional and can be delivered into other cells resulting in molecular signaling such as immune responses (22, 23) and oncogenesis (24, 25). Proteins released from microvesicles shed from tumor cells have been found to promote tumor metastasis and invasion (26, 27). Irradiation of human–hamster hybrid GM10115 cells with low-LET X rays and high-LET iron (Fe) ions showed increased induction of micronucleus and cell killing. Alterations in repeat elements, global DNA methylation and differences in miRNA were also observed, these results suggest that epigenetic changes occur without initiation of chromosomal instability (28).

Studies on exosomes could play a major role in biomarker identification for various diseases at early stages using noninvasive methods. Exosomes have been found in blood plasma (29) and may be transported between organs and be involved in systemic circulation. They were also identified in the serum of cancer patients and could play a major role in tumor progression (30). Quantification of urinary exosomes from patients undergoing standard radiotherapy resulted in the identification of prostate cancer markers, prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) in most of the patients when

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compared with healthy donors (31). The protein B7-H3 has been detected in exosomes released after radiation-induced senescence in 22 RV1 prostate cancer cells (32). Increased expression of B7-H3 protein has been identified as a potential marker for the diagnosis of prostate cancer (33). It has been reported that proton irradiation of HeLa cells does not influence the secretion of exosomes, but those exosomes contain an increased amount of survivin protein compared to controls (34). Studies on exosomes recovered from the supernatant of irradiated PC-3 and DU-145 human prostate cancer cell lines revealed the presence of heat shock protein (HSP72), a stimulator of pro-inflammatory cytokine production (35). Conditioned medium from lung, isolated from whole-body irradiated mice, was found to be capable of transferring microvesicles containing lung specific mRNAs to bone marrow cells (36). Exosomes have recently been found to be involved in radiation induced bystander effects and genomic instability as measured by DNA damage following addition of conditioned media to breast cancer MCF7 cells. RNase was found to abrogate the DNA damage indicating that exosomes containing RNA may play a role in mediating radiation induced bystander effects and genomic instability (37).

In the current study, the role of exosomes was investigated in radiation-induced bystander signaling in human keratinocyte cells (HaCaT cells). Exosomes were characterized using light scattering analysis (LSA) and scanning transmission electron microscopy (STEM). Western blotting was performed to confirm the presence of an exosomal protein marker. The role of exosomes in radiation-induced bystander signaling was investigated by real time calcium imaging, the alamar Blue cytotoxicity assay and a reactive oxygen species assay.

MATERIALS AND METHODS

Cell Culture

Human keratinocyte cells were cultured in Dulbecco's modified Eagle medium (DMEM): F-12 Ham (1:1) (Sigma Aldrich, Dorset, UK) containing 10% fetal bovine serum (FBS) (Sigma Aldrich), 5,000 IU/ml of penicillin streptomycin (Gibco Biocult, Irvine, UK) solution and 1 µg/ml of hydrocortisone (Gibco Biocult). The cells were maintained in an incubator at 37°C, with 95% humidity and 5.0% CO₂. Subculture was routinely performed when cells were 75–80% confluent using 0.25% trypsin (Sigma Aldrich) and 1 mM versene (Sigma Aldrich) at a ratio of 1:1.

Irradiation

Culture flasks (T-25 flasks, Sarstedt, Wexford, Ireland) were sealed and irradiated (0.005, 0.05 and 0.5 Gy) using a cobalt-60 teletherapy source at St. Luke's Hospital, Dublin. For the 0.5 Gy dose point the source to sample distance was 80 cm, for the 0.05 Gy and 0.005 Gy dose points, the source to sample distance was 191.5 cm. The dose rate delivered was approximately 1.5 Gy/min during these experiments as evaluated at the 80 cm source to sample distance. Control flasks (0 Gy) were removed from the incubator and handled under the same conditions as the irradiated cells. The flasks were placed in the incubator immediately after irradiation.

Harvesting of ICCM

Donor flasks containing 200,000 cells in 5 ml DMEM: F-12 media (Sigma Aldrich, Dorset, UK) were seeded in T-25 flasks (Sarstedt, Wexford, Ireland) and irradiated 6 h after plating. Medium from both irradiated and unirradiated cells was poured off donor flasks 1 h after irradiation and filtered through a 0.22 µm filter (Nalgene/Thermo Fisher, Hereford, UK) to prevent debris and cells being transferred into the conditioned medium. After filtration, the medium was aliquoted and stored at –80°C.

Exosome Purification

To isolate exosomes, the 0, 0.005, 0.05 and 0.5 Gy ICCM was subjected to ultracentrifugation using a Beckman LX-100 ultracentrifuge (Beckman Coulter, Inc., CA.). The exosomes were pelleted at 100,000g for 1 h and washed with phosphate buffered saline (PBS) at 100,000g for 1 h using fixed-angle rotor Ti-80, all at 4°C (38). Exosomes were isolated, suspended in 100 µl PBS and stored at –80°C. The conditioned media from which exosomes were removed was also stored at –80°C for further experiments; this was termed ICCM-exosomes (ICCM-Exo).

Characterization of Exosomes Using Light Scattering Analysis

The size distribution of the isolated exosomes was analyzed using the Nanosight LM10 system (Nanosight Ltd, Amesbury, Wiltshire, UK). One microliter of the exosome suspension obtained from 0, 0.005, 0.05 and 0.5 Gy ICCM was added to 1 ml of Millipore water and introduced through a glass prism to the Nanosight system.

The exosomes that pass the red laser beam are visualized using an optical microscope, connected to a video camera that is aligned normally to the axis of the red laser beam, collecting light scattered from all particles in the field of view. This instrument is equipped with Nanoparticle Tracking Analysis software (Version 2.1), which specifically tracks the Brownian motion of the vesicles in PBS solution. The data is compiled automatically after each analysis in the form of an Excel spreadsheet (Microsoft Corp., Redmond, WA), representing the size distribution for each vesicle size.

Characterization of Exosomes Using Scanning Transmission Electron Microscopy

The exosomes were deposited on to formvar coated Nickel grids (Agar Scientific, Essex, UK) and left overnight. The grids were stained in 2% (w/v) uranyl acetate for 10 min. Grids were then washed in distilled water for approximately 30 s. Imaging was performed using a Hitachi SU6600 Field Emission Scanning Electron Microscope (FESEM) using the STEM mode at an accelerating voltage of 20 kV.

Protein Isolation and Western Blotting

Exosomes were extracted from 0 and 0.5 Gy ICCM using ExoQuick™ exosome precipitation solution (Bioscience, Cambridge, Cambridgeshire, UK). Exoquick solution was added to 0 and 0.5 Gy ICCM and the extraction procedure was performed according to the manufacturer's protocol. The exosomes were directly mixed with sodium dodecyl sulfate (SDS) loading buffer without lysing as described previously (39, 40). Prior to Western blotting the proteins were quantified using a micro BCA protein assay kit (Pierce, Dublin, Ireland). Total exosomal proteins (30 µg) were resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Carrigtwohill, Ireland). Membranes were subsequently blocked using 5% low-fat dry milk (Bio-Rad Laboratories, Blessington, Ireland). The membrane was incubated with primary antibody, tumor susceptibility gene 101 (TSG101) (Abcam,

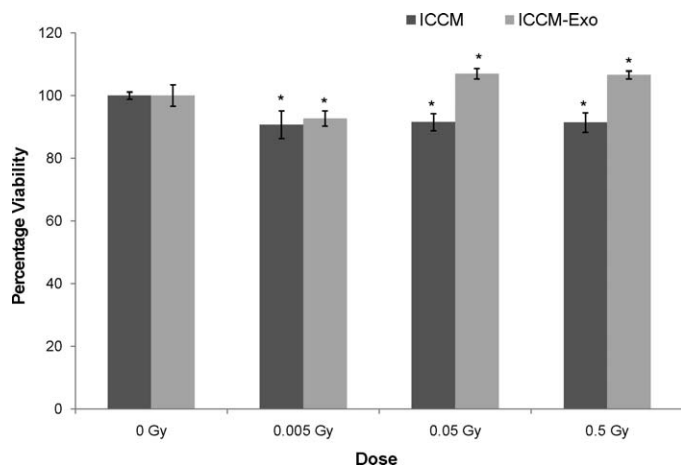


FIG. 1. Cell viability measured using the alamarBlue assay in HaCaT cells 72 h after exposure to 0, 0.005, 0.05 and 0.5 Gy ICCM and ICCM with exosomes removed (ICCM-Exo). Data is presented as mean and standard error on the mean of three independent experiments ($n = 3$, $*P < 0.05$). Control and treated values were compared using one-way analysis of variance (ANOVA) and $P < 0.05$ was considered to be statistically significant.

Cambridge, Cambridgeshire, UK) and subsequently with appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Dublin, Ireland). The proteins were visualized using chemiluminescence (Millipore). Detection was performed using Chemidoc exposure system (Bio-Rad Laboratories).

Measurement of Cell Viability Using the alamarBlue® Assay

Cell viability was measured using the alamarBlue (AB) assay as described previously for bystander cell death (41). The alamarBlue assay was performed by using alamarBlue solution (Biosource UK, Nivelles, Belgium). Approximately 7×10^4 cells/ml were seeded in each well of 96-well plates. After 24 h of cell attachment, plates were washed twice with PBS and the cells were treated with 0, 0.005, 0.05 and 0.5 Gy ICCM, and 0, 0.005, 0.05 and 0.5 Gy ICCM-Exo where exosomes and microvesicles were removed. After 72 h of incubation, the medium was discarded and cells were washed twice with PBS and 100 μ l of alamarBlue medium (5% v/v solution of alamarBlue in DMEM F-12 Ham without phenol red) were added to each well. The plates were incubated for 3 h at 37°C and measured at the wavelength of 540 nm for excitation and 595 nm for emission using a microplate reader (TECAN GENios, Grodig, Austria).

Measurement of Intracellular Reactive Oxygen Species Assay

The intracellular reactive oxygen species assay was performed immediately after the addition of ICCM, ICCM-Exo and exosomes added to fresh media (Media+Exo). Approximately 1×10^4 cells were plated in black 96-well microplates (Nunc, Roskilde, Denmark). After 24 h of plating, the cells were washed twice with PBS and loaded with CMH2DCFDA dye (5', 6'-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate) (Molecular Probes, Invitrogen, Paisley, UK) in DMEM: F-12 Ham media without phenol red (Sigma Aldrich), incubated for 30 min at 37°C and 5% CO₂. After incubation, the cells were washed twice with PBS and treated with 0, 0.05 and 0.005 Gy ICCM; 0, 0.05 and 0.5 Gy ICCM-Exo, and 0, 0.05 and 0.5 Gy Media+Exo. The fluorescence was measured at excitation and emission wavelengths of 488 and 530 nm using Tecan microplate reader (GENios, Grodig, Austria).

Measurement of Intracellular Calcium

Changes in intracellular calcium were monitored in HaCaT cells using two fluorescent indicators, Fluo 3 and Fura Red (Molecular Probes, Leiden, Netherlands) as described by Lyng *et al.* (11). The cells were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂ and 1 mM MgCl₂ (pH 7.4). The cells were incubated with 3 μ M Fluo 3 and 3 μ M Fura Red AM esters in buffer for 1 h at 37°C and then washed three times with buffer. Fluo 3 and Fura red were excited at 488 nm and emissions were collected at 525 and 660 nm simultaneously using a Carl Zeiss LSM510 confocal microscope (Carl Zeiss, Welwyn Garden City, Hertfordshire, UK). Fluorescence images and time series data of the Fluo 3 and Fura Red fluorescence intensities were recorded every 2 s for a period of approximately 5 min. After approximately 20 s, ICCM, ICCM-Exo and Media+Exo were added when a stable base line had been attained. All measurements were carried out at room temperature.

Statistical Analysis

Statistical analyses were performed using Statgraphics Centurion XV (Statpoint Technologies, Inc., Warrenton, VA). Mean values of control and treated samples were compared using one-way analysis of variance (ANOVA) where $P < 0.05$ was considered to be statistically significant.

RESULTS

Bystander Cell Death

HaCaT cells were treated with 0, 0.005, 0.05 and 0.5 Gy ICCM and the level of bystander cell death was analyzed using the alamarBlue assay (Fig. 1). Viability was expressed as mean fluorescence intensity normalized to each respective sham treated control, 0 Gy ICCM or 0 Gy ICCM-Exo. Control values were set at 100% and the absolute values were $28,030 \pm 318$ a.u. for 0 Gy ICCM and $24,920 \pm 848$ a.u. for 0 Gy ICCM-Exo. ICCM was found to induce significant bystander cell death after incubation with 0.005 Gy, 0.05 and 0.5 Gy ICCM for 72 h compared with 0 Gy ICCM (sham-irradiated controls) as previously reported (42). To investigate if exosomes plays a role in bystander cell death, the cells were incubated with 0, 0.005, 0.05 and 0.5 Gy ICCM in which exosomes were removed (ICCM-Exo). Treatment of HaCaT cells with 0.05 and 0.5 Gy ICCM in which exosomes were removed resulted in an increase in cell viability when compared with 0 Gy ICCM whereas no significant increase was observed with 0.005 Gy ICCM (Fig. 1).

Characterization of Exosomes

Exosomes were isolated from ICCM using ultracentrifugation and the pellet obtained was examined using both STEM and LSA. To assess the particle size and distribution, the NanoSight LM10 system was used for real-time visualization and assessment of vesicles in PBS solution (Fig. 2A). As all the particles were analyzed individually, the Brownian motion of the vesicles in PBS determines the simultaneous measurement of concentration, size and size distribution of the vesicles. Two different types of vesicles

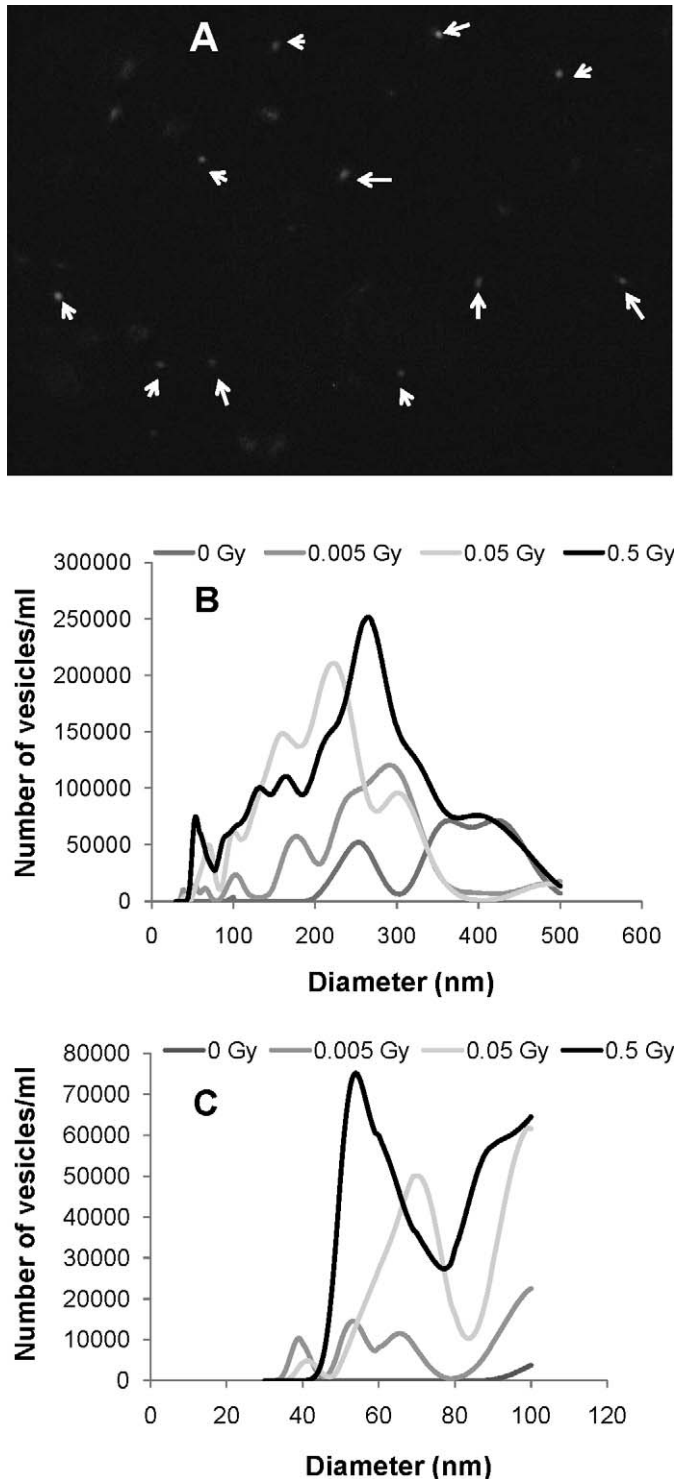


FIG. 2. Panel A: Screen shot of a video generated from the NanoSight LM10 showing light scatter from vesicles (indicated by arrows) obtained from ICCM harvested from HaCaT cells. Panel B: Vesicle size distribution (30–500 nm) measured using the NanoSight LM10 in samples prepared from 0, 0.005, 0.05 and 0.5 Gy ICCM. Panel C: Exosome size distribution (30–100 nm) in samples prepared from 0, 0.005, 0.05 and 0.5 Gy ICCM. Data is representative of five individual experiments.

were identified; exosomes which were 30–100 nm in diameter and microvesicles which were 100–500 nm in diameter as shown in Fig. 2B and C. Using the NanoSight Nanoparticle Tracking Analysis, the number of vesicles per microliter was found to increase in a dose-dependent manner with the highest concentration found in 0.5 Gy ICCM as shown in Fig. 2B and C.

STEM was used to confirm the presence of exosomes in ICCM obtained from HaCaT cells. Exosomes were visualized and the characteristic size was confirmed (30–100 nm) using Axiovision digital image processing software version 4.8 (Carl Zeiss, UK) as shown in Fig. 3A.

To confirm the presence of exosomal proteins, Western blot analysis was performed. The analysis confirmed the existence of tumor susceptibility gene (TSG101) protein which is a typical protein marker of exosomes as shown in Fig. 3B.

ROS Production

Production of ROS in HaCaT cells was measured using a fluorescent dye, CM-H₂DCFDA. The data was expressed as mean fluorescence intensity normalized to each respective sham treated control, 0 Gy ICCM, 0 Gy ICCM-Exo and 0 Gy Media+Exo. Control values were set at 100% and the absolute values were 1863 ± 43 a.u. for 0 Gy ICCM, 1838 ± 25 a.u. for 0 Gy ICCM-Exo and 2328 ± 29 a.u. for 0 Gy Media+Exo. No significant difference was observed between 0 Gy ICCM and 0 Gy ICCM-Exo but an increase in fluorescence intensity was observed for 0 Gy Media-Exo compared to 0 Gy ICCM and 0 Gy ICCM-Exo. A similar increase was observed after the addition of media containing FBS. Significant ROS production was observed after the addition of 0.005, 0.05 and 0.5 Gy ICCM compared to 0 Gy ICCM (Fig. 4A). To investigate the role of exosomes in ROS production, the cells were incubated with 0.005, 0.05 and 0.5 Gy ICCM in which exosomes were removed (ICCM-Exo). No production of ROS was observed after the addition of ICCM from which exosomes were removed (Fig. 4A). To confirm that the exosomes were involved in the ROS production, they were added to fresh media and this was added to the cells. Significant production of ROS was observed following treatment with 0.005, 0.05 and 0.5 Gy Media+Exo (Fig. 4A).

Calcium Signaling

Intracellular calcium was measured by loading calcium-sensitive dyes Fluo3 and Fura Red simultaneously in HaCaT cells. A small increase in calcium was observed after the addition of 0 Gy ICCM (Fig. 4B) and similar results were found after the addition of fresh medium containing FBS. Addition of 0.5 Gy ICCM induced a calcium flux of higher magnitude than that induced by 0 Gy ICCM (Fig. 4B). To investigate the role of exosomes in the calcium influx, further experiments were performed using 0.5 Gy ICCM in which exosomes were removed (ICCM-

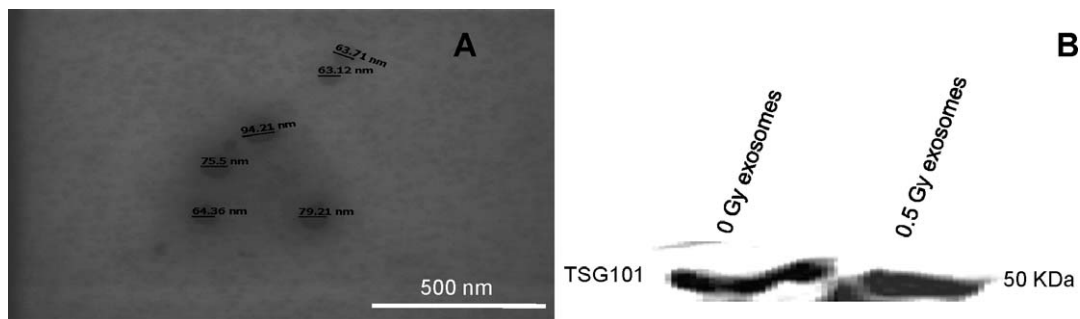


FIG. 3. Panel A: STEM image from Hitachi SU6600 Field emission scanning electron microscope (FESEM) showing exosomes isolated from ICCM harvested from HaCaT cells. The images were taken at a magnification of 80,000 $\times$ and the scale bar represents 500 nm. Individual exosomes measuring 60–95 nm are indicated on the figure. Panel B: Western blot analysis of exosomes isolated from ICCM, incubated with anti-TSG101 antibody and visualized by chemiluminescence.

Exo) and the cells showed a small increase in calcium similar to that observed for 0 Gy ICCM. To further confirm the involvement of exosomes in the calcium signaling, the exosomes were added to fresh medium and an increase in calcium was observed which was similar to that for 0.5 Gy ICCM (Fig. 4B). Similar results were observed for 0.005 and 0.05 Gy ICCM (data not shown).

DISCUSSION

This study has shown the isolation and basic characterization of exosomes from ICCM harvested from irradiated HaCaT cells. Vesicles were isolated using ultracentrifugation and identified as exosomes (30–100 nm) and microvesicles (100–500 nm) using LSA. STEM was performed to identify and confirm the size of the exosomes (30–100 nm). The endosomal origin of the exosomes isolated from ICCM was confirmed by identifying the protein marker TSG101, which is a component of endosomes from which exosomes are derived (43).

Addition of ICCM resulted in calcium influx into the cells, production of ROS and reduction in cell viability. Treatment of unirradiated cells with ICCM in which exosomes were removed resulted in the abrogation of calcium influx into the cells and no production of ROS, which are key mediators of radiation-induced bystander effects. No reduction in viability was observed in cells that were treated with 0.05 and 0.5 Gy ICCM in which exosomes were removed. Addition of exosomes to fresh medium produced similar effects as ICCM.

In this study, significant cell death was observed in cells that were treated with 0.005, 0.05 and 0.5 Gy ICCM and this is similar to our previous studies (42, 44, 45). Increased viability was observed in cells that were treated with 0.05 and 0.5 Gy ICCM from which exosomes were removed compared to 0 Gy ICCM-Exo treated cells. This could suggest increased metabolic activity in these cells compared to the sham treated cells but further work would be needed to test this hypothesis. This increased viability was not observed with 0.005 Gy ICCM-Exo and could suggest that

different mechanisms operate at lower doses and that other signals besides exosomes could also play a role.

Calcium plays a wide role in the signal transduction process (46) and increases in calcium have been reported in cells upon mechanical stress and exposure to ionizing radiation (47–49). There have been many studies on membrane signaling in bystander cells (41, 50–53). Rapid membrane signaling, calcium signaling and ROS induction has been reported previously in unirradiated cells after the addition of ICCM (41). The current study has shown calcium influx into the cells immediately after the addition of 0.5 Gy ICCM. A small calcium influx was also observed in HaCaT cells upon addition of 0 Gy ICCM and ICCM in which exosomes were removed, although this was significantly less than for 0.5 Gy ICCM. Similar effects were also observed with fresh medium and this may be due to the content of the medium or fetal bovine serum. To confirm the involvement of exosomes, the exosomes were added back to fresh media and an increase in calcium was observed similar to that observed for 0.5 Gy ICCM. This clearly shows a role for exosomes in calcium signaling in HaCaT cells. Rapid transient increases in cytosolic calcium have been observed after exosome adhesion to TNF α -activated fibroblasts indicating a novel function of exosome-cellular interactions, which can trigger intracellular signaling events within a cell (54). Exosome release that was regulated in a calcium dependent manner was previously reported in K562 cells, an increase in intracellular calcium leading to an increase in exosome secretion (55).

Ionizing radiation is an important agent that induces the production of oxidative stress in cells. Long lasting intracellular ROS production has also been observed in bystander cells (12, 56). Harada *et al.* (12) has identified the involvement of different free radicals when irradiated cells are co-cultured with unirradiated cells. Chen *et al.* (57) have reported that ROS production in irradiated cells is involved in mitochondrial calcium uptake. In our present study, both calcium and ROS were confirmed to play a role in bystander signaling. There is an increase in production of ROS

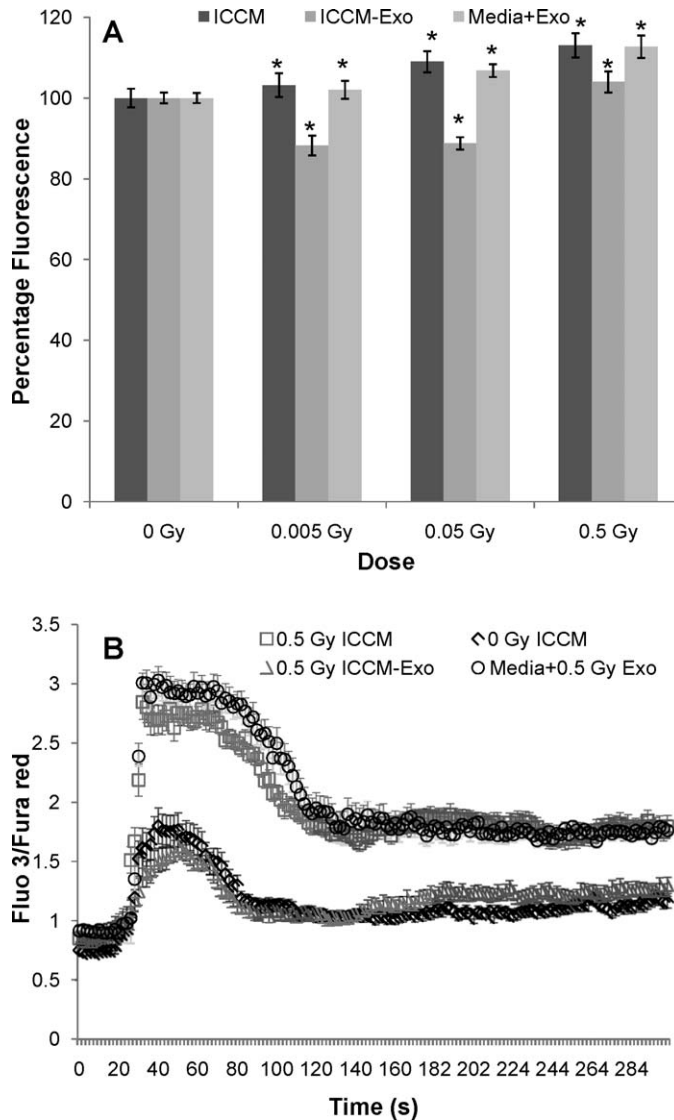


FIG. 4. Panel A: Intracellular ROS production in HaCaT cells measured using CM-H2DCFDA fluorescence in HaCaT cells after treatment with 0, 0.005, 0.05 and 0.5 Gy ICCM, ICCM in which exosomes were removed (ICCM-Exo) and fresh media to which exosomes were added (Media+Exo). Data represents the mean and standard error on the mean of three independent experiments ($n = 3$, $*P < 0.05$). Control and treated values were compared using one-way analysis of variance (ANOVA) and $P < 0.05$ was considered to be statistically significant. Panel B: Intracellular calcium levels as indicated by the ratio of fluorescence emissions from the calcium sensitive dyes Fluo-3 and Fura Red after the addition of 0 and 0.5 Gy ICCM, 0.5 Gy ICCM in which exosomes were removed (ICCM-Exo) and addition of exosomes from 0.5 Gy ICCM to fresh media (Media+Exo). Data represents the mean and standard error on the mean of three independent experiments ($n = 3$).

immediately after the addition of ICCM. Reduced ROS production was observed in cells treated with 0.005 and 0.05 Gy ICCM-Exo compared to cells treated with 0 Gy ICCM-Exo and this may be due to a change in antioxidant activity in the cells but further work would be needed to investigate this. A small but significant increase in ROS production was observed in cells treated with 0.5 Gy ICCM-

Exo and again may suggest that different mechanisms operate at higher doses where signals other than exosomes also play a role. Significantly increased ROS production was observed after the addition of 0.005, 0.05 and 0.5 Gy Media+Exo compared to 0 Gy Media+Exo. An increase in ROS was also observed after the addition of 0 Gy Media+Exo compared to 0 Gy ICCM but this may have been due to the content of the medium or fetal bovine serum as similar effects were observed with fresh medium. These increases in ROS were transient and returned to baseline levels by 5 min whereas addition of 0.005, 0.05 and 0.5 Gy Media+Exo resulted in further ROS increases which persisted for up to 3 h (data not shown). Exosomes harvested from MC/9 cells were found to induce oxidative stress in recipient cells resulting in subsequent cell death (58). The same group identified that exosomal RNA content changes according to cell origin and the condition under which the exosomes are released and mRNA from exosomes isolated from MC/9 cells has been shown to be functional using an *in vitro* translation assay (24). Furthermore, exosomes have been found to transfer from cell to cell and modulate expression of various genes in recipient cells by carrying mRNA and miRNAs (24, 59).

The role of exosomes in mediating nontargeted effects in MCF7 breast cancer cells has been reported in a recent study (37) where exosomes were shown to be involved in radiation induced bystander effects and genomic instability. DNA damage was measured after addition of conditioned media to MCF7 cells and RNase was found to abrogate this DNA damage, indicating that exosomes carrying RNA may play a role in mediating radiation-induced bystander effects and genomic instability (37).

In summary, our study has shown the role of exosomes in radiation-induced bystander signaling. Light scattering analysis showed the size distribution of the exosomes and revealed that the number of exosomes increased in a dose-dependent manner. Electron microscopy confirmed the size profile of the exosomes and Western blotting confirmed their endosomal origin. Removal of exosomes from ICCM has shown abrogation of calcium influx, ROS production and bystander cell death while addition of exosomes to fresh media showed similar effects to ICCM.

Previous work from our laboratory has shown that bystander signals released into ICCM can trigger membrane signaling and an influx of calcium followed by induction of ROS in unirradiated cells (41). The present study has shown that these bystander signals may be communicated through the secretion of exosomes or microvesicles. It is known that communication by exosomes can involve functional changes in the recipient cells related to transfer proteins, receptors and/or bioactive lipids and recent evidence suggests that exosomes shuttle mRNA and microRNA and thus may induce epigenetic changes in the recipient cells by transfer of genetic information (24). Further work on identification of the exosomal cargo, mRNA, microRNA or proteins, may help to identify the signaling molecules

that are responsible for the generation of radiation-induced bystander effects.

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