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Reactive oxygen species induced release of signalling factors in irradiated cells triggers membrane signalling and calcium influx in bystander cells

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Abstract

Purpose: The aim of this study was to elucidate the sequence of very early bystander signalling events and to determine the role of the different signaling molecules in both the production of the bystander signal and the response to this signal.

Materials and Methods: Human keratinocytes (HaCaT cell line) were irradiated (0.005, 0.05 and 0.5 Gy) using a cobalt 60 teletherapy unit, the medium was harvested one hour post irradiation and transferred to recipient HaCaT cells. Membrane permeability and levels of calcium, reactive oxygen species and nitric oxide were measured in the recipient cells immediately after the addition of irradiated cell conditioned medium (ICCM). Inhibitors of reactive oxygen species (ROS), nitric oxide (NO), calcium and membrane signalling were used in both donor and recipient cells to investigate if bystander effects could be blocked.

Results: It was found that membrane signalling followed by calcium influx was the first response in the recipient cells to addition of ICCM. ROS, NO and calcium were all found to be important signalling molecules involved in bystander responses, while ROS and calcium were found to be involved in the production of the bystander signal.

Conclusions: The data suggest that calcium and/or ROS induce irradiated cells to release long lived signalling factors which can trigger membrane signalling and an influx of calcium further inducing ROS in unirradiated cells.

Introduction

Radiation-induced bystander effects are characterised by the induction of biological effects in unirradiated cells as a result of receiving signals from irradiated cells (Prise & O'Sullivan 2009, Little 2006; Mothersill & Seymour 2006; Wright & Coates 2006; Hamada et al. 2007; Hei et al. 2008). These effects include mutations (Nagasawa & Little 1999; Zhou et al. 2000), transformation (Sawant et al. 2001), cytogenetic damage (sister chromatid exchanges, chromosomal aberrations, micronucleus formation) (Nagasawa 1992; Prise 1998; Sawant et al. 2001), changes in gene expression (Nagasawa 1992; Azzam et al. 1998; Prise 1998; Sawant et al. 2001; Klokov et al. 2004; Ghandhi et al. 2008), proliferation (Iyer et al. 2000; Belyakov et al. 2003), and cell death (Mothersill & Seymour 1997; Mothersill & Seymour 1998). Protective responses like apoptosis (Belyakov et al. 1999; Lyng et al. 2000, 2002; Lyng et al. 2006), terminal differentiation (Belyakov et al. 2002, 2006; Vines et al. 2009) and radioadaptive responses (Belyakov et al. 2002; Iyer & Lehnert 2002; Mitchell et al. 2004; Belyakov et al. 2006; Maguire et al. 2007; Vines et al. 2009) have also been described.

Although these bystander effects have been well documented over the past decade, the underlying mechanisms are still unclear. Whether through direct cell to cell communication by gap junctions or secretion of soluble factors into the cell culture medium, it is likely that several different pathways are involved.

Bystander effects have been shown to be mediated by a variety of signalling factors including reactive oxygen species (ROS) (Lehnert et al. 1997; Wu et al. 1999; Azzam et al. 2002; Lyng et al. 2006; Harada et al. 2008), nitric oxide (NO) (Matsumoto et al. 2001; Shao et al. 2002; Harada et al. 2008), second messengers like calcium (Lyng et al. 2000, 2002; Lyng et al. 2006; Shao et al. 2006), cytokines such as transforming growth factor beta (TGFβ) (Iyer et al. 2000; Shao et al. 2008) and interleukins (Osterreicher et al. 2003; Facoetti et al. 2006; Facoetti et al. 2009) and tumour necrosis factor alpha (TNF-α) and tumor necrosis (TNF)-related apoptosis-inducing ligand (TRAIL) death inducing pathways (Shareef et al. 2007; Luce et al. 2009). In addition, cyclooxygenase-2 (COX-2) (Zhou et al. 2005; Hei et al. 2008), Nuclear Factor KappaB (NFkB) (Azzam et al. 2002; Zhou et al. 2008) and mitogen-activated protein (MAP) kinase (Azzam et al. 2002; Zhou et al. 2005; Lyng et al. 2006) signalling have all been shown to be involved in bystander responses. Membrane signalling has also been shown to play an important role in bystander responses (Nagasawa et al. 2002; Shao et al. 2004; Burdak-Rothkamm et al. 2007; Tartier et al. 2007).

Our previous work has shown signalling pathways in bystander cells leading to apoptosis, such as calcium, MAP kinase, mitochondrial and ROS signalling (Lyng et al. 2000, 2002; Lyng et al. 2006). The aim of this study was to follow calcium, reactive oxygen and nitrogen species and membrane signaling singly and simultaneously to elucidate the sequence of very early signalling events in the

bystander cells. A further aim was to determine the role of the signaling molecules in both the production of the bystander signal and the response to this signal.

Materials and Methods

Cell Culture

A human keratinocyte cell line, HaCaT cells, originally obtained as a kind gift from Dr. Petra Boukamp, Deutsches Krebsforschungszentrum (DKFZ), Germany, was used for this study. These cells are immortal but non-malignant with a doubling time of 21 hours (Boukamp 1988) with a deletion in one allele of tumour protein 53 (p53) and a point mutation in the other (Lehman 1993). HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM): Nutrient Mixture F-12 Ham (F12) (1:1) medium (Sigma, Dorset, U.K) containing, 10% fetal bovine serum (Gibco, Irvine, U.K.) 1% penicillin-streptomycin solution 1000 IU (Gibco) and 1µg/ml hydrocortisone (Sigma). Cells were maintained in an incubator at 37ºC, with 95% humidity and 5% CO_2. Subculture was routinely performed when cells were 70-80% confluent, using a 1:1 solution of 0.2% trypsin (Sigma) and 1mM versene (Sigma) at 37º C.

Irradiation

Culture flasks (25 cm^2, 40 ml flasks, Nunc, Roskilde, Denmark) containing approx 2 x 10^5 cells were irradiated at room temperature using a cobalt 60 teletherapy unit delivering approximately 1.5 Gy/min during the time period of these experiments. The source to sample distance was 80 centimetres and the field size was 30 X 30 centimetres. For the 0.005Gy dose, an extended source to flask distance of 170cm was used. Thermoluminescent dosimeters (TLD) were

used to confirm that the appropriate dose was delivered. Control flasks were sham irradiated. Cells were returned to the incubator immediately after irradiation. A further set of controls included irradiated medium (with no cells) to exclude effects of medium constituents. There was no significant difference between the sham irradiated controls and the irradiated medium controls.

Generation of Irradiated Cell Conditioned Medium (ICCM)

Medium from irradiated and unirradiated cells was poured off donor flasks one hour after irradiation and filtered through a 0.22 μm filter (Nalgene/Thermo Fisher, Hereford, United Kingdom) to ensure that no cells could still be present in the transferred medium (Mothersill & Seymour 1997). The medium was then aliquoted, stored at –80^{0}C and thawed only once when required for experiments. Recipient cells were plated at a density of approx 2 x 10^5 cells per 35mm glass bottomed Petri dish (Mat Tek Corp, Ashland, MA, USA) for live cell imaging, and exposed to ICCM as detailed below.

Cell viability assay

Cells were seeded in 96-well microplates (Sarstedt, Wexford, Ireland) at a density of 1 x 10^4 cells per well. This density was found to be optimal to achieve the desired confluence at the end of the exposure period. After 24 h of cell attachment, plates were washed with 100 µl/well phosphate buffered saline (PBS) and cells treated with ICCM for 24 h. Six replicate wells were used for 0Gy, 0.005Gy, 0.05Gy and 0.5Gy ICCM per microplate. Viability was assessed

using the Alamar Blue assay. Following 24 h of exposure, ICCM was removed, cells rinsed with PBS and were incubated with 5% Alamar Blue (Invitrogen, BioSciences, Dublin, Ireland) in DMEM:F12 without phenol red (Sigma) for 3 hours and fluorescence was read at an excitation wavelength of 485 nm and an emission wavelength of 545 nm using a Tecan Genios microplate reader. Clonogenic assays could not be carried out to assess survival because no colonies formed in the flasks incubated with the different inhibitors for 7 days. No significant toxic effects were found after 24 hour incubation so the Alamar Blue assay was used as an alternative means to assess cell viability.

Live cell imaging

Intracellular calcium levels were determined relative to control levels using Fluo 3 and Fura Red (Invitrogen / Molecular Probes, BioSciences, Dublin, Ireland) as previously described (Lyng et al. 2006). Briefly, cells were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.4) and incubated with 3 μM Fluo 3 and 3 μM Fura Red acetoxymethyl esters for 1 hour in the buffer at 37 ^{0}C. Subsequently, the cultures were washed three times with buffer. Fluo 3 and Fura Red were excited at 488 nm and fluorescence emissions at 525 nm and 660 nm were recorded simultaneously using a Zeiss LSM 510 confocal microscope. Images and time series data of the Fluo 3 and Fura Red fluorescence intensities were recorded every 2 seconds. Although the ratio Fluo 3 / Fura Red is normally presented as an indicator of calcium levels, the fluorescence intensities of each dye are shown

here together with the fluorescence intensities of the other dyes used. Fluo 3 exhibits an increase in green fluorescence when bound to calcium and Fura Red exhibits a decrease in red fluorescence when bound to calcium (Lyng et al. 2006).

ROS and NO were followed in real time using the fluorescent probes 5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen / Molecular Probes) and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) (Invitrogen / Molecular Probes) respectively. Cultures were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4). Cells were loaded with 5 μM dye for 30 min in the buffer at 37 ^{0}C. Subsequently, the cultures were washed three times with buffer and incubated for an additional 15 min to allow complete de-esterification of the intracellular diacetates. Membrane signaling was followed in real time using the fluorescent probe N-(3-triethylammonium)propyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM 1-43) (Invitrogen / Molecular Probes). Cultures were washed twice with Hanks Balanced Salt Solution (HBSS) (Sigma) and cells were loaded with 5 μg/ml dye for 1 min in the buffer at room temperature. Subsequently, the cultures were washed three times with HBSS and imaged immediately. The fluorescent dyes were excited at 488 nm and fluorescence emission at 525 nm (for CM-H_2DCFDA and DAF-FM) and at 595 nm (for FM 1-43) was recorded using a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss, Welwyn Garden City,

UK). Fluorescence images and time series data of the fluorescence intensities were recorded every 2 seconds. ICCM was added after 60 seconds when a stable baseline had been established. All measurements were performed at room temperature.

In some experiments, Fluo 3, Fura Red, CM-H_2DCFDA and FM 1-43 were measured simultaneously in HaCaT cells. Reference spectra of each dye were first recorded in the HaCaT cells by recording lambda image stacks from 500–700 nm in 10 nm steps. The dyes were then loaded simultaneously into HaCaT cells and image stacks were recorded in the same wavelength range. Linear unmixing was performed using the Zeiss META software to produce time series data for each dye.

Inhibitors of ROS, NO, membrane signaling and calcium

To inhibit ROS, cultures were exposed to ICCM in the presence or absence of superoxide dismutase (SOD) (Sigma) at a final concentration of 100μg/ml and catalase (Sigma) at a final concentration of 20μg/ml. To inhibit NO, cultures were exposed to 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (c-PTIO) (Sigma) at a final concentration of 20μM. Cultures were exposed to ICCM in the presence or absence of filipin (Sigma) at a final concentration of 0.5μg/ml to inhibit membrane signalling.

Cultures were exposed to ICCM in the presence or absence of ethylene glycol tetraacetic acid (EGTA) (Sigma) at a final concentration of 5 mM to chelate extracellular calcium, bis(2-aminophenoxy)ethane tetraacetic acid acetoxymethyl

ester (BAPTA-AM) (Invitrogen / Molecular Probes) at a final concentration of 20μM to chelate intracellular calcium and verapamil (Sigma) at a final concentration of 10 μM to block voltage dependent calcium channels.

Recipient cells were pre-incubated with inhibitors for 15 mins and exposed to ICCM in the presence of the inhibitor as described for live cell imaging and the cell viability assay.

Similarly, donor cells were pre-incubated with inhibitors for 15 mins and irradiated in the presence of the inhibitor. Immediately after irradiation, this medium was replaced with fresh medium. The flasks were incubated for 1 hour and ICCM was harvested as described previously.

Statistical analysis

The data are expressed as the percentage mean ± standard error on the mean, for each of three independent experiments. Significance was assessed using the Student's t-test at $p<0.05$.

Results

Figure 1a shows strong staining of the plasma membrane by fluorescent dye FM1-43 in HaCaT cells. The fluorescence intensity decreased rapidly (within 30 seconds) after addition of 0.5Gy ICCM indicating a change in the membrane permeability (figure 1 a and b). Similar results were observed for 0.005Gy and 0.05Gy ICCM (data not shown). No change in fluorescence intensity was observed following addition of medium from unirradiated cells, 0Gy ICCM (figure 1b). The Alamar Blue cell viability assay was carried out in the presence and absence of filipin, an inhibitor of lipid rafts (figure 1c). A significant reduction in viability was observed following exposure of HaCaT cells to 0.05 Gy and 0.5 Gy ICCM for 24 hours. No significant reduction in viability was observed following exposure to 0.005Gy ICCM for 24 hours. No significant toxicity was found in the cells exposed to 0Gy ICCM in the presence or absence of filipin. An increase in the viability of the cells exposed to 0.05Gy and 0.5Gy ICCM in the presence of filipin was observed.

Levels of ROS in HaCaT cells were measured using the fluorescent dye, CM-H_2DCFDA. Figure 2 a and b shows a rapid (within 30 seconds) and sustained increase in ROS levels following addition of 0.5Gy ICCM. Similar results were obtained for 0.005Gy and 0.05Gy ICCM (data not shown). No change in ROS levels were observed following addition of medium from unirradiated cells, 0Gy ICCM (figure 2b). Cell viability was measured in the presence and absence of

SOD and catalase, inhibitors of ROS (figure 2c). No significant toxicity was found in the cells exposed to 0Gy ICCM in the presence of SOD or catalase. An increase in the viability of the cells exposed to 0.05Gy and 0.5Gy ICCM in the presence of SOD and catalase was observed.

NO levels were measured in HaCaT cells using the fluorescent dye, DAF-FM. Figure 3 a and b shows a steady increase in NO levels within 3 minutes of addition of 0.5Gy ICCM. Similar results were obtained for 0.005Gy and 0.05Gy ICCM (data not shown). No change in fluorescence intensity was observed following addition of 0Gy ICCM (figure 3b). Cell viability was measured in the presence and absence of c-PTIO, an inhibitor of NO (figure 3c). No significant toxicity was found in the cells exposed to 0Gy ICCM in the presence of c-PTIO. An increase in the viability of the cells exposed to 0.05Gy and 0.5Gy ICCM in the presence of c-PTIO was observed.

Fluorescence intensities for Fluo 3, Fura Red, CM-H_2DCFDA and FM1-43 measured simultaneously in HaCaT cells are shown in Figure 4a and b. Following addition of 0.5Gy ICCM (figure 4a) or 0.005Gy ICCM (figure 4b), a rapid increase in Fluo 3 fluorescence and a concomitant decrease in Fura Red fluorescence was observed indicating an increase in calcium levels. A simultaneous decrease in membrane permeability and a slightly later increase in ROS levels were also observed. Similar results were observed for 0.05 Gy ICCM

but no changes in fluorescence intensity were observed following addition of 0Gy ICCM (data not shown).

To elucidate whether the membrane signalling observed was due to calcium signalling, Fluo 3, Fura Red and FM1-43 were loaded simultaneously in HaCaT cells and 0.5Gy ICCM was added in the presence of BAPTA-AM, a chelator of intracellular calcium. No change in fluorescence intensity of Fluo 3 or Fura Red was observed indicating no change in intracellular calcium levels (figure 4c). However, a change in membrane permeability was still observed in these HaCaT cells (figure 4c). To check that the change in membrane permeability was not due to an influx of extracellular calcium through calcium channels, a further experiment was performed where 0.5Gy ICCM was added in the presence of EGTA, a chelator of extracellular calcium. As expected, no change in intracellular calcium levels was observed but again a change in membrane permeability was still observed in these HaCaT cells (data not shown). To further confirm that the change in membrane permeability was not due to opening of calcium channels, 0.5Gy ICCM was added in the presence of verapamil, an inhibitor of voltage gated calcium channels. Again as expected, no change in calcium levels were observed but the change in membrane permeability was still observed in these HaCaT cells (data not shown) indicating that the membrane signalling in bystander cells is a separate signalling event to calcium signalling. Cell viability was measured in HaCaT cells exposed to ICCM in the presence or absence of BAPTA-AM, EGTA and verapamil (figure 4d). A significant reduction in viability was observed following exposure of HaCaT cells to 0.05 Gy and 0.5 Gy ICCM for

24 hours. No significant reduction in viability was observed following exposure to 0.005Gy ICCM for 24 hours. No significant toxicity was found in the cells exposed to 0Gy ICCM in the presence or absence of the calcium inhibitors. An increase in the viability of the cells exposed to 0.05Gy and 0.5Gy ICCM in the presence of BAPTA-AM, EGTA and verapamil was observed. To elucidate if the calcium signalling observed was a direct result of the membrane signalling, Fluo 3, Fura Red and FM1-43 were loaded simultaneously in HaCaT cells and 0.5Gy ICCM was added in the presence of filipin, an inhibitor of membrane signalling. As expected, no change in membrane permeability was observed. No change in intracellular calcium levels was observed indicating that the increase in calcium in bystander cells results from membrane signalling (figure 4e). As seen in figure 1c, exposure to ICCM in the presence of filipin results in decreased bystander cell death.

To investigate if ROS, NO or calcium were involved in the production of the bystander signal, the irradiated (donor) cells were incubated with SOD, catalase (inhibitors of ROS), c-PTIO (inhibitor of NO) and EGTA (chelator of extracellular calcium) for 15 minutes before and during irradiation and this medium was replaced immediately after irradiation with fresh medium. The cells were incubated in the fresh medium for one hour and ICCM was harvested as described previously. Figure 5a shows the effect of inhibiting ROS in the donor cells on the bystander response. No change in fluorescence intensity of Fluo 3 or Fura Red was observed in the recipient cells indicating no change in intracellular calcium levels (figure 5a). Similarly, no change in membrane permeability was

observed (figure 5a). Cell viability was measured using the Alamar Blue assay (figure 5b). A significant increase in the viability of the cells exposed to 0.05Gy and 0.5Gy ICCM where the donor cells had been incubated with SOD and catalase was observed compared to ICCM generated under normal conditions (figure 5b).

A similar effect was observed with the extracellular calcium chelator, EGTA. No calcium influx and no change in membrane permeability was observed when the donor cells were incubated with EGTA (data not shown). A significant increase in the viability of the cells exposed to 0.05Gy and 0.5Gy ICCM where the donor cells had been incubated with EGTA was observed compared to ICCM generated under normal conditions (figure 5d).

Figure 5c shows the effect of inhibiting NO with c-PTIO in the donor cells. A calcium response (increase in Fluo 3 and decrease in Fura Red) and a change in membrane permeability was observed in the recipient cells indicating no effect of inhibiting NO in the donor cells. Cell viability was measured using the Alamar Blue assay (figure 5d). No difference in the viability of the cells was observed when the donor cells had been incubated with c-PTIO compared to ICCM generated normally (figure 5d).

Discussion

This study has elucidated for the first time the sequence of very early signalling events in HaCaT cells exposed to ICCM. Within 30 seconds of addition of ICCM, a change in the plasma membrane permeability occurs resulting in an immediate influx of calcium into the cytosol. This induces a rapid increase in ROS levels and a subsequent increase in NO levels. All of the signalling events appear to play a role in bystander induced death as when these events were inhibited, bystander cell death was blocked. In addition, ROS and calcium, but not NO, were found to play a role in bystander signal production.

Although there have been previous studies implicating membrane signalling in bystander cells (Nagasawa et al. 2002; Shao et al. 2004; Burdak-Rothkamm et al. 2007; Tartier et al. 2007), this study has shown the first direct evidence of membrane signalling within 30 seconds of addition of ICCM using a live cell imaging approach.

While there are numerous reports of bystander responses in various cell models, there is a lack of data on the mechanisms of bystander signal production in irradiated cells. Microbeam irradiation studies have clearly shown that direct DNA damage is not required in the irradiated cell to produce a bystander response (Wu et al. 1999; Shao et al. 2004). These studies showed that cytoplasmic organelles such as mitochondria could be important targets. Studies with mitochondrial DNA depleted cells have provided direct evidence of the key role of

mitochondria in bystander responses (Tartier et al. 2007; Chen et al. 2008; Zhou et al. 2008). The results from the present study showed that calcium and ROS play important roles in bystander signal production and support the idea of the involvement of mitochondria. Elevation of intracellular calcium in the irradiated cell can result in overload of calcium in the mitochondria leading to a transient loss of membrane potential and production of ROS. Chen et al (2008) recently reported that mitochondrial calcium uptake is involved in ROS production in irradiated cells. Due to the short half lives of these radical species, it is likely that long lived soluble factors are induced by these ROS and released into the medium. Cytokines such as interleukins and TGFβ have been shown to be involved in bystander responses (Iyer et al. 2000; Osterreicher et al. 2003; Facoetti et al. 2006; Shao et al. 2008; Facoetti et al. 2009) and have been shown to be induced by ROS (Iyer et al. 2000).

This study has shown that in our model system, calcium and ROS are involved in both the production of the bystander signal and in the response to this signal while NO is involved in the bystander response only. Harada et al (2008) investigated the involvement of different radical species in the bystander effect. They showed that NO was involved in bystander signal formation in the irradiated cells while hydroxyl radicals or long lived radicals were involved in the responses in the bystander cells. These differences may be due to differences in experimental design as in the present study ICCM was transferred to unirradiated

cells 1 hour after irradiation, while in the study of Harada et al (2008), irradiated cells were co-cultured with unirradiated cells immediately after irradiation. Rzeszowska-Wolny et al (2009) investigated transcript profiles in directly irradiated and bystander cells and observed common changes including groups of transcripts involved in different functional pathways, neuroactive ligand - receptor interactions, oxidative phosphorylation, cytokine-cytokine receptor interactions. Interestingly, an upregulation of the calcium signalling pathway group was also reported, consistent with the findings of the present study.

In this study, rapid membrane and calcium signaling and induction of ROS and NO have been shown in unirradiated cells following addition of ICCM. No significant differences were observed for the different doses, 0.005, 0.05 and 0.5Gy. A full signalling response was observed in all cases. Although no significant cell death was observed following addition of 0.005Gy ICCM in the present study, previous studies have shown a small but significant reduction of approx 10% in clonogenic survival in the HPV-G cell line following exposure to 0.005Gy ICCM (Seymour & Mothersill 2000; Liu et al. 2006). Reductions of approx 20-30% in clonogenic survival have also been reported previously (Liu et al. 2006) for both direct and bystander treatments at the higher doses used in the present study, 0.05Gy and 0.5Gy. The human keratinocyte HPV-G cell line shows a similar bystander response to the HaCaT cell line used in the present study. In our previous work (Liu et al. 2006), a dose threshold for bystander effects was reported with a reduction in clonogenic survival and calcium fluxes

observed above 3mGy. In the present study, cell viability was measured 24 hours after exposure whereas in our previous study (Liu et al. 2006), clonogenic survival was measured 10 days after exposure. It is interesting, however, that a significant reduction in cell viability was measurable in bystander cells after exposure to medium from cells irradiated at higher doses (0.05 and 0.5Gy). This may be due to a dose dependent expression of survival or death signals downstream of the early events reported here and will be investigated in a future study.

Although not shown in this study, it is likely that calcium overload in the mitochondria of the irradiated cells leading to ROS induction is involved in the production of the bystander signal(s). These signals can be sensed by the bystander cells through the plasma membrane. Cytokine and growth factor receptors on the plasma membrane can activate MAPK and other pathways and can transduce signals to the cell nucleus (Zhou et al. 2005; Zhou et al. 2008). In addition, plasma membrane bound NADPH oxidase can be activated leading to a long lasting production of intracellular ROS in the bystander cells (Narayanan et al. 1997; Azzam et al. 2002). In summary, the present study has elucidated for the first time the sequence of very early signalling events in HaCaT cells exposed to ICCM. A change in plasma membrane permeability results in an influx of calcium into the cytosol inducing a rapid increase in ROS levels and a subsequent increase in NO levels. Calcium and ROS were found to be involved

in both the production of the bystander signal and in the response to this signal while NO was found to be involved in the bystander response only.

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Declaration of Interest

The authors report no conflicts of interest. The authors acknowledge financial support from the FP6 Integrated Project, *Non-targeted effects of ionising radiation (NOTE)* FI6R 036465. The work was also conducted as part of the National Biophotonics and Imaging Platform of Ireland (NBIPI), funded by the Irish Government's Programme for Research in Third Level Institutions, Cycle 4 (2007-2013).

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Figure Legends

Figure 1 a) Cells were stained with the fluorescent dye FM 1-43 (5 μM, Molecular Probes) and monitored over a 5 minute period for changes in membrane permeability before and after addition of medium from irradiated cells (0.5Gy ICCM), bar = 10 μm, b) Fluorescence intensity of FM 1-43 in HaCaT cells after addition of 0Gy ICCM and 0.5Gy ICCM. ICCM was added at the time indicated by the arrow, c) % viability as measured by the Alamar Blue assay in cultures exposed to 0Gy, 0.005Gy, 0.05Gy or 0.5Gy ICCM in the presence of an inhibitor of membrane signalling, filipin. Data are presented as the mean ± standard error on the mean, n=3, * $p<0.05$

Figure 2 a) Cells were stained with the fluorescent dye CM-H_2-DCFDA (5 μM, Molecular Probes) and monitored over a 5 minute period for levels of reactive oxygen species before and after addition of medium from irradiated cells (0.5Gy ICCM), bar = 10 μm, b) Fluorescence intensity of CM-H_2-DCFDA in HaCaT cells after addition of 0Gy ICCM and 0.5Gy ICCM. ICCM was added at the time indicated by the arrow, c) % viability as measured by the Alamar Blue assay in cultures exposed to 0Gy, 0.005Gy, 0.05Gy or 0.5Gy ICCM in the presence of inhibitors of reactive oxygen species, superoxide dismutase (SOD) and catalase. Data are presented as the mean ± standard error on the mean, n=3, * $p<0.05$

Figure 3 a) Cells were stained with the fluorescent dye DAF-FM (5 μM, Molecular Probes) and monitored over a 5 minute period for levels of nitric oxide before and after addition of medium from irradiated cells (0.5Gy ICCM), bar = 10 μm, b) Fluorescence intensity of DAF-FM in HaCaT cells after addition of 0Gy ICCM and 0.5Gy ICCM. ICCM was added at the time indicated by the arrow, c) % viability as measured by the Alamar Blue assay in cultures exposed to 0Gy, 0.005Gy, 0.05Gy or 0.5Gy ICCM in the presence of an NO inhibitor, c-PTIO. Data are presented as the mean ± standard error on the mean, n=3, * $p<0.05$

Figure 4 a) Cells were stained simultaneously with Fluo 3 and Fura Red (3 μM, Molecular Probes), DCFDA and FM 1-43 and monitored over a 5 minute period for levels of calcium, ROS and for membrane permeability before and after addition of medium from irradiated cells (0.5Gy ICCM), b) Cells were stained simultaneously with Fluo 3 and Fura Red (3 μM, Molecular Probes), DCFDA and FM 1-43 and monitored over a 5 minute period for levels of calcium, ROS and for membrane permeability before and after addition of medium from irradiated cells (0.005Gy ICCM), c) Cells were stained simultaneously with Fluo 3, Fura Red and FM 1-43 and monitored over a 5 minute period for levels of calcium and for membrane permeability before and after addition of medium from irradiated cells (0.5Gy ICCM) in the presence of BAPTA-AM, an chelator of intracellular calcium, d) % viability as measured by the Alamar Blue assay in cultures exposed to 0Gy, 0.005Gy, 0.05Gy or 0.5Gy ICCM in the presence of an BAPTA-AM, EGTA and verapamil, e) Cells were stained simultaneously with

Fluo 3, Fura Red and FM 1-43 and monitored over a 5 minute period for levels of calcium and for membrane permeability before and after addition of medium from irradiated cells (0.5Gy ICCM) in the presence of filipin, an inhibitor of membrane signaling. Live cell imaging data is representative of at least 6 independent experiments. Viability data is presented as the mean ± standard error on the mean, n=3, * $p<0.05$

Figure 5 a) Cells were stained simultaneously with Fluo 3, Fura Red and FM 1-43 and monitored over a 5 minute period for levels of calcium and for membrane permeability before and after addition of medium from cells irradiated in the presence of SOD (0.5Gy ICCM), b) % viability as measured by the Alamar Blue assay in cultures exposed to medium from cells irradiated in the presence of SOD and catalase (0Gy, 0.005Gy, 0.05Gy or 0.5Gy ICCM), c) Cells were stained simultaneously with Fluo 3, Fura Red and FM 1-43 and monitored over a 5 minute period for levels of calcium and for membrane permeability before and after addition of medium from cells irradiated in the presence of c-PTIO (0.5Gy ICCM), d) % viability as measured by the Alamar Blue assay in cultures exposed to medium from cells irradiated in the presence of EGTA and c-PTIO (0Gy, 0.005Gy, 0.05Gy or 0.5Gy ICCM). Live cell imaging data is representative of at least 6 independent experiments. Viability data is presented as the mean ± standard error on the mean, n=3, * $p<0.05$