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Cell death pathways in directly irradiated cells and cells exposed to medium from irradiated cells

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Abstract

Purpose: The aim of this study was to compare levels of apoptosis, necrosis, mitotic cell death and senescence after treatment with both direct radiation and irradiated cell conditioned medium.

Materials and methods: Human keratinocytes (HaCaT cell line) were irradiated (0.005, 0.05 and 0.5 Gy) using a cobalt 60 teletherapy unit. For bystander experiments, the medium was harvested from donor HaCaT cells one hour after irradiation and transferred to recipient HaCaT cells. Clonogenic assay, apoptosis, necrosis, mitotic cell death, senescence and cell cycle analysis were measured in both directly irradiated cells and bystander cells

Results: A reduction in cell survival was observed for both directly irradiated cells and irradiated cell conditioned medium (ICCM) treated cells. Early apoptosis and necrosis was observed predominantly after direct irradiation. An increase in the number of cells in G2/M phase was observed at 6 and 12 hours which led to mitotic cell death after 72 hours following direct irradiation and ICCM treatment. No senescence was observed in the HaCaT cell line following either direct irradiation or treatment with ICCM.

Conclusion: This study has shown that directly irradiated cells undergo apoptosis, necrosis and mitotic cell death whereas ICCM treated cells predominantly undergo mitotic cell death.

Introduction

Considerable evidence exists regarding non targeted effects of radiation, such as bystander effects, where effects are observed in cells exposed to signals from irradiated cells (Mothersill and Seymour 2006; Wright and Coates 2006; Hamada et al. 2007; Hei et al. 2008; Morgan and Sowa 2009; Prise and O'Sullivan 2009). An increased occurrence of sister chromatid exchanges (SCE) (Nagasawa and Little 1992; Deshpande et al. 1996), chromosomal aberrations (Lorimore et al. 1998), activation of stress inducible signalling pathways (Azzam et al. 1998; Azzam et al. 2001, 2003), DNA damage (Nagasawa and Little 1992; Huo et al. 2001), cell death (Prise et al. 1998; Lyng et al. 2000), mitochondrial alterations (Maguire et al. 2005; Murphy et al. 2005), oncogenic transformation (Lewis et al. 2001) and changes in gene expression (Klokov et al. 2004; Ghandhi et al. 2008) have been shown in unirradiated cells that have received signals from directly irradiated cells.

To date, only a small number of studies have directly compared direct irradiation effects and bystander effects. Ojima et al (2011) investigated the number of phosphorylated ataxia telangiectasia mutated (ATM) foci in cells co-cultured with directly irradiated cells and suggested that double stranded DNA breaks induced by direct irradiation are repaired quickly when compared with those induced by bystander effects which persist for longer. Differences in DNA damage signalling in directly irradiated cells and bystander cells has also been shown (Burdak-Rothkamm et al. 2007, 2008). Studies have shown very similar changes in the transcriptome of both directly irradiated and bystander cells (Rzeszowska-Wolny et al. 2009; Herok et al. 2010) while Ghandhi et al (2010) showed that gene clusters for irradiated cells were enriched for signal transduction, cell cycle/cell death and inflammation / immunity processes whereas gene clusters for bystander cells were enriched for cell communication / motility, signal transduction and inflammation processes. Mitochondrial damage, including

mitochondrial DNA (mtDNA) damage and altered mitochondrial function, has been shown to be similar in directly irradiated cells and cells exposed to irradiated cell conditioned media (ICCM) (Murphy et al. 2005; Nugent et al. 2007; Nugent et al. 2010). Hanot et al (2009) showed similar levels of oxidative stress in both targeted and bystander cells but mitochondrial reactive oxygen species (ROS) was delayed in bystander cells compared to targeted cells. This study also showed apoptosis in targeted cells at 24 hours but not in bystander cells and increased micronuclei formation in targeted cells compared to bystander cells.

Although a number of studies have investigated the induction of apoptosis in bystander cells (Belyakov et al. 1999; Lyng et al. 2000, 2002, 2006b), other cell death mechanisms have received little attention. Cells may die by several mechanisms after direct exposure to ionising radiation; apoptosis, necrosis, mitotic catastrophe and senescence. Apoptosis is known to be the main cell death mechanism induced by irradiation in cells from lymphoid and myeloid lineages (Radford et al. 1994). Significantly less apoptosis is observed in cells of epithelial origin (Eriksson and Stigbrand 2010). Radiation induced mitotic catastrophe is often considered a type of cell death that occurs during mitosis or due to mitotic failure. It is a delayed type of cell death executed typically 2-6 days after irradiation. Mitotic catastrophe can occur as a result of DNA damage and deficient cell cycle checkpoints, which may be a result of mutation or inactivation of tumour suppressor protein (p53) (Eriksson and Stigbrand 2010) or it can be induced by hyperamplification of centrosomes which frequently occurs in cells without functional p53 and results in abnormal chromosomal segregation and the formation of cells with multiple micronuclei or multinucleated giant cells (Roninson et al. 2001; Eriksson et al. 2007). Senescence is a condition of permanent cell cycle arrest in that senescent cells do not divide but may remain metabolically active. They can secrete factors such as cytokines that can allow communication with their environment (Rodier et al. 2009).

The main aim of this study was to compare cell death pathways; apoptosis, necrosis, mitotic cell death and senescence, in both directly irradiated cells and bystander cells.

Materials and Methods

Cell culture

Human keratinocyte cells (HaCaT cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM): F-12 Ham (1:1) (Sigma Aldrich, Gillingham, Dorset, UK) containing 10% fetal bovine serum (Sigma Aldrich), 5000 international units (IU)/ml of penicillin streptomycin solution (Gibco Biocult, Irvine, Scotland, UK) and 1 µg/ml of hydrocortisone (Gibco Biocult). The cells were maintained in an incubator at 37ºC, with 95% humidity and 5.0% CO_2. Subculture was routinely performed when cells were 75-80% confluent using 0.25% trypsin (Sigma Aldrich) and 1 mM versene (Sigma Aldrich) at a 1:1 ratio.

Irradiation

Culture flasks (T-25 flasks, Nunc, Roskilde, Denmark) containing 2 X 10^5 cells were seeded and irradiated approximately 12 hours after plating (0.005, 0.05, 0.5 Gy and 8 Gy) using a cobalt-60 teletherapy source at St. Luke's hospital, Dublin. The culture medium was not changed before irradiation. The source to flask distance was 191.5 cm and the dose rate was approximately 1.5 Gy/min during these experiments. 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.

Harvesting of ICCM

Donor flasks containing 2 X 10^5 cells were seeded and irradiated approximately 12 hours after plating. The culture medium was not changed before irradiation. Medium from both irradiated and unirradiated cells were poured off donor flasks 1 hour after irradiation and filtered through a 0.22 µm filter (Nalgene/Thermo Fisher, Hereford, Herefordshire, United

Kingdom) to prevent debris and cells being transferred into the medium. After filtration, the medium was added to recipient cells immediately.

Clonogenic assay

Using the clonogenic assay technique of Puck and Marcus (1956), 400 cells were plated per T-25 flask, in 5 ml of culture medium, and incubated at 37˚C, in 5% CO_2 in air. Approximately 12 hours after plating, the cells were either directly irradiated or treated with ICCM. All flasks were checked prior to irradiation or ICCM treatment to ensure that the plated cells had not divided and that they were still single cells. Cells were allowed to grow in an incubator at 37˚C until colony formation (7 days). The colonies were then stained with carbol fuschin (BDH, Poole, UK), counted and the cell survival was determined.

Apoptosis / Necrosis assay

The plasma membrane permeability to YO-PRO and propidium iodide (PI) dye was measured in HaCaT cells after treatment using the Vybrant apoptosis kit (Invitrogen, BioSciences, Dublin, Ireland). Approximately 5 X 10^5, 3 X 10^5 and 2 X 10^5 cells were plated in T-25 flasks and directly irradiated or exposed to ICCM for 24, 48 and 72 hours respectively. After irradiation at St. Luke's hospital, the directly irradiated cells were brought to the Radiation and Environmental Science Centre (RESC) laboratory and incubated for 24, 48 or 72 hours. The ICCM was brought from St. Luke's hospital and added to the unirradiated recipient cells at the RESC laboratory and these recipient cells were incubated for 24, 48 and 72 hours. After incubation, the cells were harvested using trypsin, pelleted and re-suspended in 1 ml of phosphate buffered saline (PBS) and 1 µl of YO-PRO solution (100 µM) (Invitrogen) and PI solution (1.5 mM) (Invitrogen) was added and incubated for 30 min on ice. The analysis of stained cells was performed using flow cytometry (Partec, Munster,

North Rhine-Westphalia, Germany) and excitation was carried out at 488 nm for both Yo-PRO-1 and PI. Fluorescence emission was collected at 530 nm and >575 nm. Gating and analysis was performed using MoFlo® and CyAn™ ADP Summit version 4.3 software (Dako, Carpinteria, California, USA) to exclude the dead population from viable cells. Single-color stained controls were used for standard compensation. By using this assay, four different populations could be identified: viable cells which are negative for both YO-PRO-1 and PI, early apoptotic cells which are positive for YO-P PRO-1 but not for PI, late apoptotic cells which are positive for both YO-PRO-1 and PI and necrotic cells which are positive for PI but not for YO-PRO-1.

Mitotic cell death assay

Approximately 5 X 10^4, 2.5 X $10^4$and 1.5 X 10^4 cells were plated on coverslips in a 6-well plate and directly irradiated or exposed to ICCM and incubated for 24, 48 and 72 hours. The cells were washed with PBS and fixed using freshly prepared 4% paraformaldehyde at 24, 48 and 72 hours after treatment. The mitotic nature of the cells was determined by using mitotic protein monoclonal-2 (MPM-2) antibody (Abcam, Cambridge, Cambridgeshire, UK) diluted 1:500 in 1% bovine serum albumin (BSA) in PBS and incubated for one hour at 37 ^{o}C, followed by incubation with a secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Abcam) for 45 mins at 37˚C. The MPM-2 antibody specifically recognises phosphoproteins abundant in mitotic cells and together with morphological changes to the nucleus can be used as a marker of mitotic cell death (Wolanin et al. 2006). The cells were counter stained using PI (1 mg/ml, Sigma Aldrich) to visualise the cell nucleus and were observed using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Welwyn Garden City, Hertfordshire, UK). Excitation was carried out at 488 nm and emission was collected at 530 nm for FITC and excitation was carried out at 545 nm and emission was

collected at 590 nm for propidium iodide. Fluorescence images were recorded at 63X magnification in 10 fields of view per sample. Cells showing increased green fluorescence (MPM-2 expression) and changes in nuclear morphology and chromatin organisation such as multipolar chromosome segregation and multinucleation were scored as cells positive for mitotic cell death.

Cell Cycle analysis

Approximately 1.5 X 10^5 cells were plated for 6 and 12 hours and 2 X 10^5, 9 X 10^4 and 4.5 X 10^4 cells were plated for 24, 48 and 72 hours respectively. Cells were plated in 6 well plates and directly irradiated or exposed to ICCM and incubated for 6, 12, 24, 48 and 72 hours. After incubation, the cells were trypsinized and resuspended in cold PBS and fixed using 70% ice cold ethanol. After fixation, the cells were treated with PI (40 µg/ml, Sigma Aldrich), RNase (100 µg/ml, Sigma Aldrich) and incubated at 37ºC for 30 minutes. At this step, samples were stored at 4ºC, in the dark until analysis by flow cytometry. The samples were analysed using a CyFlow flow cytometer (Partec) and the data was analysed using MultyCycle, DNA cell cycle analysis software (32 bit version, Phoenix Flow Systems, San Diego, California, USA) to measure the DNA content in each phase of the cell cycle.

Senescence assay

The senescence-associated β-galactosidase (SA-β-gal) assay was performed using a cellular senescence kit (Chemicon International, Temecula, California, USA). Approximately 2 X 10^4 cells were plated in 35 mm wells and after treatment, the cells were incubated for 5 days and media was removed from the culture dish, washed twice using PBS. The staining reaction was performed according the instructions given by the manufacturer.

Statistical analysis

Statistical analysis were performed using Statgraphics Centurion XV (StatPoint Technologies, Inc, Warrenton, Virginia, USA). Mean values were compared using one-way analysis of variance (ANOVA) where a p value of < 0.05 was considered to be statistically significant.

Results

Cell survival

Figure 1 shows clonogenic assay data for both directly irradiated and ICCM treated HaCaT cells. Plating efficiency values for the 0 Gy control and 0 Gy ICCM control were 27.2 ± 1.8 and 28.6 ± 1.5 respectively. A slight (approximately 5%) but not significant reduction in cell survival for both directly irradiated cells and cells treated with ICCM was observed at the 0.005 Gy dose point compared to the sham irradiated control (0 Gy). A significant reduction in cell survival of approximately 20% was observed for both directly irradiated cells and ICCM treated cells at the higher dose points, 0.05 Gy and 0.5 Gy.

Apoptosis and necrosis

In the sham irradiated HaCaT cells, around 10% of cells were found to be undergoing apoptosis, whereas in directly irradiated cells, an increase in the number of early apoptotic cells was observed at 24, 48 and 72 hours after irradiation (Figure 2A-C). Significant increases in early apoptotic cells were observed at 24 hours for 0.005 Gy, at 48 hours for all dose points and at 72 hours for 0.05 Gy. Increases in necrotic cells were observed at 24 and 48 hours but only the 0.005 Gy dose point was significantly different to controls at 24 hours after irradiation.

For ICCM treated cells, approximately 5% of the sham treated cells (0 Gy ICCM) were undergoing apoptosis. A slight but not significant increase in the number of early apoptotic cells was observed 24 hours after treatment at all dose points (Figure 2D). A small but not significant increase in early apoptotic cells was observed at 48 hours for 0.5 Gy (Figure 2E) and a significant increase in early apoptotic cells was found at the 0.5 Gy dose point 72 hours

after treatment (Figure 2F). A small but not significant increase in necrotic cells was found 48 hours after incubation with ICCM specifically at the 0.005 Gy dose point (figure 2E).

Mitotic cell death

In the directly irradiated HaCaT cells, very little MPM2 expression was observed 24 and 48 hours after irradiation (data not shown). At 72 hours after irradiation, very little MPM2 expression was observed in the sham irradiated cells (0 Gy) (Table I, Figure 3A) whereas significantly increased MPM2 expression was observed in the directly irradiated cells at the 0.05 Gy and 0.5 Gy dose points (Table I and Figure 3B). Micronuclei were observed particularly in directly irradiated cells at the 0.5 Gy dose point as shown in Figure 3D. Some cells exhibited tripolar mitosis due to centromere duplication as shown in Figure 3E which later may form into multilobulated nuclei.

In the ICCM treated cells, very little MPM2 expression was observed 24 and 48 hours after treatment (data not shown). Very little MPM2 expression was observed in the sham treated cells (0 Gy ICCM) (Figure 3A, Table I) whereas significantly increased MPM2 expression was observed in the ICCM exposed cells at 0.05 Gy and 0.5 Gy dose points 72 hours after treatment (Table I and Figure 3C). Many of the cells were found with 4N DNA content and cells exhibiting tripolar mitosis as shown in Figure 3E were observed. In the case of untreated cells, the nucleus was found to be in an organised state whereas in cells that had undergone mitotic catastrophe, the nucleus was totally disorganised. Also these cells were enlarged which is an important morphological feature of mitotic catastrophe.

Cell cycle analysis

In the sham irradiated samples (0 Gy), a small percentage of cells were found to be in the G2/M phase of the cell cycle with most cells in either S phase or G1 phase. A significant increase in the percentage of cells in G2/M phase was observed 6 hours after direct irradiation at 0.005 Gy, 0.05 Gy and 0.5 Gy dose points when compared with the 0 Gy control (Figure 4A). At the same time, a significant decrease in the percentage of cells in G1 phase at all the dose points was observed. A clear shift was observed from G1 and S phase to G2/M phase at all dose points at 6 hours after direct irradiation. A similar shift was observed 12 hours after direct irradiation at the 0.05 and 0.5 Gy dose points (Figure 4B). A similar trend was observed at 24 hours for 0.05 Gy and 0.5 Gy dose points but this was not significant (Figure 4C). At 48 and 72 hours after direct irradiation, no significant increase in the percentage of cells in G2/M phase was observed compared to the sham irradiated cells (0 Gy) but a significant decrease in the percentage of cells in G1 phase was observed at 72 hours for 0.05 and 0.5 Gy (Figure 4E).

In the case of ICCM treated cells, a significant increase in the percentage of cells in G2/M phase was observed at 6 hours after treatment at the 0.05 and 0.5Gy dose points (Figure 4F). A significant decrease in the percentage of cells in S phase was also observed at 6 hours at the 0.05 Gy dose point (Figure 4F). A slight but not significant increase in the percentage of cells in G2/M phase at 12 hours for 0.005 Gy and 0.5 Gy was observed (Figure 4G). A significant decrease in the percentage of cells in S phase was observed at 12 hours at the 0.005 Gy and 0.5 Gy dose points (Figure 4G).m No significant difference in the percentage of cells in G2/M phase was observed at 24, 48 and 72 hours after incubation with ICCM as shown in Figure 4H-J.

Senescence

In both directly irradiated and ICCM treated HaCaT cells, no SA-β-gal activity was observed for 0.005 Gy, 0.05 Gy and 0.5 Gy (data not shown). Even at a higher dose of 8 Gy, no SA-β-gal activity was observed up to 5 days after irradiation. To confirm that the method was working correctly, another cell line, A549 human adenocarcinoma alveolar epithelial cell line, was tested and SA-β-gal activity was observed in this cell line 5 days after direct irradiation with an 8 Gy dose (data not shown).

Discussion

This study has shown a comparison of cell death pathways in both directly irradiated and ICCM treated cells after 24, 48 and 72 hours. A slight (approximately 5%) but not significant reduction in cell survival was observed at the 0.005 Gy dose point compared to a significant reduction of approximately 20% at the 0.05 Gy and 0.5 Gy dose points for both directly irradiated cells and cells treated with ICCM. In the directly irradiated cells, an increase in apoptosis was observed at 24, 48 and 72 hours and an increase in necrosis was observed at 24 and 48 hours. In the ICCM treated cells, a significant increase in apoptosis was observed at 72 hours for 0.5 Gy. Significantly increased MPM2 expression, which is indicative of mitotic catastrophe, was found in both directly irradiated cells and ICCM treated cells at 72 hours for the 0.05 Gy and 0.5 Gy doses. Increases in the percentage of cells in G2/M phase were observed for all doses at 6 hours and for 0.05 Gy and 0.5 Gy at 12 hours for directly irradiated cells. For ICCM treated cells, increases in the percentage of cells in G2/M phase were observed at 6 hours for 0.05 Gy and 0.5 Gy. No senescence was observed in the HaCaT cell line after either direct irradiation or treatment with ICCM.

Similar clonogenic survival data was obtained for both the directly irradiated cells and the ICCM treated cells. These results agree with those of Seymour and Mothersill (2000) who showed clearly that cell death arising from bystander effects predominates at low doses whereas at doses above 0.5 Gy, cell death arising from direct irradiation effects predominates. They showed that the bystander effect is relatively constant at all doses but that the direct effect shows a clear dose response above the 0.5 Gy dose. This explains the findings of the present study where no clear dose response was seen for either direct or bystander effects up to 0.5 Gy. Previous studies have shown a small but significant reduction of approximately 10% in clonogenic survival in the human keratinocyte HPV-G cell line following exposure to

0.005 Gy ICCM (Seymour and Mothersill 2000; Liu et al. 2006). Reductions of approximately 20-30% in clonogenic survival have also been reported previously for the HPV-G cell line for both direct and bystander treatments at 0.05 Gy and 0.5 Gy which correlate well with the results obtained in the present study for the HaCaT cell line (Liu et al. 2006). In our recent study (Lyng et al 2011) rapid membrane and calcium signaling and induction of ROS and NO were shown in unirradiated cells following addition of ICCM with no significant differences observed for 0.005, 0.05 and 0.5 Gy doses. A full signalling response was observed at all doses but a reduction in cell viability was only observed at the higher doses of 0.05 Gy and 0.5 Gy, indicating a dose dependent expression of survival or death signals downstream of the early signalling events.

The difference in the baseline apoptosis levels in the control groups (0 Gy and 0 Gy ICCM) for both directly irradiated and ICCM treated cells was most likely due to the slight differences in handling. As the apoptosis assay is performed on unfixed cells, the directly irradiated cells were transported to our laboratory immediately after irradiation. In contrast, for the bystander cells, ICCM was harvested 1 hour after irradiation and transported to our laboratory and added to recipient bystander cells. This difference may have resulted in the elevated baseline level of apoptosis in the sham irradiated control group (0 Gy).

Significant levels of apoptosis and necrosis were observed for the directly irradiated cells but significant apoptosis was only observed in the ICCM treated cells after 72 hours at the 0.5 Gy dose point. Previous work by our group showed that apoptosis is initiated in human keratinocyte cells (HPV-G cell line) exposed to ICCM by measuring calcium fluxes and loss of mitochondrial membrane potential (Lyng et al. 2000). The HPV-G cell line shows a similar bystander response to the HaCaT cell line used in the present study. Increased expression of the anti-apoptotic bcl-2 protein has also been observed previously by our group in HPV-G cells exposed to ICCM (Maguire et al. 2005) and although bcl-2 levels have not

been measured in the present study on HaCaT cells, this may explain the low levels of apoptosis observed in ICCM treated cells in comparison with directly irradiated cells. Activation of extracellular signal-regulated kinase (ERK) has also been observed in HPV-G cells following ICCM treatment (Lyng et al. 2006a) and the downstream action of ERK could result in the activation of anti-apoptotic proteins (Valerie et al. 2007). This again, may explain the low levels of apoptosis observed in the ICCM treated cells. Previously Olsson et al (2010) showed increased cell death following α particle irradiation in directly targeted human hepatoma HepG2 cells compared to bystander cells in the dose range 0.2-1.8 Gy. Cell death by necrosis was measured by scoring the number of propidium iodide positive cells. Caspase 3/7 activity was also observed peaking at 24 hours but no comparison between directly targeted and bystander cells was performed. In addition, Hanot et al (2009) showed increased apoptosis using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay at 24 and 48 hours after α particle irradiation in directly targeted normal osteoblastic MC3T3-E1 cells compared to bystander cells.

Mitotic catastrophe was found in both directly irradiated and ICCM treated cells at 72 hours for 0.05 Gy and 0.5 Gy. During mitotic catastrophe, cells can enter a transient G2 arrest, as observed in the present study with increased percentages of cells in G2/M phase at 6 and 12 hours after direct irradiation or ICCM treatment. The cells with damaged chromosomes can then progress to mitosis prematurely where the mitotic checkpoint or spindle assembly checkpoint is activated blocking progression through mitosis. Although not measured in this study, chromosomal aberrations have been reported previously in cells exposed to ICCM (Lorimore et al 2008). Apoptosis pathways can be activated during metaphase or cells can adapt to the mitotic checkpoint and exit the arrest but fail cytokinesis and enter the next G1 phase with tetraploid DNA content resulting in giant polyploid cells with aberrant nuclei,

multiple nuclei and micronuclei as observed in the present study (Weaver and Cleveland 2005).

Along with apoptosis, necrosis and mitotic catastrophe, the HaCaT cells were assayed for senescence using the SA-β-gal assay. No SA- β-gal activity was observed in these cells even for doses of 8 Gy and up to 5 days after irradiation. This may be due to a mutation in the p53 protein in HaCaT cells (Lehman et al. 1993) so another cell line with wild type p53, A549 cells (Lehman et al. 1991), was tested and SA-β-Gal activity was observed in these A549 cells. This indicates that in our hands the HaCaT cell line does not undergo senescence in response to exposure to ionising radiation or to bystander factors.

Table II shows a summary of the cell death occurring at 24, 48 and 72 hours following direct irradiation. The table shows that apoptosis and necrosis occurs at 24 hours at 0.005 Gy and apoptosis occurs at 48 hours at all dose points while apoptosis and mitotic cell death occur at 72 hours after irradiation. Total cell death has been estimated by summing the values for apoptosis, necrosis and mitotic cell death and this correlates very well with the data for clonogenic cell death, ie. approximately 4%, 21% and 25% cell death for 0.005 Gy, 0.05 Gy and 0.5 Gy respectively. Table III shows the corresponding data for ICCM treated cells and shows that mitotic cell death accounts for most of the cell death occurring at 72 hours for 0.05 Gy while apoptosis and mitotic cell death occur at the 0.5 Gy dose point. The total cell death values at 72 hours are lower than the clonogenic cell death values, ie. approximately 5%, 20% and 20% cell death at 0.005 Gy, 0.05 Gy and 0.5 Gy respectively. Clonogenic cell death was measured at 7 days so this difference indicates that further apoptosis and mitotic cell death may occur up to 7 days after ICCM treatment.

In summary, this study has shown that the main cell death mechanism for cells exposed to ICCM is mitotic catastrophe, predominantly occurring at 72 hours after exposure and

preceded by G2/M arrest at 6 hours. ICCM treated cells were also found to undergo apoptosis at 72 hours at the higher dose of 0.5 Gy. Directly irradiated cells also undergo mitotic cell death at 72 hours preceded by G2/M arrest at 6 and 12 hours as well as significant apoptosis at 24, 48 and 72 hours and necrosis at 24 hours. These different mechanisms of cell death in bystander cells and in directly irradiated cells could potentially be exploited for radiotherapy.

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Declaration of Interest

The authors report no conflicts of interest. The authors acknowledge financial support from the European Sixth Framework Programme (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 Percentage survival for directly irradiated cells and cells treated with ICCM at 0.005 Gy, 0.05 Gy and 0.5 Gy as measured by clonogenic assay 7 days after treatment. The data is presented as mean and standard error of the mean (SEM) for three independent experiments, each performed in triplicate, n=3, * Control and treated values were compared using one-way analysis of variance (ANOVA) and a p value of < 0.05 was considered to be statistically significant.

Figure 2 Percentage early and late apoptotic and necrotic cells 24 hours (A), 48 hours (B) and 72 hours (C) after treatment with direct irradiation and 24 hours (D), 48 hours (E) and 72 hours (F) after ICCM treatment using 0 Gy, 0.005 Gy, 0.05 Gy, 0.5 Gy dose points. The data is presented as mean intervals for the events and their standard error of the mean (SEM) for two independent experiments, each performed in duplicate, n=2, * Control and treated values were compared using one-way analysis of variance (ANOVA) and a p value of < 0.05 was considered to be statistically significant.

Figure 3 Representative fluorescence images of MPM2 expression (green fluorescence) and PI nuclear staining (red fluorescence) indicating mitotic catastrophe in HaCaT cells exposed to (A) 0 Gy ICCM or sham irradiation, (B) 0.5 Gy direct irradiation, (C) 0.5 Gy ICCM treatment and (D) cells showing micronuclei after 0.5 Gy direct irradiation and (E) cells showing mutlipolar mitosis after both directly irradiated and ICCM treatment. Bar = 20 μm. Three independent experiments were performed each in triplicate, n=3.

Figure 4 Percentage cells in each cell cycle phase (G1, S, G2/M) 6 hours (A), 12 hours (B), 24 hours (C), 48 hours (D) and 72 hours (E) after direct irradiation and 6 hours (F), 12 hours (G), 24 hours (H), 48 hours (I) and 72 hours (J) after ICCM treatment using 0 Gy, 0.005 Gy, 0.05 Gy, 0.5 Gy dose points. Data is expressed as mean values with standard error of the mean (SEM), for two independent experiments, each performed in duplicate, n=2 for 6 and 12 hours, and for three independent experiments, each performed in duplicate, n=3 for 24, 48 and 72 hours, * Control and treated values were compared using one-way analysis of variance (ANOVA) and a p value of < 0.05 was considered to be statistically significant.

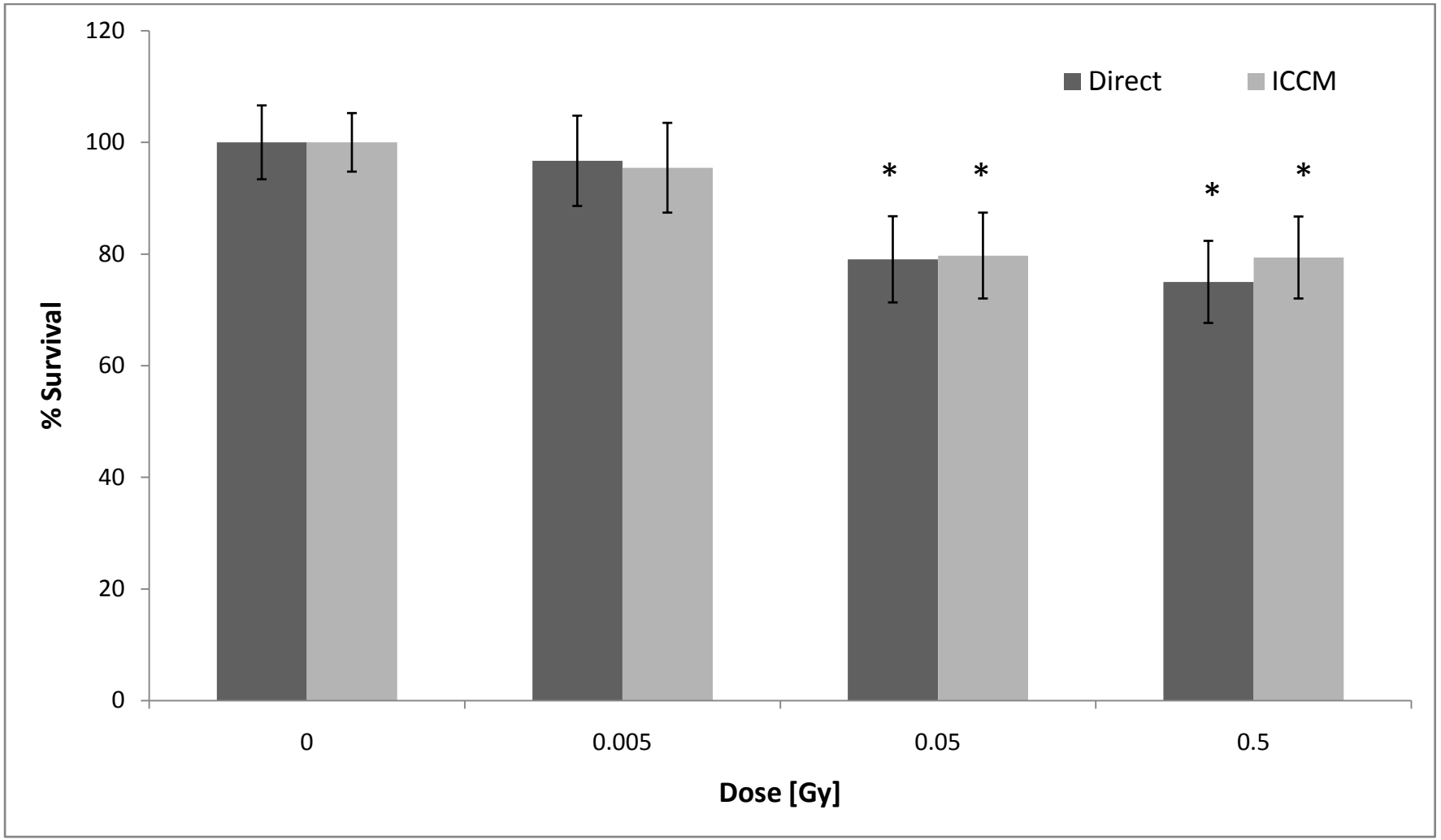

Figure 1

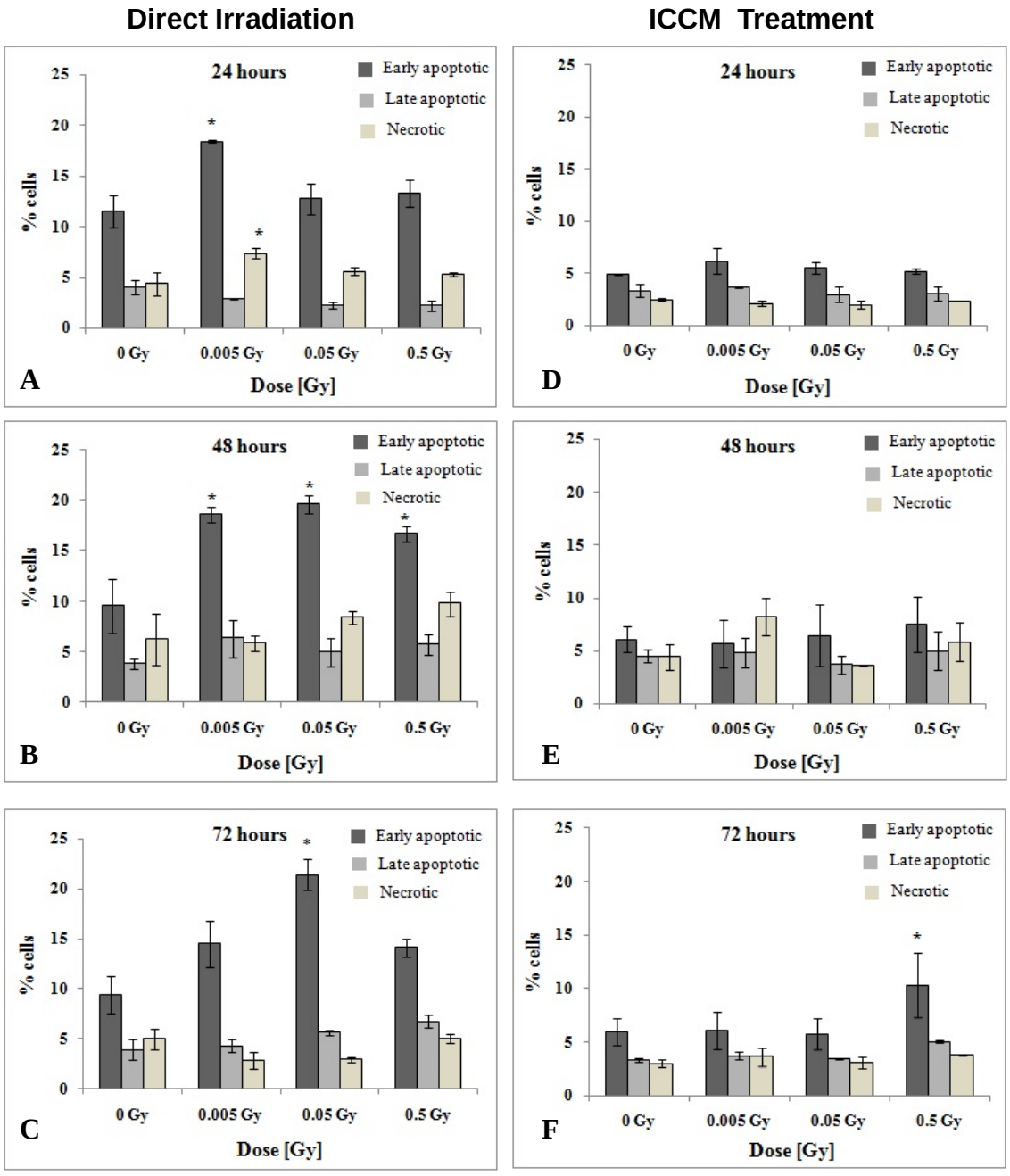
Direct Irradiation
ICCM Treatment
24 hours
48 hours
72 hours
Early apoptotic
Late apoptotic
Necrotic
% cells
0 Gy
0.005 Gy
0.05 Gy
0.5 Gy
Dose [Gy]
A
B
C
D
E
F

Figure 2

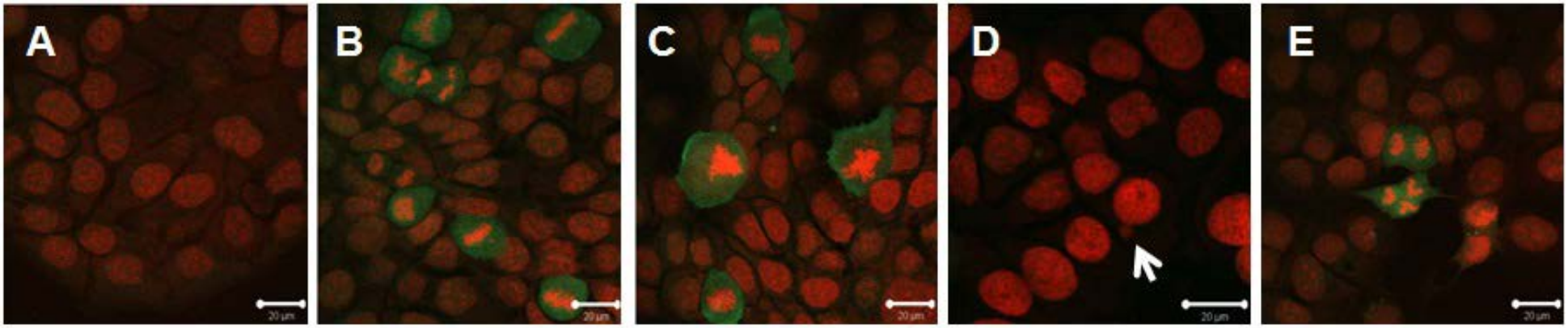

Figure 3

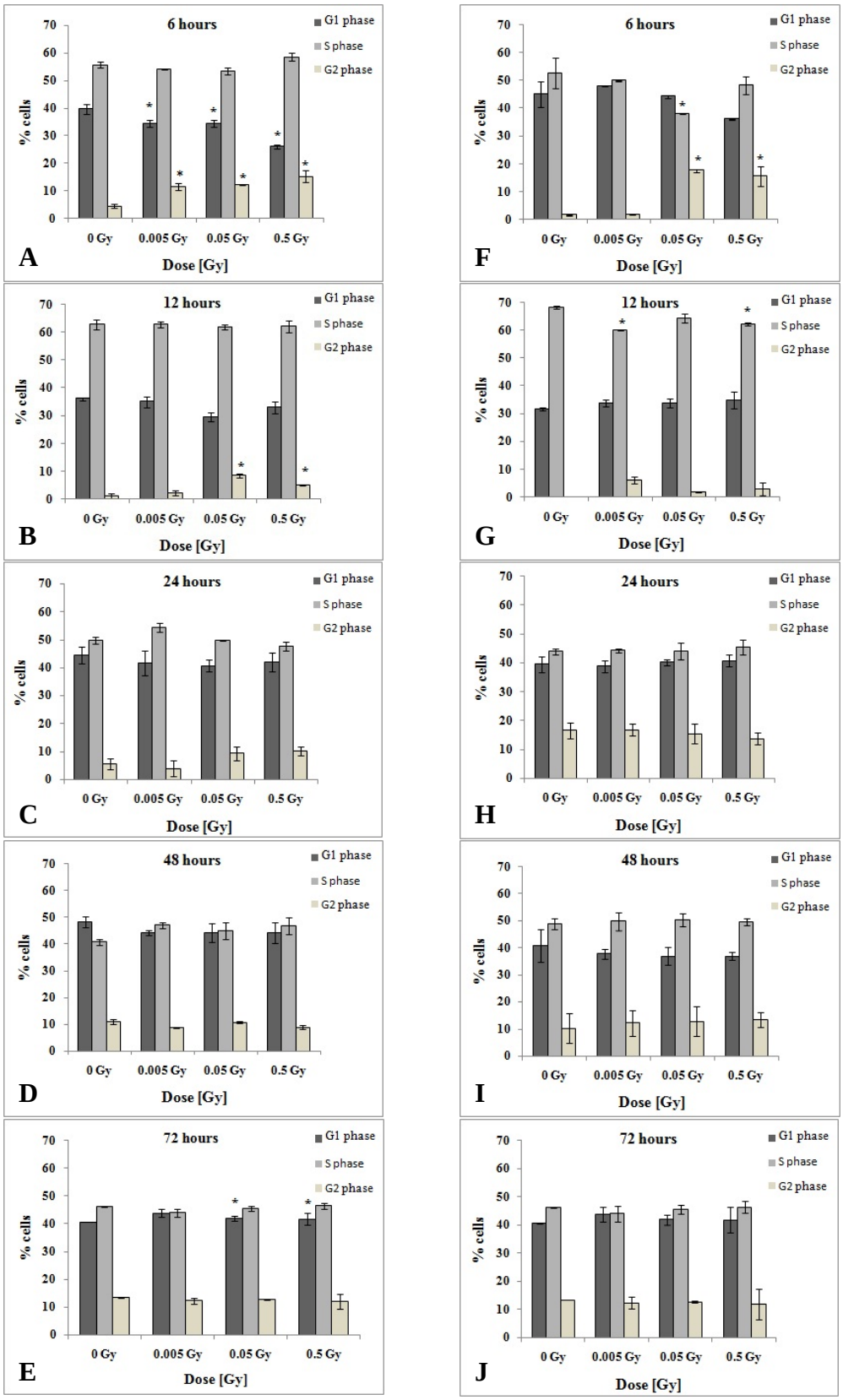
Direct Irradiation
ICCM Treatment
6 hours
12 hours
24 hours
48 hours
72 hours
G1 phase
S phase
G2 phase
% cells
0 Gy
0.005 Gy
0.05 Gy
0.5 Gy
Dose [Gy]
A
B
C
D
E
F
G
H
I
J

Figure 4

Table I Percentage HaCaT cells positive for mitotic cell death (showing increased green fluorescence (MPM-2 expression) and changes in nuclear morphology and chromatin organisation such as multipolar chromosome segregation and multinucleation) following direct irradiation and ICCM treatment. Errors indicate the standard error of the mean (SEM) for three independent experiments, each performed in triplicate, n=3, *p<0.05

Dose point	Direct irradiation	ICCM treatment
0 Gy	3.6 ± 0.2	3.9 ± 0.3
0.005 Gy	3.9 ± 0.4	5.1 ± 0.9
0.05 Gy	14.6 ± 0.7 *	12.3 ± 2.2 *
0.5 Gy	20.5 ± 1.8 *	13.8 ± 2.2 *

Table II Percentage HaCaT cells showing apoptosis, necrosis and mitotic cell death above control levels[1] following direct irradiation (data from figures 2 and table I). Total cell death is given as the sum of apoptosis, necrosis and mitotic cell death. Errors have been combined from each individual data set according to the formula, $\Delta x = \sqrt{(\Delta y)^2 + (\Delta z)^2}$, where Δy and Δz are errors on independent measurements, y and z.

	24 hours			48 hours			72 hours		
	0.005 Gy	**0.05 Gy**	**0.5 Gy**	**0.005 Gy**	**0.05 Gy**	**0.5 Gy**	**0.005 Gy**	**0.05 Gy**	**0.5 Gy**
Early apoptosis	6.9 ±1.6	1.2±2.2	1.8±2.1	9.1±2.8	10.1±2.8	7.1±2.8	5.1±2.9	12.1±2.4	4.7±2.1
Late apoptosis	-1.2±0.7	-1.8±0.7	-1.9±0.9	2.6±1.9	1.2±1.5	3.5±1.1	0.4±1.2	1.7±1.1	2.8±1.2
Necrosis	2.9±1.2	1.2±1.2	0.9±1.1	-0.4±2.6	2.1±2.6	3.5±2.8	-2.1±1.3	-2.1±1.1	0.1±1.2
Mitotic cell death							0.3±0.5	11.0±0.8	16.9±1.9
Total cell death	8.6±2.2	0.7±2.6	0.8±2.5	11.2±4.3	13.4±4.1	14.1±4.1	3.8±3.5	22.7±2.9	24.5±2.7

[1] In some instances, control levels of apoptosis or necrosis were higher than levels in the treated cells, resulting in negative values

Table III Percentage HaCaT cells showing apoptosis, necrosis and mitotic cell death above control levels[1] following ICCM treatment (data from figures 2 and table I). Total cell death is given as the sum of apoptosis, necrosis and mitotic cell death. Errors have been combined from each individual data set according to the formula, $\Delta x = \sqrt{(\Delta y)^2 + (\Delta z)^2}$, where Δy and Δz are errors on independent measurements, y and z.

	24 hours			48 hours			72 hours		
	0.005 Gy	**0.05 Gy**	**0.5 Gy**	**0.005 Gy**	**0.05 Gy**	**0.5 Gy**	**0.005 Gy**	**0.05 Gy**	**0.5 Gy**
Early apoptosis	1.3 ±1.2	0.6±0.6	0.3±0.3	-0.4±2.5	0.4±3.1	1.4±2.8	0.1±2.1	-0.2±1.9	4.3±3.3
Late apoptosis	0.3±0.6	-0.4±0.9	-0.3±0.9	0.3±1.5	-0.8±1.1	0.5±1.9	0.4±0.4	0.1±0.2	1.7±0.2
Necrosis	-0.4±0.3	-0.5±0.4	-0.2±0.2	3.8±2.1	-0.8±1.2	1.4±2.2	0.6±0.9	0.1±0.6	0.8±0.3
Mitotic cell death							1.2±0.9	8.4±2.2	9.9±2.2
Total cell death	1.2±1.4	-0.3±1.2	-0.2±0.9	3.7±3.6	-1.2±3.5	3.3±4.1	2.4±2.3	8.3±2.1	16.8±3.3

[1] In some instances, control levels of apoptosis or necrosis were higher than levels in the treated cells, resulting in negative values.