Radiation induced bystander effects are effects observed in unirradiated cells which receive signals from irradiated cells. Bystander effects include DNA damage, chromosomal aberrations, changes in gene expression, oncogenic transformation and cell death. Bystander signals can be transferred to surrounding cells either by gap junctional intercellular communication or by the production of soluble extracellular factors released from irradiated cells. Recently, exosomes have been shown to play a role in transferring signals from irradiated to non-irradiated cells. This review summarises a recent body of work on radiation induced bystander signalling, focusing on calcium, reactive oxygen and nitrogen species and on membrane signalling.

Key words: Radiation induced bystander effects, signalling, calcium, reactive oxygen species, reactive nitrogen species, membrane signalling

1. Introduction: What are radiation induced bystander effects?

The radiation induced bystander effect (RIBE) describes an effect observed in a non-targeted cell population which is induced by exposure of targeted cells to radiation. RIBE challenge the classical paradigm of radiation biology where direct deposition of energy in the cell’s nucleus was thought to be required to elicit an effect. In contrast to this classical paradigm, it has been shown that unirradiated cells can respond to signals produced by irradiated cells and exhibit responses similar to those of irradiated cells. Early reports of abscopal effects described radiation-induced effects in tissues distant from the site of radiation exposure. In 1915, Murphy and Morton1) reported reduced tumour growth in mice following irradiation of normal tissues and in 1954, Parsons et al.5) showed damage in the sternal bone marrow following irradiation of the spleen in patients with chronic granulocytic leukaemia. Similar findings have been reported over the past 50 years (reviewed comprehensively in)6).

A related phenomenon is the production of clastogenic factors in plasma following irradiation which can induce chromosome damage in unirradiated cells. In 1960, Souto4) described the induction of tumours in female rats after injection with plasma from lethally irradiated rats and sheep. Chromosomal damage was observed in cultured lymphocytes following exposure to plasma from irradiated patients4-6). Similarly, chromosomal breaks were reported in unirradiated lymphocytes from healthy individuals exposed to plasma of individuals following whole-body irradiation7). Increased chromosomal aberrations were also shown in lymphocytes exposed to plasma from irradiated individuals8). Pant and Kamada5) reported increased chromosomal breaks in leucocytes exposed to plasma from atomic bomb survivors who had been exposed over 30 years earlier while Faguet et al.9) described persistent chromosomal breaks in rats for...
at least 10 weeks following whole-body irradiation. A number of papers, from Emerit and co-workers\(^ {11-16}\) have described radiation-induced clastogenic factors in plasma from individuals who were exposed to radiation medically or accidentally. The clastogenic factor(s) were found to be of low molecular weight (1000–10000 Da) and lipid peroxidation and oxidative stress were found to be involved in production of the factor(s)\(^ {13}\).

In 1992, a paper by Nagasawa and Little\(^ {17}\) heralded the start of the ‘modern’ RIBE studies. They showed that exposure of cells to low doses of \(\alpha\) particles led to the formation of sister chromatid exchanges in 30–50% of the cell population despite the fact that only 1% of the cell nuclei would have been traversed by an \(\alpha\) particle. This was later confirmed by Azzam \textit{et al.}\(^ {18}\) who showed changes in gene expression in human fibroblast cell lines irradiated with very low fluences of \(\alpha\) particles. Mothersill \textit{et al.}\(^ {19, 20}\) showed that bystander effects could also be induced by irradiated cell conditioned medium (ICCM) where the culture medium from irradiated cells was transferred to cells that had not been irradiated. Transfer of ICCM to recipient cells was found to reduce clonogenic survival and increase the incidence of apoptosis in cells that had never been irradiated. To date, more than 900 papers have been published describing bystander effects. A very comprehensive review of radiation induced bystander effects can be found in Mothersill \textit{et al.}\(^ {3}\).

2. How are radiation induced bystander effects transferred?

Radiation induced bystander effects are mediated by signals communicated from irradiated cells to unirradiated cells (Fig. 1). The signals can be communicated by gap junctional intercellular communication when the irradiated cells and the unirradiated cells are in direct contact\(^ {18, 21, 22}\) (Fig. 1A). Gap junctions can allow transmission of signalling molecules up to 1,000–1,500 Da such as \(\text{Ca}^{2+}\) and other secondary messengers. Bystander signals can also be mediated by the release of soluble factors from irradiated cells which can be transferred through ICCM to unirradiated cells\(^ {19, 20, 23}\) (Fig. 1B). More recently, exosomes have been shown to be involved in transmission of radiation induced bystander signals\(^ {24-27}\).
Radiation induced bystander effects have been observed in most cell types for a range of endpoints including damaging endpoints such as DNA damage, mutations, transformation and cell death, and protective endpoints such as terminal differentiation, apoptosis and adaptive responses\(^{(28)}\).

### 3. Radiation induced bystander signalling

This review provides a summary of a body of work from 2000-2018 on radiation induced bystander signalling focussing on calcium, reactive oxygen species (ROS) and membrane signalling.

A media transfer protocol was employed for these studies where conditioned media from irradiated cells was transferred to unirradiated recipient cells and bystander responses were measured in the recipient cells (Fig. 2).

Lyng \textit{et al.}\(^{(23)}\) investigated the ability of ICCM to induce early events in the apoptotic cascade in HPV-G human keratinocytes. Intracellular calcium levels were measured using calcium sensitive dyes, Fluo-3 and Fura Red. Fluo-3 exhibits an increase in green fluorescence and Fura Red exhibits a decrease in red fluorescence upon binding to calcium so the ratio Fluo-3/Fura Red gives a good indication of calcium levels (Fig. 3A). Rapid calcium fluxes were observed within 30 seconds after addition of ICCM (Fig. 3B). No significant difference was observed for 0.5 Gy and 5 Gy ICCM and no change in calcium levels was observed following addition of 0 Gy ICCM. The observed calcium flux was transient, lasting only 30 – 40 seconds. Induction of ROS was measured using 2,7 dichlorofluoresce in diacetate which emits green fluorescence when oxidised by ROS. An increase in fluorescence was observed at 1, 6, 12 and 24 hours after addition of 0.5 Gy and 5 Gy ICCM indicating induction of ROS (Fig. 4). No change in fluorescence was observed after addition of 0 Gy ICCM. Mitochondrial membrane potential was measured using rhodamine 123, a green fluorescent dye that accumulates in mitochondria with high membrane potential. A decrease in fluorescence was observed at 6, 12 and 24 hours after addition of 0.5 Gy and 5 Gy ICCM indicating loss of mitochondrial membrane potential. No change in fluorescence was observed after addition of 0 Gy ICCM. Increased levels of apoptosis were observed 48 hours after addition of 0.5 Gy and 5 Gy ICCM, ~15% apoptotic cells compared to ~1% apoptotic cells after addition of 0 Gy ICCM. In addition, a ~40% reduction in clonogenic survival was observed 9 days after addition of 0.5 Gy and 5 Gy ICCM. This study showed that initiating events in the apoptotic cascade could be induced in unirradiated cells by a signal in irradiated cells which finally resulted in apoptotic cell death in the unirradiated recipient cells.

A follow on study\(^{(29)}\) investigated if ICCM from irradiated cells several passages distant from the original irradiation could initiate apoptosis in unirradiated cells. HPV-G cells were irradiated to 0.5 Gy and 5 Gy and ICCM was harvested at each passage up to the 7th passage (approx. 35 population doublings) post irradiation and transferred to unirradiated cells. Calcium fluxes, loss of mitochondrial membrane potential and ROS induction were observed in the unirradiated cells. No significant difference was observed between 0.5 Gy and 5 Gy ICCM or between ICCM from initially irradiated cells and from the progeny of these irradiated cells up to 35 population doublings. This study showed that the bystander signal could be produced by the progeny of irradiated cells for several generations.

Maguire \textit{et al.}\(^{(30)}\) further investigated mitochondrial changes in bystander cells and the role of ROS
and caspases. Mitochondrial mass was measured using MitoTracker Green which accumulates in the mitochondrial matrix where it covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues. Increased fluorescence indicating increased mitochondrial mass was observed in cells 18 hours after exposure to 0.005 Gy and 0.5 Gy ICCM but no increase in mitochondrial mass was observed after exposure to 5 Gy ICCM. In addition to the change in mitochondrial mass following exposure to 0.005 Gy and 0.5 Gy, there was a change in distribution of the mitochondria from a diffuse cytoplasmic distribution to a more densely concentrated perinuclear distribution. No significant increase in mitochondrial mass was observed in cells incubated with ICCM containing N-acetylcysteine (NAC) indicating a role for ROS. A significant increase in B-cell lymphoma 2 (bcl 2) expression was observed in cells treated with 5 Gy ICCM but not 0.005 Gy or 0.5 Gy ICCM indicating up-regulation of anti-apoptotic pathways at the higher dose. The caspase 9 inhibitor, Z-LEHD-FMK, prevented the reduction in clonogenic survival by 0.005 Gy ICCM but not by 5 Gy ICCM whereas NAC prevented the reduction in clonogenic survival at both ICCM doses. This study showed that apoptosis caused by exposure to <0.5 Gy ICCM (no significant bcl 2 expression) occurs through a caspase-dependent pathway, whereas exposure to 5 Gy ICCM (significant bcl 2 expression) occurs through a caspase-independent pathway and that the dose dependence of the bystander responses suggests that the mechanisms may be aimed at control of response to radiation at the population level through signalling pathways. Similar findings were reported showing the induction of early apoptotic events, such as mitochondrial membrane potential depolarisation, induction of ROS, expression of bcl 2 and release of cytochrome c in HPV-G human keratinocytes following microbeam irradiation with protons. No significant differences were observed between the effects in the directly irradiated cells and the bystander cells or between the effects following irradiation with 1 or 10 protons to the directly irradiated cells. This study showed that the mechanisms seemed to be similar for medium transfer and for microbeam irradiation and therefore likely to be universal.

Mothersill et al. extended these studies to investigate if bystander signals produced in vivo could induce apoptosis in reporter cells. CBA/Ca or C57BL/6 mice, known to differ significantly in radiation response, were exposed to 0.5 Gy total-body radiation, bladders were removed and explant tissue cultures were established. Irradiated tissue culture medium (ITCM) from irradiated C57BL/6 mice, but not CBA/Ca mice, were found to produce bystander signals that induced apoptosis and reduced clonogenic survival in reporter HPV-G human keratinocytes. Calcium fluxes and reduced mitochondrial membrane potential was observed following addition of ITCM from C57BL/6 mice, but not CBA/Ca mice. Pro-apoptotic (C57BL/6) and anti-apoptotic (CBA/Ca) proteins were found to play a role in determining the overall response. This study showed the influence of genetic factors on the in vivo induction of bystander signals.

Lyng et al. investigated signalling pathways underlying radiation-induced bystander cell death and the role of calcium and ROS. Mitogen-activated protein kinase (MAPK) signalling pathways were shown to be involved
in radiation induced bystander effects as extracellular signal–regulated kinase (ERK) and Jun N-terminal Kinase (JNK) were significantly activated at 30 mins and 24 hours in cells exposed to 0.5 Gy ICCM. Increased apoptosis was observed in HPV-G cells exposed to 0.5 Gy ICCM in the presence of ERK inhibitors whereas decreased apoptosis was observed in HPV-G cells exposed to 0.5 Gy ICCM in the presence of a JNK inhibitor. Chelation of extracellular calcium by ethylene glycol tetraacetic acid (EGTA) or blockade of voltage-dependent calcium channels was found to abrogate the ICCM induced calcium fluxes whereas depletion of intracellular calcium stores by thapsigargin only attenuated but did not completely abrogate the ICCM induced calcium fluxes. No change in mitochondrial membrane potential and no significant increase in apoptosis was observed in HPV-G cells pre-incubated with EGTA or verapamil and exposed to ICCM in the presence of EGTA or verapamil. Similarly, no change in mitochondrial membrane potential and no reduction in survival was observed in HPV-G cells pre-incubated with superoxide dismutase (SOD) or catalase and exposed to ICCM in the presence of SOD or catalase. This study showed that MAPK signalling pathways are triggered in bystander cells and that calcium and ROS are both important modulators of bystander responses.

Adaptive bystander responses were investigated by Maguire et al. HPV-G cells were exposed to a priming dose of 0.005 Gy or 0.5 Gy ICCM for 24 hours followed by a challenge dose of 0.5 Gy or 5 Gy ICCM. An increase in survival compared to cells exposed to 0.5 or 5 Gy ICCM only was observed, indicating an adaptive response and ROS were found to be involved in the bystander-induced cell death. Calcium fluxes were observed but were found to vary in magnitude and a significant number of the primed cells did not respond to the challenge ICCM dose. No significant loss of mitochondrial membrane potential compared to cells exposed to 0.5 or 5 Gy ICCM only was observed, again indicating an adaptive response. Interestingly, increased mitochondrial mass which has been linked to genomic instability was observed following the challenge ICCM dose. This study showed that bystander signals produced by irradiated cells induce an adaptive response in unirradiated cells to a further exposure to bystander signals. Vines et al. further extended the studies on murine bladder tissue and investigated markers of apoptosis and terminal differentiation following direct irradiation or exposure to ITCM from the directly irradiated cultures. Increased Bcl-2 expression was observed after direct irradiation to 5 Gy and after exposure to 0.5 Gy and 5 Gy ITCM. No change in avian myelocytomatosis virus oncogene cellular homolog (c-myc) expression was observed following direct irradiation but a dose dependent increase was observed in the ITCM exposed cultures. Similarly, no change in uroplakin expression was observed following direct irradiation but a dose dependent increase was observed in the ITCM exposed cultures. The responses appeared to involve maintenance of the tissue integrity and could be seen as protective or damaging depending on the dose and on the balance of bcl-2 and c-myc proteins.

The sequence of the early bystander signalling events was elucidated by Lyng et al. Plasma membrane permeability was measured using the fluorescent dye FM1-43 and monitored over a 5 minute period for changes in plasma membrane permeability before and after addition of medium from irradiated cells (0.5 Gy ICCM). Images are representative of at least six independent experiments.

Fig. 5. Cells were stained with the fluorescent dye (N-(3-triethylammonium)propyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM1-43) and monitored over a 5 minute period for changes in plasma membrane permeability before and after addition of medium from irradiated cells (0.5 Gy ICCM). Images are representative of at least six independent experiments.

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**Fig. 5.** Cells were stained with the fluorescent dye (N-(3-triethylammonium)propyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM1-43) and monitored over a 5 minute period for changes in plasma membrane permeability before and after addition of medium from irradiated cells (0.5 Gy ICCM). Images are representative of at least six independent experiments.
response to ICCM followed by calcium influx, a rapid increase in ROS levels and a subsequent increase in nitric oxide (NO) levels (Fig. 6). Calcium and ROS were found to be involved in the production of the bystander signal while calcium, ROS and NO were all found to be important signalling molecules involved in bystander responses.

The role of exosomes in bystander induced signalling was investigated by Jella et al.25) Two different types of vesicles were identified in ICCM, exosomes (30-100 nm) and microvesicles (>100 nm) and the concentration of these extracellular vesicles was found to increase in a dose dependent manner. Significant bystander cell death was observed 72 hours following addition of 0.005, 0.05 and 0.5 Gy ICCM. Abrogation of this bystander cell death was observed following treatment with 0.05 and 0.5 Gy ICCM in which the extracellular vesicles had been removed. No calcium signalling and no production of ROS was observed in cells exposed 0.05 and 0.5 Gy ICCM in which the extracellular vesicles had been removed whereas significant calcium signalling and ROS production was observed in cells exposed to fresh media containing the extracellular vesicles from 0.005, 0.05 and 0.5 Gy ICCM.

Monitoring of ROS and NO bystander signalling in real time up to 24 hours was carried out by Jella et al.25) ROS production was observed within 1 min of addition of ICCM and this was sustained for 24 hours whereas NO production was observed within 1 hour of ICCM addition and this was sustained for 4 hours. Inhibition of the ERK pathway was found to completely block the ROS production whereas JNK inhibition increased ROS production. Significant increases in glutathione levels were observed up to 1 hour of ICCM addition. Increased caspase activation up to 8 hours and a reduction in cell viability up to 48 hours was also observed. This study showed a link between ROS production and cell survival pathways with persistent production of ROS and NO in bystander cells following exposure to ICCM.

4. Summary

Overall, this body of work has shown the early signalling events in bystander cells. Membrane signalling was shown to be the first response to the bystander factor(s) present in ICCM, followed by calcium, ROS, NO signalling. Figure 7 illustrates these signalling pathways. An overload of calcium in the mitochondria of the irradiated cells leading to production of ROS mostly likely results in the production of the bystander factor(s). The bystander factor(s) are then sensed by the unirradiated recipient cells through the plasma membrane. MAPK and other pathways can be activated by cytokine and growth factor receptors on the plasma membrane. Plasma membrane bound NADPH oxidase can be activated which can lead to a long-lasting production of intracellular ROS in the bystander cells and this has been shown previously22). Bystander effects could have implications both for radiotherapy, for example abscopal or out of field effects, and for radiation protection, where they could have an impact on risk assessment for low dose exposures.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References


