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The use of radiation microbeams to investigate the bystander effect in cells and tissues

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Abstract

Microbeams are ideally suited to the study of so-called 'non-targeted' phenomena that are now known to occur when living cells and tissues are irradiated. Non-targeted effects are those where cells are seen to respond to ionising radiation through pathways other than direct damage to the DNA. One such phenomenon is the 'bystander effect'; the observation that unirradiated cells can be damaged through signalling pathways initiated by a nearby irradiated cell. The effect leads to a highly non-linear dose–response at low doses and is forcing a rethink of established models used to estimate low-dose radiation risk, which are largely based on linear extrapolations from epidemiological data at much higher doses. The bystander effect may also provide an opportunity for improvements in the treatment of cancer by radiotherapy, as it may be possible to chemically influence the bystander response in such a way as to enhance cell killing in tumour cells or to protect healthy tissue.
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1. Introduction

Very soon after the discovery of ionising radiation, it became evident that exposure to its rays could cause cancer. Over a century later, it is perhaps surprising that the risks associated with occupational and environmental levels of radiation exposure remain poorly understood. Also within months of its discovery, radiation was being used to treat cancer and improvements in the use of ionising radiation in cancer medicine have been sought ever since.

Radiobiology seeks to improve our understanding of the mechanisms by which exposure to radiation leads to cell killing and mutation. Such an understanding may ultimately lead to improvements in radiotherapy and better estimates of low-dose radiation risk. A major breakthrough to our understanding was the discovery of DNA and subsequently, its importance as a critical target for radiation damage. Over time, the study of DNA damage led to the 'dogma' that direct damage to the DNA helix (through bond-breaks) is a necessary requirement for the induction critical biological effects. However, recent experimental data are challenging this direct relationship between cell killing or mutation and DNA damage. 'Non-targeted' effects are those where cells appear to respond to ionising radiation though pathways other than direct damage to the DNA. One such effect currently of great interest is the so-called 'bystander-effect', where unirradiated cells exhibit damage in response to signals transmitted by irradiated neighbours [1]. The bystander effect predominates at low doses where it can lead to a highly non-linear dose–response. This has important implications for estimates of the risk associated with low-dose exposure to radiation, which are based largely on a linear extrapolation of known risks at higher doses (the Linear, No-Threshold Model). With regard to radiotherapy, it is possible that the treatment of cancer by radiation could be improved though selective modification of the radiation response of either the tumour or the healthy

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tissue by chemical action directed at the signalling molecules involved in the bystander effect, leading to an overall therapeutic benefit.

Using a radiation microbeam, it is possible to selectively irradiate individual cells within a cell population. Therefore the microbeam technique is ideally suited to studying the bystander-effect. Despite this, the number of facilities in routine use remains low, due partly to the difficulty in solving a range of technical issues that arise from applying this technique to living cells [2]. The Gray Cancer Institute (GCI) has developed two types of microbeam; one using collimated light ions and another that uses focussed low-energy X-rays. Both facilities have been fully operational for a number of years and used to gain insight into non-targeted effects.

2. The bystander effect

The bystander effect was first reported by Nagasawa and Little [3], who observed chromosome damage in 30% of cells following exposure to a broad field of α-particles such that only 1% of cell nuclei are actually hit. Since then, the bystander effect has become one of the most widely studied of the non-targeted effects, which also include genomic instability, adaptive responses, low-dose hypersensitivity, the inverse dose-rate effect and the regulation of genes at low-doses [4,5].

Since the pioneering work of Nagasawa and Little, subsequent studies have reported evidence for the bystander effect using a range of cell types and end-points, including chromosome damage, cell death, mutation and transformation (an *in vitro* measurement of carcinogenesis). Common to all studies is the observation that the effect dominates the dose–response at low doses (<200 mGy) but saturates as the dose is increased (see Fig. 1). A number of methods have been used to study the bystander effect, including the use of a low-fluence of α-particles (such that only a fraction of cells are hit) and media-transfer experiments, where a bystander effect can be induced by transferring medium from a dish of irradiated cells to a dish of unirradiated cells. Masks and grids that partially shield the cell dish can also be used, as can dishes that allow physically separated co-cultures within the same dish (such that only one culture is irradiated). However, it is the use of microbeam methods that has provided most versatility for the design and execution of investigations into the bystander effect. A microbeam makes it possible to irradiate just a single cell within a population of cells. In the case of particles, microbeams can been used to irradiate a cell with exactly one ion. If helium ions are used, it is possible to mimic the effect of environmental exposure to radon and its daughters. Radon is one of the main contributors to our environmental exposure to ionising radiation. The exposure from radon is mainly to lung epithelial cells and exposure levels are such that it is very unlikely that hit cells will receive more than one α-particle traversal.

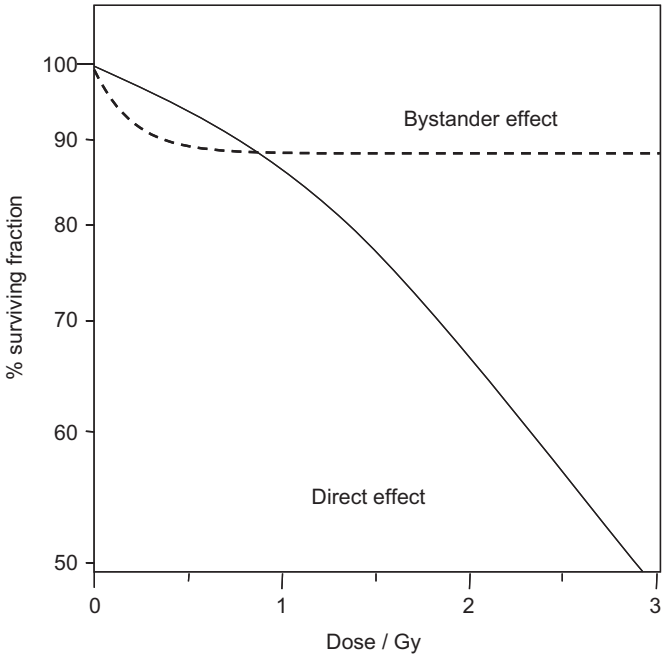

Fig. 1. Typical cell survival curves. The direct effect is a consequence of the damage that arises when all cells are targeted. The bystander effect is observed when only a few calls are targeted. Typically, the bystander effect dominates at low doses and saturates at high does.

With sufficient targeting accuracy, it is also possible to select which part of a cell to irradiate, i.e. either the cell nucleus or the cell cytoplasm. Conventional wisdom would indicate that irradiating just the cell cytoplasm (which does not contain genomic DNA) should be relatively ineffective. Microbeams are being used to see if this assumption also applies to bystander effect.

3. Microbeam methods for irradiating cells

Radiobiological microbeams have been developed using charged-particles [6], low-energy X-rays [7] and low-energy electrons [8]. Most particle microbeams use light ions; either protons or helium ions because of their radiobiological relevance. However, studies using heavier ions are also of interest for their application to risks associated with long-term space travel and there is increasing interest in connection with particle radiotherapy using carbon ions rather than protons. Light-ion microbeams have the disadvantage that the ions will be significantly scattered by the vacuum exit window and transmission detector such that probe sizes less than 1–2 μm are difficult to achieve, irrespective of the focussing or collimation method used. By contrast, low-energy X-rays interact almost entirely through the photoelectric effect and are therefore not scattered. The 'fineness' on an X-ray probe is ultimately limited by the range of the secondary electrons it sets in motion, which are typically much less than a micron for X-rays of a few keV, or less. A few groups have developed electron microbeams. However, they are the least favourable with regard to probe size, because the electron energies

required to penetrate the cell (>15 keV) produce long-range secondary electrons within the cell itself.

At GCI, a fully operational ion microbeam has been in use since the mid-1990s [9,10] and makes use of a purpose-built beamline from our 4 MV VdG accelerator. Fig. 2 shows the arrangement for micro-targeting cells. The accelerator can be used to generate energetic protons, or $^3He^{2+}$ ions, which are steered vertically upward to the cell irradiation apparatus, mounted on an optical table. At the end of the beamline is a 1 μm diameter bore silica capillary collimator. A dish of cells is located on a micro-positioning stage above the collimator. The cells are attached to a 3 μm thick Mylar membrane that forms the base of the dish, also containing cell culture medium. To irradiate cells, they are located, in turn, above the collimator and exposed to an exact, predefined number of particles. The effect of scattering is minimised by arranging for the collimator to be as close as possible to the cell. In fact, the top of the collimator assembly is motorised to move vertically by a small amount (~500 μm) and is driven upward such that it just touches the base of the cell dish prior to each exposure. A 12 μm thick plastic scintillator is 'sandwiched' between the collimator exit and the cell dish and a photo-multiplier (PM) tube mounted just above the dish detects the pulse of light due to the passage of a particle through this scintillator. A fast electrostatic shutter then terminates the irradiation of each cell once the preset number of particles has been delivered. Using this arrangement, a targeting accuracy of ±2 μm, with >99% detection efficiency is achieved [11].

To align the cells to the collimator, an epi-fluorescent microscope fitted with a CCD camera views the cell dish from above *in situ*. Automated procedures are used to identify targets and assign co-ordinates. All targets are identified and their locations are stored prior to the irradiation step, as the microscope objective is replaced by the PM tube during this phase. Typically, it takes a few minutes to identify up to several thousand targets (typically cell nuclei) on one dish, after which targets are automatically aligned and irradiated at a rate of 2–3 per second (i.e. up to 10,000 cells per hour).

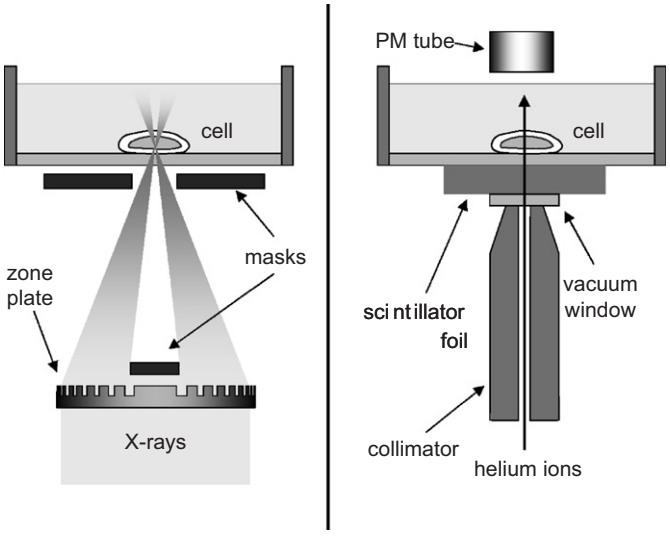

Fig. 2. The two methods used at GCI to micro-irradiate cells. Left: Low-energy X-rays are focused using zone-plate diffractive optics. Right: Energetic ions are collimated using a glass capillary and detected using a scintillator foil and photomultiplier (PM) tube.

The GCI X-ray microprobe [6] uses a tabletop X-ray microfocus source to generate low-energy X-rays that are subsequently focussed to a micron-sized spot using a small diffraction lens (zone-plate). Fig. 2 depicts the schematic arrangement. Our latest X-ray microprobe is in the final stages of development and will be capable of delivering focussed C_K (0.28 keV), Al_K (1.48 keV) and notably, Ti_K (4.5 keV) X-rays [12]. Unlike C_K and Al_K X-rays, Ti_K X-rays are capable of penetrating well beyond the first cell layer and are therefore much better suited to studies involving tissues and multi-cellular layers.

In our new design, electrons up to 15 kV are generated by a custom-built gun and focussed using a permanent neodymium–iron–boron magnet assembly onto a target whose characteristic-K radiation is required. This generates a 'point-source' of X-rays that are then focussed by a zone-plate optical assembly mounted to the end of a hollow vertical tube that can be precisely positioned above the X-ray source. A cell positioning stage and microscope similar to that used with our ion microbeam is mounted above the source to locate and align cells to the focussed X-ray spot.

4. Microbeam studies of the bystander effect

Microbeam investigations of the bystander effect have sought to address the prevalence, magnitude and mechanisms that underpin this phenomenon. At its most extreme, it can be shown that irradiating just a single cell *in vitro* can induce a significant bystander response within a cell population over millimetre distances. For example, in an experiment using the GCI particle microbeam, a single Chinese hamster V79 cell in a non-confluent cell population has been targeted with counted 3.2 MeV protons and the level of bystander-induced cell killing in a 5 × 5 mm area of the dish measured using a colony-forming assay [13]. Irradiating one cell with 5 or more protons reduced the cell survival of the whole population by about 5–7% and was independent of dose up to the maximum dose used (50 protons through one cell). A similar experiment using helium ions shows that a single helium ion through one cell is sufficient to induce a bystander effect of about the same magnitude. By contrast, a single proton through a single cell did not appear to increase the level of cell killing compared to the control. The reason that helium ions are more effective is that they are more densely ionising. For the energies used here, a helium ion deposits about 6–8-fold more energy than a proton as it traverses the cell.

The observation that a single proton does not induce a bystander response shows that there is a dose threshold for this effect. Schettino et al. [14] have used the GCI X-ray

microprobe to carefully explore the bystander effect at very low doses, between 0.05 and 0.2 Gy. If the data from many experiments is averaged, then a gradual bystander induced dose–effect between zero dose and saturation (which occurs at about 0.2 Gy) is observed. However, if the individual data points are not averaged, then it is apparent that there is a tendency for cell populations to exhibit either the full bystander effect (i.e. about 5–7% cell kill) or no effect above background, with an increasing probability of full effect as the dose is increased. This suggests that the bystander effect is a 'binary-response' that exhibits either no response, or is triggered to maximum response once a threshold dose (which may vary slightly from cell to cell) is exceeded.

Another interesting observation is that the bystander signal can extend over considerable distances. To investigate this, a single cell within a non-confluent cell population (with an average distance between cells of 150 μm) was irradiated and the positions of cells that exhibit a bystander response recorded, up to a distance of 3 mm from the targeted cell [15]. No correlation with distance was apparent, however, the distribution of damage did not appear to be random, but instead showed a tendency for damaged cells to be clustered. One possible explanation for this is that cells damaged by the bystander signal may then release a further signal, leading to a sustained chain reaction.

There is naturally considerable interest in elucidating the signalling pathways that lead to bystander effects. Several studies have shown at the bystander response involve cytokines (such as tumour necrosis factor α) and reactive oxygen species (ROS) such as hydrogen peroxide. In tissues and confluent cell systems, it is possible for cell to cell communication to take place via 'gap–junctions' such that the disruption of membrane signalling pathways relevant to gap–junction communication can suppress the bystander effect.

In studies using the GCI particle microbeam, Shao et al. [16] have shown that both nitric oxide (NO) and ROS are involved. AG01522 (AG0) primary human fibroblasts were co-cultured in alongside the T98G glioma cells in separate regions 5 mm apart. Targeting one or more cells in just one of the populations with a single $^{3}He^{2+}$ ion produced a significant increase in the production of DNA damage within the other (un-irradiated) population, demonstrating that bystander responses can be induced across genotypes. However, with NO scavengers present, the bystander effect was inhibited in the case of T98G cells being targeted and partially inhibited when AG0 cells were targeted, showing that NO is involved in the signalling process. Similarly, adding anti-oxidants that inhibit the effects of ROS completely suppressed the bystander effect in both cases.

Shao et al. have also shown that the bystander response can be induced by irradiating just the cell cytoplasm [17]. They measured the induction of DNA damage induced in a population of T98G glioma cells, after targeting the cytoplasm of one cell close to the centre of the population with a single $^{3}He^{2+}$ ion. They find that the overall yield of DNA damage increased from 13.5% in the control experiments, to 18.3% when the cytoplasm of one cell was irradiated, and with no increase in the yield when a greater fractions of cells were targeted through their cytoplasm. These findings show that direct damage to the genomic DNA is not necessary to initiate the bystander effect.

Another non-targeted effect to be widely investigated is the phenomenon of genomic instability, characterised by the occurrence of chromosome aberrations and lethal mutations in the progeny of irradiated cells that appear viable after exposure and remain so for many cell divisions. Using the GCI microbeam, Moore and colleagues irradiated a precise fraction of a human lymphocyte cell population with a single ion, then looked for aberrations that appear in cells after about 12–13 population doublings [18]. One finding is that there is roughly a 2-fold increase in the number of aberrations scored throughout the cell population when just 15% of cells are irradiated and that this did not increase as more cells were targeted (up to 100% cells hit). This shows that bystander-induced instability is involved, supporting a suggestion that the bystander effect is the main pathway to instability [19].

5. The bystander effect and risk models

While the bystander effect (and other non-targeted effects) has been demonstrated in cultured cell systems, it remains unclear how these findings apply to living organisms. Clearly, this needs to be addressed if we are to understand the potential impact of the bystander effect on radiation risk models. Experiments have been performed using ex-vivo tissue models. In one experiment, Belyakov et al. [20] used the GCI microbeam to microirradiate a section of ureter (either human or porcine) with one or more $^{3}He^{2+}$ ions. The sample comprises four to five cell layers containing both fully differentiated cells and undifferentiated cells. After irradiation, the tissue is cultured and an explant outgrowth formed. When the explant is stained to highlight terminally differentiated cells, a significant rise in the fraction of differentiated cells is observed in the irradiated sample (about 15% above that of an unirradiated sample). One speculative interpretation of this finding is that it is a protective mechanism, preventing the proliferation of damage as cells divide.

If it can be demonstrated that the bystander effect dominates the low-dose response in humans, then the current models of radiation risk are seriously undermined. Underpinning the Linear No-Threshold Model is the concept that direct damage to DNA is a necessary requirement for critical biological effects. While it is clear that the bystander effect challenges this dogma, what remains to be established is how the effects seen in cultured cell models relate to the overall response of a living organism to damage by ionising radiation.

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