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New insights into the cellular response to radiation using microbeams

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Abstract

Micro-irradiation techniques continue to be highly relevant to a number of radiobiological studies, due to their ability to deliver precise doses of radiation to selected individual cells (or sub-cellular targets) in vitro. The Gray cancer institute (GCI) ion microbeam uses a 1 μm diameter bore glass capillary to vertically collimate protons, or helium ions accelerated by a 4 MV Van de Graaff. Using $^{3}He^{2+}$ ions, 99% of cells are targeted with an accuracy of ±2 μm, and with a particle counting accuracy >99%. Using automated cell finding and irradiation procedures, up to 10,000 cells per hour can be individually irradiated.

Microbeams are now being used to study a number of novel 'non-targeted' responses that do not follow the standard radiation model based on direct DNA damage and are now known to occur when living cells and tissues are irradiated. One such response is the so-called 'bystander effect' where unirradiated cells are damaged through signalling pathways initiated by a nearby irradiated cell. This effect predominates at low doses and profoundly challenges our understanding of environmental radiation risk. Furthermore, we now have evidence that simple molecules (such as nitric oxide) are involved in the signalling process, such that it may be possible to chemically influence the bystander response. If so, then this could eventually lead to improvements in the treatment of cancer by radiotherapy. Other studies have shown that the bystander effect is induced with equal effectiveness if either the nucleus or the cytoplasm of a cell is targeted.
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1. Introduction

The application of nuclear microprobes in the field of radiation biology continues to be of great

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interest for investigating a number of responses currently of concern to the radiobiological community. In particular, it is the study of so-called 'non-targeted' effects [1] that are benefiting most from the use of micro-irradiation techniques. Non-targeted effects are those where cells are seen to respond *indirectly* to ionizing radiation and are in conflict with the conventional view of cellular radiation damage, which assumes that *direct* damage to the DNA helix is necessary to induce critical effects (either through direct ionization of the DNA, or through the action of reactive radical species from the ionization of water close to the DNA molecule). The conventional representation of radiation damage (i.e. one that does not consider non-targeted effects) is consistent with the 'linear no-threshold' model used to estimate the risk associated with exposure to occupational and environmental levels of radiation. This model is based on a linear extrapolation of known risks at higher doses to the low-dose region where reliable data from epidemiology or experiments are not currently available. However, if non-targeted effects are considered, then their effects will predominate at low doses and our confidence in the linear no-threshold is dramatically undermined. Non-targeted effects are also of potential relevance to the advancement of the treatment of cancer by radiotherapy. This form of treatment relies on maximising the damage to the tumour while minimizing damage to the surrounding healthy tissue. We now have evidence that some non-targeted effects are induced by signalling processes that involve simple molecules, such as nitric oxide. This raises the possibility of increasing the therapeutic benefit by selectively modifying the response of either the tumour, or the healthy tissue to radiation by chemical action directed at the signalling molecules.

2. The role of microbeams for studying non-targeted effect in cells

A number of non-targeted and anomalous low-dose effects have been reported. These include: *Adaptive responses* – where cells subjected to a low priming dose are subsequently challenged with a higher dose and show a lesser response [2]. *Low-dose hypersensitivity* – whereby cells exhibit increased radiation sensitivity a low doses [3]. *Genomic instability* – which is the observation in some cell lines of chromosome changes and mutation in the surviving progeny of irradiated cells. Note that this effect is highly dependent on radiation quality [4]. *Inverse dose–rate effect* – where increased levels of mutations or transformations are seen at very low dose rates (normally, biological effect decreases with decreasing dose–rate) [5]. *Gene expression* – which is the up, or down regulation of genes at doses below levels of significant of DNA damage, suggesting that direct DNA damage is not a prerequisite for these effects [6]. *Bystander effects* – this is the observation of damage in unirradiated cells through signalling processes arising from irradiated cells and can occur both through factors released into the cell culture medium and through gap-junctions of adjacent cells [7].

Of these non-targeted effects, it is the so-called 'bystander effect' that has generated the most interest. This phenomenon was first reported in a report by Nagasawa and Little [8], 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. Subsequently, Deshpande et al. [9] reported a similar finding in primary human fibroblasts, while Hickman et al. [10] showed greater than expected levels of p53 (a tumour suppressor protein) in α-particle irradiated epithelial cells. Since then, microbeams have become the source of choice for studying bystander effects. Our own microbeams have been used extensively for investigating this effect in both cell and tissue models. Through our studies, we have been able to gain some insight into both the magnitude of the effect and the signalling mechanisms that underpin it.

As a consequence of the increasing interest in microbeams, a number of groups are now actively engaged in developing microbeams for radiobiological applications [11,12], or in adapting existing analytical microprobes for radiobiological use. Most recently, a consortium ('CELLION') for the development and biological applications of microbeam was established through the Marie

Curie Research – Training Network under the Sixth Framework Programme of the European Community (2002–2006). This consortium involves the participation of ten European partners, and the development of a number of ion microbeams in the UK, France, Germany, Poland, Sweden and Italy. Currently, there are ten biological ion microbeams in Europe at various stages of advancement (including two that are not part of CELLION). Worldwide, there are a number of proposed, developing or established ion microbeams, located in the USA (two facilities), Japan (two facilities), Canada (one facility) and China (one facility).

3. The Gray cancer institute charged-particle microbeam

The GCI has been involved in the development and radiobiological application of both ion microbeams [13] and focused X-ray microprobes [14] for a number of years. A fully-operational ion microbeam has been in use since the mid-1990s and its development has been reported previously [13,15]. Briefly, the ion microbeam makes use of a purpose-built beamline from our 4 MV VdG accelerator to transport particles, either protons, or $^{3}He^{2+}$ ions, vertically upward through the floor of the laboratory above to the cell irradiation apparatus, mounted on an optical table at bench height. Note that $^{3}He^{2+}$ ions are radiobiologically equivalent to $^{4}He^{2+}$ ions of the same ionisation density, but have greater penetration. A fine radiation beam is formed using a 1 μm diameter bore fused silica capillary collimator, mounted at the end of the beamline. Cells to be irradiated are attached to a thin plastic membrane that forms the base of a cell dish containing cell culture medium. The dish is located on a three-axis micro-positioning stage above the collimator. During irradiation, each cell (or sub-cellular target) is located, in turn, above the collimator and exposed to an exact, predefined number of particles. The particles incident on the cells are counted using a photo-multiplier (PM) tube mounted just above the cell dish. The PM tube detects the pulse of light (due to the passage of a particle) from a thin scintillator 'sandwiched' between the collimator exit and the cell dish. A fast electrostatic shutter terminates the irradiation of each cell once the preset number of particles has been delivered. The targeting accuracy is limited primarily by particle scattering from the vacuum window and scintillator. The effect of scattering is minimised by arranging for the collimator to be as close as possible to the cell. In fact, the collimator just touches the base of the cell dish prior to each exposure.

We have determined the targeting accuracy and particle counting efficiency of our collimated facility using CR-39 track-etch plastic [16]. Our measurements show that for protons, we can hit 90% of targets with an accuracy of ±2 μm, or 96% of cells with an accuracy of ±5 μm. Using $^{3}He^{2+}$ ions (which are less easily scattered), 99% of cells are targeted with an accuracy of ±2 μm. When single particle counting, the detection efficiency is greater than 99%, with no missed particles and less than 1% false positives. While studies of the bystander effect may require just a few, or often only a single cell to be irradiated, there are nevertheless occasions when it is necessary to irradiate many thousands of cells on a dish to establish the underlying dose-effect with sufficient accuracy. It is essential therefore that the process of target identification, alignment and irradiation are both automated and rapid. In this regard, the GCI facility has advanced cell recognition and alignment capabilities. Typically, up to 10,000 cells per hour can be located and irradiated using our facility.

4. Studies of the bystander effect in cellular systems using the GCI microbeam

Our initial studies of the bystander effect involved the targeted exposure of the cell nucleus of just a single cell within a population of ~800 cells (primary human fibroblasts) seeded onto a cell dish [17]. The nucleus was targeted with between 1 and 15 counted $^{2}He^{3+}$ ions and the amount of damage assessed by scoring micronuclei throughout the cell population. Micronuclei are a form of DNA damage expressed in cells in the first cell division post irradiation and are quantified by treating the cells with cytocholasin (which inhibits

cell division, but not nuclear division) and assaying for bi-nucleate cells with micronuclei. On average, an additional 100 cells expressing micronuclei were measured throughout the dish when just a single cell is targeted, representing a 2–3 fold increase over background. Furthermore, the level of damage observed was independent of the number of particles traversing the cell, suggesting that a single helium ion is sufficient to induce a full bystander response. Another observation is that the bystander response is always seen when this experiment is performed, from which it can be concluded that every cell within the population can produce a bystander signal, but not every cell will respond to the signal.

In a subsequent experiment [18], a single Chinese hamster V79 cell within a cell population has been targeted with counted 3.2 MeV protons and the level of bystander-induced cell killing in a 5×5 mm^2 area of the dish measured using a colony-forming assay. Above five protons targeted through a cell, the reduction in cell survival is about 5–7% and independent of dose up to the maximum dose used (50 protons through one cell). However, unlike helium ions, a single proton through a cell did not appear to increase the level of cell killing compared to the control. Note that the dose from the single proton is about 6–8 fold lower than that from a helium ion and indicates that a threshold dose for the bystander effect exists that is greater than the dose deposited by a single energetic proton, but less that that deposited by a single helium ion. In further studies performed this year, Schettino et al. (publication in preparation) have used our X-ray microprobe to carefully explore the doses around the threshold region (between 0.05 Gy and 0.2 Gy) and have observed 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 a greater probability of full effect at higher doses. This suggests that rather than exhibiting a gradual dose-effect between zero dose and saturation, the bystander effect is triggered to maximum effect once a threshold dose is exceeded.

Another issue of interest is the spatial dependence of the bystander response and how the induction of bystander damage depends on the position of the cell relative to the targeted cell. Since our microbeam stores the co-ordinates of all the cells on the dish, it is possible to map the distribution of damaged and undamaged cells (measured using the colony-forming assay) post irradiation. Schettino et al. [19] have analysed the probability of a cell exhibiting a media-borne bystander response as a function of the distance from the irradiated cell in a non-confluent cell population (with an average distance between cells of 150 μm) and find no correlation with distance, up to 3 mm (the maximum distance studied). However, the distribution of damage did not appear to be random, but instead showed a tendency for clustering amongst damaged cells. One explanation for this is that cells damaged by the bystander signal may then release a further signal, leading to a chain reaction.

5. Factors involved in the bystander signal

There is now a great deal of interest in identifying the factors involved in mediating the bystander response. As very few fully operational biological microbeams exist, many of the studies to date have been performed using either α-particle sources at very low fluences, or by transferring cell culture medium from irradiated cell dishes to unirradiated cell dishes. Using such methods, a number of factors have been identified, including cytokines [20], reactive oxygen species [21], membrane signalling [22], and nitric oxide (NO) [23]. Most recently, Shao et al. have used the GCI ion microbeam to investigate the role of NO-mediated signalling in the bystander response of individually targeted T98G glioma cells [24]. In one experiment, an exact fraction of the total number of cells (out of about 1200 cells) was irradiated with a single $^3He^{2+}$ ion and the production of micronuclei scored after 1 h incubation. The results show that the number of micronuclei induced rises sharply as the fraction of cells irradiated increases to 20% of the total, then only slightly between 20% and 100% of the cells irradiated (between 20% and 100% of the cells irradiated, about 28–31% of the cells yielded micronuclei). In other words, irradiating just 1 in 5 cells on the dish produced

almost as much damage as irradiating all the cells on the dish. To investigate the role of NO-mediated signalling in the observed response, the experiment was repeated in the presence of c-PTIO, an NO-specific scavenger. It was found that the addition of the NO scavenger reduced the micronuclei yields to those expected if only direct effects were being produced, indicating that the bystander response had been inhibited when the NO signalling pathway was blocked.

Clearly, the observed non-linear dose-effect challenges established estimates of radiation risk at low doses, suggesting that the cancer risk associated with low radiation doses may be greater than currently indicated. However, the involvement of simple molecules such as NO in mediating the bystander response in tumour cells points the way to potential new approaches to improve the efficacy of cancer treatment by radiotherapy. If the mechanisms that underpin the bystander response can be controlled, then it may be possible to develop methods that lead to enhanced cell killing in tumour cells, or increased protection in surrounding healthy tissue. This would be particularly beneficial in the case of T98G glioma cells, which are known to be radioresistant.

6. The bystander response: cytoplasmic versus nuclear irradiation

Much of data on the bystander response reported so far has been obtained by the random targeting of cells with low particle fluences, or through media-transfer experiments, both of which do not target a specific sub-cellular compartment. In contrast, a microbeam with sufficient accuracy can be used to selectively target either the cell nucleus or the cell cytoplasm. Microbeam studies have been performed which demonstrate that explicit irradiation of the cytoplasm can cause genetic mutations and cell killing [25]. However, until recently, it was not known if the bystander response could be induced though cytoplasmic irradiation. A new study by Shao and colleagues using the GCI microbeam has addressed this question [26]. In this study, the induction of micronuclei induced in a population of about 1000 T98G glioma cells was assessed, after targeting the cytoplasm of one cell near to the centre of the 5 mm × 5 mm area containing the cell population. It was found that the overall yield of micronuclei increased from 13.5% in the non-irradiated control experiments, to 18.3% when the cytoplasm of one cell was irradiated with a single targeted $^{3}He^{2+}$ ion. Furthermore, when greater fractions of cells were targeted through their cytoplasm (either one cell, ten cells, or the whole population), no increase in the yield of micronuclei was seen in the cell population. This is in contrast to what is observed if the nucleus is targeted, where an increased fraction of micronuclei are produced as a greater fraction of cell nuclei are irradiated. This may be because in this instance, an increased fraction of micronuclei are being induced by the direct genotoxic effect of the particle traversal. In another experiment, AG01522 (AG0) primary human fibroblasts were co-cultured in alongside the T98G glioma cells in separate regions 5 mm apart. Targeting the cytoplasm of a single T98G cell with one $^{3}He^{2+}$ ion produced a 78% increase in the production of micronuclei within the non-irradiated AG0 population, demonstrating that bystander responses can be induced across genotypes. As with earlier studies [24] the NO-specific scavenger, c-PTIO could be used to verify that NO is involved in the bystander signal following irradiation of the cytoplasm, both when just T98G cells were used and in the co-culture cell experiments. In all instances, the addition of c-PTIO blocked the bystander response.

7. Summary

Microbeams continue to be a powerful and versatile tool in the armoury of the radiation biologist. In particular, the ability to precisely target individual cells with single particles is of great use in understanding non-targeted effects in cells. Although these effects have been studied for over a decade, there is still a great deal that we do not know about the underlying mechanisms, or their relevance to biological effect at low doses. Recent studies using the GCI ion microbeam have shown that nitric oxide has a role in mediating

the bystander signal, indicating a possible pathway for modifying the radiosensitivity of tissues during radiation therapy. With suitable control of the signalling process, improvements in the efficacy of cancer therapy may be possible, either by reducing the bystander signal in healthy tissue, or by amplifying its response in tumours.

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