Status of Charged Particle Microbeams for Radiation Biology

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Abstract. The Gray Cancer Institute is one of a small number of laboratories worldwide routinely using particle microbeam techniques for radiobiological applications. Cellular micro-irradiation methods have been used to provide experimental opportunities not possible with typical ‘broad-field’ irradiation methods. Using microbeams, it is possible to deliver precise doses of radiation to selected individual cells, or sub-cellular targets in vitro. This technique continues to be applied to the investigation of a number of phenomena currently of great interest to the radiobiological community. In particular, it is the study of so-called ‘non-targeted’ effects (where cells are seen to respond indirectly to ionizing radiation) that are benefiting most from the use of microbeam approaches. One important non-targeted effect is the ‘bystander-effect’ where it is observed that unirradiated cells exhibit damage in response to signals transmitted by irradiated neighbours.

1. Introduction

Radiotherapy remains an important method for the treatment of many cancers and improvements in the use of ionising radiation in cancer medicine continue to be sought. However, it is also the case that exposure to ionising radiation can cause cancer and despite a century of research, the risks associated with occupational and environmental levels of radiation are not well understood. To address these issues, radiobiological research seeks to improve our understanding of how ionizing radiation interacts with living systems. An important branch of radiobiology studies the effects of ionising radiations at the cellular and tissue level. Historically, this is achieved by irradiating and observing biological effects in cultured cell populations. However, there is now considerable interest in the use of cellular micro-irradiation techniques that make it possible to deliver precise doses of radiation to selected individual cells, or sub-cellular targets in vitro.

The initial impetus for the application of microbeams in radiation biology was to address the issue of the risk associated with environmental exposures to low doses of α-particles. This arises largely through exposure to radon, which occurs naturally in granite-rich regions. Using a microbeam, it is possible to reliably deliver a single α-particle to a single cell (in environmental exposures, it is very unlikely that a cell will experience more than one α-particle traversal in its lifetime). More recently, microbeam methods have been shown to be particularly useful to the study of ‘non-targeted effects’ [1] such as the ‘bystander-effect’ where it is observed that unirradiated cells exhibit damage in response to signals transmitted by irradiated neighbours [2]. In general, non-targeted effects are those where cells are seen to respond to ionizing radiation through pathways other than direct damage to their DNA. There is now considerable interest in the bystander-effect and other non-targeted effects as they undermine the current estimates of the risk associated with low-dose exposure to radiation, which
are largely based on a linear extrapolation of known risks at higher doses. The bystander effect is also of potential relevance to the advancement of the treatment of cancer by radiotherapy. One possibility being considered is to increase 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 involved in the bystander effect.

The Gray Cancer Institute (GCI) charged particle microbeam makes use of energetic protons or helium ions, accelerated using a 4MV Van de Graaff accelerator, to irradiate individual mammalian cells with counted and collimated particles, such that it is possible to precisely deliver a single particle to a single cell. The collimator is a 1\(\mu\)m bore glass capillary, and the overall targeting accuracy is \(\pm 2\mu\)m. Using a transmission scintillation detector, single particles can be detected with an efficiency >99%. Automated cell finding and micropositioning systems are used to locate and align cells, such that up to 10,000 cells per hour can be individually irradiated. Through our studies, we have been able to gain some insight into both the magnitude of non-targeted effects and the mechanisms that underpin them.

Worldwide, a number of other groups are developing or using microbeams for radiobiological applications [3]. The interest in this technique is such that number of facilities in use, or under construction has increased from three in the mid-1990’s, to over thirty in 2006. Despite this, the number of facilities in routine use remains low, reflecting in part, the difficulty in overcoming a range of technical issues that arise when applying this technique to living cells.

2. Methods
The first generation of microbeams were developed in the 1990’s and included our own facility at GCI, the Radiological Research Accelerator Facility (RARAF), Columbia University, New York and the heavy ion facility at the Japan Atomic Energy Research Institute, Takasaki, Japan. All these facilities made use of collimation to define a microbeam of charged particles. More recently, several facilities have been developed that are an adaptation of an existing focussing micro-irradiation facility, such as an analytical microprobe. Examples of focussed facilities are those being developed at GSI, Darmstadt and CENBG, Bordeaux and a new facility at RARAF. See [3] for a comparison of the different approaches used to develop particle microbeams. In general, the collimated microbeams tend to be oriented vertically, while those based on analytical microprobes are constrained to a horizontal configuration. However, working with a horizontal beam means that the design of the cell dish must allow for the dish to be mounted sideways, which is less straightforward for living cells in media.

The development of the GCI charged-particle microbeam has been reported previously [4]. A fine radiation beam is formed using a 1\(\mu\)m diameter bore fused silica capillary collimator, mounted at the end of a vertical particle beamline. The source of radiation is a 4MV VdG accelerator, producing either protons, or \(^3\)He\(^{2+}\) ions, that are transported upward through the floor of the microbeam laboratory to the cell irradiation apparatus, mounted on an optical table. Note that \(^3\)He\(^{2+}\) ions are radiobiologically equivalent to \(^4\)He\(^{2+}\) ions of the same ionization density, but have greater penetration. A schematic diagram of the charged-particle microbeam is depicted in figure 1.

The cells 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 3-axis micro-positioning stage above the collimator. During irradiation, each cell is located, in turn, above the collimator and exposed to an exact, predefined number of particles. These are counted and controlled using a photo-multiplier tube mounted just above the cell dish. When a particle traverses, the photo-multiplier tube detects the pulse of light from a thin scintillator ‘sandwiched’ between the collimator exit and the underside of the cell dish. Prior to each exposure, the collimator is automatically raised slightly, such that it just presses against the base of the cell dish. This brings the collimator as close as possible to the targeted cell, so as to minimise the effects of scattering. The total path length from the exit of the collimator to the cell is 24\(\mu\)m (3\(\mu\)m thick window, 18\(\mu\)m thick scintillator and 3\(\mu\)m thick cell dish base).
3. Results

3.1 Microbeam performance

Three aspects of the microbeam performance are particularly important when used in a radiobiological application. These are the targeting accuracy, the detection efficiency and the cell throughput.

To assess the performance of the collimator, CR39 track-etch plastic has been used to determine the amount of scattering that occurs as the particles pass through the vacuum window, the scintillator foil and the base of the cell dish. The CR39 is located in a cell dish so that it rests with its lower face in the plane where the cells would normally be. Clusters of 10 particles are then delivered to an array of locations on the plastic (typically a 10x10 grid, 20\(\mu\)m spacing). Measurements show [5] that using \(^3\text{He}^{2+}\) ions, 98% of cells are targeted with an accuracy of \(\pm 2\mu\text{m}\). Given that the nucleus of a typical mammalian cell has diameter of about 10\(\mu\)m, or greater, this is considered sufficient for reliable targeting of cell nucleus. However, overall targeting accuracy must also take into account the ability of the system to identify and align the target to the beam. To do this, CR39 pits are etched to about the same size as a cell nucleus and the cell-finding software is configured to automatically locate and align these pits to the beam. Each pit is then targeted at its centre automatically with a single particle and the CR39 etched a second time. Although further data are needed to reliably quantify the automated targeting accuracy, most targets are hit to within \(\pm 2\mu\text{m}\), showing that scattering is the main source of inaccuracy. As a further verification of targeting accuracy, we have used staining techniques for visualizing H2AX phosphorylation in irradiated cells. H2AX phosphorylation occurs at the site of DNA damage and is readily observed in cell nuclei that have experienced a particle traversal. Figure 2 shows a mammalian cell that has been targeted with a single \(^3\text{He}^{2+}\) ion.

CR39 is also used to ascertain the efficiency of the particle detection and shuttering system. In a typical experiment, clusters of exactly three particles, slightly spaced from each other are delivered to an array of locations on the plastic and after etching, each location is scrutinized for either an excess of hits or a lower than expected number of hits. When single particle counting, the detection efficiency is greater than 99%, with no missed particles and less than 1% false positives.
A mammalian cell, about 10μm diameter, that has been targeted with a single helium ion. The site of DNA damage is the bright spot towards the top of the nucleus. It is visualized using a stain that highlights regions of H2AX phosphorylation (involved in DNA repair).

A high cell throughput is essential for some studies, as it may be necessary to individually irradiate many thousands of cells per dish. It is essential therefore that the process of target identification, alignment and irradiation are both automated and rapid. Therefore the GCI facility has advanced cell recognition and alignment capabilities such that up to 10,000 cells per hour can be individually located and irradiated. The cell alignment system makes use of an epi-fluorescent microscope to view stained cells supported on a computer-controlled, three-axis micropositioning stage.

3.2 Biological studies using microbeams
One of the first key studies to make use of microbeams was completed using the RARAF facility in New York. Millar et al. [6] measured the transformation frequency in cells irradiated with an exact, or an average number of α-particles. In the important case of a single α-particle traversal, they find that the transformation frequency of a single particle is not statistically different to that from no traversals. If such a finding could be shown to also apply to living systems, then this would imply that environmental exposures to α-particles (such as from radon) presents no added risk to the organism. This is in conflict with current guidelines based on the linear no-threshold (LNT) model which assumes that risk is linearly proportion to dose at all doses.

Microbeams have also been used to investigate non-targeted effects, such as the bystander effect. For example in an experiment at GCI, single Chinese hamster V79 cells with a cell population have been targeted with counted 3.2 MeV protons and the level of bystander-induced cell killing in a 5 x 5mm area of the dish measured using a colony-forming assay [7]. Irradiating just a single cell with 5 or more protons caused a reduction in cell survival of the whole population of about 5-7% and was independent of dose up to the maximum dose used (50 protons through one cell). However, unlike helium ions, a single proton through a single 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 that that from a helium ion and indicates that a threshold dose for the bystander effect exists that is greater that the dose deposited by a single energetic proton, but less that that deposited by a single helium ion.

Shao and colleagues have used the GCI particle microbeam to study the induction of micronuclei induced in a population of T98G glioma cells, after targeting the cytoplasm of one cell close to the centre of the population [8]. They find that the overall yield of micronuclei (small extra nuclei expressed in damaged cells during the first cell division) 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 3He2+ ion. Furthermore, no increase in the yield of micronuclei was seen when greater fractions of cells were targeted through their cytoplasm (one cell, ten cells, or the whole cell population).

In another experiment [9], AG01522 (AG0) primary human fibroblasts were co-cultured in alongside the T98G glioma cells in separate regions 5 mm apart. Targeting one or more T98G cells
with a single $^3\text{He}^{2+}$ ion produced a significant increase in the production of micronuclei within the non-irradiated AG0 population. A similar finding was seen in the T98G cells when AG0 cells were targeted, demonstrating that bystander responses can be induced across genotypes. Some information about the signalling processes involved in the bystander response was gained by adding nitric oxide scavengers. With NO scavengers present, the bystander effect was inhibited in the case of T98G cells being targeted, but only partially inhibited when AG0 cells were targeted. Showing that NO is involved to some extent in the signalling process. Similarly, adding anti-oxidants that inhibit the effects of reactive oxygen species (ROS) completely suppressed the bystander effect in both cases, shown that ROS production is involved in bystander signalling.

The bystander effect has also been shown to have a role in genomic instability. Moore and colleagues have used the GCI microbeam to irradiate a fraction (1 cell, 15% 50%, 100%) of a human lymphocyte cell population with a single ion, then look for aberrations that appear in cells after about 12-13 population doublings [10]. One finding is that when they irradiate 15% or more cells, there is roughly a two-fold increase in the number of aberrations scored and that irradiating just 15% of cells produces as much instability as irradiating all the cells, showing that a bystander induced instability is involved. In fact, it has been proposed that the bystander effect is the main pathway to instability [11].

The bystander effect has been shown to be susceptible to modification through an adaptive response [12]. The adaptive response manifests itself as a reduction in the effect of a high dose of radiation when a small (<0.2 Gy) priming dose is given first, typically a few hours ahead on the high dose. This observation undermines traditional thinking with regard to radiation effects and has been linked to radiation hormesis; the concept that radiation at low doses may actually be beneficial. Mechanistically, the adaptive response is poorly understood, but it has been suggested that the stimulation of repair processes and anti-oxidants are involved.

4. Conclusions

Clearly, the observed non-linear dose-effects at low doses challenge established estimates of radiation risk based on the LNT model. However, it remains unclear as to exactly how non-targeted effects influence risk at low doses. Depending on how the data are interpreted, it is possible to deduce a range of models for the low dose risk response: an increased risk, a decreased risk, a zero risk or indeed, a ‘negative risk’, i.e. that exposure to a low dose of ionizing radiation is beneficial (see figure 3).

Further findings, such as the involvement of NO and ROS 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.

**Figure 3.** Possible models of non-linearity at low doses due to the influence of non-targeted effects. Key: Solid line: LNT Model 1. Increased risk at low doses. 2. Reduced risk at low doses. 3. Zero risk at low doses 4. ‘Negative risk’
References

[1] Ward J F 2000 Radiat. Res. vol 2 ed M Moriarty, C Mothersill, C Seymour, M Edington, J F Ward and R J M Fry, (USA: Allen Press) 379

[2] Prise K M, Folkard M and Michael B D 2003 Radiat. Prot. Dosimetry 104 347

[3] Folkard M, Vojnovic B, Gilchrist S, Prise K M and Michael B D 2003 Nucl. Instr. Meth. in Phys. Res. B. 210 302

[4] Folkard M, Vojnovic B, Prise K M, Bowey A G, Locke R J, Schettino G, Michael B D 1997 Int. J. Radiat. Biol. 72 375

Folkard M, Vojnovic B, Hollis K J, Bowey A G, Watts S J, Schettino G, Prise K M and Michael B D 1997 Int. J. Radiat. Biol. 72 387

[5] Peng S, Folkard M, Gilchrist S, Locke R J, Yu Z and Michael B D 2001 Nucl. Instr. and Meth. B 179 145

[6] Miller R C, Randers-Pehrson G, Gear C R, Hall E J and Brenner D J 1999 Proc. Natl. Acad. Sci. 96 19

[7] Prise K M, Belyakov O V, Newman H C, Patel S, Schettino S, Folkard M and Michael B D 2002 Radiat. Prot. Dosimetry 99 223

[8] Shao C, Folkard M, Michael B D and Prise K M 2004 Proc. Natl. Acad. Sci. 101 13495

[9] Shao C, Folkard M, Michael B D and Prise K M 2005 Int. J. Cancer 116 45

[10] Moore S R, Marsden S, Macdonald D, Mitchell S, Folkard M, Michael B D, Goodhead D T, Prise K M and Kadhim M A 2005 Radiat. Res. 163 183

[11] Lorimore SA, Kadhim MA, Pocock DA, Papworth D, Stevens DL, Goodhead DT, Wright E G 1998 Proc. Natl. Acad. Sci. 95 5730

[12] Sawant S G, Randers-Pehrson G, Metting N F and Hall E J 2001 Radiat. Res. 156 177