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MICROBEAMS IN RADIATION BIOLOGY: REVIEW AND CRITICAL COMPARISON

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Microbeams have undergone a renaissance since their introduction and early use in the mid-60s. Recent advances in imaging, software and beam delivery have allowed rapid technological developments in microbeams for use in a range of experimental studies. Microbeams allow the effects of single radiation tracks to be determined in a highly quantified way. They offer a unique tool for following DNA damage and repair in a highly controlled way. More importantly, they allow radiation to be targeted to specific regions within a cell to probe subcellular radiosensitivity. They are also playing an important role in our understanding of bystander responses, where cells not directly irradiated can respond to irradiated neighbours. Although these processes have been studied using a range of experimental approaches, microbeams offer a unique route by which bystander responses can be elucidated. Without exception, all of the microbeams currently active have studied bystander responses in a range of cell and tissue models. Together, these studies have considerably advanced our knowledge of the underpinning mechanisms. Much of this has come from charged particle microbeam studies, but increasingly, X-ray and electron microbeams are starting to contribute quantitative and mechanistic information on bystander effects. A recent development has been the move from studies with 2-D cell culture models to more complex 3-D systems where the possibilities of utilising the unique characteristics of microbeams in terms of their spatial and temporal delivery will make a major impact.

INTRODUCTION

Microbeams are an important experimental tool in modern radiation biology research. Several types are in routine use including those utilising X-rays, electrons and charged particles. Charged-particle microbeams have been used since the 1960s for quantitative elemental analysis of geological, historical and biological samples[1] where two-dimensional elemental maps can be obtained by scanning a small ion beam across a sample and monitoring the X-rays produced by the sample elements[2]. However, it was only towards the end of the 1990s that microbeams were developed into specific tools to investigate the effect of ionising radiation on living samples. Modern 'radiobiological microbeams' are instruments capable of delivering accurate predetermined doses of ionising radiation to individual cells (or a part of a cell) and can assess the damage induced on a cell-by-cell basis. The advantages of deterministic irradiation achieved by targeting and analysing cells individually have been recognised as being a powerful approach. Using a polonium tipped needle, Zirkle and Bloom[3] tried to correlate radiation-induced cell damage to the type and energy of radiation, the number of ions per cell and even the subcellular compartment irradiated. Despite the limited control and precision offered by their approach, important observations were made regarding nuclear and cytoplasmic radiosensitivity strengthening the hypothesis that considerable benefit could be achieved with a deterministic irradiation approach. However, it has only been recently that improvements in radiation production, detection and delivery, image processing and micropositioning, have enabled the required precision and speed to be achieved to successfully develop radiobiological microbeam facilities.

RATIONAL FOR MICROBEAMS

The rationale for the use of microbeams has previously been ascribed to three key attributes.

(1) They allow the precise metering of dose to individual cells. This is especially true for charged particle microbeams where it is possible to deliver single particles to each cell with high reproducibility and determine the effects of these ultimate lowest possible doses. With conventional particle exposures, to determine the effects of single particle traversals, the best that can be done is to deliver an average of one particle due to the Poisson distribution. This means 37 % of the cells receive no particle traversals, 37 % receive one particle traversal and 26 % receive greater than one. With a microbeam, a single particle can be delivered uniformly to each cell one at a time. This allows the effects of environmental and occupational exposures where the effects of individual radiation tracks are important to be clearly defined.
(2) With the increased precision of delivery of radiation, it is now possible to make choices

regarding the sites of irradiation within cells and tissues. In particular, it is possible to map radiosensitive sites within cells and tissues[4]. The degree of targeting is a function of the size of the beam spot which can be produced by the microbeam relative to the size of the target which needs to be irradiated.

(3) Finally, the ability to select out individual cells or regions of tissues for localised irradiation is key to determining the role of intra- and intercellular signalling especially bystander signalling. Various patterns of irradiation can be used to allow cell–cell signalling to be determined in various contexts.

BIOLOGICAL STUDIES

The initial success of the first microbeam facilities was related to the ability to measure radiation effects at very low doses with great accuracy. The charged particle microbeam at Columbia University was used to measure the oncogenic transforming efficiency of the nuclear traversal of exactly one alpha particle delivered to each cell[5]. It was found to be significantly lower than that predicted by a Poisson-distribution delivery of an average of one alpha particle. Such findings suggested that multiple traversals dominate the biological response and extrapolation from multiple particle traversals may overestimate the single traversal risk. However, similar experiments[6] highlighted how even a single alpha particle traversal could have a considerable toxic (~20 %) and mutagenic probability (average 110 mutants per 10^5 survivors). Similarly, using 3.2 MeV protons, Prise *et al.*[7] at the Gray Laboratory used the micronucleus assay as a measure of predominantly lethal chromosome damage showing a linear dose response in the range 1–30 protons per nucleus with a single proton responsible for micronuclei formation in less than 2 % of exposed cells. A single particle traversal was also shown to induce a significant increase in the proportion of aberrant human T-lymphocyte cells, 12–13 population doublings after exposure[8]. The unstable phenotype indicated by the high level of chromatid-type aberrations suggested that a single alpha particle through the cell nucleus can also induce genomic instability.

Although radiation microbeams are playing an important role in studies of direct DNA damage-mediated effects, a major advantage is the ability to target different regions within cells. This has been utilised by several groups interested in responses to low dose targeted irradiation. The standard paradigm for radiation effects has been based on direct energy deposition in nuclear DNA driving biological response[9]. Previous studies using radioisotope incorporation have shown that the DNA within the nucleus is a key target as ^{131}I-conconavalin A bound to cell membranes was very inefficient at cell killing, in contrast to ^{131}I-UdR incorporated into the nucleus[10]. These authors also found that dose delivered to the nucleus, rather than cytoplasm or membranes, determined the level of cell death. Recently, it has been shown that irradiation of cytoplasm alone can induce an effect. Wu *et al.*[4] found increased levels of mutations in AL cells after cytoplasmic irradiation using an alpha particle microbeam. By comparing the mutant fraction induced by nuclear and cytoplasmic alpha traversals for an equitoxic dose, cytoplasmic irradiation was found to be as much as seven times more mutagenic (due to the low cell killing) than nuclear exposure and therefore potentially more harmful. The types of mutations were similar to those that occurred spontaneously in unirradiated cells and were formed as a consequence of increased ROS species.

A major interest in radiation biology has been the elucidation of bystander responses where cells respond to their neighbours being irradiated[11]. Microbeam approaches have been used extensively to elucidate these. Using a charged particle microbeam, it has been shown that bystander responses are induced in radioresistant glioma cells even when only the cell cytoplasm is irradiated, proving that direct damage to cellular DNA by radiation is not required to trigger the effect[12]. Under conditions of cytoplasmic-induced bystander signalling, disruption of membrane rafts also inhibits the response[12]. More recently, several groups have reported an involvement of mitochondria in the signalling pathways involved in both cytoplasmically irradiated and bystander cells[13–15]. This is an expanding area of research which is beginning to understand subcellular radiosensitivity. Moreover, by targeting the cytoplasm, microbeams have shown that intracellular signalling between the cytoplasm and the nucleus can also cause DNA damage, undermining, therefore, the fundamental paradigm of radiobiology which considers direct DNA exposure a prerequisite for the manifestation of the radiation effects. The importance of cytoplasmic irradiation has also been highlighted by other microbeam experiments directed to investigate the bystander phenomenon. Using both alpha particles[12] and soft X-rays, increase in micronuclei formation and decrease in clonogenic survival have been measured following the irradiation of one or a few cells through their cytoplasm. This finding showed that direct DNA damage is not required for switching on of important intra cell-signalling mechanisms. Another advantage offered by the microbeam approach is the possibility to assess radiation damage on a cell by cell basis thus avoiding the statistical uncertainty which affects some conventional assays. For conventional assays, accurate

measurements of the radiation effect may be limited by both the uncertainty in the number of samples exposed and the dose delivered. This is a particular problem in the low dose region where only small effects are expected. However, using the Gray Laboratory charged particle microbeam, it has been possible to precisely measure the survival of V79 cells exposed to 3.2 MeV protons at doses below 1 Gy[16]. As a result of the precise particle delivery system and knowing the number of cells exposed, it was possible to detect small variations in the initial slope of the survival curve indicating the presence of a hypersensitivity region had been shown previously using X-rays[17] and proving that microbeams are ideally suited to investigate cell survival at very low doses.

A recently growing area of interest for the use of microbeams is in probing the spatial and temporal evolution of radiation damage. It is widely accepted that the biological effectiveness of ionising radiation is determined by the ionisation pattern (i.e. track structure) produced inside cells or tissues[18, 19]. Understanding the extent and pattern of DNA damage induced[20, 21] and their spatio-temporal evolution is therefore of critical importance for assessing biological risks of radiation exposure. Double-stand breaks (DSBs) are considered the most critical DNA lesion induced by radiation due to the complexity of cellular mechanisms involved in the correct rejoining of physically separated DNA sections. The DNA damage caused by a charged particle traversal is the result of a complex clustering of ionisations which occur along the particle path itself (core) and radially due to secondary electrons (penumbra). Track structure simulations[22] and more recently experimental measurements in live cells[23] have determined how the spatial distribution of ionisation critically depends on the mass and energy of the particle. As a consequence, charged particle beams are expected to induce clusters of DNA breaks which result in the formation of complex DNA–DSBs due to radical clustering[24]. Despite the final endpoint being the physical separation of the DNA double helix, DNA–DSBs arising from cluster of DNA lesions present a more difficult challenge for the cell repair mechanisms. Nikjoo *et al.*[25] calculated that although the number of breaks per unit dose remains nearly constant with the LET, their complexity varies significantly. Whilst for low energy electrons only 20–30 % of DNA–DSBs can be considered complex, this proportion increases to 70 % for high LET alpha particles and to 90 % when base damages are included. In general, the complexity of the DNA breaks is rapidly enhanced with increasing the LET.

Crucially, DSBs resulting from multiple damage sites are often associated with the loss of genetic material and high probability of incorrect rejoining which are responsible for late effects such as chromosomal aberrations and genetic mutations including carcinogenesis. Despite their clearly fundamental role in determining the fate of the irradiated cell, little is known about the spatio-temporal evolution of DSBs and their related repair events. There are currently two main aspects of great interest of the spatio-temporal evolution of DSBs: the first is related to breaks mobility within the cell nucleus, while the second concerns the dynamic interaction and alternation of DNA repair proteins. Theoretical attempts to describe how ends from different DSBs meet to form chromosome aberrations have led to two conflicting theories. While the 'contact first' theory proposes that interactions between chromosome breaks can only take place when DSBs are created in chromatin fibres that co-localise, the 'breakage first' theory is based on DSBs moving over large distances before interacting. Extensive DSB migration and interaction is therefore the centre of open debates[26, 27]. Using microbeams, it is possible to induce DSBs in precise locations inside the cell nucleus (recent biological developments allow staining of chromosome domains in live cells[28]) at precise times and investigate their spatio-temporal evolution. Being able to control the site and time of the damage induction allows investigations of the DSB mobility using conventional immunofluorescence techniques. Correlating this data to the extent of the effect induced can then provide critical information on how DSBs mobility affects DNA repair and subsequent cellular response.

As well as direct DNA damage, an understanding of the sequential steps followed by the DNA repair proteins in time is needed to further understand mechanisms of cell response. The dynamic interaction and exchange of DNA repair proteins at the site of damage are a critical aspect as it provides clues to the necessary steps, functionality and requirements of the different enzymatic activities involved in the repair process. The current knowledge of repair/misrepair events that follow DSBs induction by ionising radiation relies on immunofluorescence assays (i.e. γ-H2AX or other damage response proteins) on fixed cells[23, 29, 30]. Despite some contradictory indications of chromatin movement and subsequent formation of repair clusters[27, 29], these data provide only a static view of a selected point in time from which is very difficult to draw dynamic conclusions. Studies looking at the dynamics of DNA repair recruitment are currently being attempted[30, 31] using high atomic number charged particle irradiations (which form highly clustered ionisations) and high resolution microscopy. Modern microbeams are also equipped with state of the art imaging stations in order to accurately monitor the cell response to specific

radiation insults. Moreover, the high precision in the delivery of radiation damage to subcellular sites using a wide range of LET radiations (from X-rays to heavy ions) and the single cell nature of the experiments represent a natural approach to follow cellular reactions to radiation insults in time. Using microbeam approaches, the spatio-temporal details of the irradiation of each sample within a population can be precisely controlled and the cellular response assessed on a cell by cell basis. Combined with the use of GFP-tagged proteins, these features make radiation microbeams a unique tool for the analysis of the spatio-temporal evolution of the DSBs repair processes.

FROM 2-D TO 3-D

Several groups have now extended studies from cell-culture models to more complex tissue models and *in vivo* systems. One challenge with more complex 3-D systems is that multiple cells are irradiated even with microbeam approaches. However, these are providing convincing evidence for a role for bystander responses of relevance to the *in vivo* situation. The original work done in this area used human and porcine ureter models. The ureter is highly organised with four to five layers of urothelium, extending from the fully differentiated uroepithelial cells at the lumen to the basal cells adjacent to the lamina propria or supporting tissue. Using a charged particle microbeam, it was possible to locally irradiate a single small section of ureter such that only four to eight urothelial cells were targeted. The tissue was then cultured to allow an explant outgrowth of urothelial cells to form. When micronucleated or apoptotic cells were scored in this outgrowth, a significant bystander response was observed. Also, a significant elevation in the number of terminally differentiated urothelial cells was detected. Overall, this involves a much greater fraction of cells than those which were expressing damage. Typically, in the explant outgrowth, 50–60 % of the cells are normally differentiated, but this increases by 10–20 % when a localised region of the original tissue fragment is irradiated with the microbeam[32]. Therefore, in this model, the major response of the tissue is to switch off cell division which may be a protective response where proliferation leading to additional damage propagation is prevented[33]. Further studies with microbeams have been done in other tissue models. In recent work in commercially available skin reconstruct models, it has been possible to use localised irradiation with microbeam approaches and measure the range of bystander signalling. After localised irradiation of intact 3-D skin reconstructs, these can be incubated for up to 3 days before being sectioned for histological analysis of sections at different distances away from the irradiated area. With this approach, it was observed that both micronucleated and apoptotic bystander cells could be detected up to 1 mm away from the originally irradiated area[34]. Further studies have utilised other tissue reconstruct models including ones aiming to mimic radon exposure in the lung[35] and observed similar long-range effects. The role of cell-to-cell communication either directly via gap junctional intercellular communication (GJIC) or indirectly via autocrine and paracrine factors may be highly tissue specific and unlikely to be exactly mimicked in an *in vitro* test system, so a combination of studies with both *in vitro* and *in vivo* models will need to be developed in the future.

SUMMARY

Microbeams have provided important tools for radiation biology studies both in terms of understanding direct effects and also for probing new mechanisms of response such as bystander signalling. Future developments in the technology for producing microbeams will enable these to be probed in more complex 3-D models and ultimately *in vivo*.

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REFERENCES

1. Watt, F. and Grime, G. W. *Principles and Applications of High-Energy Ion Microbeams* (Bristol: Hilger) (1987).
2. Watt, F., Grime, G. W., Blower, G. D. and Takacs, J. *The Oxford 1micron proton microprobe*. Nucl. Instrum. Methods **197**, 65–77 (1982).
3. Zirkle, R. E. and Bloom, W. *Irradiation of parts of individual cells*. Science **117**, 487–493 (1953).
4. Wu, L. J., Randers-Pehrson, G., Xu, A., Waldren, C. A., Geard, C. R., Yu, Z. and Hei, T. K. *Targeted cytoplasmic irradiation with alpha particles induces mutations in mammalian cells*. Proc. Natl Acad. Sci. USA. **96**, 4959–4964 (1999).
5. Miller, R. C., Randers-Pehrson, G., Geard, C. R., Hall, E. J. and Brenner, D. J. *The oncogenic transforming potential of the passage of single alpha particles through mammalian cell nuclei*. Proc. Natl Acad. Sci. USA. **96**, 19–22 (1999).
6. Hei, T. K., Wu, L.-J., Liu, S.-X., Vannais, D., Waldren, C. A. and Randers-Pehrson, G. *Mutagenic effects of a single and an exact number of a-particles in mammalian cells*. Proc. Natl Acad. Sci. USA **94**, 3765–3770 (1997).
7. Prise, K. M., Folkard, M., Malcolmson, A. M., Pullar, C. H., Schettino, G., Bowey, A. G. and Michael, B. D.

Single ion actions: the induction of micronuclei in V79 cells exposed to individual protons. Adv. Space Res. **25**, 2095–2101 (2000).
8. Kadhim, M. A., Marsden, S. J., Goodhead, D. T., Malcolmson, A. M., Folkard, M., Prise, K. M. and Michael, B. D. *Long-term genomic instability in human lymphocytes induced by single- particle irradiation*. Radiat Res. **155**, 122–126 (2001).
9. Prise, K. M., Schettino, G., Folkard, M. and Held, K. D. *New insights on cell death from radiation exposure.* Lancet Oncol. **6**, 520–528 (2005).
10. Warters, R. L. and Hofer, K. G. *Radionuclide toxicity in cultured mammalian cells. Elucidation of the primary site for radiation-induced division delay.* Radiat. Res. **69**, 348–358 (1977).
11. Prise, K. M., Schettino, G., Vojnovic, B., Belyakov, O. and Shao, C. *Microbeam studies of the bystander response.* J. Radiat. Res. (Tokyo) **50**(Suppl. A), A1–A6 (2009).
12. Shao, C., Folkard, M., Michael, B. D. and Prise, K. M. *Targeted cytoplasmic irradiation induces bystander responses.* Proc. Natl Acad. Sci. USA **101**, 13495–13500 (2004).
13. Zhou, H., Ivanov, V. N., Lien, Y. C., Davidson, M. and Hei, T. K. *Mitochondrial function and nuclear factor-kappaB-mediated signaling in radiation-induced bystander effects.* Cancer Res. **68**, 2233–2240 (2008).
14. Chen, S., Zhao, Y., Han, W., Zhao, G., Zhu, L., Wang, J., Bao, L., Jiang, E., Xu, A. and Hei, T. K. *et al. Mitochondria-dependent signalling pathway are involved in the early process of radiation-induced bystander effects.* Br. J. Cancer **98**, 1839–1844 (2008).
15. Tartier, L., Gilchrist, S., Burdak-Rothkamm, S., Folkard, M. and Prise, K. M. *Cytoplasmic irradiation induces mitochondrial-dependent 53BP1 protein relocalization in irradiated and bystander cells.* Cancer Res. **67**, 5872–5879 (2007).
16. Schettino, G., Folkard, M., Prise, K. M., Vojnovic, B., Bowey, A. G. and Michael, B. D. *Low-dose hypersensitivity in Chinese Hamster V79 cells targeted with counted protons using a charged-particle microbeam*. Radiat. Res. **156**, 526–534 (2001).
17. Marples, B. and Joiner, M. C. *The response of Chinese Hamster V79 cells to low radiation doses: evidence of enhanced sensitivity of the whole cell population*. Radiat. Res. **133**, 41–51 (1993).
18. Goodhead, D. T. *The initial physical damage produced by ionising radiations.* Int. J. Radiat. Biol. **56**, 623–634 (1989).
19. Prise, K. M., Folkard, M., Newman, H. C. and Michael, B. D. *The effect of radiation quality on lesion complexity in cellular DNA*. Int. J. Radiat. Biol. **66**, 537–542 (1994).
20. Jenner, T. J., deLara, C. M., O'Neill, P. and Stevens, D. L. *Induction and rejoining of DNA double-strand breaks in V79–4 mammalian cells following g and a-irradiation*. Int. J. Radiat. Biol. **64**, 265–273 (1993).
21. Prise, K. M., Pinto, M., Newman, H. C. and Michael, B. D. *A review of studies of ionizing radiation-induced double-strand break clustering*. Radiat. Res. **156**, 572–576 (2001).
22. Paretzke, H. G. *Physical events of heavy ion interactions with matter.* Adv. Space Res. **6**, 67–73 (1986).
23. Jakob, B., Scholz, M. and Taucher-Scholz, G. *Biological imaging of heavy charged-particle tracks.* Radiat. Res. **159**, 676–684 (2003).
24. Prise, K. M., Davies, S. and Michael, B. D. *Evidence for induction of DNA double-strand breaks at paired radical sites.* Radiat. Res. **134**, 102–106 (1993).
25. Nikjoo, H., O'Neill, P., Wilson, W. E. and Goodhead, D. T. *Computational approach for determining the spectrum of DNA damage induced by ionizing radiation*. Radiat. Res. **156**, 577–583 (2001).
26. Anderson, R. M., Stevens, D. L. and Goodhead, D. T. *M-FISH analysis shows that complex chromosome aberrations induced by alpha-particle tracks are cumulative products of localized rearrangements.* Proc. Natl Acad. Sci. USA **99**, 12167–12172 (2002).
27. Nelms, B. E., Maser, R. S., Mackay, J. F., Lagally, M. G. and Petrini, J. H. J. *In situ visualisation of DNA double-strand break repair in human fibroblasts.* Science **280**, 590–592 (1998).
28. Essers, J., Houtsmuller, A. B., van Veelen, L., Paulusma, C., Nigg, A. L., Pastink, A., Vermeulen, W., Hoeijmakers, J. H. and Kanaar, R. *Nuclear dynamics of RAD52 group homologous recombination proteins in response to DNA damage.* EMBO J. **21**, 2030–2037 (2002).
29. Aten, J. A., Stap, J., Krawczyk, P. M., van Oven, C. H., Hoebe, R. A., Essers, J. and Kanaar, R. *Dynamics of DNA double-strand breaks revealed by clustering of damaged chromosome domains.* Science **303**, 92–95 (2004).
30. Asaithamby, A., Uematsu, N., Chatterjee, A., Story, M. D., Burma, S. and Chen, D. J. *Repair of HZE-particle-induced DNA double-strand breaks in normal human fibroblasts.* Radiat. Res. **169**, 437–446 (2008).
31. Jakob, B., Rudolph, J. H., Gueven, N., Lavin, M. F. and Taucher-Scholz, G. *Live cell imaging of heavy-ion-induced radiation responses by beamline microscopy.* Radiat. Res. **163**, 681–690 (2005).
32. Belyakov, O. V., Folkard, M., Mothersill, C., Prise, K. M. and Michael, B. D. *Bystander-induced apoptosis and premature differentiation in primary urothelial explants after charged particle microbeam irradiation*. Radiat. Prot. Dosim. **99**, 249–251 (2002).
33. Belyakov, O. V., Folkard, M., Mothersill, C., Prise, K. M. and Michael, B. D. *Bystander-induced differentiation: a major response to targeted irradiation of a urothelial explant model.* Mutat. Res. **597**, 43–49 (2006).
34. Belyakov, O. V., Mitchell, S. A., Parikh, D., Randers-Pehrson, G., Marino, S. A., Amundson, S. A., Geard, C. R. and Brenner, D. J. *Biological effects in unirradiated human tissue induced by radiation damage up to 1mm away.* Proc. Natl Acad. Sci. USA **102**, 14203–14208 (2005).
35. Sedelnikova, O. A., Nakamura, A., Kovalchuk, O., Koturbash, I., Mitchell, S. A., Marino, S. A., Brenner, D. J. and Bonner, W. M. *DNA double-strand breaks form in bystander cells after microbeam irradiation of three-dimensional human tissue models.* Cancer Res. **67**, 4295–4302 (2007).