Microbeam Studies of the Bystander Response

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Abstract

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.

The resurgence in the use of microbeams since the mid 90s has coincided with major changes in our understanding of how radiation interacts with cells. In particular, the evidence that bystander responses occur, where cells not directly irradiated can respond to irradiated neighbours, has brought about the evolution of new models of radiation response. 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 internationally have studied bystander responses in a range of cell and tissue models. Together these studies have considerably advanced our knowledge of bystander responses and 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 utilizing the unique characteristics of microbeams in terms of their spatial and temporal delivery will make a major impact.

INTRODUCTION

A range of responses of cells and tissues to irradiation have been classified as being non-targeted effects as they do not fit the standard DNA damage driven responses. Of these, radiation-induced bystander responses, where cells respond to their neighbours being irradiated, have attracted significant effort to understand the mechanisms underpinning these. Although a range of techniques have been used to study bystander effects, a key approach has been the development of microbeams which allow isolation of the effects of radiation exposure to individual cells within a population.

APPROACHES FOR STUDYING BYSTANDER RESPONSES

A range of approaches are available for studying bystander responses and have made significant contributions to our understanding of the mechanisms. The seminal work by Little and colleagues used a low fluence approach whereby a monolayer of cells were...
irradiated with a very low fluence of α-particles such that only 1% of the population was exposed. Under these conditions 30% of the cells showed chromosomal changes in the form of sister chromatid exchanges. This approach was also successfully utilized to show that direct cell to cell communication via gap junctions plays a role as addition of the GJIC inhibitor lindane prevents bystander signalling in primary human fibroblasts. Another extensively used approach has been the use of media transfer. Here cells are irradiated and then medium removed filtered and added to non-exposed cells. Many of the studies showing a role for factors released into the medium of bystander cells have used this approach.

A related approach has been to irradiate cells in the well of a multiwell dish and then add a second population of cells to the well contained within a membrane insert. Insert systems allow easy separation of the two communicating populations and if poorly penetrating charged particles are used, both the irradiated and bystander population can be sharing the same medium at the time of irradiation. Similar to this approach, again with charged particles several groups have used partial shielding options to allow directly irradiated and bystander cells to be sharing the same medium at the time of irradiation, where either part of a dish is shielded or regions have thicker bases through which the particles cannot traverse.

RATIONAL FOR MICROBEAMS

The development of microbeams has come about not solely as a tool for the studying of bystander responses. The rationale for the use of microbeams has previously been ascribed to three key attributes (Figure 1).

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. 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 localized 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.

MICROBEAM STUDIES OF BYSTANDER SIGNALLING

Microbeams have been a useful tool in probing bystander mechanisms and without exception all of the current generation of operational microbeams have been active in this area. The first reported microbeam studies of the bystander effect showed, that individually irradiated primary human fibroblasts could induce bystander responses, after localized irradiation with counted helium-3 ions, measured as increased micronuclei formation and...
This was then quickly followed by studies showing that microbeam irradiated cells could produce bystander responses leading to a range of outcomes including mutations\(^{11}\) and transformation\(^{12}\) in hamster fibroblasts. In follow-up studies, in primary human fibroblasts, it was shown that these effects could be observed after only a single cell was irradiated with a single helium ion\(^ {13}\). Although the early studies in this area have used essentially light ions, more recent work has extended bystander studies with higher LET radiations to heavier ions and includes studies reporting bystander effects, measured as micronuclei induction, after irradiation of a single cell within a cell monolayer with a single \(^ {40}\)Ar (approximately 1260 keV/\(\mu\)m) or \(^ {20}\)Ne (approximately 380 keV/\(\mu\)m) ion\(^{14}\). Other studies have measured responses after carbon ion microbeam irradiation, showing no significant effect of LET on the cell cycle mediated induction on CDKN1A (p21) in bystander cells\(^ {15}\), but significant temporal differences in the timescale of bystander mediated cell inactivation\(^ {16}\).

In general, work with the current generation of charged particle microbeams have contributed significantly to our understanding of the mechanism underpinning bystander responses. For example, using the unique targeting ability of microbeams, 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\(^ {17}\). Under conditions of cytoplasmic-induced bystander signalling, disruption of membrane rafts also inhibits the response \(^ {17}\). More recently several groups have reported an involvement of mitochondria in the signalling pathways involved in bystander signalling in both irradiated and bystander cells \(^ {18-20}\).

Although direct DNA damage is not required to trigger the bystander response, DNA damage is induced in bystander cells after microbeam irradiation either via reactive oxygen\(^ {21}\) or nitrogen species \(^ {22}\). A key modulating role is also played by calcium signalling processes\(^ {23}\) alongside an important role for cytokine signalling, especially release of TGF\(\beta\)\(^ {24}\). Studies have shown that removal of this damage requires the presence of a fully active non-homologous end-joining pathway for repair of DNA dsbs \(^ {25-27}\) and that mismatch repair, which checks for miscoding errors in the DNA, may also play a role \(^ {28}\). For the production of sister chromatid exchanges in bystander cells, fully functional homologous repair (HR) is required. However, HR is not capable of repairing the damage induced in NHEJ deficient cells \(^ {29}\). A key feature of studies with repair deficient mutants is that changing the repair proficiency of the irradiated cells has no significant effect on the ability of these cells to release bystander signals \(^ {30, 31}\). Significant evidence now suggests that accumulation of damage within the bystander cells could lead to difficulties during S-phase of the cell cycle, where stalled replication forks may lead to the production of dsbs \(^ {32, 33}\).

Several groups have also developed X-ray microbeams\(^ {34}\) and some of these have been applied to the study of bystander responses, although in a more limited way in comparison to charged particle studies so far. Two approaches to X-ray microbeams have been reported. One approach is to use synchrotron-based X-rays which are restricted to a small slit or aperture to produce localised irradiations with either 5.335 or 12.5 keV\(^ {36}\) X-rays. Another approach is to use characteristic soft X-rays\(^ {15}\) and focussing approaches. Using carbon-K characteristic soft X-rays it has been possible to focus these to submicron spot sizes using devices called zone-plates which act as diffraction lenses. Individual cells within populations can then be precisely irradiated using the same approaches developed for charged particle microbeams. For clonogenic survival, a significant bystander response has been reported in hamster fibroblasts, even when only a single cell was irradiated. The dose response was similar to many other studies in that after an initial increase with dose to the targeted cell a plateau region is observed\(^ {37}\). Further analysis of the response showed that...
cells actually exhibited a binary behavior for triggering of the bystander response. Essentially, the probability of triggering a bystander response increases approximately linearly with the dose delivered to the single selected cell, reaching 100% above about 0.3 Gy. The magnitude of the bystander effect, when triggered, is approximately constant with the dose and results in an overall approximately 10% reduction in cell survival. This suggests that the event that triggers the emission of the bystander signal by the hit cell is an all-or-nothing process. Extrapolation of the data indicates that when a single fast electron traverses a single hamster V79 cell, there is a probability of approximately 0.3% that the cell will emit the bystander signal38).

Microbeams have also been developed capable of delivering localised doses of relatively low energy (80keV) electrons39, 40). To date however, no studies have been reported of bystander responses after localised electron irradiation. Whether this reflects on important radiation quality differences for the activation of bystander responses needs to be further clarified.

**TISSUE-MODEL STUDIES**

Several groups have now extended studies from cell-culture models to more complex tissue models and in vivo systems. 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 4-5 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. Sections of ureter were isolated and placed on microbeam dishes with the urothelium nearest to the dish surface. Using a charged particle microbeam, it was possible to locally irradiate a single small section of ureter such that only 4 – 8 urothelial cells were targeted. The tissue was then cultured to allow an explant outgrowth of urothelial cells to form41, 42). 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. This leads to an additional $5 \times 10^4$ differentiated cells in the explant outgrowth 43). Therefore, in this model, the major response of the tissue is switching off cell division. This suggests that in intact tissues, bystander responses may be protective responses where proliferation leading to additional damage propagation is prevented 44). These findings also add to continuing debate regarding the relevance of isolated cell culture systems to the multicellular tissue environment in vivo. 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 1mm away from the originally irradiated area 45). Further studies have utilised other tissue reconstruct models including ones aiming to mimic radon exposure in the lung 46) and observed similar long-range effects. The role of cell to cell communication either directly via 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.
FUTURE APPROACHES

Bystander responses are being extensively studied using microbeam approaches and key attributes of these responses have been determined. Further work is making significant contributions to our understanding of the mechanisms underpinning bystander signalling. A key challenge is to test for interactions between bystander signalling and other non-targeted responses, particularly at low dose. Future developments will include extensive development of these approaches in tissue models and in vivo, particularly in model systems. An initial study of targeted irradiation in the nematode *C. Elegans* has already been reported using a heavy ion microbeam[47]. *C. Elegans* provides a highly characterised whole organism model, where the genetic, cellular and tissue make-up is precisely understood. They can be easily targeted with both charged particle and X-ray microbeams. Further advances in deep tissue imaging on the existing microbeams will allow these approaches to develop and contribute to the ongoing debate of the role of bystander responses in vivo.

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References


Figure 1. Rationale for the use of microbeams
Panel A shows the comparison between the conventional exposure yielding an average of 1 track per cell relative to the microbeam approach which delivers exactly one particle per cell. Panel B shows the ability to target different parts of a cell and Panel C shows the ability to deliver localised irradiation within a cell population or tissue. All three of these approaches can contribute to studies of bystander responses.