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Microbeam Irradiation of the *C. elegans* Nematode

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Microbeam/*C. elegans*/Bystander/*In vivo* models.

The understanding of complex radiation responses in biological systems, such as non-targeted effects as represented by the bystander response, can be enhanced by the use of genetically amenable model organisms. Almost all bystander studies to date have been carried out by using conventional single-cell *in vitro* systems, which are useful tools to characterize basic cellular and molecular responses. A few studies have been reported in monolayer explants and bystander responses have been also investigated in a three-dimensional normal human tissue system. However, despite the well-know usefulness of *in vitro* models, they cannot capture the complexity of radiation responses of living systems such as animal models. To carry out *in vivo* studies on the bystander effect we have developed a new technique to expose living organisms using proton microbeams. We report the use of a nematode *C. elegans* strain with a Green Fluorescent Protein (GFP) reporter for the hsp-4 heat-shock gene as an *in vivo* model for radiation studies. Exposing animals to heat and chemicals stressors leads to whole body increases in the hsp-4 protein reflected by enhanced fluorescence. We report here that γ-rays also can induce stress response in a dose dependent manner. However, whole body exposure to stress agents does not allow for evaluation of distance dependent response in non targeted tissues: the so-called bystander effect. We used the RARAF microbeam to site specifically deliver 3 MeV protons to a site in the tail of young worms. GFP expression was enhanced after 24 hours in a number dependent manner at distances > 100 μm from the site of irradiation.

INTRODUCTION

Accumulated evidence shows that the biological effects of ionizing radiation can be expressed in un-exposed neighboring cells to an irradiated cell or group of cells.[1–2)] This so called "bystander effect" challenged the dogma that cellular damage is restricted to directly irradiated cells. Proposed mechanisms to explain this phenomenon are centered on the importance of intercellular communication. Therefore, the use of advanced tools to study inter- and intra-cellular mechanism of damage signal transduction is of critical importance.

Such inter-cellular studies have often been undertaken using microbeam irradiation of individual cells plated in 2-D monolayers[1–6)] and more recently in 3-D tissue-like construct with extensive and complex cell-to-cell communication and extra-cellular matrix interactions. Pioneer studies using 3-D models started at the Gray Laboratory in the UK, where an organotypic explant model technique was used. This organ culture was selected because it maintains the *in vivo* structural and functional integrity under controlled conditions.[7)] Efforts to expand the use of more reproducible culture models were undertaken at the Columbia University Radiological Research Accelerator Facility (RARAF) microbeam, using a highly reproducible commercially available three dimensional normal re-constructed human skin tissue systems containing both epidermis and dermis or epidermal layer alone using α-particles.[8–10)] While useful, single cell *in vitro* systems do not have a realistic multicellular morphology, and *in vitro* studies using 3-D tissues do not necessarily mimic inter-cellular communication which involves tissue-level stress responses, often among multiple cell types, and mediated by microenvironment signaling. Therefore, studies using whole organisms, targeting specific cells, cell groups or organs are needed to elucidate mechanisms of radiation-induced long-distance effects in a realistic model.

A few studies have described the use of whole organisms for microbeam studies. Yang *et al.* and Tanaka *et al.*, used

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Arabidopsis thaliana specifically targeting selected region of the plant.[11–13] Recently, living animals were used for microbeam studies. Fukamoto *et al*. exposed the instar silkworm *Bombyx mori* larvae to carbon ion microbeam.[14] Sugimoto *et al*., employed the nematode *C. elegans* as in vivo system exposing young gravid worms to carbon microbeam to study cell cycle arrest and apoptosis.[15]

C. elegans has several advantages for *in vivo* microbeam studies. Among the advantages are: simple culture conditions and maintenance, rapid life cycle, short life span, fully sequenced genome, transparent body, adult organism has only 959 somatic cells and its anatomy is invariant from one animal to the next.[16] Moreover *C. elegans* shares cellular and molecular structures and control pathways with higher organisms, thus, biological information learned from *C. elegans* may be directly applicable to more complex organisms. From a practical perspective it is small enough to be compatible with microbeam irradiation since the diameter of its body is ~ 50 μm and the full length is ~ 1 mm. Thus, based on these features we designed our animal model system for *in vivo* microbeam experiments. Specifically, we used the *C. elegans* strain SJ4005 *hsp-4::gfp(zcls4)V*, which has a GFP reporter for the *hsp-4* heat-shock gene. Heat shock proteins (HSPs) are a ubiquitous family of gene products present in cells under unstressed conditions for which they function as molecular chaperones. They are expressed in much higher concentration owing to the presence of stress. The HSPs play a critical role in normal homeostasis to assist protein folding, direction of newly formed proteins to target organelles, the assembly or disassembly of protein complexes, inhibition of improper protein aggregation, such as may occur owing to crowding or thermal denaturation and activation of the initial immunological system in response for selected disease. In response to stress, HSPs assist in refolding and repair of denaturated proteins as well as facilitating synthesis of new proteins to repair damage.[17] HSP stress responses can be induced by diverse stressing agents including heat, UV irradiation, γ-rays irradiation and chemicals.[18]

The *C. elegans* strain SJ4005 has been developed and used to study chemical-induced stress responses. Calfon *et al.*, showed that the transcription of *hsp-4* gene is induced in the gut and in the hypodermis upon endoplasmic reticulum chemical stress or heat shock,[19] leading to dramatically increased levels of the protein in a time dependent manner. Consequently, we used this well characterized system to develop a model for *in vivo* microbeam studies.

MATERIALS AND METHODS

C. elegans strain and culture conditions

The *C. elegans* strain SJ4005 *hsp-4::gfp(zcls4)V* which has a transcriptional reporter for the *hsp-4* heat-shock protein gene was used for our *in vivo* microbeam studies. The baseline expression of the GFP has been previously characterized.[19] In normal/non stressed conditions, the GFP expression is most prominently localized in spermathecae and to a lesser degree, in the tail and pharynx regions; Fig. 1 shows a control worm imaged with Nomarski optics and in fluorescence with the basal GFP expression.

Worms were cultured and manipulated using previously described methods.[20] Briefly, they were cultured on growth medium (NGM) agar on Petri plates supplemented with *E. coli* (OP50) as food source. Worms were continuously fed for many generations and maintained at 20°C in a temperature-controlled incubator.

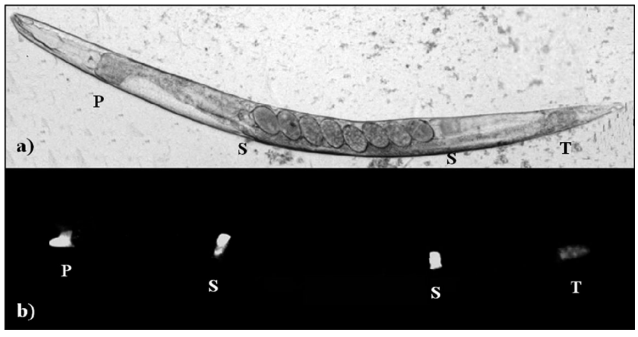

Fig. 1. **a)** Young gravid *C. elegans* SJ4005 strain imaged using Normarski optics. **b)** Fluorescent image of same worm. It is visible the basal GFP expression localized in the pharynx (P), spermathecae (S) and in the tail (T).

Whole body gamma irradiation

Twelve hours before exposure, L4 larvae were selected in order to obtain a homogeneous population of young adult hermaphrodite worms at the time of the microbeam irradiations. Young adult *C. elegans* were exposed to 0, 3 and 10 Gy of gamma rays using a Gammacell 40 ^{137}Cs irradiator (dose rate 0.8 Gy/min). Worms were irradiated at room temperature; the longest irradiation time was ~ 12 minutes. A minimum of 20 worms were used in each experimental group. After 24 hours the worms were imaged using a 10X objective of a Nikon Eclipse 600FN microscope equipped with a digital ICCD camera AG-5765EF-3U (Pulnix, America).

Site specific microbeam irradiation

Young adult *C. elegans* hermaphrodites were exposed individually to 3 MeV protons microbeams using the Columbia University RARAF charged-particle accelerator. The RARAF charged-particle microbeam delivers defined numbers of charged particles with a beam diameter as low as 0.5 μm. A detailed description of the microbeam is given in ref. 21.

The maximum penetration of the protons used was ~140 μm with an entrance LET of 12.5 keV/μm and a beam diameter of 1 μm.

For microbeam irradiations, worms were anesthetized with 10 mM sodium azide (NaN_3) in M9 buffer and placed in a customized microbeam dish with a micro cover-slip for individual exposure. Before exposure, worms were individually imaged using an epifluorescent microscope. Worms were exposed to 0, 25, 50 and 75 protons per target area. Animals were irradiated at the tail, in the center of the GFP expression region. Control worms were mock-irradiated, by targeting the microbeam just outside the worm (~ 200 μm), keeping the same set-up time for anesthetic exposure and concentrations. A minimum of 20 worms were used in each experimental group. Worms were exposed at room temperature. Room temperature during set up and irradiation was closely monitored in order to detect potential thermal stress; the temperature range during the experiments was 20 ± 2°C.

After exposure, the micro cover-slips were removed using a micro aspirating pump and the worms were washed with M9 buffer and re-cultured in standard agar/covered Petri dishes. Based on well established protein kinetic studies,[22–23] and our preliminary expression time studies, we selected a twenty four hour time point for GFP expression evaluation.

Pre- and post-irradiation imaging of GFP expression

Epifluorescent images were acquired using the 20X objective of an inverted microscope (Olympus IX70) equipped with a Hoffman modulation contrast system (Modulation Optics Inc., Greenvale, NY, USA) and a Hamamatsu Orca high-resolution, high-efficiency digital camera. In order to evaluate the stress response induced by protons after microbeam irradiation the exposed worms were imaged and recorded for later site specific fluorescence quantification.

RESULTS

Stress response after whole body gamma irradiation

The basal/normal GFP expression in the control worms was localized in pharynx, spermathecae and tail (Fig. 2A). Worms exposed to gamma rays showed a dose response increase of GFP expression. The overall GFP expression in 3 Gy exposed worms was slightly increased. Conspicuously, an over-expression region was observed in the middle section of the body corresponding to the vulva region (Fig. 2B). An extensive and marked over-expression was observed in 10 Gy exposed worms (Fig. 2C). In particular, the posterior intestine and the terminal and anterior bulbs of the pharynx showed strongest expression. Moreover, the gamma rays exposed worms showed a reduced locomotion as demonstrated in previous studies.[24]

Stress response after site specific microbeam irradiation

Microbeam exposed worms showed a different GFP stress response in terms of intensity and localization, compared with the control group. Increase in expression was noted only after delivering 50 and 75 protons. Mock irradiated worms did not exhibit any GFP stress response after 24 hours (Fig. 3a). No apparent stress response was detected when 25 protons were delivered (Fig. 3b). However, when the worms were exposed to 50 or 75 protons a strong stress response in the posterior intestine was observed, between the spermatheca and the microbeam targeted area (Fig. 3c–d). A one micrometer diameter proton beam was able to induce tail region *in situ* GFP over-expression as well as distal stress response as far as > 150 μm away from the irradiated spot. No stress response was seen in other regions of the body of exposed worms and only basal levels of GFP expression were detected at the pharynx and spermathecae regions.

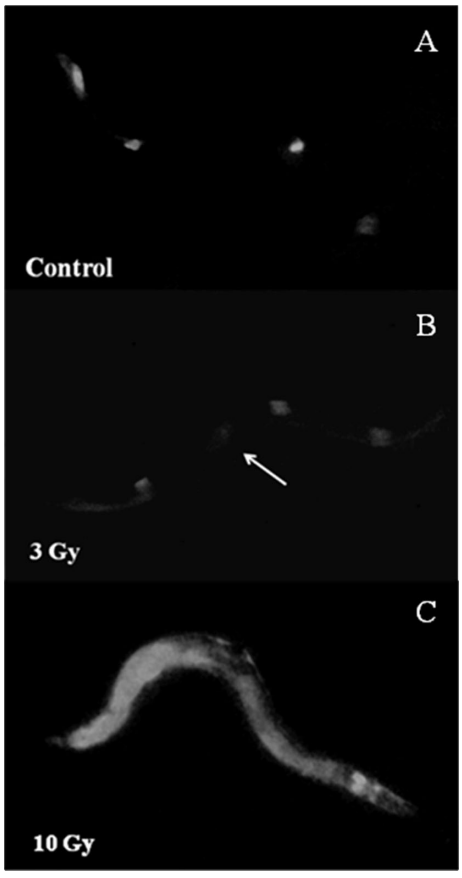

Fig. 2. GFP expression in the *C. elegans* SJ4005 strain following gamma-irradiation. The imaged worms have the same orientation: on the right is showed the pharynx and on the left the tail. **A.** A control worm showing the basal GFP expression. **B.** Worm exposed to 3 Gy of gamma rays showed a slightly increased basal GFP expression. The arrow indicates the presence of a conspicuous GFP over expression detected after irradiation. **C.** Worms exposed to 10 Gy of gamma rays showed a widespread GFP over expression mostly localized in the posterior intestine and in the pharynx.

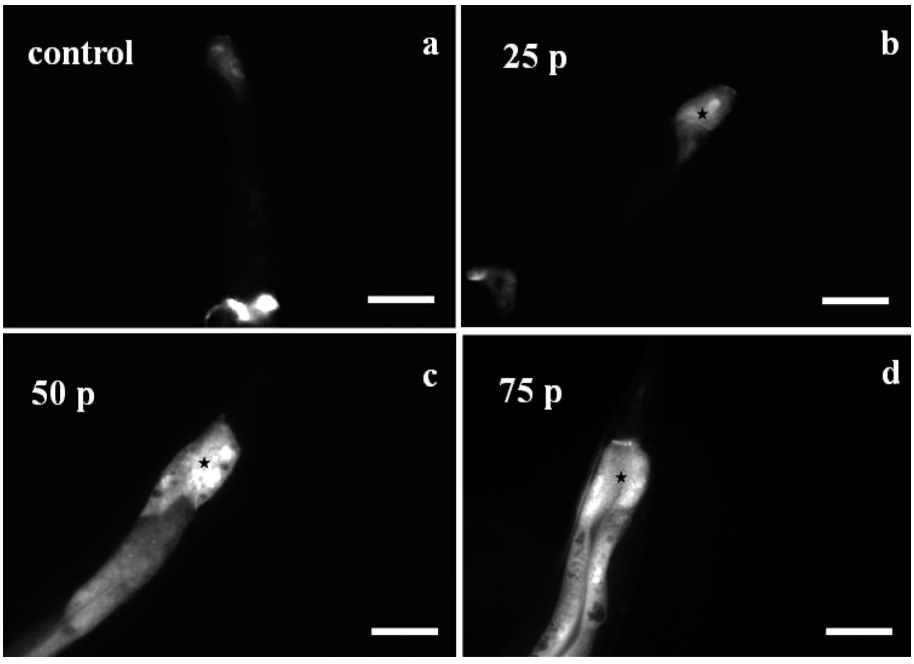

Fig. 3. *hsp-4::GFP* stress response in the *C. elegans* tail 24 hours after microbeam irradiation. **a)** Control. **b)** Worm tail irradiated with 25 particles. No stress response was detected. **c–d)** Stress response following microbeam irradiation with 50 and 75 protons. A strong stress response is visible in the posterior intestine up to 150 μm from the irradiated area. Scale bar indicates 50 μm. The black star indicates the irradiated area with 1 μm proton beam.

DISCUSSION

In summary, we have shown the suitability of *C. elegans* as a model for microbeam *in vivo* studies to investigate potential bystander effects in whole organisms. Sub-cellular proton microbeams can induce *in vivo* local and distal stress responses in this nematode. The advantages of *C. elegans* as a research tool are well established, being a multicellular eukaryotic organism that is simple enough to be studied in great detail. From a practical perspective it is small enough to be compatible with microbeam irradiation and a wide variety of mutants and transgenics are readily available, as is a large community of *C. elegans* researchers. Significantly, many cell types in *C. elegans* are shared with humans; these include neuronal, gut, excretory and muscle cells. Notably, many genetic pathways and cellular mechanisms are conserved from human to nematode. Consequently work with *C. elegans* has had a major impact on understanding fundamental biological processes important to humans such as development, apoptosis, aging and immunity.[25] Therefore, the use of engineered *C. elegans* containing reporter genes under the control of heat shock protein (HSP) promoters is an attractive model to study radiation induced bystander effect/stress responses using microbeam. This model has been previously used to study the stress responses induced by microwaves, chemicals and electromagnetic fields.[26–28] Therefore, we selected the SJ4005 *C. elegans* strain for microbeam irradiation.

Wild type and a mutant strain *C. elegans* have been used previously as an *in vivo* model for microbeam irradiation.[19] L4 stage and young gravid *C. elegans* were irradiated with collimated ~ 20–50 μm diameter microbeam 220 MeV carbon particles (LET of 120 keV/μm). In these experiments worms were exposed to 1500 carbon ions delivered to extended worm body areas. While these irradiation techniques are useful for organ or regions exposures, they do not allow the exposure of a restricted number of cells or small tissue sections in a living organism. Our technique is capable of delivering, with high accuracy, small numbers of particles at sub-cellular and cellular level. Since many phenomena under study using microbeams, such as the bystander phenomenon, must involve cell-to-cell communication, extracellular environment and functional integrity, the capability of targeting individual tissue components makes this technique more compelling.

In order to characterize the radiation-induced stress response in the selected strain, we exposed worms to graded doses of whole body gamma radiation. We demonstrated that sub-lethal doses of gamma rays induced a global stress response patterns similar to the reported response induced by chemical treatment.[19]

For our *in vivo* microbeam irradiation, we developed a technique that allows us to deliver precisely defined number

of particles at precisely defined locations. Our studies show that low doses of protons delivered at the tail region, where different organs and cell populations are located, were capable of inducing both a local and distal GFP over-expression. This response is remarkable since the microbeam used is estimated to traverse a series of spatially co-localized cell structures. How this spatially restricted stressor is capable of inducing a distal stress response is not understood. However, it is worth noting that among the organs potentially irradiated are the intestine and the stomato-intestinal muscle. Cell-cell communications as well as tissue damage signaling are potential mechanisms involved in these responses. Belyakov *et al.*, suggested that autocrine/paracrine mechanisms or juxtacrine signaling are potential bystander pathways to explain long range bystander phenomena in complex tissues.[8] Future studies will be focused on the identification of cell targets and signal mechanisms using different biological endpoints.

To summarize, an optimized irradiation system has been developed and tested to irradiate site specifically transgenic worms with a charged particles microbeam. Use of this targeted irradiation method provides a new tool to investigate complex long-range biological responses such as the bystander effect in living organisms. Developments of this technique will include a microfluidics based worm clamp for microbeam irradiation without anesthesia as well as microbeam modulation techniques to adjust the position of the Bragg peak at different locations within the target organism.

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