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Use of synchrotron medical microbeam irradiation to investigate radiation-induced bystander and abscopal effects in vivo



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ABSTRACT

The question of whether bystander and abscopal effects are the same is unclear. Our experimental system enables us to address this question by allowing irradiated organisms to partner with unexposed individuals. Organs from both animals and appropriate sham and scatter dose controls are tested for expression of several endpoints such as calcium flux, role of 5HT, reporter assay cell death and proteomic profile. The results show that membrane related functions of calcium and 5HT are critical for true bystander effect expression. Our original inter-animal experiments used fish species whole body irradiated with low doses of X-rays, which prevented us from addressing the abscopal effect question. Data which are much more relevant in radiotherapy are now available for rats which received high dose local irradiation to the implanted right brain glioma. The data were generated using quasi-parallel microbeams at the biomedical beamline at the European Synchrotron Radiation Facility in Grenoble France. This means we can directly compare abscopal and "true" bystander effects in a rodent tumour model. Analysis of right brain hemisphere, left brain and urinary bladder in the directly irradiated animals and their unirradiated partners strongly suggests that bystander effects (in partner animals) are not the same as abscopal effects (in the irradiated animal). Furthermore, the presence of a tumour in the right brain alters the magnitude of both abscopal and bystander effects in the tissues from the directly irradiated animal and in the unirradiated partners which did not contain tumours, meaning the type of signal was different.

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Introduction

Non-targeted effects including bystander (effects in unirradiated cells receiving signals from irradiated cells) and abscopal effects (effects in unirradiated tissues following irradiation of a different tissue in a distant location) are known to occur following both low and high doses of radiation and other stressors both in vitro and in vivo. Most in vivo data involve shielding part of an animal and are complicated by systemic factors such as blood and endocrine factors, making it difficult to resolve mechanistic questions related for example to the role of the immune system or the inflammatory response in the process [1—4]. While the existence of

both bystander effects and abscopal effects are widely accepted, they remain poorly understood. By definition abscopal effects occur in vivo, usually as a result of targeted radiotherapy to another part of the body [5,6]. Bystander effects have been demonstrated in vitro in numerous cell lines across all species groups and in vivo in rodent models [7], fish [8,9], amphibians [10] and yeast [11]. In much of the literature, abscopal and bystander effects are thought to share common mechanisms and to be mediated by similar signals [1,12]. However most bystander research is conducted using low doses of mainly low LET radiation delivered to the entire organism or cell culture, while abscopal effects are detected following high doses of targeted radiotherapy to precise areas of the body which usually contain tumour tissue [1,2,13]. This makes it difficult to ascertain whether common mechanisms are involved or whether both mechanism are related.

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A further limitation of research in the field is that in vivo "bystander" experiments usually use shielding of part of the body to demonstrate effects in non-irradiated areas [14–16]. This means that the hematopoietic, neural, immune and endocrine systems could be irradiated and could either pass through the unirradiated area (blood and endocrine effectors) or share common neuronal connections leading to detection of distant effects. Additionally, scatter and out of field doses may contribute sufficient radiation to trigger bystander effects, which have thresholds in the 2–3 mGy dose range [17–24]. It is unclear whether an animal that received the estimated scatter dose as whole body irradiation, is a sufficient control to cover these possibilities for reasons which will be discussed later in the paper. Clearly there could be confusion in determining separate mechanisms involved in bystander and abscopal effects in vivo.

In the past we have successfully used an approach where nonirradiated companion animals are placed in close proximity to irradiated animals. Our group have conducted several experiments with irradiated fish [25-27] sharing aquarium water with unirradiated fish, and Surinov's group in Russia [28,29] and our group [7] have also demonstrated communication between irradiated mice and their cage mates with subsequent signal expression in the nonirradiated animals. These experiments parallel in vitro "medium transfer" experiments in that the unirradiated animals receiving signals from irradiated animals were never anywhere near the radiation source and never had any part of their bodies exposed to Xrays. This precludes systemic effects due to the circulating blood, or endocrine or neural components being affected by exposure of part of the body to irradiation. The fish experiments involved whole body exposure to very low X-ray doses and confirmed a role of serotonin and calcium in the production of the bystander signal in vivo [30,31] as was seen in vitro [32–34].

The development of microbeam radiation therapy (MRT) using synchrotron generated kilovoltage energy X-rays is based on the concept that sparing of normal tissues will occur in the dose valleys between the peak dose tracks [35–37]. MRT, a still experimental form of spatially fractionated radiotherapy, has been developed for the treatment of small and otherwise intractable brain and spinal cord tumours [38–43]. Bystander effects are thought to play a role in the dose valleys where the absorbed X-ray dose is generally lower than in the peak dose zones by more than one order of magnitude [44–46]. However the precise nature and role of these effects is unclear especially since the valley dose greatly exceeds the threshold of 2-3 mGy established for the induction of bystander signalling processes in low dose in vitro irradiation [22-24]. The current experiments were performed using the biomedical beamline ID17 at the European Synchrotron Radiation Facility (ESRF) in Grenoble as part of a wider study of the use of microbeam and pencil beam therapy in the treatment of malignant brain tumours in small animal models [7,45-49].

Preliminary experiments [7], with normal tumour-free rats, have shown that bystander signals were being communicated from irradiated rats to unirradiated rats. Tissues from the unirradiated rats when cultured, gave rise to conditioned medium, which reduced the clonogenic survival of reporter cells. However the influence of tumour tissue on this process was not examined.

There is evidence from earlier in vitro experiments by our group, that some tumour cells — particularly those which are radioresistant or have mutant or dysfunctional p53 do not produce death inducing bystander signals [50–53]. Two glioma cell lines which serve as experimental models for glioma in rodents were considered for these experiments; the F98 glioma cell line, which was developed in the Fisher rat and the C6 glioma cell line, which was originally developed in the Wistar rat [54–56]. The C6 line was used in the preliminary experiments [7]. There is a divergence of

opinion in the literature concerning the p53 status of F98 cells with one author claiming they have wild type and another claiming mutant status [57,58]. The consensus at present is that the line F98 contains mutant p53. C6 cells are reported to have wild type p53 [56,59–61]. The results of several studies suggest that activating p53 expression using various drugs enhances apoptotic death after radiation exposure in both these cell lines, presumably because of dysfunctional operation of up-stream or downstream elements of the pathways involving p53 which should be activated following radiation exposure [58,62–66]. For these experiments the decision was made to use the F98 glioma cell line in Fisher rats so that the effect of a p53 mutant tumour could be examined.

In the experiments to be described here, F98 cells were stereotactically inoculated into the brain of Fisher rats. The rats were irradiated using the microbeam synchrotron radiation at the ESRF after which they were put in cages with unirradiated rats for 48 h. Samples from all rats and various controls were taken and examined in a reporter assay for evidence of bystander signal production.

Methods

Animals

Male adult Fisher rats in the weight range 260–280 g (Charles River, France) were used as the animal model in our experiments. Animals were housed and cared for prior to the experiments by the ESRF Animal Facility in accordance with French and Canadian Animal Care Protocols.

Tumour inoculation

The F98 glioma cell line was selected for our studies because of its mutant p53 status and because it shares a wide range of characteristics with the highly malignant human brain tumour glioblastoma multiforme (GBM) [56]. Once injected into the brain, F98 glioma cells rapidly proliferate forming a solid, highly invasive malignant tumour, delineated by a rim of activated astrocytes and small groups of infiltrating tumour cells [56]. This tumour model has been used in multiple studies involving conventional radiotherapy and synchrotron radiation.

For these experiments, F98 cells were obtained from ATCC and maintained in T75 cm² flasks using Dulbecco's Modified Eagle Medium (Gibco, France) supplemented with 10% FBS (Gibco, France) and 5 ml Penicillin-Streptomycin (Gibco, France). Cells from a 90% confluent culture were detached by incubation with 20 ml of calcium and magnesium free Hank's Balanced Salt Solution (Gibco, France) for 20 min at 37 °C in an atmosphere of 5% CO₂ in air. The cell suspension was centrifuged at 1000 rpm for 4 min, the pellet was re-suspended in 1 ml of fresh growth medium and cells were counted using a haemocytometer.

Fisher rats were subjected to general anaesthesia (2–2.5% isofluorane in 2 L/min compressed air) and placed in a stereotactic frame. An incision of 1–1.5 cm length was made on the scalp following the sagittal midline. A burr hole was placed in the skull over the right hemisphere, 3 mm to the right from the sagittal midline and 3 mm posterior from the coronal suture. Then 100,000 F98 cells suspended in 10 μl were slowly injected into the brain 3 mm below the cortical surface over 4 min, using an automated syringe pump (KDS 320, GENEQ). Once the injection was finished and the needle removed, the hole was sealed with bone wax and the incision was closed. Rats were maintained for 7 days to allow tumour development.

Irradiation

In preparation for the irradiation, rats were deeply anesthetised using 3% isofluorane in 2 L/min compressed air and maintained with an intraperitoneal injection of a Ketamine-Xylazine cocktail (Ketamine: Xylazine = 1: 0.625; Ket 1000 and Paxman from Virback France). Animals were transported from the animal facility to the biomedical beamline ID17 in less than 5 min. Each irradiation group had 5 rats, which were individually positioned on the goniometer and the corresponding radiation dose for its treatment group was applied in anterior—posterior position to the tumour location in the right cerebral hemisphere by off-setting one edge of the irradiation field 2 mm towards the right from the midline (Fig. 1). The left nonirradiated cerebral hemispheres and the urinary bladder served as fields for study of abscopal effects in the directly irradiated animals. After irradiation, all rats were put in individual cages with a marked, unirradiated normal (tumour free) rat, meaning that 5 irradiated rats were matched with 5 non-irradiated rats for the study of bystander effects.

Animals irradiated in MRT mode were exposed in a single treatment session to 20 or 200 Gy skin-entry doses. The skin entrance dose corresponds to the peak dose at 3 mm depth and is determined as described hereafter. Absolute dose measurements for preclinical experiments are performed using a pinpoint ion chamber (PTW 31014) in a solid water phantom (Gammex) to measure the dose rate in Gy/s/mA for a 2 cm \times 2 cm field size at 2 cm depth. All corrections, like for temperature and pressure, the polarization between the electrodes, the calibration of the electrometer, a correction factor for our energy spectrum and the ion recombination correction according to the IAEA 398 protocol are included.

The peak skin entrance dose is then converted with the help of Monte Carlo pre-calculated output factors depending on the desired beam-size chosen for the irradiation. In order to translate the experimentally determined dose rate within the MRT GUI (Graphical user interface) into a vertical displacement to extend the 520 micron height microbeams into an array of 50 quasi-parallel 14 mm high rectangular planar microbeams, the MRT goniometer speed is calculated to deliver the desired peak entrance dose, depending on the electron current (mA) in the storage ring. A 10 mm wide array of multichromatic beamlets was generated by a multislit collimator [67] with a mean energy of 105 keV by filtering the white Synchrotron beam with several filters including 1.5 mm of Aluminium and 1 mm of Copper. The typical dose rate during

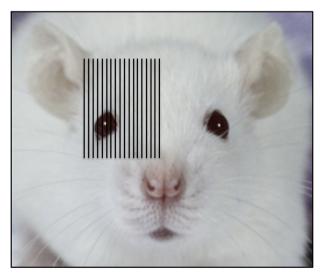


Figure 1. Graphical representation of the incident synchrotron microbeam.

these experiments was ~14,000 Gy/s. The valley dose is computed by Monte Carlo calculations and more recently a Treatment Planning System (TPS) with an analytical approach is used to calculate the valley dose based on CT data from a rat applying the irradiation parameters in these experiments [68,69]. Benchmarking of the calculated dose is still ongoing, but results with Gafchromic film dosimetry confirm an agreement within 10% between the computed and measured valley dose values [70].

Although multi-directional treatment is more successful in increasing survival, the geometry of the unidirectional beam works better for understanding bystander effects. Unidirectional irradiation creates a less complicated 3D geometrical pattern of dose peaks and dose valleys within the brain tissue than bidirectional irradiation and therefore makes it easier to study how the normal tissue between the microbeams is involved in the induction of bystander effects.

In order to determine whether scatter radiation places a role in the induction of bystander and abscopal responses, 5 rats and 5 cage mates were selected as scatter controls. A PTW semiflex ion chamber (PTW, Freiburg, Germany) was used to measure the scatter dose received at the urinary bladder after brain irradiation with 200 Gy delivered in MRT mode. The dose at the site of the urinary bladder was calculated as 3.31 mGy for MRT. An X-ray generator was adapted with different additional filters to obtain an adequate dose rate, in order to deliver the whole body dose of 3.31 mGy to the rats. HD-610 and MD-55 Gafchromic Films (ISP Advanced Materials, http://online1.ispcorp.com/) were used to verify all irradiation doses and modalities applied.

Untreated controls stayed in the ESRF animal facility and never left the cage. One group received anaesthesia before euthanasia (sham control) and another group received no anaesthesia to exclude potential effects of the anaesthetic. These control rats were also paired with cage mates and were held two to a cage similar to the other experimental groups. We previously demonstrated that a sham irradiation did not induce abscopal effects or affect the protein expression of brain compared to controls [46].

All irradiated rats were transported back to the ESRF animal facility after irradiation. At 48 h after irradiation, the animals were deeply anesthetised, beheaded and dissected.

Dissections and sampling for explant culture

Dissection of the brain was performed in a biosafety cabinet. Two pieces of brain tissue (approximately 5 mm \times 5 mm \times 3 mm) were taken from both the right and the left cerebral hemispheres using sterile instruments. The tissue sample from the right (irradiated) hemisphere was taken from the centre of the irradiation array and the sample from the left (unirradiated) hemisphere was taken from the corresponding contralateral location. Samples were placed in a 5 ml sterile tube containing 1 mL of Roswell Park Memorial Institute (RPMI 1640, Gibco) growth medium, supplemented with 10% FBS, 5 ml of Penicillin-Streptomycin (Gibco), 5 ml of Lglutamine (Gibco), 0.5 mg/ml of Hydrocortisone (Sigma-Aldrich), and 12.5 ml of 1 M HEPES buffer solution (Gibco). Samples were immediately transported on ice to the tissue culture laboratory to be prepared for explant culture. The remaining brain tissue was snap-frozen in liquid nitrogen and stored at -80 °C for proteomic studies. The entire extracted urinary bladder was also placed in a sterile 5 ml tube containing 1 ml of complete growth medium and used to set up tissue explant cultures.

Explant tissue culture and culture medium harvest

Explant tissue culture was performed in the biosafety level 2 laboratory of the ESRF biomedical beamline. Brain and urinary

bladder tissues were cut in 3 equal-size pieces of approximately 2 mm 3 in a biosafety cabinet. The pieces were plated as single explants in the centre of a 25 cm 2 growth area in a 50 ml volume flask (Falcon), containing 2 ml of complete growth medium. Flasks were then placed in a tissue culture incubator set at 37 °C, with an atmosphere of 5% CO2 in air and 95% humidity left undisturbed for 24 h. Growth medium from each of the three explant pieces (total approximately 5 ml) was harvested 24 h later by pouring it off into a sterile plastic container. This was then filtered through a sterile 0.22 μm filter (Acrodisc Syringe Filter with HT Tuffryn Membrane, Pall Life Sciences) to ensure that cells or other debris were not present in the harvested medium, and placed in a 7 mL tube. Conditioned growth medium was kept in 4 °C until all media were collected and then transported to McMaster University for clonogenic reporter bioassays.

Clonogenic reporter cell line

HaCaT cells have been used as reporters for explanted tissue assays by our group in Canada and earlier in Ireland for over 15 years [71]. The cell line consists of epithelial cells, which became immortal spontaneously. They were derived originally from normal human skin from a patient with a melanoma [72] and have been used in a wide range of experiments due to their reliable and stable response to bystander signals. They show a reduction of around 40% in colony survival in response to addition of autologous irradiated cell conditioned medium (ICCM) over a wide range of donor cell radiation doses [73]. HaCaT cells have 3 p53 point mutations; 1 in codon 179 of exon 5 on one allele, and 2 consecutive mutations in codons 281 and 282 of exon 8 on the other allele [74]. In spite of its mutations, data show that p53 in HaCaT cells remains functional with respect to inducing apoptosis [75]. In our hands they behave like wild-type cells with respect to bystander effect reporting. Unlike true p53 mutant or null cells where signal is produced but the cells cannot respond to signal. This leads us to suspect that the critical p53 function in determining whether response to bystander signals happens, is located in the wild type codons.

The HaCaT cells were cultured in T75 flasks (Falcon) with RPMI 1640 supplemented as above. Once the cells reached about 90-95% confluence they were detached using 1:1 (v:v) solution of 0.02% Trypsin/EDTA (1 mM) (Gibco) and Dulbecco's Phosphate-Buffered Solution (1x) (Gibco). The concentration of cells was determined using a Coulter Counter (Beckman Coulter model $Z_{\rm D}$).

Clonogenic HaCaT cell reporter bioassay

Upon arrival at McMaster University, the conditioned medium harvested in France was transferred into 25 cm² flasks containing the HaCaT reporter cells. Reporter flasks were seeded with 500 cells and set up 6 h prior to the medium transfer from T75 flasks which were 90–95% confluent. Plating efficiency and medium transfer controls were also set up. The flasks were then placed in an incubator for 10–12 days to allow for colony formation using the Puck and Marcus technique [76]. Once colonies reached a suitable size they were stained using 2 mL of a 1:4 solution of Carbol Fuchsin in water.

Colonies were counted using a 50 cells threshold and the percentage survival fraction was calculated using the plating efficiency (PE) of the reporter cells as shown below:

 $Survival \ Fraction = \ \frac{PE \ of \ treated \ cells}{PE \ of \ control \ cells} \ x \ 100$

Fura-2 measurements to determine intracellular free calcium in HaCaT cells

The cells were seeded in glass bottomed dish (MatTek) at a density of approximately 500,000 cells and incubated at 37 °C and 5% CO $_2$ for 18–24 h prior to measurement to achieve 50% confluence. Cells were washed 3 times with buffer (130 mM NaCl, 5 mM KCl, 1 mM Na $_2$ HPO $_4$, 1 mM CaCl $_2$, 1 mM MgCl $_2$ and 25 mM Hepes (pH 7.4)) followed by incubation with 1 ml of 8.2 μ M Fura-2/AM (aminopolycarboxylic acid which binds to free intracellular calcium) (Sigma) at 37 °C for 30 min. Cells were washed 3 times with buffer to remove residual Fura-2/AM and 300 μ L of fresh buffer added to the dish for imaging. An Olympus 1X81 microscope was used with a 40X oil objective and Fura filter cube with 510 nm emission. Fura-2 was excited at 380 and 340 nm and the ratio images were recorded every 4 s for 5 min with addition of 100 μ l of ICCM or control media after a stable baseline was reached approaching 30 s. All measurements were conducted in the dark at room temperature.

Statistical analysis

Data are presented as standard error of the mean for the specific n value of each experiment. Significance between and within groups was determined using the Tukey multi-comparison test after a two-way ANOVA. In all cases p values \leq 0.05 were selected as significant. Pearson correlations and linear regressions were done using SPSS and Prism 6.0.

Results

The clonogenic assay reports the bystander signal strength measured as the ability of the signals to reduce the clonogenic survival of the well characterised keratinocyte cell line. Figure. 2 shows the signal strength from the directly irradiated right brain (1a) of normal and tumour bearing rats receiving 20 and 200 Gy irradiation from the MRT beam and the abscopal effects in the left brain (1b) and distant bladder (1c) in these animals. All the data are normalised to the relevant sham control. There was no significant effect of the anaesthesia on clonogenic survival (nonsignificant p-values for right brain, left brain and urinary bladder respectively: p = 0.495; p = 0.989; p = 0.993). The statistical analyses were therefore performed between the sham irradiated and the irradiated groups. Because of the high doses used in these experiments and the reported low threshold dose for triggering bystander signalling in vitro [22-24], a control group was included which received a whole body dose equivalent to the highest calculated scatter dose of 3.31 mGy after delivery of 200 Gy to the head. The data in Fig. 2a show that there was no statistically significant difference between the different controls. There is however a significant difference in signal strength between the controls and the healthy rats which received direct irradiation of 20 Gy or 200 Gy to the right brain. The effect on clonogenic survival after 200 Gy is similar to that seen after 20 Gy MRT. The effect of direct irradiation to the tumour bearing right brain (Fig. 2d) is much more visible showing a very strong effect on clonogenic survival of the reporter cells after 20 Gy and an even stronger suppression of clonogenic growth after 200 Gy irradiation. Looking at the effects of signals from the left brain hemisphere (Fig. 2b), which received only a scatter dose, it is apparent that the only statistically significant effect is from the healthy animals where the tumour bearing right brain received 20 Gy MRT. The 200 Gy signals from the healthy animal are weaker but still present although not statistically significant. No significant effects are seen from the tumour bearing rats (Fig. 2e) when compared to their own controls. The data for the distant urinary bladder are shown in Fig. 2c. Once again there are no significant

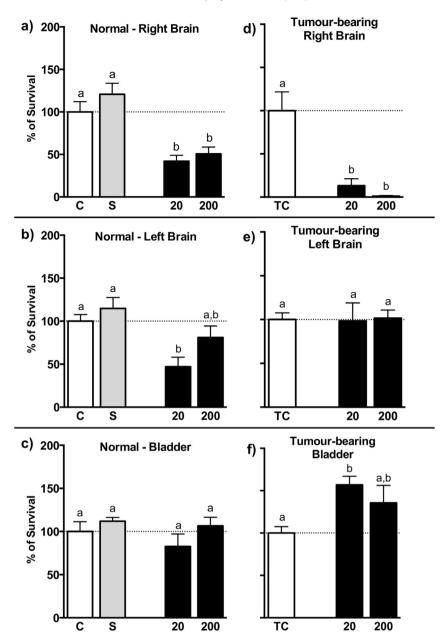


Figure 2. Shows clonogenic survival induced by normal and tumour-bearing rats. C = Control; S = Scatter; TC = Tumour control; black bars: irradiated rats (20 = 20 Gy; 200 = 200 Gy). Letters a, b & c indicate significant differences between groups. Error bars show SEM.

effects on clonogenic survival seen in the scatter group. The signals from the bladders of the 20 Gy irradiated healthy animals are not significantly different to those in the control group. However again the 20 Gy group signals are stronger than those in the 200 Gy group. When the tumour bearing animals are considered (Fig. 2f), it appears that relative to their own controls, the signals from the bladders of the groups receiving irradiation to the right brain are actually stimulating clonogenic survival.

Fig. 3 shows the results of the true bystander animals, which merely shared a cage for 48 h with irradiated rats. All the unexposed companion cage mates were tumour free but were paired with either a tumour bearing (d-f) or a tumour free animal (a-c). In Fig. 3a the clonogenic survival of reporter cells receiving signals from the right brain of the unirradiated cage mates is presented. Clearly the effect in the unexposed companion cage mate's right brain tissue is much stronger when they are paired with tumour free irradiated rats. When viewed in comparison to their own

control, there are no significant differences in signal strength when the irradiated rats have tumours (Fig. 3d). The same pattern is seen when signal strength is monitored in left brain cage mate tissue (Fig. 3b, e). In the unexposed companion cage mates bladder (Fig. 3c), signal strength is not significantly different from the controls when the irradiated rat was tumour free but the tumour bearing rat bladder (Fig. 3f) signals stimulate the clonogenic survival of the reporter cells as was seen with the directly irradiated rats.

To better understand the overall influence of the presence of the tumour, Fig. 4 shows a comparison of the clonogenic survival of normal and tumour-bearing rats relative to the unirradiated normal control. Direct-irradiated normal rats (Fig. 4a) shows clearly the escalating decline in signal strength after 20 and 200 Gy, which is significantly different from the normal control group in the right and left hemispheres after 20 Gy, and only in the right hemisphere after 200 Gy. Direct-irradiated rats harbouring tumour showed a

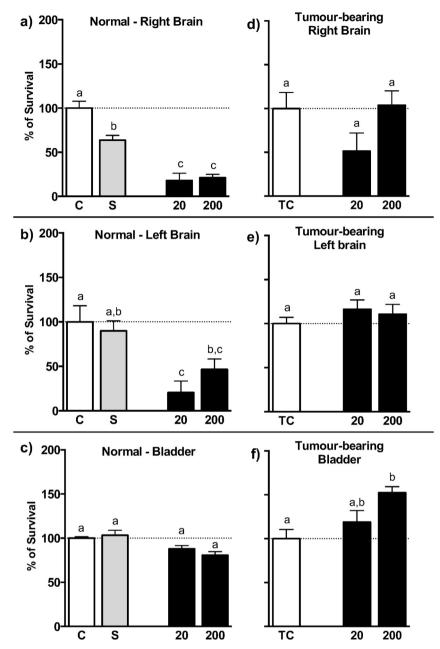


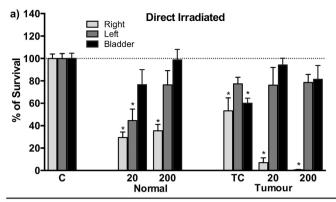
Figure 3. Shows clonogenic survival induced by unexposed companion cage mates (Bystander rats). C = Control; S = Scatter; TC = Tumour control; 20 = 20 Gy; 200 = 200 Gy. Letters a, b & c indicate significant differences between groups. Error bars show SEM.

similar pattern of escalating decline in signal strength at 20 and 200 Gy, but it was only significant in the right brain hemisphere. The unirradiated tumour control rats clearly induced a decrease in survival in the reporter cell line relative to the unirradiated normal control rats, and this decrease was significant in both right brain hemisphere and bladder.

The bystander animals (Fig. 4b) paired with normal irradiated rats show similar escalating decrease in survival at 20 and 200 Gy, which is significant in all brain tissues but not in bladder. The bystander animals paired with tumour-bearing rats followed almost the same pattern as their direct-irradiated mates but the only significant group was 20 Gy. The unirradiated tumour control rats also seemed to influence their cage mates but the decrease in clonogenicity was not significant.

The results of the calcium flux analysis are presented in Fig. 5(a-f) and Fig. 6(a-f) and on Table 1. The data show the

relationship between the amount of clonogenic cell death in reporters and the calcium flux seen in the reporter cells. This is because calcium flux (measured as area under the curve) is usually considered to be the trigger, which induces the response pathway in the reporter cells. In Figs 5 and 6 the data were modelled with a linear regression between % of cell death and Calcium flux induced by ICCM from irradiated and cage mate rats. Table 1 shows the Pearson Correlation statistics for these data. The conditioned medium from explants of the right brains of both normal and tumour bearing rats which were directly irradiated show weak positive, but statistically significant, correlations between the amount of clonogenic cell death of the reporter cells and the calcium flux in these cells. The right and left brains of unirradiated companion cage mates of normal rats without tumour show a very weak inverse correlation for right brain and a weak direct correlation for left brain which are statistically significant. Companion cage mates



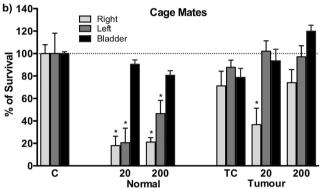


Figure 4. Comparison of clonogenic survival relative to the normal control. Letter "a" correspond to direct-irradiated rats, while "b" refers to the unirradiated Cage Mates or bystander animals. "*" Indicates significant difference against the unirradiated normal control rat. Error bars indicate SEM.

paired with animals with tumour show a negative significant correlation only in the left brains. None of the bladder tissues showed any significant effects.

Discussion

A key aim of this work was to look at the strength and type of signalling occurring within irradiated rats and between irradiated rats and their unexposed companion cage mates. This work builds on previously published data and preliminary results showing that there is a distinction between the effects of direct irradiation in an organism or a cell culture and the effects of the signals passed by that organism or cell culture to others. Clearly if the mechanisms of radiation action are to be fully understood, it is important to understand the signalling (indirect effects as well as the direct effects). The early literature on bystander effects was almost all concerned with effects in vitro [77]. These were mostly negative effects and the bystander effect was considered to be an extension of the negative effects of radiation. When in vivo experiments confirmed remote cell killing, transformation or mutational effects [78-80], the consensus was that bystander effects were bad and represented a spreading of the damage induced by the direct dose deposition. However there were also reports of adaptive responses [81–83]. These particularly occurred in cells treated with medium from irradiated cells before being themselves irradiated [84]. However, the groups using microbeams also occasionally reported adaptive and protective effects. Further extension of this to fish swimming with irradiated fish and tadpoles swimming with irradiated tadpoles [26,27,85] suggested that bystander effects could be positive as well as negative. We consider this to be an important key for understanding radiotherapy outcomes.

The data presented here for normal Fisher rats and their cage mates contradicts those published for normal Wistar rats [7]. In the Wistar rat study, the abscopal effects in the directly irradiated rats became weaker as the distance from the targeted brain tissue increased. This was not attributable to declining scatter dose because the highest scatter dose calculated was given to rats as a whole body X-ray dose and had no effect. However in the unexposed companion cage mate group all the tissues in these unexposed animals produced the same response in the reporter cells. We thus deduced that the signals from the exposed animals produced a common systemic effect in the cage mates. The proteomic evidence [86] suggests this effect is protective. Similar proteomic data suggesting upregulation of protective proteins, were obtained with fish where irradiated fish were partnered with unirradiated fish [25]. In the experiments reported here however, while there are small effects induced in the left brain from the group in which the right brain was directly exposed to 20 Gy, there are no significant signals from the bladder suggesting a weak or absent abscopal signalling mechanism. In the cage mates, it appears that the induced signalling is strongest in the right brain, weaker in the left brain and absent in the bladder. This suggests tissue specific signals rather than the homogeneous effect seen in the Wistar rats. We conclude that there must be a strain difference and draw attention to studies with CBA and C57Bl6 mice [87] where similar strain differences in production of and response to bystander signals were

What is very evident in this study is that the presence of an F98 glioma in the directly irradiated rats prevents or counteracts the signalling in the other tissues of the rat and also in all the tissues of the tumour-free unexposed companions (cage mates). Moreover, instead of stimulating growth, the non-irradiated rats harbouring tumour induced a decrease in survival relative to the normal controls. This suggests that the presence of a tumour does not boost reporter cell growth; but it rather seems to counteract the bystander signal. In fact, in the bladder tissues, the inducible effect in both directly irradiated and cage mates that seems stimulatory is always under or near the 100% value of survival observed in the unirradiated normal control tissues (Fig. 4). Since the tumour develops from F98 cells implanted in the brain rather than evolving naturally over time within a supporting microenvironment, it is hard to argue that the animal's microenvironment is incapable of producing the signal. Most of the brain in the track of the microbeam is composed of normal tissue and in the healthy rats this produced signals. While strain and cell line differences in bystander signalling support the existence of a neutral or blocking effect associated with genomic instability [88], cancer phenotype [89], cancer susceptibility [90], mutant p53 [67,91,92] and radioresistance [93-95], this appears to be the first report that bystander effects in the healthy cage mate and abscopal effects in unirradiated tissues of the directly exposed rats can both be blocked by the presence of a glioma in the brain of the rat receiving direct irradiation. Our conclusion is that the presence of tumour in the brain actively counteracts signal production.

The work raises a number of key questions about non-targeted effects in vivo. First and most important is the lack of cell killing by bystander signals when the tumour is present in the directly irradiated brain. We hypothesize that this likely stems from antideath signals expressed by the tumour rather than absence of signals. Possibly the stress response pathways such as mitogenactivated protein kinases (MAPK), which are normally activated in response to radiation or receipt of bystander signals and which lead to apoptosis are somehow actively neutralised. Previous research [53] has demonstrated that mutant p53 cells are not able to respond to bystander signals although in the situation where p53 is mutated or knocked out, they can produce signals. In the model used here,

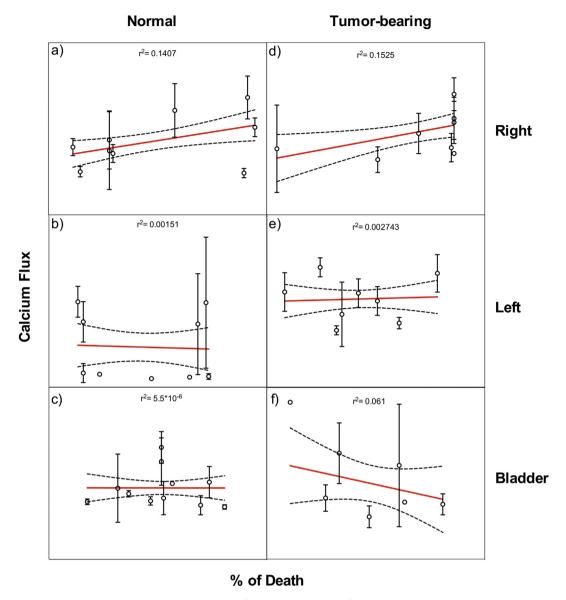


Figure 5. Scatter plots showing the relationship between Calcium Flux and % of death produced by ICCM from Irradiated Rats. Red line corresponds to the linear regression with a 95% of confidence intervals. R² values show goodness of the fit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the host rat is p53 wild type but the inoculated tumour is mutant. Thus it is necessary to postulate that the secretion of a systemic signal from the tumour capable of "disarming" the stress sensing mechanisms in the normal cells would bypass apoptosis in the cells damaged by radiation. While it might be plausible to predict this in the directly irradiated rats it is difficult to see why this would be communicated to unirradiated healthy cage mates. However, in the literature, reference can be found to have whole body irradiation causing the secretion of volatiles, which make the irradiated rats socially unattractive to the cage mates [96]. The effects of the volatiles caused the cage mates to develop compromised immune responses [97,98]. Possibly, the irradiation of tumour tissue releases different volatiles, which neutralise the other bystander effects on the normal tissues as part of a strategy of the tumour to evade being attacked by the immune system. A key to resolving these questions might be to estimate the relative volume of tumour to normal tissue irradiated in the microbeam protocol.

Analysis of calcium flux in cells in vitro [23,99] suggested that a sharp transient calcium flux triggered the response in cells

receiving bystander signals. Investigation of various stress pathways suggested a role for the MAPK pathway leading to induction of apoptosis [34]. Therefore in these experiments calcium flux was measured to see if the flux was associated with reporter cell death where the tissue generating signals had or had not been directly irradiated. By analysing the directly irradiated rats and the unirradiated companion cage mates, we hoped to distinguish bystander effects in the companions from abscopal effects in the irradiated animal and to confirm a role for the presence of tumour cells in determining the response. As expected, the data in Table 1 do show positive correlations between death and calcium flux where the right brain tissue was directly irradiated. However the left brain and bladder tissues in the directly irradiated rats show no correlations either for normal or tumour bearing tissues. This suggests that the abscopal effects seen in these tissues are not related to the calcium flux pathway. Moreover, the calcium data versus % of cell death from Table 1 shows significant positive correlations for the right brain, while the linear regressions show very low r² values. Therefore, the biological significance of a linear relationship across

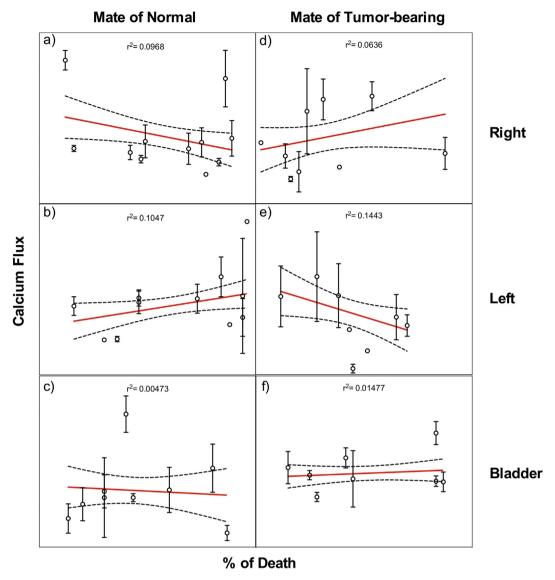


Figure 6. Scatter plots showing the relationship between Calcium Flux and % of death produced by ICCM from Cage Mate rats. Red line corresponds to the linear regression with a 95% of confidence intervals. R² values show goodness of the fit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

all doses may be spurious, a conclusion that extends to the cage mate data. The calcium flux versus fractional cell death data for the cage mates are much more difficult to explain. While companions of irradiated normal rats showed significant negative and positive

Pearson Correlation between % of cell death and Calcium flux induced by ICCM from irradiated and cage mate rats.

Group	Tissue	Pearson correlation	Significance (2-tailed)
Normal	Right	*0.375	(0.014)
	Left	-0.39	(0.817)
	Bladder	-0.02	(0.988)
Tumour	Right	*0.39	(0.022)
	Left	0.052	(0.769)
	Bladder	-0.249	(0.264)
Mate of normal	Right	*-0.311	(0.037)
	Left	*0.324	(0.047)
	Bladder	-0.069	(0.681)
Mate of tumour	Right	0.252	(0.164)
	Left	*-0.38	(0.042)
	Bladder	0.122	(0.501)

^{*}Correlation is significant at the 0.05 level.

correlations for the right and left brain respectively, the companion cage mate showed a reverse effect when a tumour was present in the irradiated rat. These observations suggest that the tumour modifies the pathways for abscopal and bystander effects. The data for the bladder explants from directly irradiated and unirradiated companion animals where the clonogenic survival suggested that the signals in the conditioned medium caused a significant positive growth stimulating effect reveal no correlation between calcium flux and growth stimulation. This again suggests that the pathways involved in the abscopal and bystander effects in these experiments do not involve the calcium flux which is associated with the directly irradiated right brain tissues. It is likely that downstream secondary signalling pathways are induced and that primary calcium signalling is confined to the tissue that actually received direct radiation energy deposition.

Another interesting result is the strain difference between Wistar and Fisher rats. While strain differences in radiation response are well known as was discussed earlier, this is a strain difference not just involving the tissues of the directly irradiated rats but involving the communication between the irradiated rats

and their cage mates and the level of induced response in the cage mates. In one case the response is the same in all tissues of the cage mates but in the other it varies in the same way as the abscopal response. This could have major implications for research into individual radiosensitivity.

Finally since synchrotron microbeam irradiation is mainly tested in the treatment of aggressive brain tumours giving the therapist the opportunity to focus a very high X-ray dose in a small tissue volume in such a way that maximal protection of normal tissue is achieved, it is important to consider the implications of our findings for this type of therapy. Early use of MRT considered the peak and valley doses to be key to achieving normal tissue sparing [44,100]. The existence of bystander effects was well known and communication of bystander signals was considered in the field but not at all understood. In our experiments the peak entrance dose of 200 Gy to cells in the path of the beam is associated with a 20 Gy dose in the "valleys" between the microbeam tracks. Both these doses are several orders of magnitude larger than the in vitro threshold doses of around 2-3 mGy for triggering bystander effects. Bystander effects are known to saturate at a dose of about 0.5 Gy and a further increase in dose (at least up to 10 Gy) does not increase the level of signal [101]. This means that bystander signalling will be saturated in the dose valleys as well as in the peak dose zones. In terms of impacts therefore, the question arises whether bystander signals from normal tissue are amplifying the harmful effects of radiation or are enabling beneficial effects and whether counter effects expressed by irradiated tumour cells (peak or valley) are having any effect. Both harmful and beneficial effects have been reported but the factors determining which response occurs are not known. Clearly more work is needed using biomarkers for damage such as YH2AX already identified as a useful marker [102] and for repair/protective effects using markers such as 53bp1 which indicate induction of repair [103].

In conclusion, the work reported here suggests that the presence of tumour tissue in the irradiated brain can modulate the abscopal effect in other organs of the directly irradiated animal and modify bystander response in unirradiated companion cage mates. The data taken together with earlier studies also suggest strain differences in these in vivo bystander responses. The implications for targeted radiotherapy using MRT are unknown and in need of further study.

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