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Exposure to low level chronic radiation leads to adaptation to a subsequent acute X-ray dose and communication of modified acute X-ray induced bystander signals in medaka (Japanese rice fish, *Oryzias latipes*)

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Abstract

Purpose: **To determine the effect of acute high dose X-rays on the direct and bystander response of chronically exposed medaka in vivo using the fish communication model.**

Methods: **Medaka were obtained from the Low Dose Rate Irradiation Facility (LoDIF) located at the Savannah River Ecology Laboratory (SREL), University of Georgia, Aiken, South Carolina, USA where they had been exposed over 264 days to cumulative total doses of 0, 0.03, 0.66 and 5.88 Gy. They were exposed to the acute dose at McMaster University and then allowed to swim with unexposed medaka. All groups were sacrificed and fins were cultured as explants and assayed using an established technique and reporter assay.**

Results: **Directly irradiated medaka with no chronic exposure showed a classic in vivo bystander response. Chronic pre-exposure resulted in a chronic dose-dependent increase in reporter cell survival in directly exposed fish. A 'pro-survival' response was also seen in the bystander fish. The proteins bcl-2 (b cell lymphoma 2) and c-Myc (myelocytomatosis oncogene cellular) in tissue explants were good predictors of pro-life or pro-death signals.**

Conclusions: **Environmentally relevant chronic exposure to medaka in vivo results in adaptive responses in fish subsequently irradiated with high acute doses and in communication of protective signals to fish swimming with exposed fish. The data have implications for interpretation of radiation effects in biota.**

Keywords: Environmental radiobiology, medaka, chronic radiation dose, biomarkers, bystander effects

Introduction

Radiation-induced bystander effects (RIBE) are now well accepted to occur in vitro and in vivo and are induced by low and high Linear Energy Transfer (LET) radiations, Ultraviolet Light (UV), and Electromagnetic Frequency (EMF) (Banergee et al. 2005, Dahle et al. 2005, Mothersill and Seymour 1997, Mothersill et al., 2007, Nagazawa and Little 1999, Whiteside and McMillan, 2009). The field has recently been reviewed by Hei et al. (2008), Prise and O'Sullivan (2009) and Mothersill and Seymour (2010). RIBE are now classed as one of a group of so-called Non-Targeted Effects (NTE) of radiation which include a number of low dose effects which are like stress responses. The NTE currently accepted include RIBE, Genomic Instability (GI), Adaptive Responses (AR) and low dose hypersensitivity/induced radioresistence (HRS/IRR). The interrelationship between these effects and the mechanisms which underlie them are not clear, nor is their impact on radiation risk although it is likely that bystander mechanisms drive the other low dose non-targeted response through production of signals capable of activating responses in both hit and non-hit cells (see the reviews cited above). Recently it has been shown that bystander signals can be transmitted between vertebrate animals (Mothersill et al. 2007, 2009, Surinov 2004), invertebrates, (Sarapultseva and Bychkovskaya 2010), and plants (Biedrzycki et al. 2010). However, most studies have investigated the effects of acute doses in the mGy–Gy range and the relevance of bystander or stress signals at environmentally relevant chronic low doses is largely unknown. Some experiments using variable but still acute dose rates suggest that protracted dose delivery reduced the magnitude of the 'toxic' bystander effect (Gow et al. 2008, Salbu et al. 2008). Audette-Stuart et al. (2005) showed that frogs taken from a tritium waste pond at Atomic Energy Canada Ltd, Chalk River, Ontario, Canada, had a much less severe effect when subsequently exposed to a high acute challenge dose of 4 Gy, than frogs taken from a clean pond suggesting that a form of adaptive response may be induced by exposure to a chronic dose. Audette-Stuart also found that if tadpoles from the contaminated pond were allowed to swim with unexposed tadpoles, they conferred a protective effect on the clean pond tadpoles (Audette-Stuart, Atomic Energy Canada Ltd. (AECL), Chalk River, Ontario, Canada personal communication with permission). Similar results

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where found in vitro when medium from repair proficient cell lines could 'protect' repair deficient cells from the damaging effects of radiation (Mothersill et al. 2006). This work was confirmed in experiments using repair proficient and deficient Japanese rice fish (medaka; *Oryzias latipes*) (Mothersill et al. 2009). Epidemiological evidence also suggests that people from high background areas have an induced tolerance to acute irradiation (Scott and Di Palma, 2006) but the relevance of bystander mechanisms is unknown. The AR has been defined as 'a biological phenomenon in which resistance to a challenging dose of radiation is established by one or several very small preceding doses' (reviewed in Wolff 1998) or as a 'protective effect after exposure to a low priming dose of a stress inducer' (Olivieri et al. 1984). An adaptive response is thought to be characterised by a reduction in the radiological response of cells pre-treated with a chronic dose before challenge with a higher acute dose (Zhou et al. 2002, Zhou et al. 2004), such as increased cloning efficiency (Skov 1999, Smith and Raaphorst 2003 reviewed in Kadhim et al. 2004). The majority of AR investigations have been carried out using cultured cell lines (reviewed by Joiner et al. 2001 and Marples et al. 2004) and, even in these in vitro models, AR is not consistent (Raaphorst & Boyden 1999). For example; pre-exposing HPV-G cells (Human Papilloma Virus transfected human Keratinocytes) to 'priming' doses of 5 mGy or 0.5 Gy, negated the reduction in cell survival caused by 'challenge' doses of 0.5 Gy and 5.0 Gy, respectively (Maguire et al. 2007) whilst, in contrast, the reduction in RTG-2 and CHSE-214 cell lines (rainbow trout gill and chinook salmon embryo, respectively) cell survival, caused by 2.0 Gy and 5.0 Gy, respectively, was increased if the cells lines were first given a 0.1 Gy priming dose (Ryan et al. 2008a).

The priming dose rates employed in the study reported herein are considerably lower than those used to induce AR in the majority of studies on cultured cells. For example; in addition to the above, 1.7 Gy min^{-1} and 1.8 Gy min^{-1} have been used in CHSE, RTG-2 and ZEB-2J (Zebrafish Embryo) cells (Ryan et al. 2008a), and HPV-G cells (Maguire et al. 2007), respectively, whilst 0.2 (priming) and 0.9 (challenge) Gy min^{-1} have been used in human lymphocytes (Wolff et al. 1991) and 0.42 (priming) and 0.9 (challenge) Gy min^{-1} have been used in human-hamster hybrid cell line (Ueno et al. 1996). Olivieri et al. (1984) were the first to report the AR phenomenon in 1984 using human lymphocytes in vitro. The AR has subsequently been studied by many workers both in vivo and in vitro, including Cregan et al. 1999, Maguire et al. 2007, Ryan et al. 2008a, Smith and Raaphorst 2003, Zhou et al. 2002, Zhou et al. 2004. The field has been well reviewed in Wolff 1996. The relatively few investigations that have been carried out *in vivo* have been largely restricted to mouse embryos (reviewed by Streffer, 2004) or the lifespan studies performed by Mitchel et al. (2008) at AECL. Mostly the term 'adaptive response' applies to specific experiments where a small (10–100 mGy) conditioning acute dose has been given about 5 h before a large acute dose and in radiobiology the term is not usually used to describe the process of evolutionary adaptation to a chronic low dose of radiation (although see Mitchel 2006).

In the experiments reported in this paper, we attempt to extend these investigations by determining the impact of chronic exposure to low doses of cesium-137 on the generation of adaptive responses and bystander signalling in medaka in vivo.

Materials and methods

Fish, husbandry and chronic exposure

Normal healthy fertilised eggs of Japanese medaka were divided into three groups and chronically exposed in the Low Dose Rate Irradiation Facility (LoDIF; Hinton et al. 2004) located at the Savannah river Radioecology Laboratory (SREL), University of Georgia, Aiken, South Carolina, USA, where fish can be maintained outdoors in natural conditions (shallow warm water exposed to natural sunlight; Figure 1). The LoDIF is arranged so that mesocosms have ^{137}Cs sources suspended above them which deliver continuous doses of gamma radiation to the fish. Table I summarises the chronic doses delivered over a 264 day chronic exposure period to the fish at egg, larva and as adult medaka . An additional group was kept in the laboratory as an unexposed control. This was important to establish baseline values for fish because the proximity of the outdoor controls to the tanks where irradiation was being delivered (see Figure 1). The eggs hatched, developed and matured into adult fish under continuous irradiation (refer to Table I) and then were transferred to McMaster University.

Following the chronic radiation exposure regime, described in Table I, the medaka were shipped, by air, to McMaster University. Shipping took 48 h and upon arrival

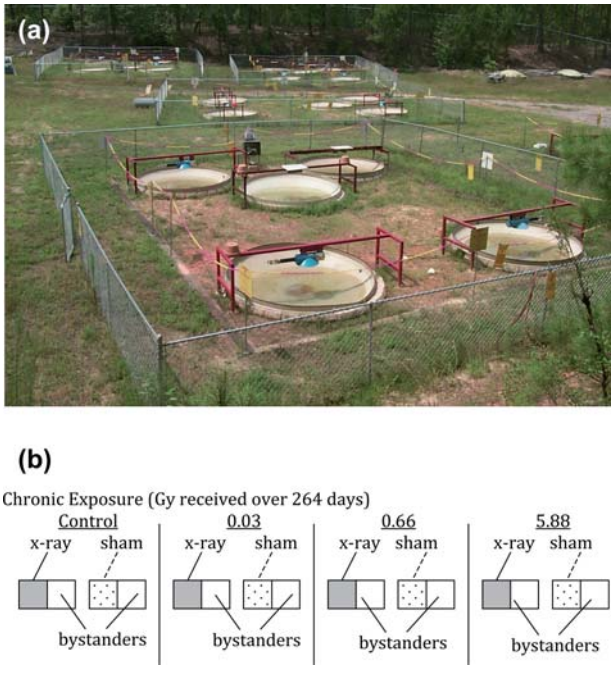

Figure 1. (a) The 'LoDif' chronic irradiation facility at the Savannah River site in South Carolina. (b) Acute X-ray scheme of medaka. Fish were chronically exposed for 264 days, resulting in total doses of 0.03, 0.66 and 5.88 Gy, plus a group of unexposed controls. Cohorts from each chronically exposed group were acutely X-rayed to an additional 0.5 Gy dose, and then placed in aquaria (divided by a mesh screen) with fish that did not receive an X-ray (bystanders). Additional fish were sham X-rayed and also placed in divided aquaria with bystanders.

Table I. Caesium source chronic exposure regime.

Developmental stage	Days of exposure	Control dose rate (mGy day^{-1})	Low dose rate (mGy day^{-1})	Medium dose rate (mGy day^{-1})
Egg	10	0	3.8	34.1
Hatchling	30	0.01	3.1	28.1
Adult	224	0.13	2.3	21.0
Total dose delivered over 264 days (Gy)		0.03	0.66	5.88

the medaka were transferred to 40-litre aquaria. The water in these aquaria was maintained at 26°C, by the use of immersion aquarium heaters (TopFin aquarium products), constantly aerated by means of a diaphragm type air pump (Elite aquarium air pumps) and an airstone diffuser (TopFin aquarium products) and filtered, through external filters (Aquaclear power filter), containing mechanical, chemical and biological components, before being returned to the aquarium via a 'waterfall' water inflow. The fish were held in these aquaria for 2 weeks before starting the X-ray treatment to ensure they had recovered from the shipping stress. Thus, there was a 16-day interval between the end of the chronic radiation exposure and the acute radiation treatment. Note; all aquarium products listed above were supplied by a local retailer (Petsmart, Hamilton, ON).

Acute X-ray treatment and bystander effect induction

A single 0.5 Gy X-ray dose was administered using a portable X-ray machine (Faxitron X-ray Corporation cabinet X-ray system; Wheeling, Illinois, USA), delivering 100 mGy per min, exactly as previously described (Mothersill et al. 2009). This acute exposure took 5 min; i.e., the acute X-ray exposure dose rate was 0.1 Gy min^{-1}. Once X-rayed, the medaka were placed, in groups of five, in plastic holding containers (Ziplock), each containing 500 ml of constantly aerated water (as described above), also maintained at 26°C, by immersion of the holding containers in a temperature-controlled water bath. After a 2-h interval (the time span was chosen based on previous investigations to allow bystander signals from the fish to build up in water) the X-rayed fish were transferred to identical experimental containers (Ziplock), partitioned by a mesh screen (partition mesh size; approximately 3 mm). The X-rayed fish were placed on one side of the partition and a group of five non-irradiated (i.e., zero chronic irradiation and no X-ray exposure) medaka, from the SREL lab control population were placed on the other side to test for partner bystander effects transmitted through the shared water media. These experimental containers also contained 500 ml of constantly aerated water and were again maintained at 26°C (as described above). After 2 h both the irradiated and non-irradiated fish were sacrificed by spinal transection, using protocols approved by McMaster University's Animal Care Committee. Under asceptic conditions the caudal fins were immediately removed (at the peduncle) and placed in ice cold RPMI-1640 (Roswell Park Memorial Institute) culture medium (Gibco Biocult, VWR, Burlington, Ontario, Canada).

Processing of these tissue samples, primary explant culture, assessment of the direct effects of radiation and the bystander effect on apoptosis of the HPV-G reporter cells, and immunostaining the primary cell cultures for bcl-2 (B-cell lymphoma 2) and c-Myc (a transcription factor produced by the myelocytomatosis oncogene) activity have all been previously described and validated (Mothersill et al. 2006, Mothersill et al. 2007) and brief descriptions only are included below.

To evaluate the handling and confinement stress associated with the X-ray procedure and transfer of fish to the holding and bystander containers a full series of sham controls from the appropriate chronic exposure group or the SREL laboratory control population were also included. Sham X-rayed fish were placed in the X-ray cabinet for 5 min (the time required to deliver a 0.5 Gy dose) but without the X-ray being switched on. These sham X-rayed medaka were then used to generate sham partner bystander fish (as described above). Sham tissue samples were collected and processed in the same way. An additional series of bystander fish were generated by removing the x-rayed fish from the water after 2 h and placing non-X-rayed fish from each chronic exposure group in the water. This was to test for waterborne bystander effects and to confirm that visual cues were not leading to the observed effects in the non-exposed fish. The experimental design is depicted schematically in Figure 1b.

Explant cell culture

Explants of medaka caudal fins were established as described previously (Mothersill et al. 2009). Briefly, tissues were dissected asceptically and three equal sized pieces, (approximately 1 mm^2) were plated as single explants in the centre of 25 cm^2 growth area, 50 ml volume flasks (Falcon, VWR, Burlington Ontario, Canada) in 2 ml growth medium. Flasks were left undisturbed for 48 h at 19°C in a refrigerated incubator. All tissue was handled according to biosafety guidelines at McMaster University.

Reporter cell culture

The HPV-G cell line was originally given to us by Dr J DiPaolo, National Institutes of Health (NIH), Bethesda, Maryland, USA, it was obtained at an early passage and expanded and frozen in our laboratory. The cell line has a well-characterised and stable bystander response, showing a reduction in cloning efficiency of ~40% over a wide range of radiation doses, and well-characterised calcium fluxes and mitochondrial effects (Lyng et al. 2002, Maguire et al. 2007). This makes it ideal as a reporter system. All cell culture procedures were performed in a class II biosafety cabinet. The cells were grown in RPMI medium containing 60 ml pre-screened foetal bovine serum (FBS), 5 ml penicillin-streptomycin, 5 ml L-glutamine, 15 mM Hepes buffer, and 1 mg/ml hydrocortisone. All reagents were manufactured by Gibco, Biocult and obtained from VWR, Burlington, Ontario, Canada. The serum was pre-screened to ensure it supported a bystander effect when tested using a positive control x-ray exposure of HPV-G cells.

Clonogenic assay technique and bystander protocol

Cell cultures that were 85–90% confluent and that had received a medium change the previous day were selected.

Cells were removed from the flasks using 0.25% w/v trypsin/1 mM Ethylenediaminetetraacetic acid (EDTA) solution (1:1) obtained from VWR, Burlington, Ontario, Canada. When the cells had detached, they were resuspended in medium, and an aliquot was counted using a Z2 Coulter particle count and size analyser (Beckman Coulter Electronics, Mississauga, Ontario, Canada. Appropriate cell numbers (~500) were plated for the recipient or bystander flasks to optimise the ratio of signal molecules to cell number. Forty eight hours after set up of the explants, medium was poured off from the flasks containing the explants. The medium was filtered through a 0.22-mm filter to remove any debris from the explant culture medium and then added to the cells in the reporter flasks from which the original medium had been removed. Ten days later, colonies of reporter cells were stained with Carbol Fuchsin (Ziehl Nielsson, Sigma) and colonies were counted to determine reporter cell survival.

Immunostaining for bcl 2 and c-Myc activity in explant cultures

Explant cultures were fixed in 10% unbuffered formalin and stored at 21°C until processed. Processing always took place within 7 days of fixation. The culture was processed *in situ* on the flask bottom. Cultures were stained for expression of bcl-2 and c-Myc. The bcl 2 and c-Myc primary antibodies used were mouse monoclonals obtained from DAKO Corporation, Carpentaria, California, USA and Novocastra Laboratories, Richmond Hill, Ontario, Canada, respectively. All were recommended for immunohistochemistry with mouse tissues but have been previously shown to work for rainbow trout and zebrafish (Mothersill et al. 2006, 2007) and were used previously by our group in Ireland to stain a wide range of tissues and species including invertebrates (Lyons, PhD thesis, Trinity College, Dublin, Ireland 1997, O'Dowd PhD thesis, Dublin Institute of Technology, Dublin, Ireland 2010 and personal communication with Dr O'Dowd now at the National C for Marine Biotechnology, Galway, Ireland). It was not possible to confirm definitively that the antibodies worked with medaka using immunoprecipitation or competitive protein binding due to the small size of the medaka tissue and the very limited number of fish available for these experiments. Similarly the small amounts of tissue available precluded a wide screen for up- or down-regulated proteins and we looked therefore for proteins we knew responded from previous studies. Immunochemistry was performed using an appropriate Vectastain Avidin/Biotin System (ABC) Elite kit, Vector Laboratories (Burlingame, USA). Diaminobendizine (DAB) was used to express the positive reaction and cultures were lightly counterstained with Mayers Haematoxylin (both reagents obtained from Sigma). Over 200 cells were scored over five fields using an Olympus image analysis system (ProDiscovery). The detection threshold for positivity was set using positive control sections from positive tissue blocks obtained from the Cell Pathology Service. Positive and negative control sections were carried with every immunocytochemistry run to correct for run variability. This method was established in the laboratory several years ago and is discussed fully in Mothersill et al. (2001). Samples of stained cultures are available as supplementary material (Figures S1 and S2 – online only).

Calculations and statistical analysis

All data are expressed at mean values ± standard deviation (SD). The effects of direct irradiation and bystander effect HPV-G clonogenic data, and survival enhancement factor data, were analysed by analysis of variance (ANOVA) followed by Least Squares Difference (LSD). The figures are therefore annotated to show which data are similar and which are statistically significant; i.e., annotation with the same letter indicates similar data whilst annotation with different letters indicates a statistically significant difference. The relationship between HPV-G clonogenic survival and bcl-2 or c-Myc expression was analysed by linear regression. All statistical analysis was carried out using Statistix analytical software and in all cases a probability of < 0.05 was considered statistically significant.

Results

HPV-G clonogenic survival and data normalisation

The overall plating efficiency of the HPV-G cells used in this study was 32%. This remained unchanged when the cells were treated with explant media from completely untreated control fish; mean (±standard deviation) = 32.5 ± 3.7% (min/max = 28/37%). The plating efficiency of HPV-G cells treated with explant media from non-chronically exposed fish subjected to a sham X-ray and to the corresponding sham waterborne and sham partner bystander fish was 20.5 ± 1.3 (19/22), 23.3 ± 10.2 (14/36) and 23 ± 4.9 (19/29), respectively. Although these plating efficiencies differed numerically with the completely untreated control fish a comparison of the data by ANOVA, revealed these were not statistically significant differences ($P = 0.068$). This allowed the HPV-G survival data to be normalised to either the completely untreated control fish or to the corresponding sham treatments, as appropriate, to fully resolve the effects of the chronic and acute components of the irradiation regimes.

Direct effects and bystander effects induced by the combined chronic/acute irradiation regime

In order to emphasise the direct effect of the acute X-ray exposure on fish which had previously been exposed to the chronic radiation doses the combined irradiation effects data are normalised to the corresponding chronic dose effect. Figure 2 therefore shows direct X-ray HPV-G survival data normalised to the corresponding sham X-ray treated fish. Exposure of medaka to an acute 0.5 Gy X-ray dose only (i.e., no prior chronic radiation exposure) resulted in explant media which reduced HPV-G reporter cell survival (Figure 2). However, when fish which had been chronically exposed to 0.03, 0.6 and 5.88 Gy were then given an acute 0.5 Gy X-ray dose, the general trend was for the effect of the acute dose to be reduced, suggesting that the chronic irradiation induced an adaptive response (Figure 2).

In order to distinguish the bystander effects from the direct effects of exposure to the combined chronic/acute irradiation regime bystander effect data were normalised to completely untreated fish (Figure 3). Media from partner bystander fish, swimming with the medaka which had received the

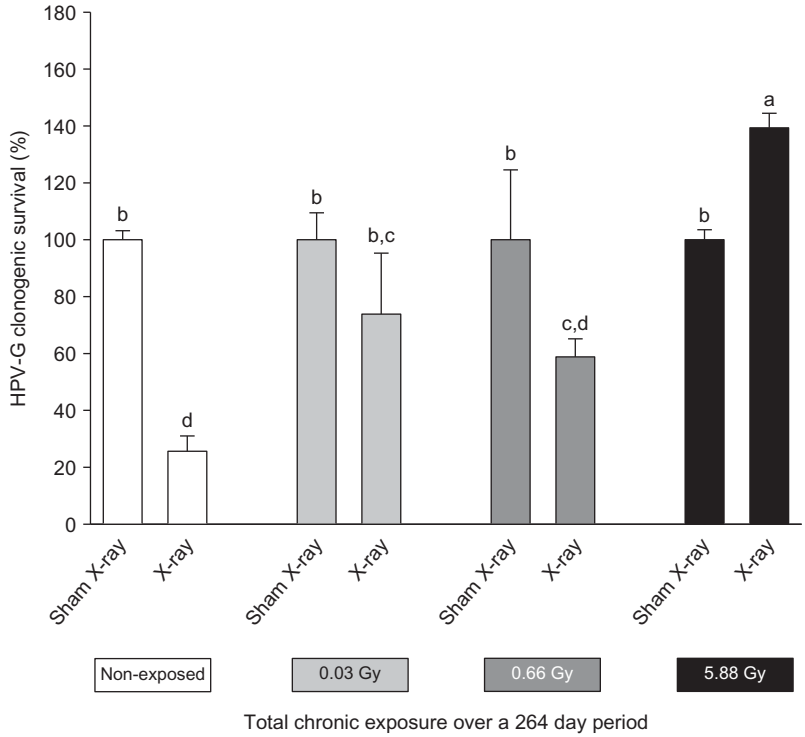

Figure 2. Clonogenic survival of HPV-G reporter cells treated with media from caudal fin epidermis explants from medaka directly exposed to chronic radiation doses, delivered over 264 days, followed by a single 0.5 Gy X-ray treatment, or to explant media from chronically exposed fish receiving bystander signals from medaka exposed to the chronic dose/X-ray treatment regime. Similar letters indicate statistically similar data, different letters indicate a statistically significant difference

acute X-ray dose only (Figure 3a) caused a similar response in the HPV-G cells as fish directly exposed to the X-ray only (Figure 2). However waterborne bystander fish placed in water in which the 0.5 Gy X-ray dose-only irradiated fish had been swimming, but had been removed prior to the introduction of the bystander fish, did not cause a response in the reporter cells (Figure 3b).

Thus, as was the case with the effect of direct exposure to chronic and acute irradiation (Figure 2), the bystander effect induced by these medaka also appeared to be an adaptive response; this applied to both the partner and waterborne bystander fish (Figure 3a and b, respectively). The adaptive effect was most clearly seen in the waterborne bystander group where survival of the reporter cells was significantly greater that the normalised control 100% ($P = 0.0001$). Note; primary data are available online as supplementary information.

In order to illustrate these adaptive responses further, in Figure 4 (a–c) the data are plotted to show the effect of increasing chronic dose to the medaka on the survival of the reporter cells. In each case the data were normalised to the relevant 0 Gy chronic and 0 Gy acute dose sham control. Values for clonogenic survival greater than 100% reflect the fact that the control plating efficiency (PE) for the reporter cells is approximately 32%. Therefore treatments which result in PE greater than 32% result in normalised survival in excess of 100% (i.e., an anti-apoptotic response). For example if a treatment PE is 50%, or 50 colonies returned per 100 cells plated, while the control PE is 32 colonies per 100 cells plated, then the normalised PE relative to the control will be $100/32 \times 50 = 156\%$.

The data show a clear adaptive response in that in directly irradiated fish (Figure 4a) even the 0.03Gy chronic total dose (which was actually a control outdoor tank measuring the scatter dose form the other tanks) leads to production of a significantly increased survival of reporter cells compared with the 0.5 Gy acute dose alone. As the chronic dose is increased the effect of the acute dose is to increase rather than reduce survival. The effect of signals generated by medaka exposed to the chronic dose alone is also to increase reporter cell survival in non-irradiated bystander fish, irrespective of whether the irradiated fish were present (partner bystander; Figure 4b) or had been removed from the water prior to the introduction of the bystander fish (waterborne bystander; Figure 4c).

Bcl-2 and c-Myc expression

In Table II the mean bcl-2 and c-Myc expression is presented for the explant cultures of fish tissue from each treatment group. These proteins have previously been identified by our group as good predictors of the type of radiation response (Mothersill et al. 2001, 2007). In non-irradiated medaka bcl-2 and c-Myc expression were affected by the sham X-ray treatments; bcl-2 expression was reduced and c-Myc expression was increased. This indicates a stress response associated with handling and is discussed later. Treatment with 0.5 Gy X-ray dose only (i.e., no prior chronic radiation exposure) caused a further decrease in bcl-2 expression and a further increase in c-Myc expression. In fact bcl-2 expression was reduced to the point of being undetectable and c-Myc expression was elevated to the extent

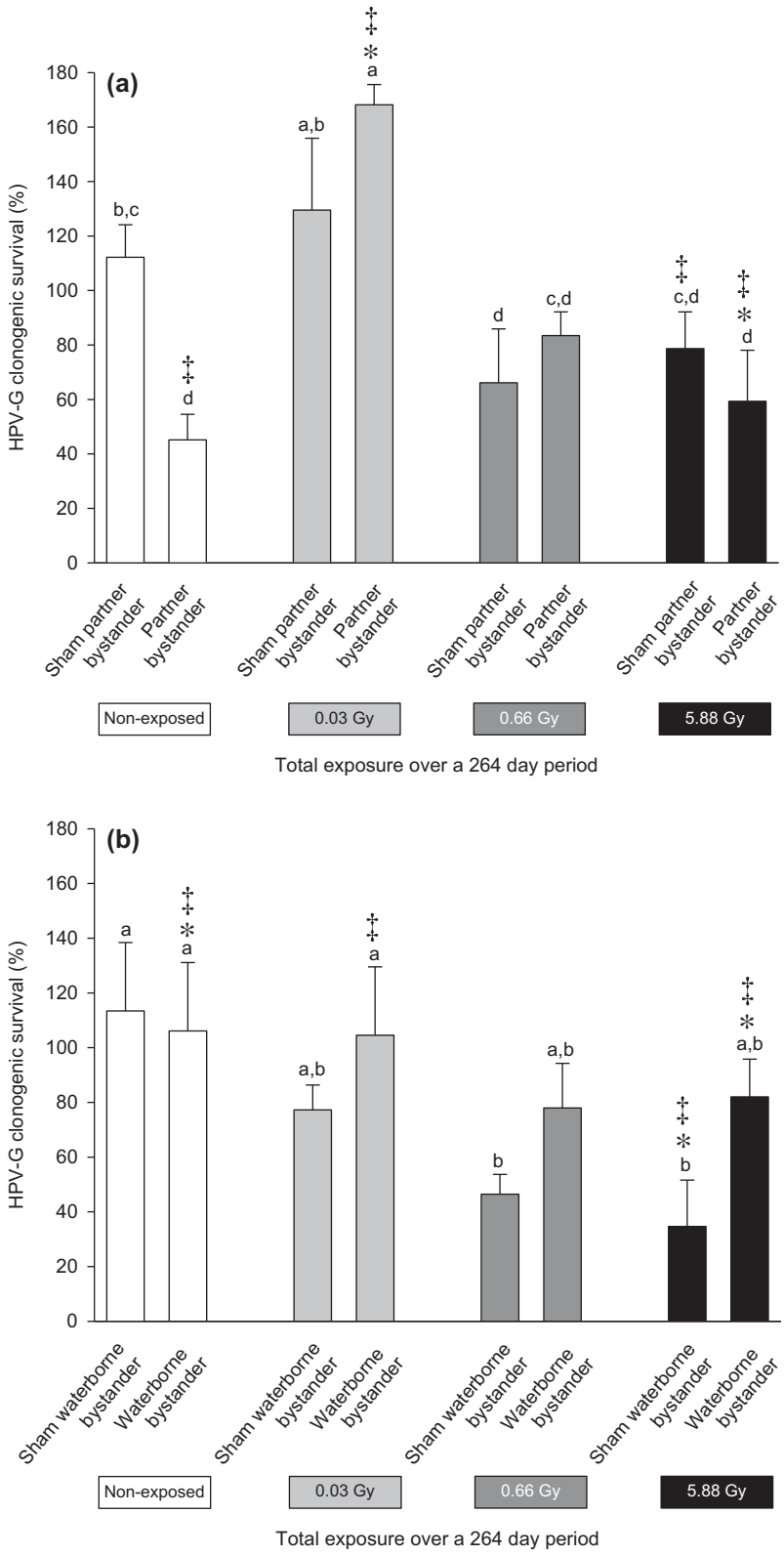

Figure 3. (a) Clonogenic survival of HPV-G reporter cells treated with media from caudal fin epidermis explants from bystander medaka partnered with directly irradiated medaka. Similar letters indicate statistically similar data, different letters indicate a statistically significant difference. * = statistically different clonogenic survival to the corresponding direct irradiation (refer to Figure 2). ‡ = statistically different clonogenic survival to the corresponding waterborne bystander data (refer to Figure 3b). (b) Clonogenic survival of HPV-G reporter cells treated with media from caudal fin epidermis explants from bystander medaka placed in water which had previously held directly irradiated medaka. Similar letters indicate statistically similar data, different letters indicate a statistically significant difference. * = statistically different clonogenic survival to the corresponding direct irradiation (refer to Figure 1). ‡ = statistically different clonogenic survival to the corresponding partner bystander data (refer to Figure 3a).

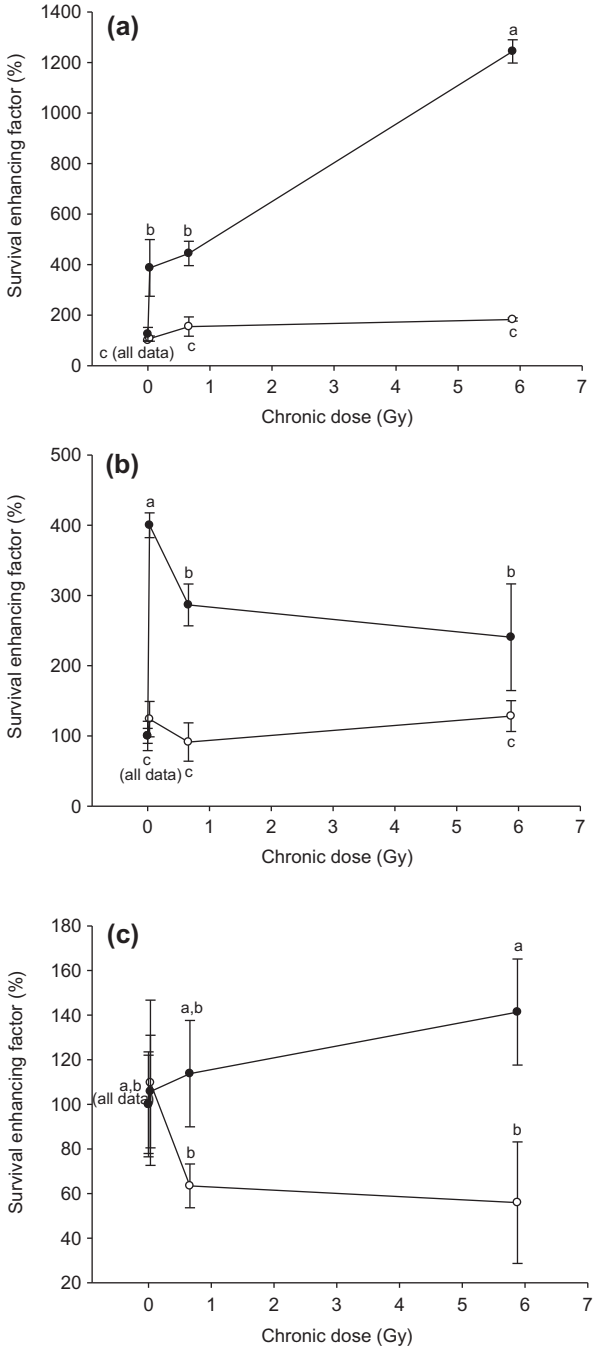

Figure 4. Effect of chronic dose only and chronic dose plus X-ray dose on HPV-G survival. (a) direct irradiation, (b) waterborne bystander effects, (c) partner bystander effects. ○ = chronic dose only. ● = chronic plus X-ray dose. Similar letters indicate statistically similar data, different letters indicate a statistically significant difference.

of being detected in 100% cells counted in explants from the X-rayed fish. Exposure to X-ray only-induced bystander signals resulted in a similar response; bcl2 expression was decreased and c-Myc expression was increased correlating with the clonogenic data from the reporter cells.

In medaka pre-exposed to a chronic radiation dose the sham X-ray treatments had no effect on bcl-2 or c-Myc expression, irrespective of the dose. X-ray treatment of fish previously exposed 0.03 and 0.66 Gy had a similar effect to that observed in non-exposed fish; bcl-2 expression was reduced and c-Myc expression was increased relative to the control but the trend was for the degree of bcl 2 reduction to decrease with increasing chronic dose and the degree of c-Myc increase to decrease with increasing chronic dose. This is consistent with the clonogenic data. In medaka pre-exposed to 5.88 Gy subsequent treatment with a 0.5 Gy X-ray dose markedly increased bcl-2 expression and decreased c-Myc expression.

The response in bystander fish which were previously exposed to a chronic radiation dose differed from those with no chronic exposure. When all data from each of the chronic radiation doses (irrespective of subsequent X-ray or bystander treatment) are combined the bcl-2 expression in the medaka explants was found to be positively correlated and c-myc expression negatively correlated, with HPV-G survival, irrespective of the chronic radiation dose (Figure 5). Thus an increase in bcl-2 expression (Figure 5a, b, c, d) was mirrored by an decrease in c-Myc expression (Figure 5e, f, g, h) and vice versa.

Discussion

Methodology

The main purpose of this paper was to address the question of how chronic irradiation could modify radiation-induced bystander effects using our in vivo fish model (Mothersill et al. 2006). Before discussing the data in this paper, it is important to summarise the advantages of the fish model for determining bystander responses in vivo in small organisms. The technique exploits the sensitive apoptotic pathway of the HPV-G cells (Lyng et al. 2002) which requires fewer and smaller explants to generate meaningful data (Mothersill et al. 2001). In the context of the present investigation this is a considerable advantage since, with small fish such as medaka, the amount of available tissue is very limited. Although there has been debate regarding whether HPV-G clonogenic survival data accurately reflects the response of the cells directly exposed to radiation or to the bystander signal (Mothersill et al. 2001, Mothersill et al. 2006), a recent investigation in vitro has demonstrated the response of the HPV-G cell line to irradiated cell conditioned medium (ICCM) from other cell lines is similar to the response of autologous cells (Ryan et al. 2009). Furthermore the positive and negative correlations between increased HPV-G cell survival and anti-apoptotic bcl-2 expression, and c-Myc expression (which in our experiments is always associated with high levels of apoptosis in the explant cells) confirms our findings in human bladder explants in vitro (Harney et al. 1995), zebrafish in vivo (Mothersill et al. 2007) and in medaka in this paper (Figure 5). This does suggest that the reporter cell line survival did reflect the potential outcome of direct radiation and/or bystander signal exposure on the medaka epidermis. The great advantage of the technique is that using fish to fish transmission of bystander signals, allows the separate mechanisms of bystander response and direct irradiation signal generation to be separately studied in vivo without compromising the mechanistic data by scatter doses or systemic effects which occur when part of an animal only is irradiated.

Table II. Bcl-2 and c-Myc expression in caudal fin epidermis explants from medaka directly exposed to a 264-day chronic radiation dose (as detailed) followed by a single acute 0.5 Gy X-ray treatment, and from bystander medaka exposed to the chronic dose only and then to bystander signals emitted by the medaka exposed to the chronic dose/X-ray treatment irradiation regime. Superscript lettering and Roman numerals indicate statistical differences and similarities in bcl-2 or c-Myc expression within each chronic exposure group. *indicates statistical difference with completely untreated medaka.

	% Bcl-2 expression		% c-Myc expression	
	Mean ± SD	Min/Max	Mean ± SD	Min/Max
Untreated medaka	58.2 ± 16.8	24/76	35.5 ± 21.6	15/76
Zero chronic exposure				
Sham X-ray	$32.0 \pm 2.5^{a,*}$	29/34	$67.0 \pm 2.6^{b,*}$	64/70
Sham X-ray bystander	$30.5 \pm 4.7^{a,*}$	26/37	60.5 ± 8.4^{b}	52/70
X-ray	$0 \pm 0^{c,*}$	0/0	$100 \pm 0^{b,*}$	100/100
Bystander	$11.3 \pm 9.2^{b,*}$	2/21	$92.3 \pm 9.0^{b,*}$	54/100
0.03 Gy chronic exposure				
Sham X-ray	49.8 ± 9.3^{B}	40/60	$58.3 \pm 11.4^{A,B}$	47/69
Sham X-ray bystander	$58.3 \pm 21.4^{A,B}$	40/88	45.5 ± 16.5^{B}	23/61
X-ray	$13.0 \pm 10.2^{C,*}$	0/24	$88.3 \pm 8.7^{A,*}$	80/100
Bystander	73.0 ± 8.9^{A}	60/80	$17.3 \pm 7.4^{C,*}$	7/24
0.66 Gy chronic exposure				
Sham X-ray	50.5 ± 29.2^{i}	25/91	47.8 ± 27.0^{ii}	10/72
Sham X-ray bystander	48.3 ± 28.9^{i}	19/75	58.5 ± 27.2^{ii}	31/87
X-ray	$21.5 \pm 14.8^{i,*}$	0/31	$91.3 \pm 6.6^{i,*}$	84/100
Bystander	48.3 ± 6.29^{i}	41/54	44.8 ± 10.1^{ii}	32/56
5.88 Gy chronic exposure				
Sham X-ray	$60.3 \pm 8.3^{II,III}$	52/70	36.0 ± 12.9^{II}	19/47
Sham X-ray bystander	67.3 ± 23.5^{II}	32/80	$42.8 \pm 21.6^{I,II}$	29/75
X-ray	$100 \pm 0^{I,*}$	100/100	$1.0 \pm 2.0^{III,*}$	0/4
Bystander	38.8 ± 22.2^{III}	21/68	71.3 ± 28.9^{I}	30/97

Combined chronic and acute irradiation, adaptive response (AR) and the bystander effect

The main results from this study are that chronic irradiation at any dose, changes the response of subsequently acutely exposed fish and their bystander fish.

The 'classic' direct irradiation and bystander response, previously seen in X-ray exposed trout (Mothersill et al. 2006) and zebrafish (Mothersill et al. 2007), is seen only in the fish which were not exposed to chronic radiation prior to the acute X-ray dose. It is also interesting that a persistent waterborne bystander signal was not detected. Instead the bystander fish had to be swimming with the directly irradiated fish in order to cause a bystander response in the reporters. This result differs from that seen in trout and zebrafish (using similar biomass:volume ratios) where the signal was retained in water and communicated to a fish placed in that water (Mothersill et al. 2006, Mothersill et al. 2007). We could speculate that the medaka signal is more volatile or is produced in smaller quantities so that a longer time of swimming in proximity is required to induce the bystander response.

Where a chronic dose administered over 264 days precedes acute irradiation the bystander effect (as defined by reporter cell survival) is reduced. This could mean that less bystander signal is produced or that the response to the signal is reduced. It could also mean that an adaptive response leads to the signal inducing pro-life signals in the reporter cells rather than death signals, the result being higher clonogenic survival. The bcl 2 and c-Myc data may support this last hypothesis since there is a trend in the X-ray data for c-Myc expression in the fish tissue to decrease with increasing chronic dose and for bcl-2 expression to increase. While c-Myc has many functions in cell metabolism and can be a pro- or anti-apoptotic protein (Soucek and Evan 2010), in our experience with radiation-induced changes in tissues from many tissues we consistently find bcl-2 expression associated with high post irradiation survival and c-Myc expression associated with low post irradiation survival, in both the human and fish systems we have developed (Harney et al. 1995, Mothersill et al. 2001, Mothersill et al. 2007).

There is clearly a stress response associated with handling the fish because the protein pattern in the sham zero control group is different to the unhandled fish. While this is not ideal (experimentally speaking), it is important to present these control data so that the handling effect can be clearly documented. Induction of an adaptive response by low dose radiation is well documented but as stated in the introduction it normally refers to delivery of a small 'conditioning' dose to the tissue or cells, followed some hours later by a large dose and is thought to result from the fact that conditioned cells have their DNA repair enzymes induced and ready at the time they get the challenge dose (Wolff 1998). However, the situation is reversed in the bystander fish because here the bcl2 expression falls with increasing chronic dose and the c-Myc expression rises. If the explanation for the changes in protein expression is truly induction of an adaptive response, then it is necessary to postulate that an adaptive response for directly irradiated fish is expressed as a pro-survival response in these tissue cells; i.e., decreased expression of apoptosis-related proteins while in the bystanders an adaptive response means induction of an increasing expression of apoptotic proteins with increasing chronic dose. The data for sham treatments and untreated groups supports the idea that active balancing of the two proteins exists until the challenge dose of acute irradiation is applied or communicated because these values are quite similar for all groups. Different gill proteomic responses have been seen in directly irradiated and bystander fish, in trout (Smith et al. 2007) and in medaka (Smith et al. 2011). In both species the bystander fish induced functionally

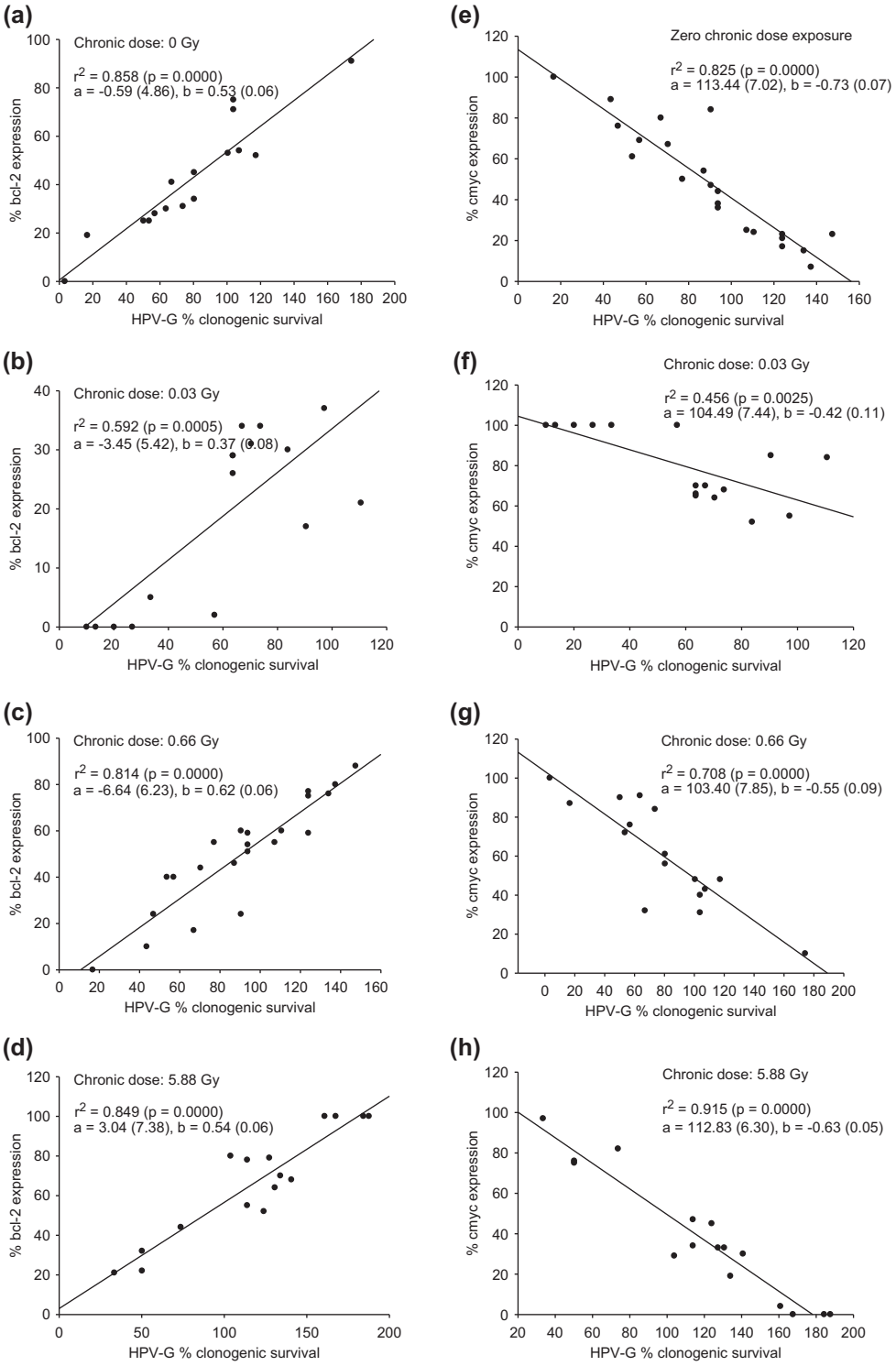

Figure 5. Relationship between bcl-2 and c-Myc expression in medaka fin cells and clonogenic survival of the HPV-G reporter cell line treated with media from fin cell explant cultures. (a) 0 Gy chronic dose bcl-2 expression, (b) 0.03 Gy chronic dose bcl-2 expression, (c) 0.66 Gy chronic dose bcl-2 expression, (d) 5.88 Gy bcl-2 chronic dose expression, (e) 0 Gy chronic dose c-Myc expression, (f) 0.03 Gy chronic dose c-Myc expression, (g) 0.66 Gy chronic dose c-Myc expression, (h) 5.88 Gy c-Myc chronic dose expression.

protective proteomic responses, while directly irradiated fish induced responses associated with tumourgenesis, apoptosis, growth and energy metabolism.

An interesting point for discussion is the fact that the waterborne bystander fish and the partner bystander fish have different levels of response, with the waterborne bystanders showing a greater response following the high chronic radiation dose exposure. At face value this might appear counter-intuitive since partner bystander fish are being constantly exposed to an ever increasing amount of newly generated bystander signal. However one must also recognise that at the time of pairing the irradiated and partner bystander fish are placed in clean water; i.e., there is no signal already present and the signal then builds

up, presumably to similar levels present in the waterborne bystander scenario, over a 2-h period. However when a waterborne bystander fish is placed in water which has already contained an irradiated fish for 2 h (and the signal has already accumulated in the water) it is instantly exposed to elevated levels of bystander signal. Also the water into which the waterborne group of fish are introduced will only contain signals emitted by the directly irradiated fish while the bystander partner fish could have feedback signals from themselves and the directly irradiated fish complicating the situation (which is why the two protocols were done in the first place). There are also interesting issues to consider in relation to the awareness of partner fish of the presence of another fish even though physical contact does not happen. We can speculate that the bystander effects may differ according to the dynamics of bystander signal exposure. More importantly this result illustrates how little we do know about bystander effect induction for different irradiation and different induction regimes.

Hyperradiosensitivity (HRS), adaptive response (AR) and increased radioresistance (IRR)

In addition to adaptive responses and bystander effects induced by radiation the third phenomenon classified as a low dose non-targeted effect HRS, occurs when exposure to a very low dose is more effective in terms of cell killing than larger doses or dose rates (e.g., Joiner et al. 1996). However the data presented here suggest there was no evidence for HRS following chronic exposure only and therefore provide additional evidence that, in spite of the extensive evidence of HRS in vitro (reviewed by Joiner et al. 1996 and by Marples et al. 2004), there is no conclusive evidence of HRS in vivo (Joiner et al. 2001) and that HRS is not universal (Joiner et al. 1996, Marples et al. 2004). Therefore the data presented here are potentially important since chronic exposure to 0.03–5.88 Gy over 264 days did cause an AR in medaka challenged with an acute 0.5 Gy X-ray exposure. This investigation also supports the suggestion that HRS and AR may not be homologous phenomena (Tapio and Jacob 2007).

The effects of the three chronic irradiation doses do follow the classic increased IRR deviation from the linear quadratic model seen at low dose exposure (e.g., Ko et al. 2006, Skov 1999), suggesting IRR was induced in medaka following a chronic exposure of between 0.66 and 5.88 Gy. This compares favourably with the notion that IRR induction occurs at radiation doses of greater than 0.3 Gy (Joiner et al. 1996).

This study also demonstrates that IRR and the classic apoptosis inducing bystander effect are not due to the same mechanism. This is because the increase in pro-life bystander effect correlates with the increase in adaptive (pro-life) response of the directly irradiated fish. This conclusion was also reached by Mothersill et al. (2002) and Ryan et al. (2009) who screened cell lines known to show HRS/IRR at doses where IRR would be induced. These cells never showed bystander effects at these doses suggesting a negative correlation between IRR and pro-death bystander effects. This suggests that IRR, AR and pro-life bystander effects have different underlying mechanisms and consequences to pro-apoptotic bystander effects and HRS.

Conclusion

It is important to distinguish between a bystander effect caused by acute irradiation following direct exposure to low dose chronic radiation and a bystander effect caused by acute irradiation of a non-irradiated bystander to chronic irradiation. The present investigation represents the first of these scenarios, both of which have been investigated in vitro (e.g., Zhou et al. 2002 and Iyer and Lehnert 2002, respectively). Making this distinction is significant since, in radiosensitive human-hamster hybrid cells (A_L), the overall bystander effect, resulting from exposure to low dose X-rays radiation followed by a higher acute dose from the Columbia microbeam, was modulated by the AR to the low irradiation dose; i.e., the priming dose negated the challenge dose (Zhou et al. 2002). Consequently the AR and the bystander effect have been described as conflicting phenomena (Zhou et al. 2002, Zhou et al. 2004) and a weak inverse relationship has been shown between AR and the bystander effect in 13 human cell lines (Mothersill et al. 2002) and in Ryan et al. (2009). Again it is important to stress that classic adaptive response experiments described in the literature deal with acute low dose priming radiation exposure followed by acute high dose challenge a short time later. In these experiments the interval between the 'priming' chronic exposure and challenge dose (16 days) is considerably longer than used for in vitro investigations; e.g., 24 h (Maguire et al. 2007), or 6 h or less (e.g., Ryan et al. 2008b, Schwartz 2007, Ueno et al. 1996; also reviewed by Kadhim et al. 2004 and by Joiner et al. 1996).

The adaptive response described in this paper is more like an evolved adaptive response to environmental conditions. Whatever the underlying mechanisms may be, this paper presents evidence of a chronic-radiation-induced bystander effect in vivo at radiation exposures relevant in the environment.

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Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Figures S1 and S2 and Raw data