Radiation Measurements 44 (2009) 1077–1080

Contents lists available at ScienceDirect

Radiation Measurements

journal homepage: www.elsevier.com/locate/radmeas

Alpha-particle-induced bystander effects between zebrafish embryos *in vivo*

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ARTICLE INFO

Article history:
Received 13 October 2008
Received in revised form
18 May 2009
Accepted 18 October 2009

Keywords:
Bystander effect
Alpha particles
Zebrafish embryos
PADC

ABSTRACT

Dechorionaed embryos of the zebrafish, *Danio rerio*, at 1.5 h post-fertilization (hpf) were irradiated with alpha particles from an ^{241}Am source. Thin polyallyldiglycol carbonate (PADC) films with a thickness of 16 μm were used as support substrates for holding the embryos and recorded alpha-particle hit positions, and thus enabled calculation of the dose absorbed by the embryos. The irradiated embryos were subsequently incubated with naïve (unirradiated) embryos in such a way that the irradiated and naïve embryos were spatially separated but the medium was shared. Acridine orange was used to perform in vital staining to show cell deaths in the naive embryos at 24 hpf. Our results gave evidence in supporting the existence of alpha-particle-induced bystander effects between zebrafish embryos *in vivo*, and a general positive correlation between the cell death signals in the naive embryos and the alpha-particle dose absorbed by the irradiated embryos.

1. Introduction

Radiation-induced bystander effects in cells refer to biological effects that the unirradiated cells respond as if they have been irradiated, when they are put in contact with the irradiated cells or in the medium previously holding the irradiated cells (e.g., Yang et al., 2005). Radiation-induced bystander effects have aroused immense interests because they can involve new biological mechanisms and can have significant implications on radiological protection. For example, low doses of ionizing radiation can become more harmful than previously thought due to the presence of bystander effects Hall and Hei 2003; Lorimore et al. 2005).

There is a large amount of literature describing or reviewing the radiation-induced bystander effects *in vitro* (see e.g., Morgan 2003a; Mothersill and Seymour 2004; Lorimore and Wright 2003; Little and Morgan 2003). However, the *in vivo* relevance and/or *in vivo* persistence are always questioned. Furthermore, *in vitro* experiments cannot be used to study allelopathic effects among individual animals or dynamic *in vivo* processes, e.g., temporally and spatially regulated patterns of gene expression. As such, it is always tempting to study the effects through *in vivo* experiments. Recently, there have been researches on bystander effects *in vivo* in mice and in fish (Morgan 2003b; Surinov et al., 2004; Mothersill et al. 2006, 2007). In particular, Mothersill et al. (2006, 2007) demonstrated X-ray-induced *in vivo* bystander effects in two non-related freshwater fish, namely, the rainbow trout (*Oncorhynchus mykiss*, W) and the zebrafish (*Danio rerio* L), and suggested that communication signals involved secretion of a chemical messenger into the water, which could then be passed from the "irradiated" fish to the "naive" fish to cause the bystander effects.

With the observations of such X-ray-induced bystander effects, it is pertinent to study whether alpha particles can also induce *in vivo* bystander effects, which forms the main objective of the present work. Alpha particles are ubiquitous in our environment. For example, alpha particles are emitted from members of the naturally occurring radioactive series.

In the present work, embryos of the zebrafish, *Danio rerio*, were employed. One advantage was their rapid development so the effects can be assessed within 24 h post-fertilization (hpf). The first challenge was the dechorionation of the embryos before alpha-particle irradiation, since the chorions would absorb the alpha-particle energies. The second challenge was the characterization of the alpha-particle dose absorbed by the embryo cells. The dechorionated embryos were first transferred into a custom-made thin-PADC-film based holder. Polyallyldiglycol carbonate (PADC) films (commercially available as CR-39 films) were used as the substrate because they were biocompatible (e.g., Li et al., 2006) and their thickness could be controlled precisely through chemical etching to control the residual alpha-particle energy incident onto the embryos (Yum et al., 2007). Most importantly, the alpha particles incident on the embryo cells could be recorded through their tracks revealed upon subsequent chemical etching, so the absorbed alpha-particle dose could be quantified. PADC films are one of the most commonly used solid-state nuclear track detectors

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doi:10.1016/j.radmeas.2009.10.025

(SSNTDs), on which a recent review has been given by Nikezic and Yu (2004).

The present work also aimed at a further development in assessing *in vivo* radiation-induced bystander signals. Here, the bystander effects were characterized by the number of cell deaths in the whole naive zebrafish embryos at 24 hpf revealed by vital dye staining. This avoided the relatively tedious procedures to use and culture explants from affected fish using *in vitro* clonogenic reporter-cell-line assay, as stipulated by Mothersill et al. (2006, 2007). With the direct assessment of bystander effects in the naive fish themselves, we also tried to identify the relationship between the absorbed alpha-particle dose and the bystander effect.

2. Materials and methods

2.1. Alpha-particle irradiation of zebrafish embryos

To control the incident alpha-particle energy, the alpha particles should pass through the PADC substrate which was in contact with the embryo cells (see Fig. 1), instead of passing through the fluid layer above the embryos, which had a variable thickness. We prepared thin (16 μm) PADC films from commercially available CR-39 films with a thickness of 100 μm (from Page Mouldings (Pershore) Limited, Worcestershire) by chemical etching in NaOH/ethanol (Chan et al., 2007). The film thickness was monitored using a micrometer (Mitutoyo, Japan) with an accuracy of ±1 μm. Most of the background counts in PADC films was due to defects present on the surface, and would thus be effectively eliminated on such heavy etching (Mishra et al., 2005).

The thin PADC films were glued by an epoxy (Araldite® Rapid, England) to the bottom of a custom-made holder made of acrylic resin with 8 × 6 holes drilled at the bottom. The holes had a diameter of 2 mm, and the holes were separated at 8 mm.

Alpha-particle irradiation of the dechorionated zebrafish embryos were made with a planar ^{241}Am source (with an alpha particle energy of 5.49 MeV under vacuum and an activity of 0.1151 μCi) from the side of the PADC film (air distance = 1 mm) at 1.5 hpf for 4 min. At this developmental stage, the cells have not assumed differentiated cell fates. This time point (1.5 hpf) is also within the cleavage period of embryogenesis (0.7–2.2 h) (Kimmel and Law 1985; Kimmel et al. 1995). Walker and Streisinger (1983) found that embryos older than 3 h were more resistant to γ-rays, which suggested a possible repair mechanism after cleavage stages.

2.2. Bystander signal exposure

After irradiation, the embryos were transferred to an agarose plate, which was then incubated at 37 °C with naïve embryos (unirradiated embryos having partnered with the irradiated embryos) in the same plate containing 3 ml medium. The irradiated and naïve embryos were accommodated in two shallow regions dredged in the agarose, separated by a small ridge. This physical setting separated the irradiated and naïve embryos spatially but allowed sharing of the same medium. This enabled subsequent studies on the irradiated and naive embryos separately while at the same time bystander signals, if any, could be transferred from the irradiated embryo to the naive embryos through the water medium. There were two stages of experiments. In the first stage, in which we aimed at proving the existence of alpha-particle-induced bystander effects between zebrafish embryos *in vivo*, 8 irradiated embryos were incubated with 8 naive embryos in the agarose plate. In the second stage, in which we aimed at identifying the relationship between the alpha-particle absorbed dose and the bystander effect, one irradiated embryo was incubated with 5 naive embryos in the agarose plate.

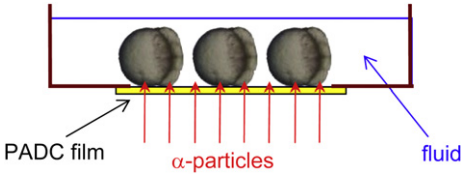

Fig. 1. Alpha-particle irradiation of zebrafish embryos through a PADC-film based holder.

2.3. Cell deaths revealed through staining with acridine orange

At 24 hpf, the embryos were collected and examined for cell death by vital dye staining according to the method described by Chan and Cheng (2003). Briefly, the embryos were transferred to a culture medium containing 5 μg/ml of acridine orange. Embryos were stained for 60 min followed by thorough washing in the culture medium for three times. Embryos were then anaesthetized with 0.016 M tricaine (Sigma, St. Louis, MO, USA). Images of the stained embryos were captured under a florescence microscope. The number of cell deaths for each embryo was counted with the help of the software MetaMorph Version 7.0r0 (1992–2006 Molecular Devices). Before 24 hpf, the untreated zebrafish embryos undergo high apoptotic activities as part of the organogenesis processes (Chan and Cheng 2003). The 24 hpf endpoint was also used by Bladen et al. (2005) who commented that increasing pigmentation after 24 hpf might obscure the signals from the cell deaths.

2.4. Determining the absorbed dose of the embryos

The dose absorbed by embryo cells was required in our second stage experiments. Before the embryos were irradiated, their images were captured using a digital camera attached to an inverted microscope with a magnification of 200×. Our experimental setup was specially designed in a way that the lens of the microscope and the radioactive source holder (below the sample) could be interchanged without disturbing the sample (embryos). In this way, after the images of the embryos were captured, by simply changing the lens of the microscope to the radioactive source, irradiation was performed.

After the irradiated embryos had been transferred from the PADC-film-based resin holder to the agarose plates, the PADC films were etched in 6.25 N NaOH at 70 °C for 3 h. Images of the etched PADC films with visible alpha-particle tracks were captured using the same digital camera with the same magnification of 200×. The images of the embryos and of the alpha-particle tracks were superimposed using Ulead® Photoimpact® Version 8.0 (1992–2002, Ulead Systems, Inc).

The number of alpha-particle tracks in the areas of the embryo cells was counted using the software MetaMorph Version 7.0r0 (1992–2006 Molecular Devices). Although this procedure was tedious and time consuming, this was indispensable since the size and shape of each cell, and the contact area with the PADC film varied significantly. Considering the distribution of residual energies of alpha particles which could or could not generate visible tracks on the PADC film under our etching conditions, we calculated through Monte Carlo simulations the average exit energy (after crossing the PADC film) per one registered alpha-particle track as 3.4 MeV. This is not very different from the exit energy of an alpha particle normally incident onto the 16 μm PADC film, which is 3.49 MeV determined using the SRIM program (Ziegler, 2003). The zebrafish embryos at 1.5 hpf are having 2 layers of 4 cells, i.e., a total of 8 cells. The average mass of a zebrafish embryo at 1.5 hpf was

determined from 300 dechorionated embryos (see Hagedorn et al. 1997) as 222 ± 6 μg (one standard error). The absorbed dose for each embryo was then obtained by dividing the absorbed energy with the average mass. In our experiments, the absorbed doses for the embryos were smaller than 2 mGy.

2.5. Relationship between alpha-particle absorbed dose and the bystander effect

With the proof of alpha-particle-induced bystander effects between zebrafish embryos *in vivo*, and with a feasible method to characterize the alpha-particle dose absorbed by the embryo cells together with a direct technique to assess the bystander effects in the naïve zebrafish embryos, we are in a good position to explore the relationship between the alpha-particle dose absorbed by the irradiated embryos and the bystander effect in naïve zebrafish embryos. Three sets of experiments were performed on three separate days.

3. Results and discussion

3.1. Bystander effects between zebrafish embryos in vivo

As described in Section 2.2, 8 irradiated embryos were incubated with 8 naive embryos in the agarose plate. There were two independent experiments here and the results are shown in Table 1.

For the set-1 experiment, the irradiated embryos showed significantly more cell death signals than the control embryos (unirradiated embryos not having partnered with irradiated embryos) ($p < 0.04$) while at the same time the naive embryos also showed significantly more cell death signals than the control naive embryos (unirradiated embryos having partnered with the control embryos) ($p < 0.05$).

For the set-2 experiment, the irradiated embryos also showed more cell death signals than the control embryos although the difference was not statistically significant ($p = 0.10$), which was due to the small number of remaining irradiated embryos. Nevertheless, the naive embryos still showed significantly more cell death signals than the control naive embryos ($p < 0.05$). These results gave evidence to the existence of alpha-particle-induced bystander effects between zebrafish embryos *in vivo*.

3.2. Relationship between alpha-particle absorbed dose and the bystander effect

There were three independent experiments and the results are shown in Fig. 2. The positive slopes shown by the best-fit lines (although only that for dataset 1 was significant) suggested an increase in the cell death signals in the naive embryos with the alpha-particle dose absorbed by the irradiated embryos. The relatively wide 95% confidence intervals were in part due to the small sample size.

Table 1
Average number of cell deaths (N) obtained for different embryos (Control, Irradiated, Control naive and Naive) in two sets of experiments. n: number of embryos used in the analyses ($n < 8$ in some cases due to death of embryos); p: p values obtained using t-tests by comparing with the corresponding controls (i.e., Irradiated embryos compared with Control embryos, and Naive embryos compared with Control naive embryos). The irradiated embryos were obtained by irradiating the embryos using a 0.1151 μCi ^{241}Am source for 4 min.

		Control	Irradiated	Control naive	Naive
1	N	71.8	111.6	56.4	79.6
	n	8	8	7	8
	p		0.0358		0.0469
2	N	69.4	98.0	77.9	137.6
	n	8	4	8	6
	p		0.1038		0.0498

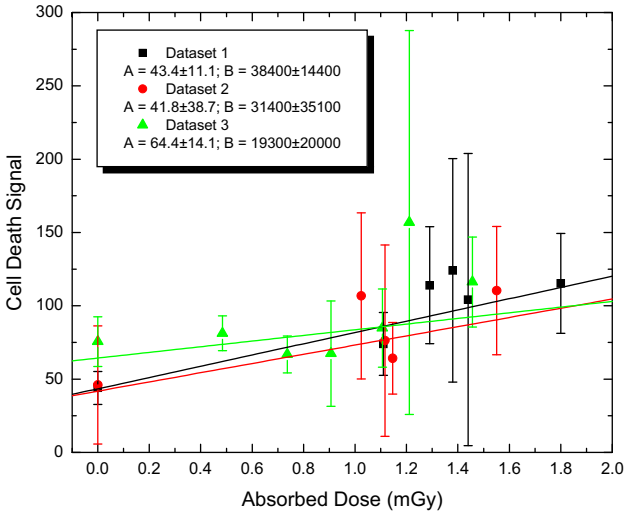

Fig. 2. The results for three sets of experiments on the relationship between the cell death signals in the naive embryos with the alpha-particle dose absorbed by the irradiated embryos. The linear best-fit lines are also shown with the their parameters (A as intercepts and B as slopes) and the corresponding 95% confidence intervals.

It is natural to consider combining the data. However, for different sets of experiments, the number of cell deaths for the control naïve embryos (i.e., those corresponding to 0 mGy alpha-particle irradiation), might present a challenge. The numbers of cell deaths in the control naïve embryos for datasets 1 to 3 were 44, 46 and 75.6, respectively. Dataset 3 might need some transformations before it could be combined with the other two datasets. Without better proposals, we subtracted all the values in dataset 3 by 30.6 ($=75.6 - 45$), so that the value for its control naïve embryos became coincident with the mean value (45) for the control naïve embryos for datasets 1 and 2. The data and results were shown in Fig. 3. The positive slope is evident and the corresponding 95% confidence interval has become narrower. This further supported the increase in the cell death signals in the naive embryos with the alpha-particle dose absorbed by the irradiated embryos.

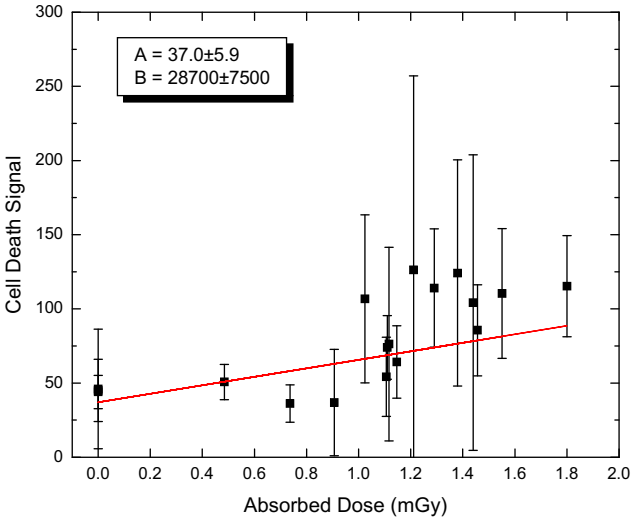

Fig. 3. Results on the relationship between the cell death signals in the naive embryos with the alpha-particle dose absorbed by the irradiated embryos, by combining data from three sets of experiments. The linear best-fit line is shown with the parameters (A as intercept and B as slope) and the corresponding 95% confidence intervals.

Despite the positive correlation identified between the cell death signals in the naive embryos and the alpha-particle dose absorbed by the irradiated embryos, a different trend in the low-dose region (i.e., <1 mGy) could not be ruled out because of the relatively smaller number of data points in this region. Data points here were not substantially higher than the values for the control naïve embryos; two were even lower. Previous research also indicated different biological effects of radiation at small doses. For example, Miyachi et al. (2003) attempted to study the effect of low doses of X-ray on zebrafish development and found a significant decrease in time to hatching following exposures of the zebrafish embryos to 0.025-Gy X-ray irradiation during the cleavage period (1.5 hpf), but this radiation-induced effect was eliminated when the dose was increased to 0.15 Gy. Bladen et al. (2005) found an absence of ectopic apoptotic cell death in zebrafish embryos irradiated to 0.15 Gy gamma rays at 6 hpf, but a large amount at a 3-fold higher dose, and the authors suggested the existence of a threshold below which radiation-induced cell death did not occur. On the other hand, Ryan et al. (2008) found that cells derived from mid-blastula-stage zebrafish (*Danio rerio*) embryonic stem cells exceedingly sensitive to ~0.1 Gy gamma radiation. Further studies and probably a much larger amount of data for the low dose regime would be necessary before a more definite conclusion on the relationship between alpha-particle absorbed dose and the bystander effect for this low dose regime can be made.

4. Conclusions

Our results showed the existence of alpha-particle-induced bystander effects between zebrafish embryos *in vivo*, and a positive correlation between the cell death signals in the naive embryos and the alpha-particle dose absorbed by the irradiated embryos. These supported that communication signals leading to a radiation response could be passed between zebrafish embryos and that the bystander factors involved chemical messengers secreted into the water medium.

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