Development and use of an adoptive transfer method for detecting radiation-induced bystander effects *in vivo*

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Doctor of Philosophy

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Summary

Ionising radiation can cause damage to DNA that can result in gene mutations contributing to carcinogenesis. Radiation-protection policy currently estimates cancer risks from exposures to radiation in terms of excess risk per unit dose. At very low radiation dose-rates, where not all cells are absorbing radiation energy, this formula carries the inherent assumption that risk is limited to those cells receiving direct energy depositions. Numerous studies have now called this assumption into question. Such low dose-rates are in the relevant range that the public receives from natural background and man-made sources, and, if this fundamental assumption proves unfounded, current estimations of radiation-induced cancer risk at low doses will be incorrect. Accurate predictions of stochastic cancer risks from low-dose radiation exposures are crucial to evaluating the safety of radiation-based technologies for industry, power generation and the increasing use of radiation for medical diagnostic and screening purposes.

This thesis explores phenomena known as radiation-induced bystander effects. The term bystander effects, as used here, describes biological responses to ionising radiation (hitherto observed *in vitro)* in cells not directly traversed by an ionising track, due to intercellular signals received from neighbouring cells that did receive energy depositions. This study aimed to determine whether radiation effects are communicated between irradiated and unirradiated cells *in vivo*, and if so, whether this effect alters current estimations of cancer risk following low-dose radiation exposures. In order to answer these questions, an *in vivo* experimental system for studying bystander effects in mice was developed. The method was based on the adoptive transfer of irradiated splenocytes into unirradiated hosts with simultaneous

identification of irradiated donor cells, and biological endpoints in unirradiated bystander cells *in situ* using fluorescence microscopy and image analysis.

Splenocytes from donor mice were radiolabelled with ^{3}H-thymidine or received an acute X-ray dose. The irradiated donor cells, labelled with a fluorescent probe, were then adoptively transferred into unirradiated recipient mice via the tail vein, whilst control mice received sham-irradiated donor cells. A proportion of the cells lodged in the recipient mouse spleens where they remained for a period before the tissues were cryopreserved. The locations of donor cells were identified in frozen spleen sections by the fluorescent probe, and the levels of apoptosis and proliferation were simultaneously evaluated *in situ* in the surrounding unirradiated bystander cells using fluorescence-based assays. Transgenic pKZ1 recipient mice were also used to quantify chromosomal inversions in bystander cells. Since three-dimensional spatial relationships were preserved, responses could be measured in the local area surrounding irradiated cells as well as further afield. Following the development of the irradiated-cell adoptive transfer protocol and validation of the sensitivity and reproducibility of the biological assays *in situ*, a series of experiments was performed. In the initial experiments, 5×10^{5} radiolabelled cells ($0.33\ \mathrm{mBq.cell^{-1}}$) were injected into recipient mice and the spleen tissues were isolated 22 h later. No changes in apoptosis or proliferation were detected in local bystander spleen cells or throughout the spleen, compared to mice receiving sham-radiolabelled donor cells. In subsequent experiments, the effects of a number of experimental conditions were explored including the injection of tenfold more donor cells, analysis of spleen tissues after three days lodging *in vivo*, radiolabelling of donor cells with 100-fold higher ^{3}H dose-rate and irradiation of donor cells *ex vivo* with 0.1 or 1 Gy X-rays. In each case, no changes in apoptosis or proliferation were observed.

The *in vivo* method described here was designed to simulate the conditions of a bystander scenario from low dose-rate exposures relevant to public radiation protection. Contrary to the many reports of bystander effects *in vitro*, experiments using this sensitive method for examining the *in vivo* responses of unirradiated cells to neighbouring low-dose irradiated cells, have so far shown no changes in bystander cells in the spleen. This adoptive transfer method is the first *in vivo* method for examining the effects of known irradiated cells exposed to low radiation doses at low dose-rates, on neighbouring cells *in situ* that are truly unirradiated. Both the irradiated and bystander cells are normal, non-transformed primary spleen cells functioning in their natural environment. This *in vivo* experimental system allows the examination of tens of thousands of bystander cells and has shown a remarkable sensitivity, with statistical power to rule out changes in apoptosis >10% from the control.

The relevance of *in vitro* bystander findings is unclear. Many reported bystander effects are more analogous to the systemic communication of abscopal effects from highly irradiated tissues. Disagreement between experimental systems and difficulty in reproducing key results between laboratories further complicate the translation of bystander data *in vitro* to human risk-estimation. The radiation protection community has expressed its need for *in vivo* validation of the bystander phenomenon before it can be included into the appraisal of carcinogenic risk. This adoptive transfer method is now available to study a range of bystander endpoints and potential signalling mechanisms *in vivo*, and provides a way to translate the wealth of data previously collected *in vitro* into findings directly relevant to human risk-estimation.

Candidate's Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Benjamin Blyth

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Abbreviations

BrdU	Bromodeoxyuridine
CHO	Chinese hamster ovary
CMRA	CellTracker™ Orange CMRA
ConA	Concanavalin A
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
FITC	Fluorescein isothiocyanate
GJIC	Gap-junctional intercellular communication
HPRT	Hypoxanthine-Guanine Phosphoribosyl Transferase
ICCM	Irradiated cell–conditioned medium
ICRP	International Commission on Radiological Protection
LD_{50}	Lethal dose (50%)
LET	Linear energy transfer
LNT	Linear no-threshold
LPS	Lipopolysaccharide
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
ROS	Reactive oxidative species
RPMI 1640	Rose Park Memorial Institute cell culture medium #1640
SCE	Sister chromatid exchange
SCM	Splenocyte culture medium
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
γ-H2AX	γ-variant of histone H2AX

Publications & abstracts arising during PhD candidature

Publications

Zeng, G, Day, TK, Hooker, AM, Blyth, BJ, Bhat, M, Tilley, WD and Sykes, PJ. 2006. "Non-linear chromosomal inversion response in prostate after low dose X-radiation exposure." *Mutation Research* 602(1-2): 65-73.

Hooker, AM, Grdina, D, Murley, J, Blyth, BJ, Ormsby, R, Bezak, E, Giam, K and Sykes, PJ. 2009. "Low doses of amifostine protect from chromosomal inversions in spleen in vivo when administered after an occupationally relevant X-radiation dose." *International Journal of Low Radiation* 6(2).

Publications in preparation

Blyth, BJ, Azzam, EI, Howell, RW and Sykes, PJ. "A Novel *in vivo* Method to Detect Radiation-Induced Bystander Effects in Normal Mouse Spleen"

Blyth, BJ, Ormsby, RJ, Staudacher, AH, Dreimanis, M and Sykes, PJ. "Chronic low-dose irradiation from incorporated radionuclides does not alter the fate of bystander cells in mouse spleen"

Oral Presentations

'Identifying the fate of low dose irradiated cells *in vivo*' BJ Blyth & PJ Sykes, *Radiation 2006- Australian Institute for Nuclear Science and Engineering*, Sydney, Australia (April, 2006)

'Can bystander signalling really change the carcinogenic risk of unirradiated cells *in vivo*?' BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *DOE Low Dose Radiation Research Investigators' Workshop VII*, Washington DC, USA (January, 2008)

'Bystander Effects: A Risk or an Opportunity?' BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *Modelling of Tumours Meeting*, Adelaide, Australia (June, 2008)

'Determining the impact of radiation-induced bystander effects on low dose radiation protection *in vivo*' BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *Australian Radiation Protection Society Conference*, Canberra, Australia (September, 2008)

'Bystander Signalling *In Vivo*: Rising above the noise' BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *LOWRAD 2008*, Lisbon, Portugal (November, 2008)

Poster Presentations

'Identifying Non-linear Radiation Dose Responses In Vivo: Exploring Bystander Effects' BJ Blyth, TK Day, PJ Sykes, *BELLE Conference*, Amherst, USA (May, 2006)

'Low dose radiation exposure: exploring bystander effects *in vivo*' BJ Blyth & PJ Sykes, *DOE Low Dose Radiation Research Investigators' Workshop VII*, Washington DC, USA (August, 2006)

'An *in vivo* model for detecting radiation-induced bystander effects: shedding light on tissue responses to low dose radiation' BJ Blyth & PJ Sykes, *Australian Society for Medical Research SA Scientific Meeting*, Adelaide, Australia (June, 2007)

‘Low dose radiation-induced bystander effects in the spleen’ BJ Blyth, EI Azzam, RW Howell & PJ Sykes, *International Conference of Radiation Research*, San Francisco, USA (July, 2007)

‘Low dose radiation-induced bystander effects in the spleen’ BJ Blyth & PJ Sykes, *Conference on the Normal Tissue Radiation Effects*, Las Vegas, USA (July, 2007)

‘Determining cancer risks after low-dose radiation exposures’ BJ Blyth, AL Cochrane & PJ Sykes, *Lorne Cancer Conference*, Lorne, Australia (February, 2008)

‘Studying intercellular signalling after low dose radiation exposures *in vivo’* BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *Australian Society for Medical Research SA Scientific Meeting*, Adelaide, Australia (June, 2008)