Development and use of an adoptive transfer method for detecting radiation-induced bystander effects \textit{in vivo}

A thesis submitted in fulfilment of the requirements of the

\textit{Doctor of Philosophy}

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Contents

FIGURES .................................................................................................................. III

TABLES ..................................................................................................................... V

SUMMARY ................................................................................................................. VI

CANDIDATE’S DECLARATION ................................................................................ IX

ACKNOWLEDGEMENTS .......................................................................................... X

ABBREVIATIONS ...................................................................................................... XII

PUBLICATIONS & ABSTRACTS ARISING DURING CANDIDATURE ..................... XIII

CHAPTER 1: PROBLEM STATEMENT ................................................................. 1

Problem Statement .................................................................................................. 1
Aim and Scope ......................................................................................................... 1
Overview of the Study .............................................................................................. 2

CHAPTER 2: INTRODUCTION AND BACKGROUND .......................................... 4

Ionising radiation ..................................................................................................... 4
The radiation risk assessment paradigm .................................................................. 6
Radiation-induced bystander effects ....................................................................... 15
Bystander effects: moving from phenomenon to risk ............................................. 40
Summary .................................................................................................................. 44

CHAPTER 3: RESEARCH INTENT AND DESIGN .............................................. 45

Précis ......................................................................................................................... 45
Research questions ................................................................................................. 45
Research method and hypotheses .......................................................................... 46
Summary .................................................................................................................. 49

CHAPTER 4: DEVELOPMENT OF THE ADOPTIVE TRANSFER METHOD FOR DETECTING BYSTANDER EFFECTS IN VIVO ..................................................... 50

Précis ......................................................................................................................... 50
Initial decisions in the development of the in vivo bystander method ......................... 50
Donor splenocytes: Chronic radiolabelling experiments ......................................... 59
Donor splenocytes: Acute X-irradiation ................................................................... 90
Recipient Mice ......................................................................................................... 95
Pilot adoptive transfer experiments .......................................................... 99
Analysis of recipient mouse spleen tissues ........................................... 105
Biological endpoints as candidates for induction in bystander cells ........ 116
Experimental design ........................................................................... 139

CHAPTER 5: USE OF THE ADOPTIVE TRANSFER METHOD TO STUDY BYSTANDER EFFECTS IN VIVO ................................................... 143

Précis ........................................................................................................ 143
Detection of bystander effects from chronically irradiated lymphocytes lodged in unirradiated mouse spleens .......................... 144
Detection of bystander effects from increased numbers of chronically irradiated lymphocytes ...................................................... 157
Detection of bystander effects from chronically irradiated lymphocytes after longer-term lodging in vivo ........................................ 164
Detection of bystander effects from high dose-rate chronically irradiated lymphocytes ................................................................. 174
Detection of bystander effects from splenocytes exposed ex vivo to X-radiation .............................................................. 178
Summary ................................................................................................. 181

CHAPTER 6: EVALUATION OF THE ADOPTIVE TRANSFER BYSTANDER METHOD AND ITS INITIAL FINDINGS .............................................. 184

Précis ........................................................................................................ 184
Assessing the research findings .............................................................. 184
Suitability of the adoptive transfer method ........................................... 186
Reliability of the adoptive transfer method ........................................... 198
Generalisability of the results obtained using the adoptive transfer method ................................................................. 216
Significance and implications of not detecting a bystander effect in vivo 221
Recent developments ........................................................................... 240
Conclusions ........................................................................................... 244

REFERENCES .......................................................................................... 248
Figures

Figure 4.1: Starting method for chronic radiolabelling experiments ............... 59
Figure 4.2: Proliferative response to concanavalin A in donor splenocytes .......... 64
Figure 4.3: Viable cell numbers of donor T lymphocytes after ConA stimulation.... 71
Figure 4.4: Cell-cycle progression of donor T lymphocytes after ConA stimulation ................................................... 72
Figure 4.5: Effect of cell density on donor cell growth in culture ..................... 73
Figure 4.6: Effect of atmospheric CO$_2$ on donor cell growth in culture ............ 74
Figure 4.7: Representative growth of donor cells in culture ............................ 75
Figure 4.8: Incorporation of BrdU into donor T cells ....................................... 78
Figure 4.9: $^3$H-thymidine incorporation into donor cells ................................. 81
Figure 4.10: CMRA labelling of donor cells ................................................... 87
Figure 4.11: Final protocol for the chronic radiolabelling adoptive transfer method ................................................... 89
Figure 4.12: Initial strategy for the acute irradiation adoptive transfer method .... 90
Figure 4.13: Final protocol for the acute irradiation adoptive transfer method .... 94
Figure 4.14: Pre-warming of cages to dilate mouse tail veins ........................... 97
Figure 4.15: Recipient mouse tail vein injection setup ..................................... 97
Figure 4.16: Radiolabelling and fluorescent labelling of donor cells prior to adoptive transfer .................................. ................................................100
Figure 4.17: $^3$H-thymidine radiolabelled donor cells identified lodged in recipient mouse spleen ................................................... 101
Figure 4.18: Locations of donor cell lodging in representative spleen section .... 102
Figure 4.19: Z-stack projection of 3-dimensional cluster of lodging donor cells .... 104
Figure 4.20: Representative global field ............................................................. 111
Figure 4.21: Area surveyed in 20 representative global fields .............................. 112
Figure 4.22: Donor cells surveyed in 25 representative local fields ..................... 114
Figure 4.23: Region included in the area around donor cells in a representative local field ................................................................. 115
Figure 4.24: Pseudo-coloured overlay of a local screening field from TUNEL–stained spleen section ................................................................. 121
Figure 4.25: Fragmented TUNEL–stained cell nucleus ....................................... 123
Figure 4.26: Phagocytic cell and ingested TUNEL-positive debris ....................... 124
Figure 4.27: Manual apoptosis scoring in local screening field from TUNEL–stained spleen section ................................................................. 125
Figure 4.28: Estimating total number of cells per field ..................................... 126
Figure 4.29: Pseudo-coloured overlay of a local screening field from Ki-67 assay .... 131
Figure 4.30: Levels of Ki-67 staining in local fields ........................................... 132
Figure 4.31: Automatic proliferation scoring in local screening field from Ki-67 stained spleen section ................................................................. 133
Figure 4.32: pKZ1-encoded β-galactosidase activity detected in X-gal stained spleen sections ........................................................138
Figure 4.33: Workflow for adoptive transfer experiments ..............................................141
Figure 4.34: Data analysis workflow .........................................................................142
Figure 5.1: Donor cell lodging frequencies in the spleens of recipient mice ..........146
Figure 5.2: Local and global apoptosis frequencies in mice receiving sham-radiolabelled or radiolabelled donor cells .............................................................148
Figure 5.3: Local and global proliferation indices in mice receiving sham-radiolabelled or radiolabelled donor cells ..............................................................151
Figure 5.4: Very high levels of Ki-67 staining in Mouse #44 ........................................153
Figure 5.5: Apoptosis and proliferation within mice receiving radiolabelled donor cells in local fields with only $^3$H-negative donor cells, versus fields with confirmed $^3$H-positive donor cells ........................................155
Figure 5.6: Lodging patterns in spleens of mice receiving 5 or $5 \times 10^5$ donor cells ..................................................................................................................159
Figure 5.7: pKZ1 chromosomal inversion frequencies in mice receiving sham-radiolabelled or radiolabelled donor cells .................................................................161
Figure 5.8: Effect of donor cell lodging density on proliferation index in global fields ........................................................................................................................................161
Figure 5.9: Equivalent donor cell radiolabelling in duplicate trials ..............................163
Figure 5.10: Correlation between apoptosis frequency and donor cell lodging frequency for analysis of spleen tissues at one or three days ................................172
Figure 5.11: Donor cell lodging frequencies in the spleens of recipient mice ..........180
Figure 6.1: Variation in bystander apoptosis and proliferation of mice receiving sham-irradiated cells ........................................................................................................206
Figure 6.2: Model of $^3$H decay and accumulated dose over 22 hours at one disintegration per hour .........................................................................................227
Figure 6.3: Model of time elapsed since last tritium disintegration at an average exposure rate of 1 disintegration per hour .........................................................237
Tables

Table 2.1: Summary of protein expression changes in bystander cells ..................... 38
Table 5.1: Number of mice used in the initial three experiments using the adoptive transfer method ................................................................. 145
Table 5.2: Ki-67 status of radiolabelled and non-radiolabelled lodged donor cells in mice receiving radiolabelled donor cell preparations ................... 147
Table 5.3: Summary of results from local and global screening of apoptosis ............. 149
Table 5.4: Summary of results from local and global screening of proliferation ...... 152
Table 5.5: Summary of results from global screening of apoptosis, proliferation and pKZ1 inversions ................................................................. 160
Table 5.6: Summary of local and global screening results for apoptosis and proliferation .................................................................................................. 170
Table 5.7: Correlations between number of donor cells and proliferation/apoptosis in local and global fields ................................................................. 170
Table 5.8: Correlations between donor cell lodging frequency and global and local apoptosis frequencies ................................................................. 171
Table 5.9: Summary of global screening results for apoptosis and proliferation ...... 177
Table 5.10: Summary of local and global screening results for apoptosis and proliferation .................................................................................................. 179
Table 5.11: Summary of experimental conditions ..................................................... 182
Table 5.12: Effect sizes for apoptosis and proliferation across the series of experiments conducted to detect a radiation-induced bystander effect in vivo ........................................................................................................ 183
Table 6.1: Number of bystander cells and fields scored in the local screens ......... 203
Table 6.2: Number of bystander cells and fields scored in the global screens ...... 203
Table 6.3: Predicted sample sizes based on set prospective statistical power ...... 209
Table 6.4: 95% confidence limits on effect sizes ..................................................... 211
Table 6.5: 95% confidence limits on effect sizes, Acute X-ray Experiment ......... 214
Table 6.6: Bystander effect experiments conducted in vitro with low-LET radiations ........................................................................................................ 225
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, \(5 \times 10^5\) radiolabelled cells (0.33 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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Final thanks go to my Dad, whose curiosity and fascination with the world inspires my love of science; and to whom I dedicate this thesis.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CMRA</td>
<td>CellTracker™ Orange CMRA</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dUTP</td>
<td>Deoxyuridine triphosphate</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GJIC</td>
<td>Gap-junctional intercellular communication</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-Guanine Phosphoribosyl Transferase</td>
</tr>
<tr>
<td>ICCM</td>
<td>Irradiated cell-conditioned medium</td>
</tr>
<tr>
<td>ICRP</td>
<td>International Commission on Radiological Protection</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Lethal dose (50%)</td>
</tr>
<tr>
<td>LET</td>
<td>Linear energy transfer</td>
</tr>
<tr>
<td>LNT</td>
<td>Linear no-threshold</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxidative species</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Rose Park Memorial Institute cell culture medium #1640</td>
</tr>
<tr>
<td>SCE</td>
<td>Sister chromatid exchange</td>
</tr>
<tr>
<td>SCM</td>
<td>Splenocyte culture medium</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>γ-variant of histone H2AX</td>
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Publications & abstracts arising during PhD candidature

Publications


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


Poster Presentations


‘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)