



2002-01-01

Initiation of Apoptosis in Cells Exposed to Medium from the Progeny of Irradiated Cells: a Possible Mechanism for "Bystander" induced Genomic Instability.

Fiona Lyng

Technological University Dublin, Fiona.lyng@dit.ie

B. Maguire

Dublin Institute of Technology

B. McClean

St. Luke's Hospital, Dublin

C. Seymour

St. Luke's Hospital, Dublin

Carmel Mothersill

Dublin Institute of Technology

Follow this and additional works at: <https://arrow.dit.ie/radart>



Part of the [Radiology Commons](#)

Recommended Citation

Lyng, F. et al. (2002) Initiation of Apoptosis in Cells Exposed to Medium from the Progeny of Irradiated Cells: a Possible Mechanism for "Bystander" induced Genomic Instability. *Radiation Research*, Vol.157, 2, pp.365-370. doi:10.1667/0033-7587(2002)157[0365:IOAICE]2.0.CO;2

This Article is brought to you for free and open access by the Radiation and Environmental Science Centre at ARROW@TU Dublin. It has been accepted for inclusion in Articles by an authorized administrator of ARROW@TU Dublin. For more information, please contact yvonne.desmond@dit.ie, arrow.admin@dit.ie, brian.widdis@dit.ie.



Initiation of apoptosis in cells exposed to medium from the progeny of irradiated cells: A possible mechanism for “bystander” induced genomic instability?

FM Lyng, CB Seymour, C Mothersill

Radiation and Environmental Science Centre, Dublin Institute of Technology, Kevin St,
Dublin 8, IRELAND

Number of copies submitted: 3

Number of figures: 6

Number of tables: 0

Running head: Apoptosis in cells exposed to “bystander” medium from the progeny of irradiated cells

Person to whom all correspondence should be sent:

Dr Fiona Lyng

Radiation and Environmental Science Centre

Dublin Institute of Technology

Kevin St

Dublin 8

IRELAND

Tel: ++ 353 1 4022818

Fax: ++ 353 1 6620884

Email: fiona.lyng@dit.ie

Lyng, F.M., Seymour, C.B., Mothersill, C. Initiation of apoptosis in cells exposed to medium from the progeny of irradiated cells. Radiat Res.

Genomic instability and bystander effects have recently been linked experimentally both in vivo and in vitro. The aim of the present study was to determine if medium from irradiated cells several passages distant from the original exposure could initiate apoptosis in unirradiated cells.

Human keratinocytes (from the HPV-G cell line) were irradiated at 0.5 Gy and 5 Gy. Medium was harvested at each passage up to the 7th passage (approx 35 population doublings) post-irradiation and transferred to unirradiated keratinocytes. Intracellular calcium levels, mitochondrial membrane potential and the level of reactive oxygen species were all monitored for a period of 24 hours following medium transfer.

Rapid calcium fluxes (within 30 secs), loss of mitochondrial membrane potential and increases in reactive oxygen species (from 6 hours after medium transfer) were observed in the recipient cells. There was no significant difference between medium generated by cells irradiated to 0.5 or 5Gy. The effect of medium from progeny was the same as the initial effect reported previously and did not diminish with increasing passage number.

The data suggest that initiating events in the apoptotic cascade are induced in unirradiated cells by a signal produced by irradiated cells and that this signal can still be produced in the progeny of irradiated cells for several generations.

Introduction

In recent years it has become apparent that low doses of radiation can cause delayed effects in cells surviving the radiation dose which may not become apparent for many cell generations. Delayed effects, such as lethal mutations or delayed reproductive cell death in the progeny of irradiated cells that persist for many generations (1 – 5), de novo chromosomal aberrations (6 – 9) and gene mutations (10 - 12) arising at high frequency in the progeny of cells irradiated many cell divisions previously, are defined as genomic instability. The mechanisms are unclear but oxidative stress induced by the initial radiation exposure has been shown to persist and could predispose surviving cells to sustain mutations more easily (13). Mechanisms of induction and transmission of genomic instability appear to involve indirect untargeted interactions between cells and signal transduction processes.

Considerable evidence has accumulated recently suggesting that bystander effects can occur in cells which were not themselves irradiated but were either in the vicinity of irradiated cells (14 – 21) or exposed to medium from irradiated cells (22, 23). Very low doses of α particles have been shown to lead to the formation of sister chromatid exchanges (14, 15), chromosomal aberrations (16) and changes in protein expression (17) in considerably more cells than would have been traversed by an α particle. Irradiation of cells with a microbeam, which permits the traversal of one cell or part of a cell in a field with a charged particle beam, has shown that effects of single cell irradiation are not limited to the exposed cell but affect other cells in the vicinity. Micronucleus formation (18, 19), mutation (20) and oncogenic transformation (21) have all been shown in cells distant from the target cell. Medium from epithelial cells irradiated with γ rays transferred to cultures that have not been irradiated has been shown to induce cell death by apoptosis in the unirradiated cells (22, 23). Decreased cell survival

has also been reported in cells in contact with cells internally irradiated with ^3H β particles in a three dimensional tissue culture model (24, 25).

The bystander effect, once induced appears to persist in the progeny of irradiated cells which survive the irradiation (16, 26, 27). Lorimore et al (16) demonstrated chromosomal instability in the progeny of nonirradiated hemopoietic stem cells caused by interactions between the irradiated cells and the nonirradiated cells, ie. a bystander effect. Seymour and Mothersill (26) showed that medium from irradiated cells can induce delayed effects in the progeny of cells that survive the initial exposure to the medium. Watson et al (27) demonstrated a link between a radiation induced bystander effect and the induction of genomic instability in vivo by transplanting a mixture of nonirradiated and irradiated hemopoietic stem cells into mice. Chromosomal instability was demonstrated in the progeny of nonirradiated stem cells.

Our group has previously shown that medium from irradiated cells can induce early events in the apoptotic cascade, such as mobilisation of intracellular calcium, loss of mitochondrial membrane potential and an increase in reactive oxygen species, in unirradiated cells (28). All these events have been clearly linked with induction of apoptosis (29 – 32). The aim of the present study was to investigate if medium from the progeny of irradiated cells could also initiate apoptosis in unirradiated cells thus defining a mechanistic link between radiation-induced bystander processes and genomic instability.

Materials and Methods

Cell Culture

HPV keratinocytes were cultured in Dulbecco's MEM:F12 (1:1) containing 7% foetal calf serum, 5ml penicillin streptomycin solution, 25mM Hepes buffer and 1 μ g/ml hydrocortisone (all from Gibco Biocult Ltd. Irvine Scotland) and were maintained in an incubator at 37°C in an atmosphere of 5% CO₂ in air. Subculture was routinely performed using a 1:1 solution of 0.25 % trypsin and 1mM EDTA in Earle's balanced salt solution at 37°C.

Irradiation

Culture flasks (25 cm², 40 ml flasks, Nunc, Denmark) were sealed and irradiated at room temperature using a cobalt 60 teletherapy unit delivering approximately 2.0 Gy/min during the time period of these experiments. The source to flask distance was 80 centimetres and the field size was 30 X 30 centimetres. Flasks were returned to the incubator immediately after irradiation.

Bystander Medium

Medium from irradiated and unirradiated cells was poured off donor flasks one hour after irradiation and filtered through a 0.22 μ m filter to ensure that no cells could still be present in the transferred medium. The medium was then stored at -80°C until required for experiments. Recipient cells were plated onto glass coverslips embedded into 35mm plastic culture dishes and were grown to approx. 80% confluence. Culture dishes, containing the minimum amount of buffer (130 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 1 mM MgCl₂ and 25 mM HEPES (pH 7.4)) required to just cover the cells on the glass coverslip, were then mounted on the stage of a Zeiss 510 confocal

microscope. Medium from irradiated and unirradiated cells, as well as fresh medium, was added directly to the culture dish while continuously scanning an area of interest. The medium from irradiated cells used in these experiments has been shown to reduce clonogenic survival in recipient cells (33).

Ratiometric measurement of calcium

Intracellular calcium levels were determined relative to control levels using two visible wavelength calcium sensitive dyes, Fluo 3 and Fura Red (Molecular Probes, Leiden). Fluo 3 exhibits an increase in green fluorescence upon binding to calcium whereas Fura Red exhibits a decrease in red fluorescence upon binding to calcium. The ratio Fluo 3 / Fura Red is a good indicator of intracellular calcium levels. Cultures were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 1 mM MgCl₂ and 25 mM HEPES (pH 7.4). Cells were loaded with the calcium sensitive dyes by incubation with 3 μM Fluo-3 and 3 μM Fura Red AM esters for 1 hour in the buffer at 37 °C. Subsequently, the cultures were washed three times with buffer. Fluo 3 and Fura Red were excited at 488 nm and fluorescence emissions at 525 nm and 660 nm were recorded simultaneously using a Zeiss LSM 510 confocal microscope. Ratio images and time series data of the Fluo 3 / Fura Red fluorescence intensities were recorded every 2 seconds. Medium was added after 60 seconds when a stable baseline had been established. All measurements were performed at room temperature.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined using rhodamine 123, a green fluorescent dye that accumulates in active mitochondria with high membrane potentials. Cultures were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 1 mM MgCl₂ and 25 mM HEPES (pH 7.4). Cells were loaded

with 5 μ M Rhodamine 123 for 30 min in the buffer at 37 $^{\circ}$ C. Subsequently, the cultures were washed three times with buffer. Rhodamine 123 was excited at 488 nm and fluorescence emission at 525 nm was recorded using a Zeiss LSM 510 confocal microscope. The mean fluorescence intensity (or mean grey value) was determined using the software package, NIH Image (National Institutes of Health, USA).

Measurement of reactive oxygen species

Induction of reactive oxygen species was measured using 2,7 - dichlorofluorescein diacetate. Once inside a cell, the acetate group is cleaved by cellular esterases leaving dichlorofluorescein which emits green fluorescence when oxidized by the reactive oxygen species, hydrogen peroxide and nitric oxide. Cultures were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 1 mM MgCl₂ and 25 mM HEPES (pH 7.4). Cells were loaded with 5 μ M 2,7 dichlorofluorescein diacetate for 30 min in the buffer at 37 $^{\circ}$ C. Subsequently, the cultures were washed three times with buffer. Dichlorofluorescein was excited at 488 nm and fluorescence emission at 525 nm was recorded using a Zeiss LSM 510 confocal microscope. The mean fluorescence intensity (or mean grey value) was determined using the software package, NIH Image (National Institutes of Health, USA).

All measurements are presented as mean values \pm S.E. of 4 independent experiments. Significance of differences was determined by a student's unpaired t-test and the differences were considered significant if $p \leq 0.05$.

Results

Rapid calcium fluxes (within 30 secs) were observed following addition of medium both from initially irradiated cells and from the progeny of irradiated cells, up to the 7th passage (approx. 35 population doublings) post irradiation. Ratio images of calcium levels before and after addition of medium from the progeny (passage 7) of cells initially irradiated at 0.5 Gy are shown in figure 1. The images are colour coded for calcium levels; blue indicates low levels of calcium while green, yellow and red indicate progressively higher levels of calcium. There was no change in intracellular calcium levels following addition of fresh medium or of medium from densely seeded but unirradiated cells (figure 1). Figure 2 shows a rapid and transient increase in calcium levels following addition of medium from the progeny (passage 7) of cells initially irradiated at 0.5 Gy. There was no significant difference between medium generated by cells irradiated at the different doses or between medium from initially irradiated cells and progeny. There was no change in intracellular calcium levels following addition of medium from unirradiated cells or of control medium (figure 2).

Mitochondria with high membrane potentials were observed in control cells and in cells treated with medium from unirradiated cells (figure 3). A decrease in fluorescence and more unspecific staining was observed at 6 hours after addition of medium from the progeny (passage 7) of cells initially irradiated at 0.5 Gy (figure 3) suggesting a decrease in mitochondrial membrane potential. There was no significant difference between medium generated by cells irradiated at the different doses or between medium from initially irradiated cells and progeny up to passage 7 (figure 4).

Very low levels of fluorescence were observed in control cells or cells treated with medium from unirradiated cells (figure 5). An increase in fluorescence was observed at 6 hours after addition of medium from the progeny (passage 7) of cells initially irradiated

at 0.5 Gy (figure 5) suggesting an increase in reactive oxygen species. There was no significant difference between medium generated by cells irradiated at the different doses or between medium from initially irradiated cells and progeny up to passage 7 (figure 6).

Discussion

This study suggests that initiating events in the apoptotic cascade were induced in unirradiated cells by a signal produced by irradiated cells and that this signal is still produced in the progeny of irradiated cells.

Rapid calcium fluxes (within 30 secs), loss of mitochondrial membrane potential and increases in reactive oxygen species (from 6 hours after medium transfer) were observed. There was no significant difference between medium generated by cells irradiated at 0.5 Gy or 5 Gy or between medium from initially irradiated cells and the progeny of irradiated cells up to passage 7 (35 population doublings). It has previously been shown that the bystander effect appears to saturate at doses in the range 0.03 – 0.05 Gy in the human keratinocytes used in this study (33).

Considerable support for the existence of bystander effects is available in the literature but the mechanisms by which the bystander signal is produced and transduced have yet to be elucidated. Cellular stress responses or general damage signalling through a range of signal transduction pathways may be involved. Not all cells would reach the end stage of apoptosis but it seems likely that most cells would respond to the initial signal with the production of a calcium pulse. Whether the cell continues to the end stage would depend on passing the various checkpoints in the signal transduction pathway (34).

Bystander effects have been attributed to the production of extracellular factors that lead to the generation of reactive oxygen species (35 –37). Recently, Iyer and Lehnert (38) identified TGF- β 1 as a mediator of α particle induced bystander responses and Matsumoto et al (39, 40) reported that nitric oxide may be one of the factors mediating the bystander effect.

Some studies of bystander effects implicate gap junctional intercellular communication in transmission of the signal (17, 20, 25) whereas others have implicated a medium transmitted mechanism (22, 23, 38). It is possible than more than one mechanism may be involved in the transduction of the signal and it may depend on the cell type, the type of radiation and other factors including the endpoint being measured. Clearly in the experiments described here GJIC cannot be the mechanism of signal transmission since the donor and recipient cells are in separate flasks, however it is possible that the signal passes through membrane channels, such as gap junctions, via an active transport mechanism. This could easily be shut down by conditions which close gap junctions.

Genomic instability and the bystander effect have recently been linked experimentally (16, 26, 27). The present paper provides a mechanistic link because it appears that radiation induced genomic instability may be induced by the bystander factor and that a possible mechanism may involve the induction of persistent oxidative stress. This was proposed as a mechanism for perpetuation of genomic instability by Clutton et al in 1996 (13). Previous studies by our group (41, 42) showed persistent apoptosis in the distant progeny of irradiated cells, as well as a number of morphological and biochemical changes which together suggested a long-term induction of damage response pathways in progeny of irradiated cells.

In conclusion it would appear that low dose irradiation induced a long-term stress response in cells in the field, whether they received a direct "hit" or not. This stress response persists in clonal progeny for many generations and is both inducible and transmissible by an as yet unidentified factor. Apoptosis is clearly one endpoint which can be shown to increase but others such as changes in the frequency of cytogenetic abnormalities, gene/protein expression and mutation frequency occur. Many different factors may contribute to the final expression of the effects initiated in the originally irradiated cells.

Acknowledgement

We are grateful to St. Luke's Hospital, Dublin for continued access to the cobalt - 60 source. This work has been carried out (in part) within the Dublin Institute of Technology's Facility for Optical Characterisation and Spectroscopy (FOCAS), funded by the Irish Government Programme for Research in Third Level Institutions.

References

- 1 C.B. Seymour, C. Mothersill and T. Alper, High yields of lethal mutations in somatic mammalian cells that survive ionising radiation. *Int J Rad Biol* **50** 167 – 169 (1986)
- 2 L. Gorgojo and J.B. Little, Expression of lethal mutations in the progeny of irradiated mammalian cells. *Int J Rad Biol* **55** 619 – 630 (1989)
- 3 A. Mendonca, W. Kurohara, R. Antoniono and J.L. Redpath, Plating efficiency as a function of time post irradiation. Evidence for the delayed expression of lethal mutations. *Radiat Res* **119** 387 – 393 (1989)
- 4 C.B. Seymour and C. Mothersill, All colonies of CHO cells surviving gamma radiation contain mutated cells. *Mutat Res* **267** 19 – 30 (1992)
- 5 D.C. Brown and K.R. Trott, Clonal heterogeneity in the progeny of HeLa cells which survive X-irradiation. *Int J Rad Biol* **66** 151 – 156 (1994)
- 6 M. Kadhim, D.A. Macdonald, D. T. Goodhead, S. A. Lorimore, S.J. Marsden and E. G. Wright, Transmission of chromosomal instability after plutonium alpha particle irradiation. *Nature* **355** 738 – 740 (1992)
- 7 I. Sabatier, B. Dutrillaux, and M.B. Martin, Chromosomal instability. *Nature* **357** 548 (1992)
- 8 B.A. Marder, and W.F. Morgan, Delayed chromosomal instability induced by DNA damage. *Molecular and Cellular Biology* **13** 6667 – 6677 (1993)
- 9 M. A. Kadhim, S. A. Lorimore, K.M.S. Townsend, D. T. Goodhead, V.J. Buckle and E. G. Wright, Radiation induced genomic instability: delayed cytogenetic aberrations and apoptosis in primary human bone marrow cells. *Int J Rad Biol* **57** 287 – 293 (1995)
- 10 T. Stamato, R. Weinstein, B. Peters, J. Hu, and A. Giaccia, Delayed mutation in Chinese hamster cells. *Somatic Cell Molecular Genetics* **13** 57 – 66 (1987)

- 11 J.B. Little, L. Gorgojo and H. Vetrovs, Delayed appearance of lethal and specific gene mutations in irradiated mammalian cells. *Int J Rad Oncol, Biol Phys* **19** 1425 - 1429 (1990)
- 12 J.B. Little, H. Nagasawa, T. Pfenning, and H. Vetrovs, Radiation induced genomic instability: delayed mutagenic and cytogenetic effects of X rays and alpha particles. *Radiat Res* **148** 299 – 307 (1997)
- 13 S.M. Clutton, K.M.S. Townsend, C. Walker, J.D. Ansell, and E.G. Wright, Radiation induced genomic instability and persisting oxidative stress in primary bone marrow cultures. *Carcinogenesis*, **17** 1633-1639 (1996)
- 14 H. Nagasawa and J.B. Little, Induction of sister chromatid exchanges by extremely low doses of alpha particles. *Can Res* **52**, 6394 – 6396 (1992)
- 15 A. Deshpande, E.H. Goodwin, S.M. Bailey, B.L. Marrone and B.E. Lehnert, Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extra-nuclear target. *Radiat Res* **145**, 260-267 (1997)
- 16 S. A. Lorimore, M. A. Kadhim, D. A. Pocock, D. Papworth, D. L. Stevens, D. T. Goodhead and E. G. Wright, Chromosomal instability in the descendants of unirradiated surviving cells after α -particle irradiation. *Proc Nat Acad Sci USA* **95**, 5730-5733 (1998)
- 17 E.I. Azzam, S.M. de Toledo, T. Gooding, J.B. Little, Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. *Radiat Res* **150**, 497-504 (1998)
- 18 K.M. Prise, O.V. Belyakov, M. Folkard, and B.D. Michael, Studies of bystander effects in human fibroblasts using a charged particle microbeam. *Int J Radiat Biol* **74**, 793-798 (1998)

- 19 O.V. Belyakov, A.M. Malcolmson, M. Folkard, K.M. Prise, and B.D. Michael, Direct evidence for a bystander effect of ionising radiation in primary human fibroblasts. *Br J Cancer* **84**, 674-679 (2001)
- 20 H. Zhou, G. Randers-Pehrson, C.A. Waldren, D. Vannais, E.J. Hall and T.K. Hei, Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc Natl Acad Sci* **97**, 2099-2104 (2000)
- 21 S.G. Sawant, G. Randers-Pehrson, C.R. Geard, D.J. Brenner, E.J. Hall, The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells in vitro can be initiated in the unirradiated neighbours of irradiated cells. *Radiat. Res* **155**, 397-401 (2001)
- 22 C. Mothersill and C.B. Seymour, Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *Int J Rad Biol* **71**, 421-427 (1997)
- 23 C. Mothersill and C.B. Seymour, Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. *Radiat Res* **149**, 256-262 (1998)
- 24 A. Bishayee, D.V. Rao and R.W. Howell, Evidence for pronounced bystander effects caused by nonuniform distributions of radioactivity using a novel three dimensional tissue culture model. *Radiat Res* **152** 252 – 262 (1999)
- 25 A. Bishayee, H.Z. Hill, D. Stein, D.V. Rao and R.W. Howell, Free radical initiated and gap junction mediated bystander effect due to nonuniform distribution of incorporated radioactivity in a three dimensional tissue culture model. *Radiat Res* **155** 335 – 344 (2001)

- 26 C.B. Seymour and C. Mothersill, Delayed expression of lethal mutations and genomic instability in the progeny of human epithelial cells that survived in a bystander killing environment. *Radiat Oncol Investig* **5**, 106 – 110 (1997)
- 27 G.E. Watson, S.A. Lorimore, D.A. Macdonald and E.G. Wright, Chromosomal instability in unirradiated cells induced in vivo by a bystander effect of ionising radiation. *Can Res* **60** 5608 - 5611 (2000)
- 28 F.M. Lyng, C.B. Seymour and C. Mothersill, Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis, *Br J Cancer* **83**, 1223-1230 (2000)
- 29 D.M. Ojcius, A. Zychlinsky, L.M. Zheng, J.D. Young, Ionophore induced apoptosis: role of DNA fragmentation and calcium fluxes. *Exp Cell Res* **197**, 43 (1991)
- 30 G. Kroemer, N. Zamzami, and S.A. Susin, Mitochondrial control of apoptosis. *Immunology Today* **18**, 44-51 (1997)
- 31 D.R. Green and J.C. Reed, Mitochondria and apoptosis. *Science* **281**, 1309-1312 (1998)
- 32 J.M. Garland and A. Halestrap, Energy metabolism during apoptosis. *J Biol Chem* **272**, 4680 (1997)
- 33 C.B. Seymour and C. Mothersill, Relative contribution of bystander and targeted cell killing to the low dose region of the radiation dose response curve. *Radiat Res* **153** 508 – 511 (2000)
- 34 R.E. Shackelford, W.K. Kaufmann, R.S. Paules, Cell cycle control, checkpoint mechanisms and genotoxic stress. *Environ Health Perspect* **107** 5 – 24 (1999)
- 35 B.E. Lehnert and E.H. Goodwin, Extracellular factor(s) following exposure to alpha particles can cause sister chromatid exchanges in normal human cells. *Cancer Res* **57** 2164-2171 (1997)

- 36 P.K. Narayanan, E.H. Goodwin and B.E. Lehnert, Alpha particles initiate the biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Res* **57** 3963-3971 (1997)
- 37 P.K. Narayanan, K.E.A. LaRue, E.H. Goodwin and B.E. Lehnert, Alpha particles induce the production of interleukin-8 by human cells. *Radiat Res* **152** 57-63 (1999)
- 38 R. Iyer and B.E. Lehnert, Factors underlying the cell growth related bystander responses to alpha particles. *Cancer Res* **60** 1290-1298 (2000)
- 39 H. Matsumoto, S. Hayashi, M. Hatashita, H. Shioura, T. Ohtsubo, R. Kitai, T. Ohnishi, O. Yukawa, Y. Furusawa and E. Kano, Induction of radioresistance to accelerated carbon ion beams in recipient cells by nitric oxide excreted from irradiated donor cells of human glioblastoma. *Int J Radiat Biol* **76** 1649-1657 (2000)
- 40 H. Matsumoto, S. Hayashi, M. Hatashita, K. Ohnishi, H. Shioura, T. Ohtsubo, R. Kitai, T. Ohnishi and E. Kano, Induction of radioresistance by a nitric oxide mediated bystander effect. *Radiat Res* **155** 387-396 (2001)
- 41 F.M. Lyng, S. O' Reilly, D.C. Cottell, C.B. Seymour and C. Mothersill, Persistent expression of morphological abnormalities in the distant progeny of irradiated cells. *Radiation and Environmental Biophysics* **35** 273 – 283 (1996)
- 42 J.V. Harney, C.B. Seymour, D.M. Murphy, C. Mothersill, Variation in the expression of p53, c-myc, and bcl-2 oncoproteins in individual patient cultures of normal urothelium exposed to cobalt 60 gamma-rays and N-nitrosodiethanolamine. *Cancer Epidemiol Biomarkers Prev.* **4** 617 – 25 (1995)

Formatted: Bullets and Numbering

Legends

Figure 1 Ratio images of calcium levels in HPV-G cells (a) before and (b) 30 s after addition of medium from the progeny (passage 7) of unirradiated cells (0Gy), (c) before and (d) 30 s after addition of medium from the progeny (passage 7) of cells initially irradiated at 0.5 Gy. Blue indicates low levels of calcium while green, yellow and red indicate progressively higher levels of calcium. Bar = 20 μm

Figure 2 Intracellular calcium levels in HPV-G cells after addition of medium from the progeny (passage 7) of unirradiated cells (0Gy, open circles) and medium from the progeny (passage 7) of irradiated cells (0.5Gy, closed circles). Medium was added at the time indicated by the arrow. The ratio of fluorescence emissions from the calcium sensitive dyes Fluo 3 and Fura Red provides an indication of intracellular calcium levels.

Figure 3 Fluorescence images showing the level of mitochondrial membrane potential in (a) HPV-G cells after addition of medium from the progeny (passage 7) of unirradiated cells and (b) HPV-G cells 6 hours after addition of medium from the progeny (passage 7) of irradiated cells (0.5Gy) and (c) phase contrast image of the cells shown in panel (b). A decrease in rhodamine 123 fluorescence is indicative of a loss of mitochondrial membrane potential. Bar = 5 μm .

Figure 4 Mean rhodamine 123 fluorescence (indicator of mitochondrial membrane potential) following exposure of unirradiated cells to medium from initially irradiated cells and from the progeny of irradiated cells. * $p < 0.0005$

Figure 5 Fluorescence images showing the level of reactive oxygen species in (a) HPV-G cells 6 hours after addition of medium from the progeny (passage 7) of unirradiated cells and (b) HPV-G cells 6 hours after addition of medium from the progeny (passage 7) of irradiated cells (0.5Gy) and (c) phase contrast image of the cells shown in panel (a). An increase in dichlorofluorescein fluorescence is indicative of an increase in reactive oxygen species. Bar = 5 μ m.

Figure 6 Mean dichlorofluorescein fluorescence (indicator of reactive oxygen species) following exposure of unirradiated cells to medium from initially irradiated cells and from the progeny of irradiated cells. * $p < 0.0005$