Genetic Factors Influencing Bystander Signaling in Murine Bladder Epithelium after Low-Dose Irradiation In Vivo

Authors: Mothersill, Carmel, Lyng, Fiona, Seymour, Colin, Maguire, Paula, Lorimore, Sally, et al.

Source: Radiation Research, 163(4) : 391-399

Published By: Radiation Research Society

URL: https://doi.org/10.1667/RR3320

RADIATION RESEARCH **163**, 391–399 (2005)
0033-7587/05 $15.00

Genetic Factors Influencing Bystander Signaling in Murine Bladder Epithelium after Low-Dose Irradiation *In Vivo*

Carmel Mothersill,[a,b,1] Fiona Lyng,[a] Colin Seymour,[a,b,1] Paula Maguire,[a] Sally Lorimore[c] and Eric Wright[c]

[a] *Radiation and Environmental Science Centre, Dublin Institute of Technology, Dublin, Ireland:* [b] *Saint Luke's Hospital, Rathgar, Dublin, Ireland; and* [c] *Department of Molecular and Cellular Pathology, Ninewells Hospital and Medical School, Dundee DD1 9SY Scotland*

Mothersill, C., Lyng, F., Seymour, C., Maguire, P., Lorimore, S. and Wright, E. Genetic Factors Influencing Bystander Signaling in Murine Bladder Epithelium after Low-Dose Irradiation *In Vivo. Radiat. Res.* 163, 391–399 (2005).

Radiation-induced bystander effects occur in cells that are not directly hit by radiation tracks but that receive signals from hit cells. They are well-documented *in vitro* consequences of low-dose exposure, but their relevance to *in vivo* radiobiology is not established. To investigate the *in vivo* production of bystander signals, bladder explants were established from two strains of mice known to differ significantly in both short-term and long-term radiation responses. These were investigated for the ability of 0.5 Gy total-body irradiation *in vivo* to induce production of bystander signals in bladder epithelium. The studies demonstrate that irradiated C57BL/6 mice, but not CBA/Ca mice, produce bystander signals that induce apoptosis and reduce clonogenic survival in reporter HPV-G-transfected keratinocytes. Transfer of medium from explants established from irradiated animals to explants established from unirradiated animals confirmed these differences in bladder epithelium. The responses to the *in vivo*-generated bystander signal exhibit genotypic differences in calcium signaling and also in signaling pathways indicative of a major role for the balance of pro-apoptosis and anti-apoptosis proteins in determining the overall response. The results clearly demonstrate the *in vivo* induction of bystander signals that are strongly influenced by genetic factors and have implications for radiation protection, medical imaging, and radiotherapy.

INTRODUCTION

Radiation-induced bystander effects are now well accepted as a consequence of exposure of cells to low doses of ionizing radiation. The field has been reviewed comprehensively by several authors (*1–4*). Most of the recent evidence comes from *in vitro* studies, and while anecdotal evidence exists concerning persistent clastogenic or abscopal effects *in vivo*, many of these studies are old, and the results could have other explanations since the studies were conducted before radiation-induced genomic instability and radiation-induced bystander effects were recognized phenomena in radiation biology. The reason radiation-induced bystander effects are important is that they have been linked with radiation-induced genomic instability in some systems (*5, 6*). This phenomenon is also caused by low-dose exposure to a variety of ionizing and nonionizing radiations (*3*). Recent evidence suggests that bystander mechanisms may be implicated in the process of genomic instability and may determine the type of end point seen (*6, 7*). The transducing mechanism appears to involve persistent induction of oxidative stress (*8–10*). There are many manifestations of radiation-induced genomic instability, for example chromosomal instability, micronucleus formation, delayed death, delayed mutation, and delayed transformation, but all have the common factor that the effects are induced at very low radiation doses, appear to saturate at low doses, and once induced are effectively permanent features of what is really a new phenotype. There is evidence of radiation-induced genomic instability and radiation-induced induction or modulation of bystander effects by certain chemicals as well (*11, 12*). A potential mechanism for an indirect pathway *in vivo* is suggested by macrophages exhibiting the phenotype of activated phagocytes after whole-body irradiation (*13*). The characteristics of these macrophages are also features of inflammatory responses that are known to have the potential for both bystander-mediated and persisting damage as well as for conferring a predisposition to malignancy. The observations are consistent with bystander effects and instabilities reflecting interrelated aspects of inflammatory-type responses to radiation-induced stress and injury.

Given that the dose range of importance for radiation-induced bystander effects and radiation-induced genomic instability is very low [the lowest dose for induction of the processes by α-particle radiation is one track through one cell in a population of millions of cells (*14*) and the lowest γ-radiation dose tested is 2 mGy, which gives the full effect (*15*)], the mechanisms and relevance are of intense interest in radiation protection. Key questions remain, however, and one of these concerns the *in vivo* relevance of the effects.

[1] Present address and address for correspondence: Medical Physics and Applied Radiation Sciences Unit, McMaster University, 1280 W. Main Street, Hamilton, ON L8S 4K1, Canada; e-mail: mothers@mcmaster.ca.

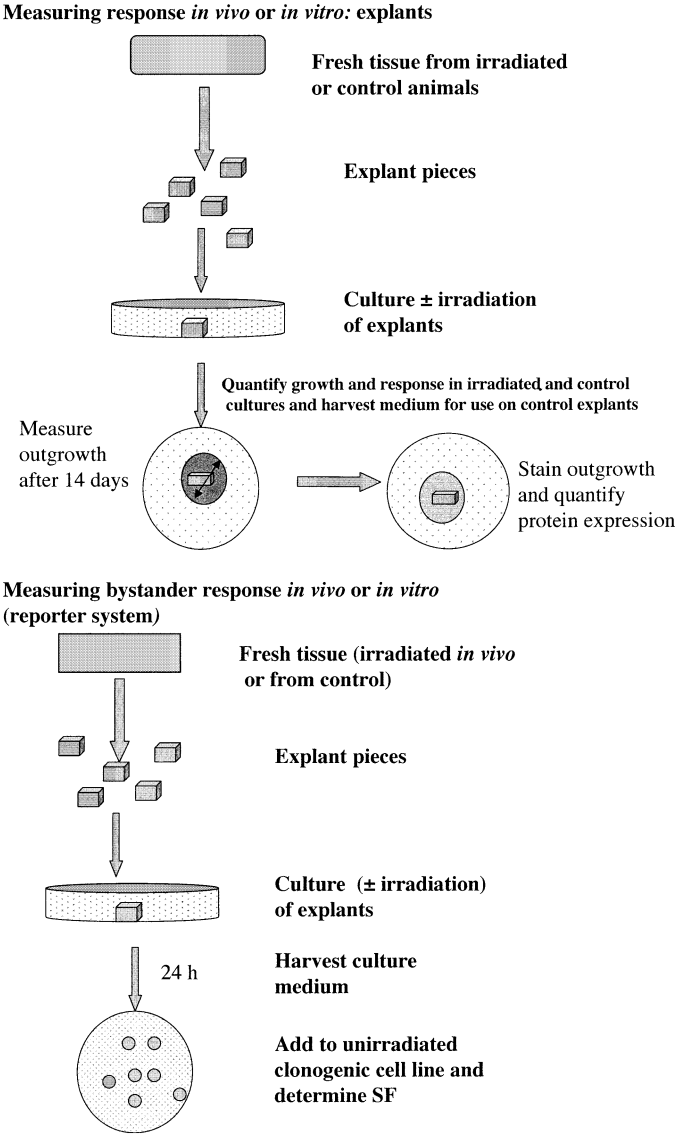

FIG. 1. Flow diagrams showing the procedures used in the experiments.

Radiation-induced genomic instability has been shown to occur *in vivo* (*16*) in mice and to have a genetic basis (*17*, *18*). Radiation-induced bystander effects are more difficult to demonstrate definitively, but studies that are highly suggestive of radiation-induced bystander effects *in vivo* include those of Watson *et al.* (*19*) and Xue *et al.* (*20*). In the present study, mouse strains previously shown to demonstrate *in vivo* induction of radiation-induced genomic instability were tested to assess whether *in vivo* whole-body irradiation led to production of signals that could induce bystander-type responses *in vitro* in an established reporter assay.

METHODS

In Vivo Irradiations

Animal studies at the University of Dundee were carried out in accordance with the guidance issued by the Medical Research Council and Home Office Project Licence PPL 60/2841. All mice were bred and housed on site to ensure consistency of health standards and to minimize genetic drift. Adult CBA/Ca or C57BL/6 mice 8 to 10 weeks old were exposed to 0.5 Gy total-body γ radiation at a fixed dose rate of 0.45 Gy/min using a CIS Bio International 637 Caesium irradiator. Bladders were surgically removed under aseptic conditions, transferred into sterile transport medium, and couriered overnight to Dublin. Controls were sham-irradiated animals that were brought to the irradiator but not exposed.

In Vitro Irradiations

Where indicated, tissue cultures or reporter cells were irradiated *in vitro* at room temperature using a cobalt-60 teletherapy unit (Theatron, Siemens) in Saint Luke's Hospital in Dublin at a flask-to-source distance of 80 cm. The dose rate during these experiments was approximately 1.8 Gy/min. These cultures served as checks for effects seen with the *in vivo*-irradiated samples that could be compared to the work done previously using explants and the reporter assay (*7*). Sham-irradiated controls were always carried along with the irradiated samples to control for disturbance effects due to movement of cultures or temperature effects. The techniques are summarized in the flow diagrams (Fig. 1).

Bladder Explant Culture

Bladders were removed from mice in Dundee 1 h after total-body irradiation (0.5 Gy) *in vivo*, using aseptic techniques. The time of 1 h was chosen because this is the interval between irradiation and medium harvest used in our *in vitro* protocol. Bladders from four to six animals per experimental group were pooled. These were placed in transport medium that consisted of Earle's Balanced Salt Solution (Gibco-Biocult, Irvine Scotland) containing 5 ml penicillin–streptomycin solution (Gibco) and 5 m*M* L-glutamine solution. The bladders were shipped on ice to Dublin using an overnight courier. On arrival, bladders were chopped into small pieces of approximately 2 mm^2 each. The explants were placed in 0.25% trypsin solution containing 10 mg/ml collagenase type IV (Sigma, Poole

TABLE 1
Cloning Efficiencies (means ± SEM, $n = 4$) for HPV-G-Transfected Reporter Cells Treated with Culture Medium Harvested from Mouse Bladder Explants Established from Nonirradiated Mice and Mice Exposed to 0.5 Gy Total-Body Irradiation

Explant medium added to HPV-G cells	Cloning efficiency of HPV-G cells (%)	Cloning efficiency of progeny of HPV-G cells (%)
Explant established from unirradiated CBA/Ca mice	24.3 ± 1.6	23.8 ± 3.6 e
Explant established from irradiated CBA/Ca mice	21.4 ± 2.7	22.5 ± 2.7
Explant established from unirradiated C57BL/6 mice	22.5 ± 2.7	21.9 ± 2.4
Explant established from irradiated C57BL/6 mice	14.6 ± 2.1*	12.9 ± 1.4*

* $P < 0.02$.

TABLE 2
Mitochondrial Membrane Potentials Measured using a Fluorescence Plate Reader 6 h after Exposure of Reporter Cells to Medium Harvested from Bladder Explants Cultured from CBA/Ca and C57BL/6 Mice Exposed *In Vivo* to 0.5 Gy Total-Body Irradiation

Mouse group	Mitochondrial membrane potential actual value	Percentage of control
CBA/Ca control	7.8 ± 0.9	100
C57BL/6 control	13.7 ± 3.8	100
CBA/Ca TBI, 0.5 Gy	10.4 ± 4	133
C57BL/6 TBI, 0.5 Gy	6.4 ± 0.2	46.7

Notes. Controls were sham-irradiated. Results are means ± SEM. * $P < 0.05$.

Dorset) for 30 min. After this time the explants were plated singly in 25-cm² 40-ml volume tissue culture flasks (NUNC, Uden, Denmark) containing 2 ml tissue culture medium [RPMI 1640, with 20% fetal calf serum, (Gibco) 100 IU insulin (Novo-Nordisk), 1 μg/ml hydrocortisone (Sigma), and penicillin-streptomycin solution (Gibco)]. Culture medium was harvested from the explants for the assays described below after 24 h. This was replaced with Clonetics serum-free medium (KGM, Clonetics Corporation, San Diego, CA) except where indicated. Cultures were maintained in KGM for 14 days. After this time they were fixed in 10% unbuffered formalin and stained to detect expression of Bcl2, Trp53 and Bax using an immunoperoxidase technique.

Transfer of Medium from Exposed to Unexposed Explants

To assess the effect of irradiated or control tissue conditioned medium (ITCM or CTCM) on unirradiated bladder explants, medium was harvested from the *in vivo*- and *in vitro*-irradiated explants 24 h after they were set up. The medium was passed through a 0.22-μm pore sterile filter (Nalgene) and added to unirradiated explants. After a further 24 h incubation, this medium was removed and the explants were placed in KGM for the remainder of the time. They were fixed as described above at the same time as the cultures from *in vivo*-irradiated mice.

Reporter Assay for Bystander Activity

The following procedure was used to set up the reporter assay. Flasks of HPV-G-transfected keratinocytes, obtained originally as a kind gift from Dr. J. DiPaolo, NIH, Bethesda, MD (*21*), were used. These were 85–90% confluent and had received a medium change the previous day. Cells were removed from the flask using 0.25% w/v trypsin/1 m*M* EDTA solution (1:1). When the cells had detached, they were resuspended in medium, and an aliquot was counted using a Coulter counter model Z_2 (Beckman Coulter Ltd., Dublin) set at a threshold calibrated for the cell line using a hemocytometer. Three hundred cells were plated for the reporter assay to determine survival using the clonogenic assay technique of Puck and Marcus (*22*). The cells were plated 6 h before they were needed. Medium was harvested from the bladder explants, pooled and then divided into aliquots. Some was reserved for assays to be described later. The remainder was transferred to the keratinocyte cultures. Each keratinocyte culture received 5 ml of medium harvested from the pooled replicate bladder cultures. Controls for medium only and *in vitro* irradiation effects were included in each experiment. Controls for transfer of unirradiated and irradiated medium from densely seeded cultures to cultures seeded at cloning densities were also always included to check that the assay was working. Cultures were incubated in 5 ml of culture medium in 25-cm², 40-ml flasks (Nunclon, Denmark) in a humidified 37°C incubator in an atmosphere of 5% CO_2 in air.

The HPV-G-transfected keratinocytes used here as reporter cells are nontumorigenic, have about 30% wild-type Trp53 expression (*23*), and have a normal epithelial pattern of cobblestone density-inhibited cell growth. They are used because when they are exposed to autologous irradiated cell-conditioned medium (ICCM), they give a stable bystander effect of approximately 40% reduction in plating efficiency over a very wide range of doses and exposure conditions (*23*). This allows comparison of bystander-inducing signal strengths when the HPV-G-transfected cells are exposed to signals from other cell lines or tissues. Response was monitored by determining the cloning efficiency and by using two assays indicative of early events in the cascade leading to apoptosis.

Protocol for Determination of Delayed Effects

The experiments were conducted as described above for the reporter assay, but the effects were determined in progeny cells of those receiving ITCM. Medium was harvested from the explant cultures and added to the clonogenic cells. These were then grown to confluence and subcultured as often as required. The end point used for instability was delayed death (persistent reduction in the cloning efficiency of progeny cells).

Immunoperoxidase Staining Technique

Expression of proteins involved in the control of apoptosis or proliferation was examined in the primary cultures after direct irradiation or exposure to ITCM from *in vivo*-irradiated animals. Cultures were fixed in 10% unbuffered formalin and stored at 4°C until processed. Processing always took place within 7 days of fixation. The explant outgrowth was processed *in situ* on the flask bottom to enable spatial distribution to be related to the type of cell and the degree of differentiation. Cultures were stained for expression of the following proteins: Bcl2, Bax and Trp53. The Trp53 and Bcl2 primary antibodies used were mouse monoclonals. BAX was a rabbit polyclonal. All were obtained from DAKO Laboratories. All were recommended for immunohistochemistry with mouse tissues. Immunochemistry was performed using an appropriate Vectastain ABC kit (Burlingame, CA). Diaminobendizine (DAB) was used to express the positive reaction, and cultures were lightly counterstained with Harris hematoxylin. At least three explant flasks were stained for each antibody and over 200 cells scored per explant over five fields using a Leica image analysis system. The detection threshold for positivity was set using positive control sections from positive tissue blocks obtained from the Cell Pathology service. The scores were calculated according to the method used in that service; it sums separate scores for intensity and for distribution of the staining. 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 ref. (*7*).

Quantification of Apoptosis

The extent of growth was assessed in the stained explant cultures on day 14 after irradiation or receipt of ITCM using a Leica Image Analysis system. Numbers of apoptotic cells were scored using morphological criteria under phase contrast using living cultures 48 and 72 h after plating and exposure to ITCM (where indicated).

Assay of Apoptosis-Inducing Activity in ITCM

The morphological indicators of apoptosis are terminal, and since they can result in cells being lost from the culture, they are indicative only. Two early indicators of apoptotic activity are an increase in the level of free calcium in the cells and a decrease in mitochondrial membrane potential. Both these end points were examined using reporter cells that had been exposed to medium harvested from the *in vivo*-exposed bladder explant cultures.

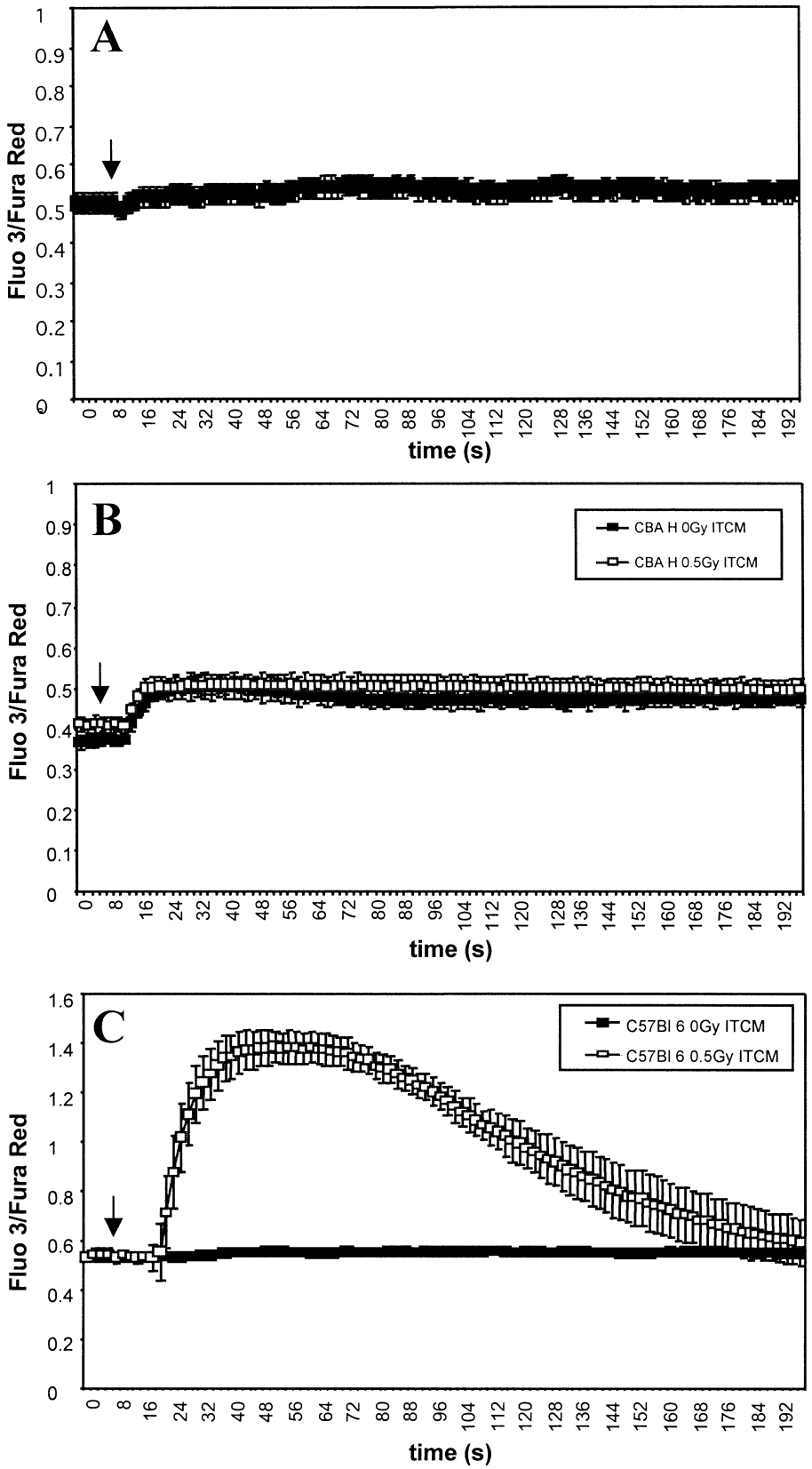

FIG. 2. Intracellular calcium levels in HPV-G cells after addition of medium from *in vivo*-irradiated C57BL/6 and CBA/Ca mouse bladder explants and their controls. Medium was added at the time indicated by the arrow. The ratio of fluorescence emissions from the calcium-sensitive dyes Fluo-3 and Fura Red provides an indication of intracellular calcium levels. Panel A shows data for unused medium, panel B is for medium derived from CBA/Ca cells, and panel C is for medium derived from C57BL/6 cells.

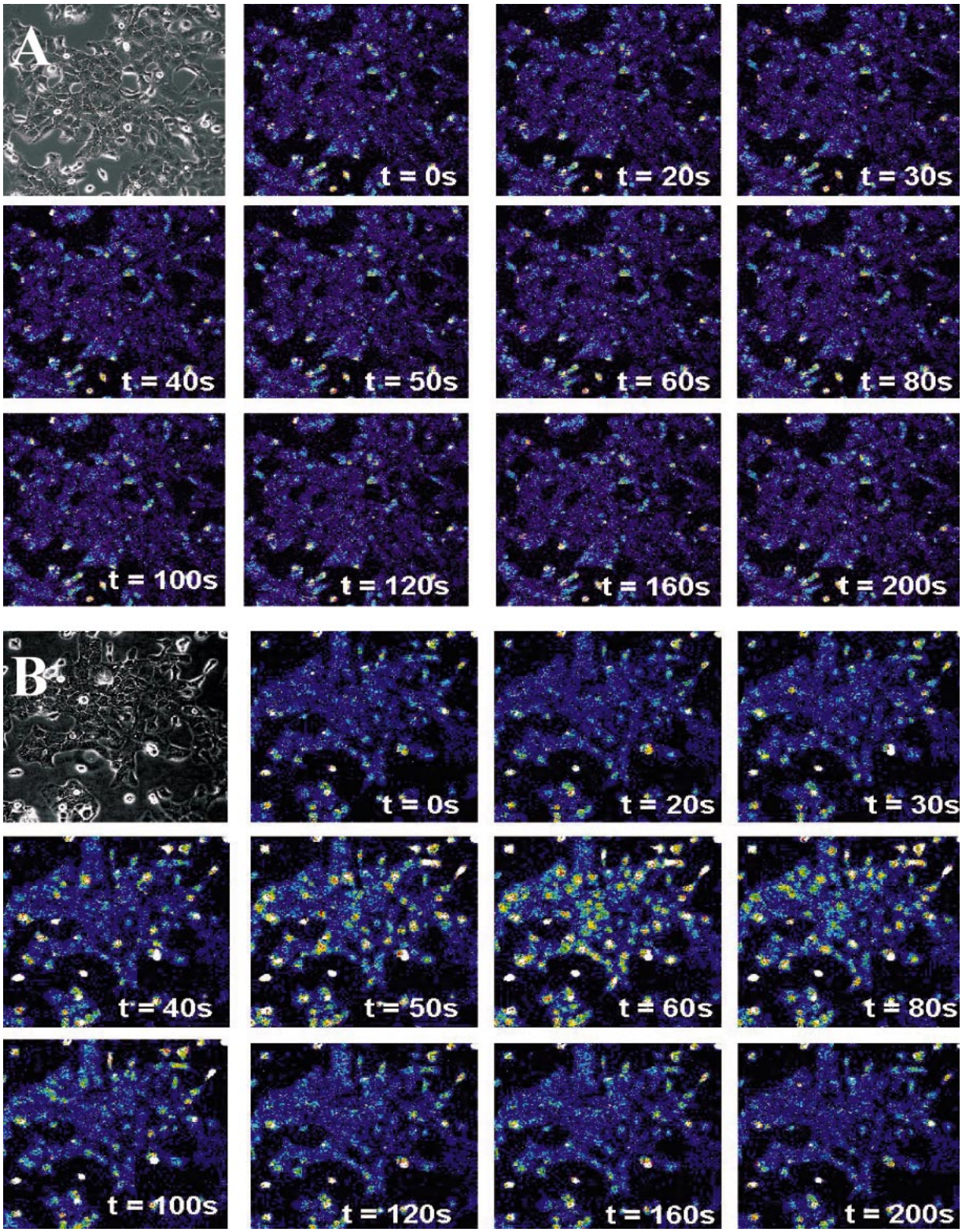

FIG. 3. Ratio images of calcium levels in HPV-G cells before ($t = 0$ s) and at various times after addition of medium from *in vivo*-irradiated CBA/Ca (panel A) and C57BL/6 (panel B) mouse bladder explants. Blue indicates low levels of calcium while green, yellow and red indicate progressively higher levels of calcium. A phase-contrast image is also shown alongside the fluorescence ratio images.

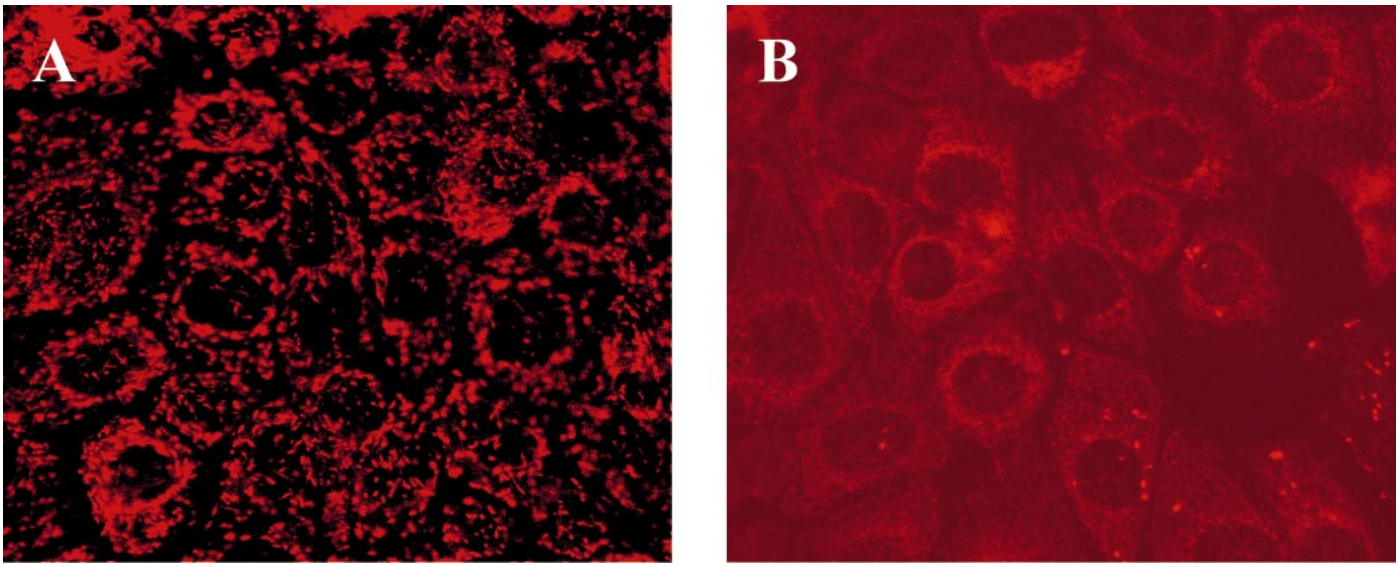

FIG. 4. Zeiss confocal image (40×) of HPV-G cells stained with JC-1 for 20 min after 6 h exposure to bystander medium. Panel A is a control exposed to medium derived from unirradiated C57BL/6 cells and is typical of ''healthy'' HPV-G cells as indicated by clear red punctate staining. Panel B is a similar image of HPV-G cells after 6 h exposure to C57BL/6 medium derived from *in vivo*-exposed animals. The depolarized mitochondrial membranes are indicated by a diffusion of the red punctate staining of the mitochondria.

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 m*M* NaCl, 5 m*M* KCl, 1 m*M* Na_2HPO_4, 1 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$ (pH 7.4). Cells were loaded with the calcium-sensitive dyes by incubation with 3 μ*M* Fluo-3 and 3 μ*M* Fura Red AM esters for 1 h 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 for the Fluo-3/Fura Red fluorescence intensities were recorded every 2 s. Medium was added when a stable baseline had been established. All measurements were performed at room temperature.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured by using a fluorescent dye, JC-1 (Molecular Probes, Leiden). JC-1, with its two-color fluorescence, clearly identifies depolarized mitochondria (*25*). HPV-G-transfected cells were seeded at 1×10^4 in 98-well plates for the fluorescence plate reader and seeded at high concentrations on glass cover slips for confocal microscopy and incubated in medium until the cells had attached. The medium was then removed and sample medium was added to the cells. The cells were left in the incubator for 6 h. The sample medium was then removed and the cells were washed in a Mg^{2+}/Ca^{2+} buffer (130 m*M* NaCl, 5 m*M* KCl, 1 m*M* Na_2HPO_4 1 m*M* $MgCl_2$, and 1 m*M* $CaCl_2$). Cells were loaded with JC-1 (5 μg/ml) for 20 min at 37°C and subsequently washed twice in buffer.

Using a fluorescence plate reader, Victor² (Perkin Elmer) or a Zeiss LSM 510 confocal microscope, JC-1 was excited at 488 nm and fluorescence emissions were recorded at 525 nm and 590 nm.

Statistical Analysis

Data are presented as means ± standard errors of the mean for four independent experiments with at least three replicate flasks per experiment. Significance was determined using the Student's *t* test.

RESULTS

In clonogenic cultures of HPV-G-transfected cells treated with medium obtained from bladder explants established from C57BL/6 mice that had received 0.5 Gy total-body irradiation, there was a significant reduction in plating efficiency from 22.5% to 14.6% (Table 1). In addition, samples of bulk cultures of HPV-G-transfected cells treated with such bladder explant medium taken after 19 population doublings exhibited a similarly reduced colony-forming efficiency. This effect was not detected in HPV-G-transfected cells after exposure to medium obtained from bladder explants established from unirradiated mice or from bladder explants established from CBA/Ca mice that had received 0.5 Gy total-body irradiation. When the HPV-G-transfected reporter cells respond to a bystander signal, they do so as a consequence of calcium signaling (Fig. 2). This is demonstrated by the rapid and transient increase in intracellular calcium after addition of medium harvested from bladder explants established from irradiated C57BL/6 mice. The CBA/Ca medium and the medium derived from the two sham-irradiated control groups were not significantly different from each other. In Fig. 3, the time sequence of the pulse is shown in the confocal images, and this allows the pulse to be tracked in individual cells. Using the same system, mitochondrial membrane depolarization was measured (Fig. 4 and Table 2); only the medium samples from

TABLE 3a
Characteristics of Explant Cultures Established from Nonirradiated Mice or Mice Exposed to 0.5 Gy Total-Body Irradiation (TBI)

Cells	Medium	Percentage apoptosis at 48 h	Percentage apoptosis at 72 h	Total cells at day 14 as percentage of control
CBA/Ca	CBA/Ca	<1	<1	100
TBI CBA/Ca	TBI CBA/CA	<1	<1	112.4 ± 2.7
CBA/Ca explants irradiated *in vitro*		<1	<1	100
C57BL/6	C57BL/6	3.2 ± 0.9	4.1 ± 0.5	100
TBI C57BL/6	TBI C57BL/6	10.3 ± 2.1	22.6 ± 3.0	100
C57BL/6 explants irradiated *in vitro*		21.6 ± 3.7	34.1 ± 2.8	56.9 ± 4.1
C57BL/6	TBI CBA/Ca	5.3 ± 2.4	4.8 ± 0.3	119.0 ± 8.6
CBA/Ca	TBI C57BL/6	2.1 ± 0.3	3.9 ± 0.5	98.6 ± 6.5

the irradiated C57BL/6 mice induced a significant effect, consistent with a downstream response after calcium release when the bystander signal is sectoring cells toward apoptosis.

To explore further the genetic basis of the genotype-dependent *in vivo* production of a bystander signal present in the explant cultures, a series of experiments was conducted in which medium harvested from cultured explants derived from *in vivo*-exposed or sham-exposed bladder tissues were added to unirradiated bladder explants of the same or the other strain, and apoptosis in the early stages of culture and total cellularity after 14 days in culture were assessed. The results (Table 3a) are consistent with the conclusion that exposure to medium from bladder explant cultures derived from *in vivo*-irradiated animals reduces the growth of C57BL/6-derived unirradiated autologous cultures but not CBA/Ca-derived cultures ($P < 0.2$). Apoptosis was evident at 72 h after explantation and was greater in C57BL/6 cells. When explant medium was exchanged between mouse strains, the CBA/Ca cells exhibited a CBA-type response to C57BL/6-derived bystander signal and the C57BL/6 cells responded as CBA explants to the medium signals produced by the CBA/Ca cells.

Using immunohistochemistry, Trp53 response and the expression of the pro-apoptosis protein Bax and anti-apoptosis protein Bcl2 were determined in the cultured bladder explants (Table 3b). The expression of Trp53 was greater in the control CBA/Ca cultures than in the cells from the C57BL/6 strain ($P < 0.05$); Bcl2 expression was also higher but not significantly so ($P = 0.08$). When the explants established from irradiated mice received medium from cells of their own strain, it was observed that Bax was up-regulated relative to the controls in the C57BL/6 cells and Bcl2 was up-regulated in the CBA/Ca cells. Again in the cross strain medium exchange experiments, the CBA/Ca response dominated.

DISCUSSION

The data presented in this paper provide direct evidence that bystander factors in the form of long-lived medium-soluble entities are produced by murine bladder tissues after irradiation at a relatively low dose *in vivo*. These factors are capable of inducing responses, including apoptosis, that are characteristic of a direct radiation response, in unirradiated cells that have not been in contact with irradiated cells. An important factor that influences direct radiation responses is the differential activation of Trp53-response genes, and it is now clear that differential induction of specific Trp53 target genes is a genetically modified process (*26, 27*).

The data for hemopoietic cells are consistent with a genotype-dependent Trp53-mediated transcriptional activation of downstream target genes after irradiation tending to re-

TABLE 3b
Immunocytochemical Characteristics of Cells at Day 14 in Explant Cultures Expressed as Percentage Positive Cells

Cells	Medium	Trp53	Bcl2	Bax
CBA/Ca	CBA/Ca	44.5 ± 8.7	3.5 ± 0.6	0
TBI CBA/Ca	TBI CBA/CA	43.6 ± 5.1	86.3 ± 1.4	2.4 ± 0.2
CBA/Ca explants irradiated *in vitro*		48.6 ± 4.1	100	5.9 ± 1.1
C57BL/6	C57BL/6	5.1 ± 1.8	0.5 ± 0.1	0
TBI C57BL/6	TBI C57BL/6	76.8 ± 4.6	7.4 ± 0.5	100
C57BL/6 explants irradiated *in vitro*		87.3 ± 4.9	10.2 ± 2.0	100
CBA/Ca	C57BL/6	44.2 ± 4.1	3.6 ± 0.6	0
CBA/Ca	TBI C57BL/6	47.1 ± 6.2	3.5 ± 0.7	0.4 ± 0.03
C57BL/6	CBA/Ca	5.3 ± 2.0	15.3 ± 2.1	0
C57BL/6	TBI CBA/Ca	10.7 ± 2.1	67.3 ± 4.8	0

duce (in CBA/Ca) or reinforce (in C57BL/6) the apoptotic program (C57BL/6).

Directly irradiated murine urinary epithelium exhibits similar genotype-dependent apoptotic responses that reflect the range of responses seen in human uroepithelium (*7*).

We have now shown a similar genetic influence on responses to bystander signals produced *in vivo*, confirming data obtained from *in vitro* studies using human and mouse uroepithelial tissues, which showed a large variation in the toxicity of bystander factors derived from irradiated explant fragments (*7*). The response to the *in vivo*-generated bystander signal exhibits genotypic differences in calcium signaling and also in signaling pathways indicative of a major role for the balance of pro-apoptosis (C57BL/6) and anti-apoptosis (CBA/CA) proteins in determining the overall response. By using a ''cross-genotype'' approach, we were able to demonstrate that while we could not detect a response of CBA/Ca cells to self-derived signals, a CBA/Ca-derived bystander signal was able to induce Bcl2 expression in C57BL/6 explants. Whether the absence of, for example, the calcium flux in CBA/Ca mice means that no signal is produced or that a different signal is produced is not known. Also, the quantitative measurements of mitochondrial membrane depolarization show an increase in potential in cells receiving CBA/Ca-derived medium but a decrease in the cells exposed to C57BL/6-derived medium. It is possible that in CBA/Ca mice an anti-apoptosis signal prevents both the calcium flux and the downstream events in the cascade leading to apoptosis. The fact that the CBA/Ca pattern of response dominates in these experiments when medium is exchanged would support this view.

The nature of the bystander signal is unknown, but it is stable during transport for 24 h at room temperature, and it persists in frozen samples. It is also persistently expressed into culture medium by irradiated cells and tissues. These observations make it unlikely that the bystander signal is simply a consequence of radiation-induced radical formation and more likely that it is an induced long-term biological response. Whatever the molecular mechanism, it is strongly influenced by genetic factors, and the demonstration of bystander signals *in vivo* means that their relevance to radiotherapy and diagnostic imaging need to be considered more seriously. It also means that if the type of genotype-dependent differences demonstrated in these studies can be extrapolated to humans, as is indicated by our previous studies of uroepithelial responses (*7*), then the response of the recipient of a low dose of radiation may be more important than the actual dose to which they are exposed. It is of course important to recognize that there may be a distinction between bystander effects from signals that result from exposed cells receiving very many hits and possibly dying (such as after the 0.5-Gy dose used in this paper) and bystander effects from signals that arise after a much lower dose when only some cells would be hit, and then only once, and would not die. These signals may be different. The latter situation could arise, for example in a whole-body exposure to low-LET radiation at a dose $\ll 1$ mGy, while the former (authors' conditions) may occur in a partial-body (therapeutic) exposure. Clearly more work needs to be done with *in vivo* systems over a range of relevant doses. The expression of bystander effects and the potential consequences of such effects in tissues appear to reflect a balance between the type of bystander signals produced and the responses of cell populations to such signals, both of which may be significantly influenced by genotype. Thus, as well as targeted effects of damage induced directly in cells by radiation, a variety of bystander effects may also make important short-term and long-term contributions to determining overall outcome after radiation exposures.

ACKNOWLEDGMENTS

The authors acknowledge support from Science Foundation Ireland, The Irish Cancer Research Advancement Board, and The EU Radiation Protection Programme. We also wish to thank Prof. Brian Harvey and Dr. Natasha Coen for allowing us to use the fluorescence plate reader.

Received: August 12, 2004; accepted: November 6, 2004

REFERENCES

1. C. Mothersill and C. B. Seymour, Radiation-induced bystander effects: Past history and future directions. *Radiat. Res.* **155**, 759–767 (2001).
2. S. A. Lorimore and E. G. Wright, Radiation-induced genomic instability and bystander effects: Related inflammatory-type responses to radiation-induced stress and injury? A review. *Int. J. Radiat. Biol.* **79**, 15–25 (2003).
3. J. B. Little, Genomic instability and bystander effects: A historical perspective. *Oncogene* **22**, 6978–6987 (2003).
4. W. F. Morgan, Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects *in vivo*, clastogenic factors and transgenerational effects. *Radiat. Res.* **159**, 581–596 (2003).
5. 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 alpha-particle irradiation. *Proc. Natl. Acad. Sci. USA* **95**, 5730–5733 (1998).
6. 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).
7. C. Mothersill, E. G. Wright, D. M. Rea, D. Murphy, S. Lorimore, C. B. Seymour and K. O'Malley, Individual variation in the production of a 'bystander signal' following irradiation of primary cultures of normal human urothelium. *Carcinogenesis* **22**, 1465–1471 (2001).
8. 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).
9. S. M. Clutton, K. M. 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).
10. 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).

11. N. Coen, M. A. Kadhim, E. G. Wright, C. P. Case and C. E. Mothersill, Particulate debris from a titanium metal prosthesis induces genomic instability in primary human fibroblast cells. *Br. J. Cancer* **88**, 548–552 (2003).

12. N. Coen, C. Mothersill, M. Kadhim and E. G. Wright, Heavy metals of relevance to human health induce genomic instability. *J. Pathol.* **195**, 293–299 (2001).

13. S. A. Lorimore, P. J. Coates, G. E. Scobie, G. Milne and E. G. Wright, Inflammatory-type responses after exposure to ionizing radiation *in vivo*: A mechanism for radiation-induced bystander effects? *Oncogene* **20**, 7085–7095 (2001).

14. M. A. Kadhim, S. J. Marsden, D. T. Goodhead, A. M. Malcolmson, M. Folkard, K. M. Prise and B. D. Michael, Long-term genomic instability in human lymphocytes induced by single-particle irradiation. *Radiat. Res.* **155**, 122–126 (2001).

15. C. Mothersill and C. B. Seymour, Bystander and delayed effects after fractionated radiation exposure. *Radiat. Res.* **158**, 626–633 (2002).

16. G. E. Watson, S. A. Lorimore and E. G. Wright, Long-term *in vivo* transmission of alpha-particle-induced chromosomal instability in murine haemopoietic cells. *Int. J. Radiat. Biol.* **69**, 175–182 (1996).

17. G. E. Watson, S. A. Lorimore, S. M. Clutton, M. A. Kadhim and E. G. Wright, Genetic factors influencing alpha-particle-induced chromosomal instability. *Int. J. Radiat. Biol.* **71**, 497–503 (1997).

18. B. Ponnaiya, M. N. Cornforth and R. L. Ullrich, Radiation-induced chromosomal instability in BALB/c and C57BL/6 mice: The difference is as clear as black and white. *Radiat. Res.* **147**, 121–125 (1997).

19. 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 ionizing radiation. *Cancer Res.* **60**, 5608–5611 (2000).

20. L. Y. Xue, N. J. Butler, G. M. Makrigiorgos, S. J. Adelstein and A. I. Kassis, Bystander effect produced by radiolabeled tumor cells *in vivo*. *Proc. Natl. Acad. Sci. USA* **99**, 13765–13770 (2002).

21. L. Pirisi, A. Batova, G. R. Jenkins, J. R. Hodam and K. E. Creek, Increased sensitivity of human keratinocytes immortalized by human papillomavirus type 16 DNA to growth control by retinoids. *Cancer Res.* **52**, 187–193 (1992).

22. T. T. Puck and P. I. Marcus, Action of x-rays on mammalian cells. *J. Exp. Med.* **103**, 653–666 (1956).

23. 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).

24. L. Formigli, L. Papucci, A. Tani, N. Schiavone, A. Tempestini, G. E. Orlandini, S. Capaccioli and S. Z. Orlandini, Aponecrosis: Morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. *J. Cell Physiol.* **182**, 41–49 (2000).

25. S. Sperandio, I. de Belle and D. E. Bredesen, An alternative, nonapoptotic form of programmed cell death. *Proc. Natl. Acad. Sci. USA* **97**, 14376–14381 (2000).

26. P. J. Coates, S. A. Lorimore, K. J. Lindsay and E. G. Wright, Tissue-specific p53 responses to ionizing radiation and their genetic modification: The key to tissue-specific tumour susceptibility? *J. Pathol.* **201**, 377–388 (2003).

27. M. Wallace, P. J. Coates, E. G. Wright and K. L. Ball, Differential post-translational modification of the tumour suppressor proteins Rb and p53 modulate the rates of radiation-induced apoptosis *in vivo*. *Oncogene* **20**, 3597–3608 (2001).