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Environmental Research 105 (2007) 200–211
Environmental Research
www.elsevier.com/locate/envres

Bystander effects of ionizing radiation can be modulated by signaling amines

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Received 18 September 2006; received in revised form 7 December 2006; accepted 18 December 2006
Available online 8 February 2007

Abstract

Actual risk and risk management of exposure to ionizing radiation are among the most controversial areas in environmental health protection. Recent developments in radiobiology especially characterization of bystander effects have called into question established dogmas and are thought to cast doubt on the scientific basis of the risk assessment framework, leading to uncertainty for regulators and concern among affected populations. In this paper we test the hypothesis that small signaling molecules widely used throughout the animal kingdom for signaling stress or environmental change, such as 5-Hydroxytryptamine (5-HT, serotonin), L-DOPA, glycine or nicotine are involved in bystander signaling processes following ionizing radiation exposure. We report data which suggest that nano to micromolar concentrations of these agents can modulate bystander-induced cell death. Depletion of 5-HT present in tissue culture medium, occurred following irradiation of cells. This suggested that 5-HT might be bound by membrane receptors after irradiation. Expression of 5-HT type 3 receptors which are Ca^{2+} ion channels was confirmed in the cells using immunocytochemistry and receptor expression could be increased using radiation or 5-HT exposure. Zofran and Kitryl, inhibitors of 5-HT type 3 receptors, and reserpine a generic serotonin antagonist block the bystander effect induced by radiation or by serotonin. The results may be important for the mechanistic understanding of how low doses of radiation interact with cells to produce biological effects.

Keywords: Novel protective agents; 5-HT; 5-HT receptors; Radiation-induced bystander effects; Radiation protection

1. Introduction

Environmental risks, especially cancer induction in humans, consequent on ionizing radiation exposure are highly controversial. Huge volumes of data can be produced to support increased risk at low doses and equal amounts to support decreased risk or zero risk at low doses. Until quite recently, radiation was thought to act only by inducing cellular DNA damage which if not faithfully repaired before the cell divided could lead to a mutation. Any mutation frequency had a concomitant and finite stochastic probability of being an initiating carcinogenic event. This led to the so-called Linear-non-threshold or LNT hypothesis, correlating in a quantitative way, the radiation dose with cancer risk in a linear fashion. Implicit in this hypothesis was the absence of any dose at which there was zero probability of a carcinogenic event, i.e. there is no safe dose. It was also implicit that all the radiation effects were due to DNA "hits" and were either repaired or mis-repaired before the cell divided. It is now known that radiation effects can persist and manifest in apparently healthy cells many generations after the initial exposure and that cellular signaling mechanisms exist which appear to coordinate response to radiation at the hierarchical level of the cell population or tissue. For reviews see Little and Morgan (2003), Lorimore and Wright (2003), Morgan (2003) and Mothersill and Seymour (2004). Very recently our group and that of Surinov has actually demonstrated that these signaling mechanisms can induce effects in unexposed litter mates or in unirradiated fish swimming in water with irradiated fish, thus showing possible coordination of stress responses at the animal population level (Surinov et al., 2005; Mothersill et al., 2006). These findings challenge the LNT hypothesis and in the absence of detailed mechanistic information, make it very difficult to

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doi:10.1016/j.envres.2006.12.003

link dose to risk in any simple way, leading to uncertainty, mistrust and huge financial costs. Radiation-induced bystander signals appear to coordinate cellular responses in cells not directly exposed to or traversed by radiation tracks. Prior to the recent characterization of bystander effects in a systematic way it was held that DNA double strand breaks and cellular survival/damage were inextricably linked and that radiation damage, possibly leading to carcinogenesis could be defined as a function of DNA double strand breaks (Kellerer and Rossi, 1972; Chadwick and Leenhouts, 1973; Barendsen, 1994; Pieffer, 1998). This is obviously challenged because of the increasing number of studies which demonstrate indirect (i.e., non-DNA related) effects and coordinated tissue responses. The non-targeted effects appear to saturate at low doses (0.003–0.5 Gy) and result in a breakdown of the dose–response relationship which dominates at high doses (Seymour and Mothersill, 2000; Kashino et al., 2004). The low-dose mechanisms may either mitigate or exacerbate the direct effects of the dose and dominate the results at doses at or below 0.5 Gy. Doses below 0.1 Gy are considered low in environmental protection. Current models of radiation dose–response do not accommodate these new findings and as long as the mechanisms remain unclear, modeling low-dose effects is difficult and uncertainty is high. While the downstream response to bystander signals is relatively well characterized (Clutton et al., 1996; Limoli et al., 1998; Albanese and Dainiak, 2000; Lyng et al., 2002; Rugo and Schiestl, 2004), the present paper reports experiments designed to investigate earlier events in signal production in cells actually irradiated. Transmission of these signals to recipient cells and inhibition of these processes were also studied. Our overall driving hypothesis is that bystander effects are really ubiquitous cell signaling systems which exist in all multicellular organisms and are not unique responses to radiation. This led us to look at candidate initiation mechanisms and signaling molecules across a wide range of animal species. We selected molecules which had been harnessed as neurotransmitters in advanced species but which also had signaling or defence roles in very early metazoa and in aquatic species. We looked at the effects of serotonin, and reserpine, which is an inhibitor of serotonin function (Ban, 2001). Also studied were L-DOPA, the precursor of dopamine and the monoamine oxidase inhibitor (MAOI), *l*-(−)deprenyl, which should increase dopamine levels. Reserpine also inhibits accumulation of L-dopamine through its inhibition of the vesicular monoamine reporter (Erickson et al., 1992). Nicotine and glycine were investigated in some very preliminary, "proof of principle" experiments, as other examples of small bioactive molecules involved in signaling. The latter are particularly interesting since there is evidence that their receptors are affected by nitric oxide (Yu and Eldred, 2005; Muller et al., 2006), which is a molecule thought to have a role in bystander signaling (Shao et al., 2005). Most of the experiments centred on defining the role of serotonin. Serotonin (5-hydroxytryptamine), a biogenic amine, is a product of tryptophan metabolism. It is a well-evaluated neurotransmitter in the central nervous system and can be found in high concentration in the chromaffin cells of the intestinal mucosa, in the platelets and the serotonergic neurones of the brain. Central-serotonergic neurones influence physiological functions such as sleep, mood, hormonal and cardio-vascular regulation. Increased serum levels can be found in association with some carcinomas, endogenous depression and schizophrenia (Costall and Naylor, 1991; Derkach et al., 1989; Tuladhar et al., 2000).

While serotonin has a well-characterized signaling role as a stimulatory neurotransmitter in the brain of mammals, it is also involved in signaling in other cells and particularly in lower organisms. In invertebrates it can be a communication, signaling or defence molecule (Kohidai, 1999; Yaguchi and Katow, 2003; Zega Pennati et al., 2005). Serotonin also has well-described effects on calcium homeostasis causing a transient calcium pulse in cells bearing 5-HT_2 or 5-HT_3 receptors (Ban, 2001). A rapid calcium flux is characteristic of cells receiving medium containing the bystander factor. L-DOPA was used in some experiments because it has similar signaling roles in invertebrates but does not appear to affect calcium homeostasis in the same way (Roth and Chuang, 1987; Burnashev, 1998; Watson, et al., 2000), although there is one report in the literature of a protective effect of dopamine and L-DOPA against serum depletion-induced cell death in PC12 cells, which is attributed to activation of the MAP kinase pathway consequent on a sustained elevation of intracellular calcium concentration (Koshimura et al., 2000). L-DOPA is also a brain neurotransmitter substance. Reduced levels are associated with Parkinson's disease. The comparison of the effects of these substances should address whether the bystander effect can be induced by signaling amines and also whether the effects are related to the ability of the molecules to modulate calcium homeostasis. The effects of specific inhibitors or modulators of biogenic amine function – *l*-deprenyl and reserpine—on bystander responses detected in cells were also considered. *l*-Deprenyl which is a monoamine oxidase B inhibitor is known to regulate dopamine levels in the brain (Ebadi et al., 2002) but it has also been shown by our group to act at much lower concentrations as an inhibitor of bystander effects and genomic instability (Seymour et al., 2003). Tatton et al. (1996) have also published a role for low concentrations of *l*-deprenyl in the modulation of mitochondrial calcium levels and maintenance of mitochondrial membrane potential. Both groups identified a role for up-regulation and relocation of bcl-2 in the mitochondrial protection. As mentioned earlier, transitorily increased intracellular calcium levels are the first sign our group has been able to detect following exposure of cells to bystander medium (Lyng et al., 2002) this calcium flux appears to initiate cell death pathways in recipients of bystander medium via changes in mitochondrial membrane potential. Thus we consider that a study of these agents may enable us to define more clearly, the nature of bystander signals, their

induction in response to irradiation and their transmission to recipient cells. Reserpine, which is an inhibitor of serotonin (Ban, 2001) has also been shown to affect calcium homeostasis (Satoh et al., 1992), blocking specific L channels by binding to serotonin receptors on the cell surface. The specific hypothesis being tested in this paper is that bystander effects involve the transient increase in intracellular calcium and that small biologically active signaling molecules capable of affecting calcium transport into (or indeed out of) cells, will alter bystander effects.

2. Methods

2.1. Cell culture

Clonogenic cell line: HPV-G cells are adherent epithelial cells derived originally from a human foreskin primary culture and immortalized by the HPV virus (Pirisi et al., 1988) They were obtained as a gift from Prof. J. DiPaolo and have been used in our laboratory as a reporter system for bystander signal production in a wide range of experiments (e.g. Mothersill et al., 2001, 2005). The cell line was grown in DMEM:F12 (1:1) obtained from Gibco Biocult, Irvine, Scotland. This was supplemented with 10% Foetal bovine serum, 1 μg/ml hydrocortisone (Sigma, Poole Dorset, UK), 5 ml penicillin:streptomycin solution and 5 ml L-glutamine solution. Hepes buffer (12.5 ml of IM solution) was added to help maintain pH. Except where indicated, all reagents were obtained from Gibco Biocult. The line was maintained in T75 flasks (NUNC Inc, Uden, Netherlands) and subcultured into T25 flasks (40 ml volume) for experiments. The cells are non-tumourigenic, have about 30% wild-type p53 expression (Cooper et al., 2003) and have a normal epithelial pattern of cobblestone density inhibited cell growth. They are used because when exposed to autologous medium harvested from irradiated cells, they give a stable bystander effect of ~40% reduction in plating efficiency over a very wide range of doses and exposure conditions (Seymour and Mothersill, 2000). This allows comparison of bystander-inducing signal strengths even when the HPV-G cells are exposed to signals from other cell lines or from explants.

2.2. Clonogenic assay for bystander activity using HPV-G cells as reporters

Flasks which were 85–90% confluent and that had received a medium change the previous day were chosen. Cells were removed from the flask using 0.25%w/v trypsin/1 mM EDTA solution (1:1). When the cells had detached they were resuspended in medium, and an aliquot was counted using a Coulter counter model D_N set at a threshold calibrated for each cell line using a haemocytometer. Appropriate cell numbers were plated for survival using the clonogenic assay technique of Puck and Marcus (1956). Flasks destined to donate medium were plated with cell numbers in the region of 1×10^5 per 5 ml medium (40,000 cells/ml) in T25 40 ml volume flasks (Nucleon, Denmark). Medium was harvested 1 h post-irradiation, which took place 6 h after plating. The harvested medium was transferred to cultures containing cloning densities of cells set up at the same time as the donors. Controls for medium only and actual irradiation effects were included in each experiment. Controls for transfer of unirradiated medium from densely seeded cultures to cultures seeded at cloning densities were also always included. Cultures were incubated in 5 ml of culture medium in 25 cm^2, 40 ml flasks (Nunclon, Denmark), in a humidified 37 °C incubator in an atmosphere of 5% CO_2 in air.

2.3. Medium transfer

The technique used has been described in detail in Mothersill and Seymour (1997). Briefly, medium was poured off donor flasks (containing explants or HPV-G cells). This was filtered through a 0.22 μm filter. This was to ensure that no cells could still be present in the transferred medium. Intact cells were not present in the filtered supernatants (as detected by examination of aliquots of medium under the microscope). Culture medium was then removed from the flasks designated to receive irradiated conditioned medium and the filtrate was immediately added to these recipient flasks. A medium change of unirradiated but similarly filtered medium from unirradiated donor flasks containing explants or seeded at the donor density of ~200,000 cells/flask was given to controls at the same time. Standard plating efficiency controls were also set up. There was never a significant difference between these two controls. Standard clonogenic survival points following irradiation were also always included, with and without a medium change at the appropriate time. No effect of changing the medium alone was found. The donor medium generated as described in this paragraph is referred to as bystander medium. The bystander medium harvested at 1 h, (a time previously determined to be optimal for expression of bystander signals), after irradiation was in some cases transferred to unirradiated autologous explants of murine bladder. Response was monitored by determining the growth in cell number, and the numbers of apoptotic cells as appropriate.

2.4. Tissue explant technique

Explants of murine bladder and rainbow trout skin were established as described previously (Mothersill et al., 2005). Briefly, the C57Bl6J mice were sacrificed by cervical dislocation 1 h after exposure to gamma irradiation, bladders were removed pooled and placed in complete growth medium. They were dissected into four equal sized pieces per bladder and incubated for 30 min at 37 °C in 0.25% w/v trypsin (Gibco Biocult, Irvine, Scotland), containing a solution of 10 mg/ml collagenase. At the end of the incubation time the explants were washed in growth medium and plated as single explants in the centre of 25 cm^2 growth area, 40 ml volume flasks (NUNC, UDEN, Denmark) in 2 ml growth medium. Flasks were left undisturbed for 48 h at 37 °C in an atmosphere of 5% CO_2 in air before irradiation. Fish skin samples were also treated similarly but the samples were incubated at 19 °C without CO_2 and were exposed in vitro to 0.5 Gy gamma irradiation 3 days after plating. All tissue was obtained and treated according to guidelines at McMaster University and the procedures were covered by AUP #05-08-44.

2.5. Preparation of chemical solutions

Serotonin (5-hydroxytryptamine) as the creatinine sulphate salt, and reserpine were purchased from Sigma Chemicals Ltd. Serotonin was made up as a concentrated aqueous solution and then serially diluted. Reserpine is very insoluble in culture medium and was prepared as a concentrated (1 mM) solutions in DMSO as recommended by the the supplies insert. The stock solutions were serially diluted so that the desired concentration of the drug could be added in a 0.1 ml volume to the 5 ml of medium in the culture flasks. A solvent control with an appropriate volume of solvent was run with every experiment. L-DOPA, *l*-deprenyl (seleginine), nicotine and glycine were also purchased from Sigma Chemicals Ltd. These were prepared in sterile PBS and diluted so that the desired concentration could be added as a 0.1 ml volume as described above. Zofran (Ondansetron) and Kitryl (Granisetron), are commercially available anti-emetics which work by inhibiting 5 HT type 3 receptor binding by 5 HT (Savastano et al., 2005). They were obtained as injectable solutions dissolved in sterile distilled water. All stock solutions were filter sterilized before use.

2.6. Irradiation of tissue cultures

Where indicated, cultures were irradiated at room temperature using a cobalt 60 teletherapy unit at flask to source distance of 80 cm. Irradiation commenced 6 h after plating for cell lines, 48 h after plating for mouse explants and 3 days after plating for fish tissues. The dose rate during these experiments was ~1.8 Gy/min.

2.7. Apoptosis scores

Numbers of apoptotic cells were scored according to morphological criteria (picnotic nuclei, cytoplasmic blebbing, shrunken cellular morphology) under phase contrast using attached living cultures 48 h after exposure to ionizing radiation or donor medium from irradiated cells (where indicated).

2.8. Fura 2 measurements to determine intracellular free calcium in HPVG cells

Fura-2 acetoxymethyl (AM) is a Ca^{2+} sensitive fluorescent dye used to assess concentrations of free cytoplasmic calcium for cultured adherent HPVG cells in a two-well chamber cover glass. Fura-2 binds to cell membranes as an ester and is hydrolysed in the cell to its Ca^{2+} sensitive acidic form. Florescence for the adherent HPVG cells is measured over time with an automated Olympus 1×81 microscope using a 40X oil objective and a Fura filter cube with 510 nm emission. Hardware control and image acquisition are performed with PTI ImageMaster software. HPVG cells are plated at 300,000 cells/well of a two-well cover glass and incubated at 37 °C and 5% CO_2 for 18–24 h. Cells were washed three times with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 25 mM Hepes (pH 7.4). Subsequently, the cells were loaded with 1 ml of 4.17 μM Fura-2 and incubated for 30 min. Cells were then washed three times with buffer and leaving 300 μl of buffer in each well for the calcium measurement. Twelve bit images are acquired with the camera binning set to 2×2 and exposure time set to 977 ms. A field of about 10–100 evenly distributed cells is selected for the measurement. Fura-2 was excited at 380 and 340 nm and the ratio images were recorded every 2 s for 4 min. one hundred microlitres of reserpine or 5-HT or appropriate solvent control was added after 120 s, when a stable baseline had been established. All measurements were performed in the dark at room temperature.

2.9. ELISA assay for serotonin in medium samples

The assay kit obtained from Labor Diagnostika Nord GmbH and Co. KG Nordhorn, Germany, provides materials for the quantitative determination of derivatised serotonin in serum, plasma, urine, platelets, tissue homogenates, cell culture supernatants and liquor. The derivatisation is part of the preparation of samples. By using an acylation reagent the serotonin is derivatised into *N*-acylserotonin.

The competitive serotonin ELISA kit uses the microtiter format. Serotonin is bound to the solid phase of the microtiter plate. Acylated serotonin and solid phase bound serotonin compete for a fixed number of antiserum-binding sites. When the system is in equilibrium, free antigen and free antigen–antiserum complexes are removed by washing. The antibody bound to the solid phase serotonin is detected by the anti-rabbit/peroxidase. The substrate TMB/peroxidase reaction is read at 450 nm. The amount of antibody bound to the solid phase serotonin is inversely proportional to the serotonin concentration of the sample.

2.10. Assay for 5-HT receptor 3

Monoclonal rabbit Anti-Serotonin 5-HT3 Receptor (Sigma, Saint Louis, Missouri product number S1561) was used to detect and quantify 5-HT3 receptor labelling in HPV keratinocytes. The antibody used is an anti-serotonin 5-HT3 receptor developed in rabbit using synthetic peptide conjugated to KLH via an *N*-terminal cysteine. This sequence corresponds to amino acids 444–457 from rat serotonin 5-HT3 receptor protein. The amino acid sequence contains one amino acid substitution with mouse and two substitutions with human sequences for the serotonin 5-HT3 receptor. The antibody from sigma has already been characterized specifically for the anti-5-HT3 receptor. The Streptavidin Peroxidase method was employed using the mouse monoclonal Vectastain ABC kit (Vectastain® Corporation, Inc., Burlingame, CA). Cultures were fixed after irradiation in 10% formalin and stored at room temperature. Immunostaining was performed within 7 d of fixation. Culture flasks were cracked open using a pair of pliers leaving only the base with the attached cells. The cultures were washed and covered with a thin layer of PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H_2O_2) in methanol for 3 min after which the cultures were rinsed in PBS. Diluted 1% bovine serum (Vectasiain® ABC kit) was then applied to the cultures for 20 min to block cross-reacting proteins. Subsequently, the cultures were washed with PBS. The primary antibody (Anti-Serotonin 5-HT3 receptor, 1:50 dilution) was then applied to the cultures for 60 min. Cultures were washed with PBS and incubated with diluted biotinylated anti-mouse reagent (Vectasiain® ABC kit) for 30 min. A wash with PBS followed. Diluted streptavidin peroxide was then applied to the cultures for 30 min, followed by a wash in PBS. 3,3′-diaminobenzidine tetrahydrochloride (DAB) was used as chromogen (10 min). Cultures were then washed with distilled water and counterstained with Harris haematoxylin (Sigma-Aldrich, Oakville, Canada) (10 s). Any excess haematoxylin was removed by running the slides in warm water. Cultures were then differentiated in 3% acid alcohol (10 s) and cover slipped and mounted in glycergel (DakoCytomation, Carpinteria, CA). A positive reaction gives a brown product. Negative controls were cultures in which all reagents were applied except the primary antibody (Anti-Serotonin 5-HT3 receptor). All incubations were performed in the dark and at room temperature. Images were obtained using an Olympus CK40 microscope and cells were scored using the Image-Pro® Discovery Version 4.5 software.

2.11. Statistical analysis

Data are presented as mean ± standard error for at least three independent experiments with at least three replicate flasks per experiment and significance was determined using Student *T*-test.

3. Results

Fig. 1 shows the effect of serotonin and its inhibitor—reserpine—on radiation-induced direct or bystander clonogenic cell death in HPV-G cells. Serotonin, in the micromolar range, reduces the survival of unirradiated or 0.5 Gy directly irradiated cells to levels obtained when bystander medium from irradiated cells is added to unirradiated cells. Increasing concentrations of serotonin above 1 up to 1000 μM do not further increase toxicity, suggesting that as with radiation, the effect saturates. Apoptosis frequency is shown in Table 1 and the data agree with the clonogenic survival data. Reserpine, at concentrations which are not in themselves toxic abolishes the bystander cell death response in recipient cells. Apoptosis data also shown in Table 1, agree with the clonogenic survival data. The results for murine bladder explants are shown (Table 2). Clearly, similar effects are produced in tissue explants and in immortalized cells.

Fig. 2 and Table 3 show similar data for L-DOPA and the monoamine oxidase inhibitor *l*-deprenyl. The results show that L-DOPA has a protective, anti-apoptotic effect on bystander cell death at low concentrations but high doses are toxic. *l*-Deprenyl prevents cell death in the nanomolar range and has a greater protective effect than L-DOPA. Again the tissue explants (Table 4) show similar effects to established cells.

Data for nicotine and glycine, which are also small signaling amines involved in neurotransmission are shown

in Fig. 3. In both cases the direct radiation doses have increasing cell killing effect with increasing concentrations of the amines (10 nM–10 μM) but the bystander effect is prevented by all concentrations of glycine. Nicotine in the nanomolar range actually reverses the bystander effect of radiation and stimulates the survival of cells receiving bystander medium.

Of the amines and inhibitors tested, Serotonin was the only one which gave by itself, a similar effect to that seen with radiation, therefore the remainder of this paper deals with a detailed investigation of this chemical. In Fig. 4 the serotonin-associated reduction in clonogenic survival is compared with data showing increasing toxicity of ICCM associated with irradiating increasing numbers of donor cells. This suggests that the "active" range of 1–100 μM serotonin is equivalent to the bystander toxicity produced by ~750,000 cells in 5 ml culture medium.

Since these results suggested that serotonin might be a candidate bystander diffusible factor, an ELISA assay was performed to see if it was increased in media harvested from irradiated cells. In Table 5(a) and (b) data showing the measurements of serotonin levels in the culture medium harvested from irradiated HPV-G cells (5(a)), and mouse in vivo exposed bladders (5(b)) are shown along with controls. Media harvested from rainbow trout fish explant experiments were also available and are included in Table 5(b) to show the universality of the serotonin mechanism. Clearly serotonin is not increased in media from irradiated cells or explant cultures or from cultures set up from tissues obtained from irradiated mice, rather it is significantly decreased in these four totally different experimental systems. In each case the depletion correlates with detection of a bystander effect (Fig. 5). The results obtained using coded samples, were repeated several times and selected points were confirmed using radioimmunoassay by another operator also using coded samples (data not shown). Unused freshly prepared DMEM:F12 or RPMI-1640 complete medium containing 10% foetal bovine serum, without cells contained ~300 ng/ml serotonin. This value was unaffected by addition of reserpine, ondansetron or granisetron showing that these antagonists did not interfere with the ELISA assay. A study of serial dilutions of serum alone in distilled water, or in medium confirmed that the source of serotonin was the serum. Serotonin levels were found to degrade quite rapidly (over 2 weeks) in medium at 37 °C and even in medium stored at 4 °C and was degraded by exposure to bright light such as that commonly used in Biological Safety Hoods. This may account for much of the reported variability in media transfer bystander experiments and also accounts for the difference in serotonin levels in medium from control flasks containing cells or tissues. Cells were always irradiated and harvested within 8 h of set up while tissues were 3–4 d old when media was harvested since the explants took time to attach.

Depletion of serotonin following irradiation in all these different systems, suggested that irradiated cells might be binding serotonin or using it in some way. To test whether serotonin was being actively bound by cells, several approaches were used; first reserpine was added to the cultures before irradiation to block serotonin binding. The ELISA result obtained is shown in Table 5(a) and in Fig. 4. Reserpine prevented the depletion of serotonin in the culture medium and also, as shown before, prevented the bystander effect. The second approach was to measure intracellular calcium directly following addition of 5-HT to cells. The results (Fig. 6) clearly show that addition of 5-HT to cells leads to a rapid increase in the level of intracellular free calcium. Since the type of calcium pulse reported by our group is inhibited also by verapamil (Lyng et al., 2006), it was considered that 5 HT type 3 receptors were the most likely to be involved in binding 5 HT. These are the receptors most often associated with peripheral and epithelial tissues (Lundeberg et al., 2002; Costall and Naylor, 2004; Slominski et al., 2003, 2005). To test this hypothesis specific 5-HT3 receptor antagonists Zofran (Ondansetron) and Kitryl (Gravistron) were added to cultures prior to irradiation and medium harvest. These drugs are widely used to stop emesis during cancer therapy and act by specifically blocking the 5-HT3 receptor (e.g. see Loewen et al., 2000; Scott et al., 2006). The results (Fig. 7) clearly show that the bystander effect induced by 0.5 Gy irradiation is prevented in cells which were treated by these receptor antigonists. Finally, immunocytochemistry was used to stain for the numbers of human keratinocytes cells expressing 5-HT3 receptor with and without exposure to radiation and or 5-HT. The results are shown in Fig. 8. Clearly receptors are present in these cells and there is expression in more cells following exposure to both 5-HT and 0.5 or 5 Gy radiation.

4. Discussion

The data in this paper suggest that biogenic amines can alter bystander effects induced by radiation. They also support the hypothesis that serotonin has a role in the production of bystander signals by irradiated cells. The evidence suggests that irradiated cells bind serotonin and that this action can be blocked using reserpine and by antagonists of the 5-HT type 3 group of receptors. The paper also reports that radiation exposure increases the expression of 5-HT3 receptors in these cells. Since serotonin is known to open L-channels permitting a rapid and transient influx of calcium into cells, the paper supports evidence from our group that calcium entry into the cell is an important transducer of bystander related cell death effects but it suggests the new idea that the pharmacological mechanism may involve the binding of serotonin by irradiated donor cells, leading to initiation of the pathway culminating in the release of diffusible "bystander signals" into the medium. The demonstration that the serotonin effect occurs in mice irradiated in vivo and in fish and human explants, suggests a mechanism which covers a wide evolutionary time span. Previous work

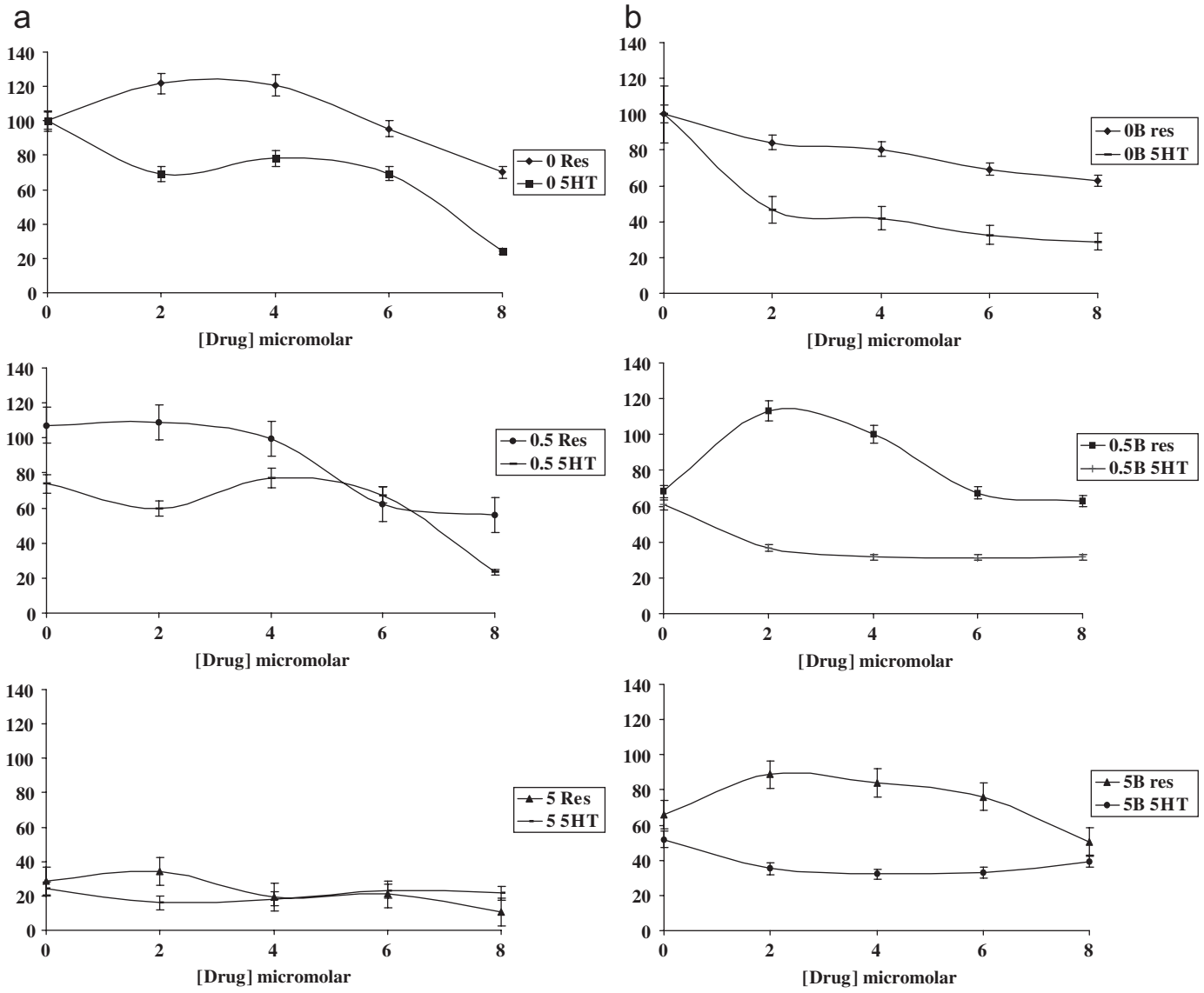

Fig. 1. Left-hand panel; % clonogenic survival, of HPV-G keratinocytes exposed to direct irradiation (0, 0.5 or 5.0 Gy Cobalt 60) with or without prior exposure to 5-hydroxytryptamine (5-HT) or reserpine. Data are means ± SEM for $n = 3$. Right-hand panel; % clonogenic survival of HPV-G keratinocytes exposed to bystander medium harvested from cells which received direct irradiation (0, 0.5 or 5.0 Gy Cobalt 60) with or without prior exposure to 5-hydroxytryptamine (5-HT) or reserpine (res). B indicates bystander medium. Data are mean ± SEM for $n = 3$.

Table 1
Apoptosis scores (%) for HPV-G cells which were exposed to reserpine (res) or serotonin (5-HT) before being directly irradiated 6 h post-plating, or which were exposed to medium harvested 1 h post-irradiation (bystander) from HPV-G cells exposed to res or 5-HT and transferred to unirradiated cultures of HPV-G cells seeded at cloning densities

[Reserpine/5HT]	0 Gy direct	0.5 Gy direct	5 Gy direct	0 Gy bystander	0.5 Gy bystander	5 Gy bystander
0	1.3 ± 0.2	1.1 ± 0.07	28.7 ± 3.3	1.0 ± 0.06	20.4 ± 2.3	21.1 ± 1.9
1 μM res	1.1 ± 0.08	1.2 ± 0.05	34.4 ± 2.9	3.7 ± 0.4	0.2 ± 0.009	1.3 ± 0.07
10 μM res	0.6 ± 0.04	1.0 ± 0.07	19.4 ± 2.4	10.9 ± 0.8	10.6 ± 0.6	12.0 ± 0.6
100 μM res	3.4 ± 0.16	10.1 ± 1.6	20.9 ± 3.1	15.3 ± 1.1	18.6 ± 1.3	12.3 ± 0.9
1000 μM res	4.1 ± 0.6	6.8 ± 1.2	10.6 ± 1.5	17.2 ± 1.6	15.5 ± 1.0	16.7 ± 1.2
1 μM 5 HT	20.1 ± 1.5	20.9 ± 1.6	21.3 ± 1.7	16.9 ± 1.1	17.9 ± 2.2	21.0 ± 1.2
10 μM 5 HT	19.6 ± 2.0	20.4 ± 2.2	20.0 ± 1.3	24.5 ± 1.8	25.4 ± 1.8	33.2 ± 2.1
100 μM 5 HT	18.3 ± 1.3	17.1 ± 1.0	19.3 ± 0.9	30.2 ± 1.7	27.8 ± 2.4	31.7 ± 2.0
1000 μM 5 HT	18.8 ± 1.6	19.2 ± 2.4	20.4 ± 1.4	30.7 ± 2.8	27.1 ± 1.9	31.1 ± 2.6

Fifty microcolonies (~4 cells each), per flask ($n = 3$) were scored at 48 h post-irradiation/ICCM treatment, giving a total scored cell number of ~600 cells.

Table 2
Apoptosis scores (%) for murine explants which were exposed to reserpine (res) or serotonin (5 HT) before being directly irradiated 48 h post-plating, or being exposed to medium harvested 1 h post-irradiation from explants and transferred to autologous explants which had not been irradiated (bystander)

[Reserpine/5 HT]	0 Gy direct	0.5 Gy direct	5 Gy direct	0 Gy bystander	0.5 Gy bystander	5 Gy bystander
0	3.2±0.3	3.5±0.5	43.9±3.6	4.1±0.7	38.4±4.0	33.1±4.6
1 μM res	2.1±0.5	2.5±0.2	25.3±1.0	2.3±0.3	2.1±0.1	2.9±0.5
1 μM res	3.7±0.4	3.7±0.3	27.5±1.9	4.4±0.3	4.5±0.4	5.8±0.6
1 μM 5 HT	32.7±2.6	23.9±2.8	36.1±2.5	30.3±2.7	32.9±2.6	39.7±1.1
10 μM 5 HT	38.0±3.7	34.8±1.2	40.1±3.7	32.8±5.7	43.2±4.7	38.2±4.4

Apoptosis was scored 48 h after irradiation/ICCM exposure. Three fields from 5 separate explant outgrowths (~500 cells in total) were scored.

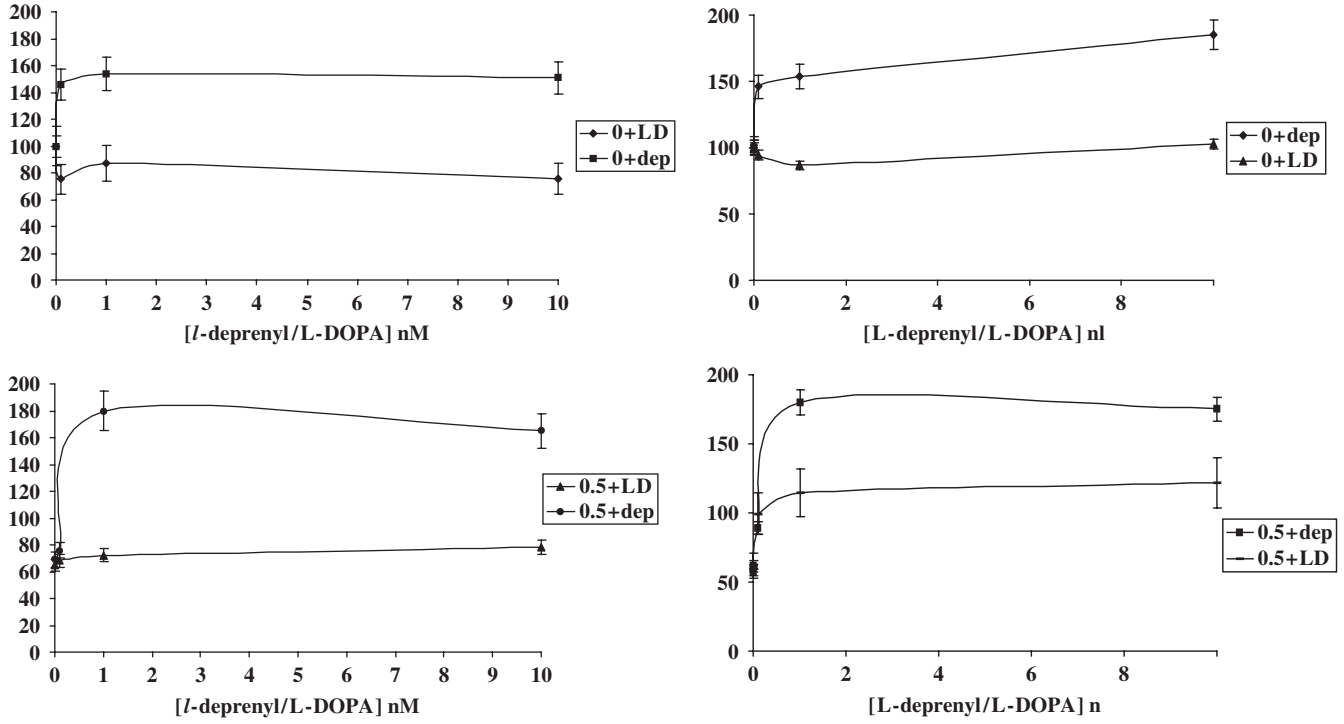

Fig. 2. Left-hand panel; % clonogenic survival of HPV-G keratinocytes exposed to bystander medium harvested from cells which received direct irradiation (0 or 0.5 Gy Cobalt 60). Right-hand panel; % clonogenic survival, of HPV-G keratinocytes exposed to medium harvested (Bystander medium) from cells which received irradiation (0 or 0.5 Gy Cobalt 60) with or without prior exposure to L-DOPA or *l*(−) deprenyl. B indicates bystander medium. Data are mean ±SEM for $n = 3$ with or without prior exposure to L-DOPA or *l*(−) deprenyl. B indicates bystander medium. Data are mean±SEM for $n = 3$.

by the group showed that a rapid transient flux of calcium into the cell occurred after exposure of unirradiated cells to medium from irradiated cells, this is associated with the apoptotic-type response to radiation and is not seen in systems not responding to radiation by initiating apoptosis (Lyng et al., 2002; Mothersill et al., 2001, 2005). Research just published by our group clearly demonstrates that the calcium goes from outside the cell into the intracellular milieu and is not solely released from intracellular stores (Lyng et al., 2006). The current paper suggests that the calcium influx may also occur upon irradiation of the donor cells and may be mediated by the serotonin present in the culture medium, although given the speed with which the flux occurs (induced and resolved within 2 min) it has not been possible to demonstrate this directly using radiation. Because of their role in signaling and defence in colonial species (Kohidai, 1999; Yaguchi and Katow, 2003; Zega Pennati et al., 2005) coupled with their effects on calcium channels (Roth and Chuang, 1987), the biogenic amines and their inhibitors were an obvious candidate group to study. The results for serotonin and its inhibitor reserpine show that medium harvested from cells treated with serotonin even in the absence of irradiation actually reduces clonogenic survival to a level similar to that produced by medium from irradiated cells. Over the range 1–100 μM this effect is constant, suggesting that toxicity of the drug is not the cause of the drop in clonogenic survival. Serotonin is known to act by opening L channels, permitting calcium to enter the cell. Reserpine specifically acts as a serotonin antagonist, blocking the

channel receptors for serotonin. The effects of reserpine are opposite to the effects of serotonin. Interestingly, the addition of medium containing serotonin or reserpine from irradiated cells, does not augment the effect. This, in addition to the fact that serotonin decreases in medium from irradiated cells excludes the possibility that it is itself "the bystander factor" because if it were then the combination of radiation and serotonin should increase the effect, but it does suggest that it could be a key transducer of signal production. This remains an issue for debate. Although an increase in the toxic effect of harvested bystander medium with increasing cell number irradiated was reported by our group (Mothersill and Seymour, 1997), the data reported here show a plateau response for both serotonin and reserpine, suggesting that the mechanism saturates. One hypothesis is that when only nanomolar concentrations of serotonin are available, (as in

Table 3
Apoptosis scores (%) for HPV-G cells which were treated with L-DOPA or *l*-deprenyl then either directly irradiated at 6 h post-plating, or exposed to medium harvested 1 h post-irradiation from HPV-G cells irradiated in the presence of L-DOPA or *l*-deprenyl and transferred to unirradiated cultures of HPV-G cells seeded at cloning densities (bystander)

[LD/dep]	0 Gy direct	0.5 Gy direct	5 Gy direct	0 Gy bystander
0	1.3±0.09	4.6±0.5	1.9±0.2	20.8±1.8
1 nM LD	5.3±1.4	4.2±0.31	1.5±0.09	0.8±0.06
10 nM LD	4.1±0.8	7.5±0.9	3.4±0.4	0
100 nM LD	6.1±0.4	7.3±0,8	0	0
1000 nM LD	Toxic	Toxic	Toxic	Toxic
0.01 nM dep	0.8±0.06	10.1±0.8	1.9±0.12	21.1±1.4
0.1 nM dep	0.6±0.07	8.3±0.5	0.8±0.07	10.4±2.3
1 nM dep	0	9.7±0.7	0	0
10 nM dep	0	0	0	0
100 nM dep	0	0	0	0
1000 nM dep	Toxic	Toxic	0	0

Fifty microcolonies (~4 cells each), per flask were scored at 48 h post-irradiation/ICCM treatment, giving a total scored cell number of ~600 cells.

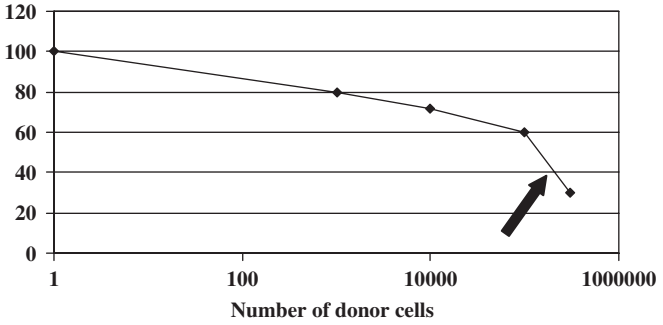

Fig. 4. Percent of clonogenic survival, of HPV-G keratinocytes exposed to medium harvested from cells which received 0.5 Gy irradiation plotted against the donor (irradiated) cell number. The arrow indicates the cell number equivalent to the reduced survival obtained with 5-HT.

Table 4
Apoptosis scores (%) for murine explants which were exposed to L-DOPA (LD) or *l*-deprenyl (dep) then directly irradiated 48 h post-plating, or exposed to medium harvested 1 h post-irradiation from explants and transferred to autologous explants which had not been irradiated (bystander)

[LD/dep]	0 Gy direct	0.5 Gy direct	5 Gy direct	0 Gy bystander	0.5 Gy bystander	5 Gy bystander
0	2.9±0.4	3.1±0.5	40.8±3.7	4.6±0.6	37.2±4.8	32.0±4.4
1 nM l-dep	0.4±0.03	2.1±0.09	1.4±0.3	2.9±0.1	1.6±0.07	1.9±0.3
10 nM l-dep	1.7±0.09	3.4±0.5	5.1±1.7	6.4±0.8	7.8±0.4	7.1±0.9
1 nM LD	3.1±0.1	2.6±0.2	24.8±2.5	2.8±0.3	2.4±0.2	3.7±0.1
10 nM LD	3.7±0.3	3.8±0.2	19.9±2.9	3.7±0.1	3.8±0.4	4.3±0.5

Apoptosis was scored 48 h after irradiation/ICCM exposure. Three fields from five separate explant outgrowths (~500 cells in total) were scored.

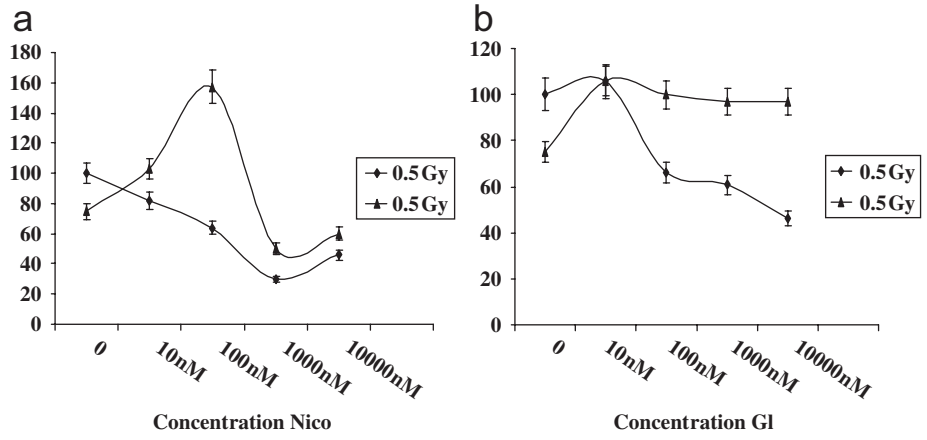

Fig. 3. Percent of control plating efficiency, for HPV-G keratinocytes exposed directly to 0.5 Gy (diamond symbols) or to medium harvested from cells (triangle symbols) treated with increasing concentrations of Glycine (3a), or Nicotine (3b), prior to radiation or bystander medium (By) exposure.

Table 5

Sample	% of control serotonin	% clonogenic survival
(a) *ELISA results for serotonin levels detected in medium harvested from HPV-G cultures exposed to 0.5 Gy irradiation*[a]		
Control DMEM medium harvested from unirradiated HPV-G cells	100 (109.6±8.59)	100% (24.4±3.4)
Control DMEM medium harvested from cells which received 10 nM reserpine	111.1±7.6, ns	100% (29.3±2.6), ns
DMEM medium harvested from HPV-G cells exposed 1 h earlier to 0.5 Gy	63.9±4.1, $p = <0.01$	62.6±4.1, $p<0.01$
DMEM medium harvested 1 h after irradiation from cells treated with 10 nM reserpine prior to radiation exposure	117.4±9.4, ns	123.8±3.4, $p<0.05$
(b) *ELISA results for serotonin levels detected in medium harvested from tissue cultures of mice and fish exposed to 0.5 Gy irradiation*[b]		
Control RPMI-1640 medium harvested from unirradiated bladder explants from C57Bl6/C129 mice	100 (87.5±5.6)	100% (14.3±0.9)
RPMI-1640 medium harvested from bladder explants obtained from in vivo irradiated (0.5 Gy) C57Bl6/C129 mice	58.2±4.7, $p<0.01$	43.7±2.6, $p<0.01$
Control RPMI-1640 medium harvested from unirradiated skin explants from rainbow trout	100 (74.28±2.6)	100% (17.2±2.3)
RPMI-1640 medium harvested from in vitro irradiated skin explants from rainbow trout irradiated in vitro to 0.5 Gy	44.4±4.17, $p<0.0001$	57.5±7.7, $p<0.01$

[a]Results are the mean±SEM for $n = 5$ experiments where flasks were set up in triplicate. Mean survival% for recipient HPV-G reporter cells from these experiments are also shown. Survivals were normalized to 100% for the relevant control, the actual plating efficiency value is shown in brackets. Serotonin concentrations were also corrected to 100% for the control, the actual concentrations in ng/ml are shown in brackets.

[b]Results are the mean±SEM $n = 3$ experiments where three flasks were set up per point. Mean survival % for HPV-G reporter cells from these experiments are also shown. Survivals were normalized to 100% for the relevant controls. Mouse tissue culture medium was harvested from 3-day-old explants which had been irradiated on day 2, i.e. 1 day before harvest. Rainbow trout tissue culture medium was harvested from 4-day-old cultures which had been irradiated on day 3.

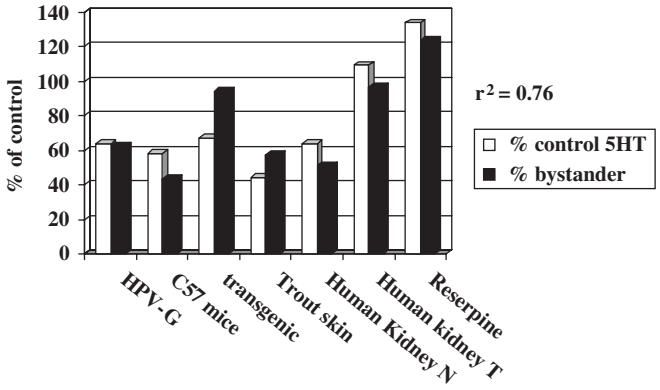

Fig. 5. Correlation between the depletion of medium serotonin following irradiation of HPV-G keratinocytes, mouse, human and fish tissues and the bystander effect. A reserpine control which blocks cellular uptake of serotonin is included. The r^2 value was derived from a pared t-test correlating the two parameters (serotonin depletion and bystander medium-induced cell death).

the standard culture medium used), then, the ionizing effect of irradiation is needed in addition to 5-HT to permit calcium entry and trigger the bystander signal production. Excess added serotonin in the medium is sufficient by itself to cause the L channels, which are ion gated, to open, permitting calcium entry. It is also possible that only particular subsets of cells (e.g. certain cell cycle phases), can respond and that accumulation of these cells is facilitated by both radiation and serotonin. It is evident that the radiation-induced bystander effect commonly described by our group which results in reduced cloning efficiency and apoptosis, can be produced by another agent which facilitates calcium entry into cells and that this agent is actively bound by cells following irradiation. To further test the "calcium hypothesis" we looked at the effects of another monoamine—L-DOPA. This also has well-known signaling roles in biology but does not appear to act by causing the rapid calcium pulse. Rather the increase in intracellular calcium caused by L-DOPA is more sustained (Koshimura et al., 2000). In our experiments it had a small protective effect and appeared to prevent apoptosis. *l*-Deprenyl, often used in the treatment of Parkinson's disease is a dopamine regulator through its action as an inhibitor of monoamine oxidase B (Ebadi et al., 2002), has been shown by our group to prevent genomic instability and the bystander-induced fall in survival (Seymour et al., 2003) by a mechanism involving bcl-2 expression in exposed cells. *l*-Deprenyl was used here over a wide concentration range and was found to be active only in a narrow nanomolar range. The MAO-B inhibitor activity of the drug is very unlikely to be involved at these levels (Magyar and Szende, 2004) but deprenyl also affects calcium homeostasis mainly by mitochondrially located mechanisms and through anti-apoptotic effects involving bcl-2 (Tatton et al., 1996; Naoi et al., 2003; Seymour et al., 2003). Mitochondrial membrane depolarization is a known downstream event following the influx of calcium (Maruyama et al., 2002). Thus the small protective effect of L-DOPA effects and the occurrence of *l*-deprenyl effects is consistent with a central role for calcium homeostasis in radiation-induced bystander signaling pathways.

One of the implications of a calcium homeostasis-type mechanism is that there could be multiple "bystander inducing factors" acting on different receptors and being interpreted by different signal transduction pathways linked to the receptors. This would perhaps explain the very varied

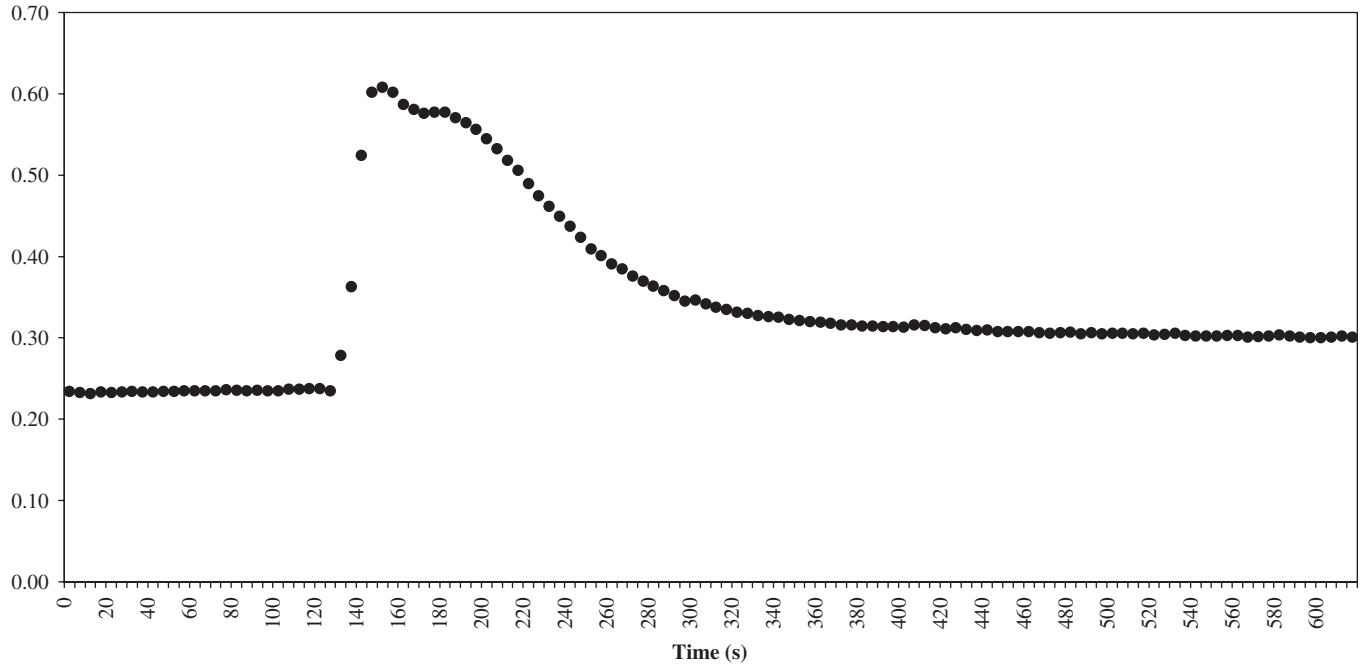

Fig. 6. Transient increase in intracellular free calcium induced in cells treated with 2 μM 5-HT. The graph is the integrated intracellular calcium level detected in seven separate fields.

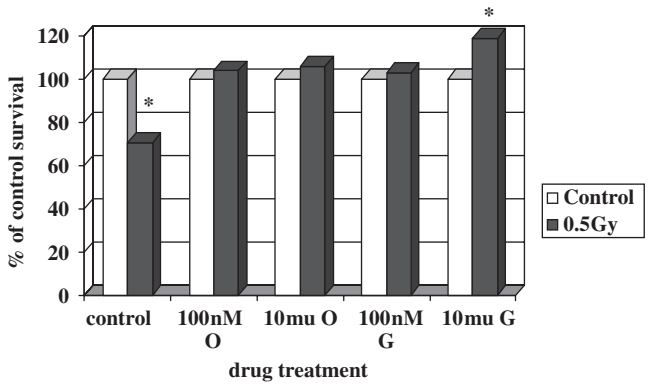

Fig. 7. Inhibition of the bystander effect induced by 0.5 Gy cobalt 60 gamma radiation in human keratinocyte cells pretreated with the 5-HT3 receptor antagonists, ondansetron (O) and granisetron (G). mu = micromolar, * = significant at the $p<0.05$.

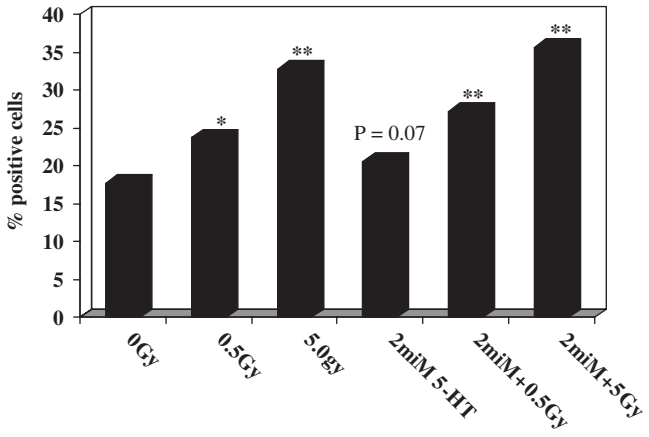

Fig. 8. Expression of 5-HT receptors in human keratinocyte cells with and without treatment with 2 μM 5-HT and 0.5 Gy radiation. * = $p<0.05$, ** = $p<0.01$.

responses seen in different cell lines and is supported by the findings of genotypic variation in bystander responses (Watson et al., 2000; Mothersill et al., 2001, 2005). There is also the possibility of a radiation quality effect since all these experiments were done with gamma radiation, but much of the bystander literature relates to high LET radiations. Clearly investigation of these pathways in after high LET would be interesting. It is known that proteins needed for functions controlled by membrane channels are found to congregate at the intracellular location of these channels, thus this mechanism could be part of a wider control of radiation response sectoring at the cellular level. It is also true that the "diffusible factors" which are so elusive may be many and varied, determined mainly by the response the cell or cells choose to mount following the calcium influx.

In conclusion, the data presented suggest a role for serotonin and other signaling amines in the initiation and transduction of radiation-induced bystander effects and further suggest a role for cell surface receptors involved in controlling calcium homeostasis in the mechanism. The results may also provide an insight into how sectoring to various radiation responses is determined. The results may, in the future, be relevant for management of radiation in the environment and may help elucidate bystander mechanisms as responses to low-dose radiation exposures to man and non-human species.

Acknowledgments

We are very grateful to our funding bodies—The Canada Chairs programme, Bruce Power, Ontario Power Generation, CANDU Owner's Group (COG) and The National Science and Engineering Research Council (NSERC). We also acknowledge Janet Denbeigh and Jen Lemon at McMaster University for providing us with bladders from irradiated mice.

Ethical statement

This is mainly a cell culture based study however for one series of experiments, tissues from mice, fish and humans were cultured and treated in vitro. All tissue was obtained and treated according to guidelines at McMaster University and the procedures were covered by AUP #05-08-44.

Acknowledgement of funding sources

The Canada Research Council Chairs programme and The National Science and Engineering Research Council (NSERC) are the government research funding agencies.

Bruce Power, Ontario Power Generation, and CANDU Owner's Group (COG) are industrial partners who matched the government funds.

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