Any apoptotic bystander effect induced by low dose radiation in spleen *in vivo* must fall within the homeostatic range.

Alexander H. Staudacher[a], Benjamin J. Blyth[a], Rebecca J. Ormsby[a], Eva Bezak[b], Pamela J. Sykes[a,c]
[a] *Haematology and Genetic Pathology, Flinders University and Medical Centre, Adelaide, South Australia, Australia*
[b] *Department of Medical Physics, Royal Adelaide Hospital, Adelaide, Australia*
[c] *SA Pathology, Flinders Medical Centre, Adelaide, South Australia, Australia*

Adaptive responses and bystander effects contradict the current linear no-threshold (LNT) model of radiation risk assessment which assumes that risk is confined to directly irradiated cells only, and that radiation doses are strictly additive. There is considerable evidence demonstrating that protective adaptive responses for carcinogenic and mutagenic endpoints occur after low dose radiation exposure *in vivo* but evidence for the existence of bystander effects *in vivo* at low doses is very limited. It is a challenge to examine bystander effects *in vivo* at relevant low doses, where only a fraction of cells in an irradiated tissue receive direct energy deposition, since it is not trivial to identify those cells which have been irradiated amidst their unirradiated neighbours. The majority of *in vivo* studies have used very high radiation doses to initiate a systemic, toxic response which is measured at sites distant to the irradiated field. The few studies which have attempted to demonstrate low dose radiation–induced bystander effects *in vivo* have required substantial manipulation of the cells studied, the use of immortalised or cancerous cells, or the use of lethally-irradiated hosts. Recently, we developed a lymphocyte adoptive transfer method to study the *in vivo* apoptotic and proliferative responses in normal unirradiated spleen cells, surrounding normal low dose irradiated cells, in normal mice (Blyth et al, *Radiation Research* 173: 125-137, 2010). No bystander effects were observed even though a number of parameters including dose, time of analysis and number of cells irradiated were explored based on previous *in vitro* bystander effects reported in the literature for these same parameters. This adoptive transfer method has the great advantage of being able to definitively distinguish irradiated cells from unirradiated cells *in situ*, however it does involve *ex vivo* manipulation of the irradiated donor cells prior to their introduction into the unirradiated hosts. In order to further test whether bystander effects occur at low doses relevant to occupational and population exposure *in vivo*, we exposed mice to very low dose whole-body X-radiation where only a proportion of cells would receive an electron track. We then analysed the apoptosis frequency *in situ* in spleen tissue sections at various time-points after irradiation and compared the apoptosis frequency with that predicted from LNT. This approach bypasses the need for *ex vivo* manipulation of cells and more closely mimics the real-life scenario of a human receiving low dose diagnostic X-irradiation or a short-term whole-body low LET occupational radiation exposure. We performed temporal studies to increase the chance of detecting a delayed apoptotic bystander effect.

We first determined the effect of high dose (1000 mGy) radiation on apoptosis at 7 h, 1, 3, and 7 days after irradiation. A significant increase in apoptosis was observed at 7 h and to a lesser extent at 1 day, after a dose of 1000 mGy. The apoptosis frequency had returned to that of sham-irradiated mice by days 3 and 7. Apoptosis was induced above endogenous levels at a rate of 1.4% apoptosis/Gy at 7 h after irradiation. Data from the 1 Gy irradiated mice was used to calculate the expected increases in apoptosis 7 h after a dose of 1 mGy or 0.01 mGy using a LNT approach based on the assumption that the rates of apoptosis induced would be

directly proportional to dose. The expected increases in apoptosis 7 h after 1 mGy or 0.01 mGy would thus be 1.4 in 10^5 cells and 1.4 in 10^7 cells respectively.

Using a highly accurate method for apoptosis detection *in situ* analysing a minimum of 110,000 cells, we first determined that the mean endogenous apoptosis frequency in sham-irradiated mice was 0.293 ± 0.044% (± SE). Power calculations (α=0.05, 1-β=0.8) demonstrate that we would need >10^4 and >10^8 mice per group if we were to expect to detect the linear-predicted increases in apoptosis described above, after 1 mGy and 0.01 mGy respectively. It is thus impractical to perform the experiments necessary to confirm such small increases in apoptosis frequency if the apoptotic responses are linear with dose. Assuming that bystander effects are of similar magnitude *in vivo* to that observed *in vitro*, we began calculations based on a nominal 5-fold bystander effect. Power calculations predicted that even at this level, detecting a significant increase from direct + bystander apoptosis would require a prohibitive number of mice (>500 mice/treatment group). In this study we used 10 mice per treatment group which would power a study to detect apoptosis increases from a >40-fold bystander effect. We did not observe any significant changes in the apoptosis frequency in mouse spleen after 0.01 mGy or 1 mGy at 7 hours, 1, 3 or 7 days after irradiation. Although the upper 95% confidence limit on the apoptosis induced by 1 mGy was 1.4 extra apoptotic cells in 10^3, even this small range only permits a potential bystander amplification of apoptosis of ≈100-fold. The upper limit on an increase in apoptosis after 0.01 mGy was similar (1.7×10^{-3}), permitting a potential bystander effect of over 10,000-fold.

An absorbed dose of 1 mGy X-radiation equates to ≈60% of cells receiving at least 1 electron track whereas at a dose of 0.01 mGy only 1 in 100 cells would be expected to be traversed by even a single electron track. These data demonstrate that since the apoptosis expected from targeted effects at these low doses is only a fraction of the natural variation in unirradiated mice, even amplification of the apoptosis due to a substantial bystander effect would be expected to fall within the homeostatic range. Whilst we did not observe any changes in apoptosis after low doses of radiation, *in vivo* studies using chromosomal inversions as an end-point in our laboratory have shown that single whole-body radiation doses of 0.01 mGy and 1 mGy do modulate chromosomal inversions and can protect from inversions caused by high dose radiation. Therefore these very low doses are not inconsequential *in vivo*. It is possible that the adaptive and potential bystander effects in response to these low doses *in vivo* are highly specific for a few rare cells which will fall within homeostatic apoptosis variations *in vivo*, and the effects may only be detected using health end-points over long periods of time as has been observed for latency of tumour formation *in vivo* in mice. If bystander effects are occurring after these low doses then they fall within the range of normal homeostatic apoptotic responses and are of lower magnitude than apoptotic responses to stresses such as mild heat stress. It may be necessary to study the effects of low doses of radiation on apoptosis via their effect on apoptosis induced by a subsequent high dose; however, this information will likely be more informative for protection from high dose radiation rather than for radiation risk assessment of low dose exposures.

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