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The role of intercellular communication and DNA double-strand breaks in the induction of bystander effects

(INTERSTANDER)

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(summary)

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A commonly accepted assumption is that the biological effects of ionising radiation are a direct consequence of DNA damage in the exposed cells. However, recent studies have demonstrated 'non-targeted' effects (i.e. effects in cells not directly exposed to radiation, but which are in the vicinity of the exposed cells) at highly significant levels. Such non-targeted effects have been shown to cause cell killing, mutations, cancerous transformation and other cellular responses associated with DNA damage in "bystander", non-irradiated cells.

These observations question the validity of the International Commission on Radiation Protection (ICRP) extrapolated estimates of cancer risk for low-dose exposures, which do not consider bystander effects. Similarly, the practice of radiotherapy assumes that damage is inflicted only in tissues within the radiation field. Radiation is not thought to affect adjacent non-exposed areas. Thus, an evaluation of the relative contribution of bystander effects to the overall response of tissues to ionising radiation and an understanding of the underlying mechanisms is a prerequisite for accurate recommendations in radiation protection and valid predictions in radiation therapy. The main objective of the present proposal was to try to further our understanding of the molecular mechanisms underlying bystander responses. One hypothesis is that bystander effects are caused by the transfer of low-molecular-weight chemical factors excreted from irradiated cells which affect non-exposed, nearby cells. An alternative hypothesis is that the transfer of small molecules occurs from irradiated cells through 'gap junctions', cell-membrane channels, through which low-molecular-weight substances can pass between adjacent cells. Such intercellular passage could for example allow damaging reactive oxygen species, or signalling molecules, to pass from irradiated to non-irradiated cells. The INTERSTANDER project focused first on the development and characterisation and then on the use of model systems to study the contributions of such extra-cellular and gap-junctional transfer of low-molecular-weight factors to the development of bystander effects.

One experimental strategy was to use intercellular communication-deficient cells to quantify the role of excreted factors in causing bystander effects. A second strategy aimed at evaluating the contribution of gap junctions by restoring the relevant 'connexins' (the building blocks of gap junctions) by introducing a genetically engineered DNA expression vector into the gap-junction-deficient cells.

A further approach was aimed at understanding the initiating signal for the bystander effect. The hypothesis was that a DNA double-strand break could be the initiating signal. Thus the strategy for testing this hypothesis involved the use of a genetically engineered cell strain in which we could make a single break in the DNA chain (at a I-Sce-I site in one specific chromosome, using an enzyme introduced into the cell by poration techniques).

Comparison of results with those from a microbeam of alpha particles (a very fine beam of ionising particles produced from a small radioactive source which can be directed at well defined numbers in specific parts of a cell, e.g. the cell nucleus) that also produces chain breaks in cells' DNA would allow us to evaluate the possible contribution of a low level of oxidative stress to the bystander effect, since in addition to 'cutting' the DNA directly, these particles also produce reactive oxygen species near the DNA. Also, microbeam irradiation of the cell away from its nucleus to the cytoplasm with low-LET microbeam protons provided a further possibility to investigate the role of oxidative stress, this time in the *absence* of DNA damage. Co-culture protocols were applied to study the development of G2 arrest and the activation of p53 (as end points) in a number of cell lines, characterised in terms of their ability to establish gap junctions.

Experiments (Essen group) carried out with bEnd3 cells did not demonstrate bystander effects when using these end points. Because bystander responses are reported to be more significant following high LET radiation, the effect of neutron irradiation was tested using the same cell line. Here again no evidence for bystander responses could be obtained despite the outstanding capability of these cells to develop intercellular communication. Other cell lines (reported as showing bystander effects) were tested using the same experimental approach. However inexplicably, in our hands bystander responses could not be demonstrated in any of these cell lines. Although the majority of the results obtained in these investigations are negative in terms of eliciting a bystander response, we believe that they are important in the field as they allow narrowing down the conditions under which their development occurs. Our observations when contrasted with reports of robust bystander responses using the same cell lines using different experimental approaches suggest that their development in the co-culture experiments may be prevented by the required manipulations such as trypsinisation. Further experiments involving co-culture of two populations under conditions that allow free exchange of soluble factors whilst preventing the mixing of the cell populations (and using micronucleus formation as an end point for bystander response) did not show a bystander response.

Results with co-cultured malignant trophoblast Jeg3 cells after exposure to 5 Gy X-rays using activation of p53 as end-point provided evidence for bystander effects. This bystander effect was not mediated via gap junctions because the effect was similar in both communicating and non-communicating Jeg3 cells, and was independent from the connexin isoform expressed. These results suggest that the observed bystander effect in Jeg3 cells operates by paracrine mechanisms and does not require active intercellular communication. In addition, differences in the results obtained between the Essen groups point to additional factors determining bystander responses that require further clarification.

As noted above, bystander effects have been reported to be more marked at high LET and are also reported to be dependent on the type of cells used. However, using cell survival as endpoint, experiments (Braunschweig group) with primary human skin fibroblasts (HSF2) exposed to 4.5 MeV alpha particles (LET = 100 keV/µm) showed no bystander effects. Surprisingly, after exposure of these human cells to 10 MeV microbeam protons (LET = 4.7 keV/µm), a significantly *higher* survival (i.e. compared to unirradiated controls) was observed for doses lower than about 0.3 Gy when all cells, or when only 10 % of all cells, were irradiated. These results indicate *protective* rather than the expected negative bystander effect. The finding that primary human fibroblasts, which are competent in forming gap junctions, show no bystander effect after high LET exposure, whereas immortalised rodent fibroblasts (C3H10T1/2 cells) do, suggests that immortalisation or cell type are significant determinants in the development of bystander effects.

As a further test for the production of bystander factors within irradiated cells, the outcome of fusing irradiated and non-irradiated cells was investigated (Athens group). For this purpose two sets of experiments were carried out. In the first set, irradiated G_0 lymphocytes were fused to exponentially growing (un-irradiated) CHO cells. In the resultant syncytium (containing the two cell nuclei), lymphocyte prematurely condensed chromosomes (PCCs) were induced when the CHO nucleus entered mitosis. However, endogenous 'bystander effects' between the irradiated lymphocyte nucleus and cellular components and the unirradiated CHO nucleus were not observed, i.e. no increase in PCC fragments was seen over background in the non-exposed CHO chromosomes. But when irradiated CHO cells

were fused to non-irradiated G_0 lymphocytes, the lymphocyte chromosomes were undamaged. However, surprisingly the frequency of aberrations in CHO chromosomes was significantly *lower* when compared with that in non-fused CHO cells, i.e. the lymphocyte component of the hybrid was exerting a type of 'protective' effect. Further experimental data suggested that the 'protection' observed in irradiated CHO cells, when fused to non-irradiated lymphocytes, may result from a delay in cell-cycle progression of the hybrids, possibly giving time for DNA repair to occur in the irradiated CHO chromosomes. When the hybrid CHO/human cells were irradiated immediately after fusion, the yield of excess PCC fragments in the human chromosomes was found to be approximately *double* that obtained when only the lymphocytes were irradiated with the same dose just before their fusion to mitotic cells, possibly indicating the presence of a damaging endogenous factor released by the CHO nucleus or cellular components, which could be equivalent to a bystander factor. However, further experiments would be required to test this hypothesis.

To study whether a bystander effect could be mediated between irradiated (male) lymphocytes and non-irradiated (female) lymphocytes via the culture medium, peripheral blood from male donors was irradiated and immediately mixed with blood from female donors. The mixed cells were then cultured in the presence of PHA, and chromosomal aberrations were scored in the female lymphocytes. No bystander effects were detected between cells from several different donors having a range of radiosensitivities.

The aim of the St Andrews University contribution to INTERSTANDER was to investigate whether DNA double-strand breaks (DSB) could be the initiating lesions in a bystander effect. A second aim was to test whether the bystander signal, or factor, is intercellular gap-junction mediated or transmitted via the growth medium. Initial experiments were carried out using a cell line (human choriocarcinoma Jeg-3, already available in Essen) conditionally expressing connexin-43-mediated gap junctions, in order to investigate the role of gap junctions in the bystander effect. However, an early problem was encountered in that it was found not possible to select for a Jeg3 clone containing the single *I-SceI* DSB-site plasmid (*pCMVI-SceI)* since this cell line was already G418 resistant, a fact that we weren't aware of at the outset of the project. Therefore as an alternative strategy, we chose to use an inter-species co-culture system employing one of our own engineered muntjac cell lines as the donor cells in the bystander experiment. This cell line contains three plasmids, one for the *I-SceI* DSB site and the other two constituting the inducible *I-SceI* endonuclease system. This cell line was shown to constitutively express connexin-43 and therefore could be used to couple with Jeg3 cells. Another significant advantage was that muntjac cells form very stable confluent mono-layers on which the recipient bystander cells could be plated in co-culture experiments. Experiments using dye markers indicated that inter-cellular gap-junction coupling was occurring between these two cell lines. As a preliminary experiment to test for a bystander effect with radiation, we mixed irradiated 'donor' muntjac cells with Jeg-3 cells and looked for the induction of sister chromatid exchanges (SCEs) in the recipient (unirradiated) Jeg3 cell population. In this experiment, following irradiation of the confluent and quiescent muntjac monolayer, the exponentially growing Jeg3 cells were overlayed on the muntjac cell layer and then labelled through two cell cycles with bromodeoxyuridine in order to detect the SCEs. Very rare muntjac cell metaphases were observed, indicating the almost totally quiescent nature of the underlayer, and could be easily distinguished from Jeg3 cells by their karyotype. We could not detect a significant increase in SCEs in the Jeg3 cells. However, we found that the frequency of SCEs in directly irradiated Jeg3 cells showed only a small increase, confirming that ionising radiation per se is not a very potent inducer of SCEs in this cell line. However, other agents are more efficient at inducing SCEs and it was possible that a

bystander factor (e.g. reactive oxygen species) might have been more efficient than ionising radiation at inducing SCEs.

As an alternative strategy to the use of SCEs, we used phosphorylated p53 as an end point. However, it was found that the immunocytochemical detection of p53 in Jeg3 cells was problematic due to a high background staining in controls, Jeg3 cells being derived from a human choriocarcinoma. It was therefore decided to adopt a different strategy, namely using CHO-CHO interaction. The kinetics of induction of CFP-tagged p53 was determined by western blot. In bystander experiments the donor population was the CHO cell line containing an I-SceI site and inducible endonuclease (meganuclease), and the recipient line was engineered to contain a plasmid expressing CFP-tagged p53 to distinguish it from the normal p53 (actually non-functional in CHO). Using western blots, induction of phosphorylated CFP-tagged p53 was observed in directly irradiated cells and some (but rather variable from experiment to experiment) induction was observed in (unirradiated) bystander cells with either co-culture of donor and recipients or simply with medium transfer from donor cultures. Experiments using the restriction endonuclease as an initiating signal was substantial and therefore this method would be well worth following up with future experiments. Thus in summary, results with SCE as an end point do not show evidence of a bystander effect. Experiments with phosphorylated CFP-tagged p53 as a novel marker of a damage response were more promising and in preliminary experiments a small bystander effect was indeed observed. However, this technique is very sensitive and requires further work to lower background induction of p53 phosphorylation (probably caused by stress on cells during the treatments), so that it is possible to distinguish a positive bystander effect from the background.

Overall, the project allowed the development and optimisation of powerful methods with potential utility in the evaluation of bystander responses, such as the cell-fusion experiments and co-culture techniques. The negative results obtained allow a better definition of the operational space for bystander responses and will facilitate the focusing of future studies.