Final Technical Report (9/15/1998-5/31/2005)

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	"Effects of Low-Dose Alpha-Particle Irradiation in Human Cells: The Role of Induced Genes and the Bystander Effect"
SC Division:	SDC-72
Program Manager:	Arthur M. Katz (301) 903-4932
Research Area:	Cell Biol

Executive Summary:

This grant was designed to examine the cellular and molecular mechanisms for the bystander effect of radiation (initially described in this laboratory) whereby damage signals are passed from irradiated to non-irradiated cells in a population. These signals induce genetic effects including DNA damage, mutations and chromosomal aberrations in the non-irradiated cells. Experiments were carried out in cultured mammalian cells, primarily human diploid cells, irradiated with alpha particles. This research resulted in 17 publications in the refereed literature and is described in the Progress Report where it is keyed to the publication list. This project was initiated at the Harvard School of Public Health (HSPH) and continued in collaboration with students/fellows at Colorado State University (CSU) and the New Jersey Medical School (NJMS).

Students/Research Fellows involved in this research:

Edward Azzam (HSPH, NJMS)
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Hatsumi Nagasawa (HSPH, CSU)
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Publications (1998-2005)

1. Azzam, E.I., S.M. de Toledo, T. Gooding, and J.B. Little. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. Radiat. Res,. 150: 497-504, 1998.

2. Azzam, E.I., S.M. de Toledo, A.J. Walker and J.B. Little. High and low fluences of alpha-particles induce a G1 checkpoint in human diploid fibroblasts. Cancer Res. 60: 2623-2631, 2000.

3. Azzam, E.I., S.M. de Toledo and J.B. Little. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha-particle irradiated to nonirradiated cells. Proc. Natl. Acad. Sci. USA 98: 473-478, 2001.

4. Nagasawa, H. and J.B. Little. Rapid Communication. Unexpected sensitivity to the induction of mutations by very low doses of alpha-particle radiation: evidence for a bystander effect. *Radiation Research* **152:** 552-557, 1999.

5. Huo, L., H. Nagasawa. and J.B. Little. HPRT mutants induced in bystander cells by very low fluences of alpha particles result primarily from point mutations. *Radiation Res.* **156:** 521-525, 2001.

6. Azzam, E.I., S.M. de Toledo, D.R. Spitz, and J.B. Little. Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from alpha-particle-irradiated normal human fibroblast cultures. *Cancer Res.* **62:** 5437-5442, 2002.

7. Nagasawa, H., A. Cremesti, R. Kolesnick, Z. Fuks, and J.B. Little. Involvement of membrane signaling in the bystander effect in irradiated cells. *Cancer Res.* **62:** 2531-2534, 2002

8. Nagasawa, H., L. Huo, and J.B. Little. Increased bystander mutagenic effect in DNA double stand break repair deficient mammalian cells. *Int. J. Radiat. Biol.* **79:** 34-41, 2003.

9. Azzam, E.I., S. M. de Toledo, and J.B. Little. Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. *Oncogene* **22:** 7050-7057, 2003.

10. Nagasawa, H. and J.B. Little. Bystander effect for chromosomal aberrations induced in wild-type and repair deficient CHO cells by low fluences of alpha particles. *Mutation Res*. **508:** 121-129, 2002.

11. Little, J.B., Nagasawa, H., Li, G.C., Chen, D.J. Involvement of the nonhomologous end joining DNA repair pathway in the bystander effect for chromosomal aberrations. *Radiat. Res.* **159:** 262-267, 2003.

12. Little, J.B., E.I. Azzam, S.M. de Toledo, and H. Nagasawa. Bystander effects: intercellular transmission of radiation damage signals. *Radiation Protection Dosimetry* **99:** 159-162, 2002

13. Azzam, E.I., S.M. de Toledo and J.B. Little. Expression of *CONNEXIN-43* is highly sensitive to ionizing radiation and other environmental stresses. *Cancer Res.* **63:** 7128-7135, 2003.

14. Glover, D, J.B. Little, M.F. Lavin, and N. Gueven. Low dose ionizing radiation-induced activation of connexin 43 expression. *Int. J. Radiat. Biol.* 79: 955-964, 2003.

15. Little, J.B. Genomic instability and bystander effects: a historical perspective. *Oncogene* **22:** 6978-6987, 2003.

16. Nagasawa, H., Y. Peng, P. Wilson, Y-C. Lio, D. Chen, J. Bedford and J.B. Little. Role of homologous recombination in the alpha particle bystander effect for sister chromatid exchanges and chromosomal aberrations. *Radiat. Res.* **164:** 141-147, 2005.

17. Little, J.B., E.I. Azzam, S.M. de Toledo and H. Nagasawa. Characteristics and mechanisms of the bystander response in monolayer cell cultures exposed to very low fluences of alpha particles. *Radiation Physics and Chemistry* **72:** 307-313, 2005.

Progress Report (1998-2005)

The first publication supported by the present grant presented evidence both by western blotting and *in situ* immunostaining that the p53 damage response pathway is upregulated in bystander cells (1). This confirmed the notion that radiation damage signals can be transmitted from irradiated to neighboring non-irradiated cells. We presented preliminary evidence for the involvement of gap junction mediated intercellular communication (GJIC) in this phenomenon, by use of specific inhibitors (1). To confirm that this signal transduction pathway was functional in bystander cells, we employed wild-type, p53 and p21 mouse knock-out cell lines to show that a p53 dependent G_1 arrest occurred in bystander cells (2). In order to provide direct evidence for the role of GJIC in the intracellular transmission of these damage signals, we utilized genetically altered cell lines examined by western blotting and in situ immunofluorescence techniques (3). These included connexin 43 mouse knockout cell lines, as well as wild-type and connexin 43 mutated isogenic cell lines. Finally we provided the first evidence for the induction of specific gene mutations in bystander cells (4). The efficiency with which each alpha track induced mutations rose significantly at very low alpha particle fluences, indicating that bystander cells as well as hit cells were at risk for the induction of mutations by very low fluences of alpha particles (4). This resulted in hyperlinearity of the dose-response curve for mutations induced by very low mean doses. Interestingly, we found that mutations induced in bystander cells were primarily point mutations, as opposed to deletions as occur in directly irradiated cells (5). We hypothesize that the mutations induced in bystander cells are the result of oxidative base damage.

In subsequent experiments, we found that proteins in the MAP kinase group of signaling pathways are activated in bystander cells (6). Activation of these pathways occurs as a result of signals arising in the cell membrane. In order to examine the possible role of membrane signaling in bystander cells, we examined the effects of specific agents known to disrupt cholesterol-rich membrane rafts, leading to disassembly of raft associated proteins and thus inhibition of membrane signaling. The induction of sister chromatid exchanges and specific gene mutations was inhibited by these agents in bystander cells, whereas they had no effect on the induction of such genetic changes in directly irradiated cells. A manuscript describing these results was published in *Cancer Research* (7).

The role of oxidative metabolism in the up-regulation of stress-inducible signaling pathways as well as the induction of micronuclei in bystander cells was investigated by immuno-blotting and in situ immunofluorescence techniques. Active superoxide dismutase enzyme (SOD) and the active catalase enzyme were shown to inhibit both the up-regulation of $p21^{Waf1}$ and the induction of micronucleus formation in bystander cells from confluent cultures of normal human diploid fibroblasts irradiated with 0.3 to 3 cGy of alpha particles. Enzyme activity assays indicated that exogenous SOD became significantly associated with the cells. Reactive oxygen species (ROS) apparently derived from a flavin containing oxidase enzyme (presumably an NAD(P)H-oxidase) appeared to be major contributors to the bystander-induced upregulation of p53 and $p21^{WAF1}$ as well as micronucleus formation, as evidenced by the inhibition of these effects by diphenyliodonium (DPI). Rapid activation of NFkB, Rafl-1, ERK1/2, JNK and p38-MAPK and their downstream effectors AP-1, ELK-1, P90RSK and ATF2 was also observed. Significant attenuation in the activation of these kinases and transcription factors occurred in irradiated cultures treated with either SOD or catalase. Overall, these results support the hypothesis that superoxide and hydrogen peroxide produced by flavin containing oxidase enzymes mediate the activation of several stress inducible signaling pathways in bystander cells. These results were reported in *Cancer Research* (7).

In order to examine the influence of DNA repair processes on the bystander effect, experiments were designed to study the induction of *HPRT* mutations in DNA repair

deficient cells. The initial experiments with Chinese hamster xrs-5cells which are deficient in DNA double strand break (DSB) repair yielded the surprising result that the bystander effect was markedly enhanced in these cells. Whereas we had previously shown a 3-4-fold enhancement in the efficiency of each alpha track in producing mutations at very low fluences, indicating that approximately three neighboring non-irradiated cells were at risk for the induction of mutations (4), the efficiency of each alpha track was increased 30-50-fold in xrs-5 cells. Interestingly, molecular structural analyses indicated that the mutations in xrs-5 bystander cells were primarily deletions. We hypothesize that oxidative damage leading to single base changes predominates in bystander cells; however, some DSB are produced perhaps as a result of opposed oxidative lesions but these are efficiently repair ed in wild-type cells. In repair deficient cells, on the other hand, these DSB remain unrepaired yielding highly mutagenic lesions that lead to large-scale genetic changes (8, 9).

The results described above indicate that mutations in wild-type bystander cells arose primarily from single base changes leading to point mutations, whereas those in repair deficient cells result from large-scale changes including partial and total gene deletions. As gross chromosomal aberrations presumably arise from large-scale genetic changes, we examined the induction of chromosomal aberrations under bystander conditions in wild-type and xrs-5 cells. We hypothesized that the frequency of such aberrations should be low in wild-type bystander cells, but considerably elevated in the repair deficient cell line. The results confirmed this hypothesis. A manuscript based on these findings was published in *Mutation Research* (10).

In order to confirm that a the large bystander effect observed in xrs-5 cells was indeed related to their DNA repair defect rather than to some intrinsic property of this hamster cell line, we examined the bystander effect for chromosomal aberrations in specific mouse knockout cell lines for Ku 70, Ku 80, DNA-PKcs or PARP. These proteins are all involved in the complex controlling the non-homologous end joining (NHEJ) pathway for the repair of DSB. The results confirm those observed with the hamster cells relating a defect in the repair of radiation-induced DSB by the NHEJ pathway to an enhanced bystander effect. A manuscript based on these findings was published in *Radiation Research* (11).

We carried out experiments employing microarray technology to examine whether differing patterns of gene expression in bystander as opposed to directly irradiated cells might offer clues as to mechanisms. Unexpectedly, we observed a marked exhancement in the expression of *Connexin 43* which mediates gap junction intercellular communication in human diploid fibroblasts. Of particular interest, markedly enhanced expression of *Connexin 43* appeared to be induced by radiation. We have now carried out a number of additional experiments to confirm this finding in several different cell lilnes by northern and western blotting. We have also examined *Connexin 43* expression in cells exposed to several other toxic stresses. These results were published in *Cancer Research* (13). This finding has been confirmed in collaborative experiments with Martin Lavin at the Queensland Institute of Medical Research; low dose radiation exposure induced transcriptional upregulation of *Connexin 43* expression involving NFA1 AP1 sites of the promoter region (14). The observation that intercellular communication may be activated or enhanced by exposure to radiation is a very new finding that could have significant implications for the biological effects of ionizing radiation *in vivo*.

The NHEJ DSB repair pathway though error-prone is active throughout the cell cycle. Homologous recombination, on the other hand, takes place only in the later phases of the cell cycle, primarily in late S and G_2. Sister chromatid exchanges (SCE) are presumably a consequence of homologous recombinational events. We examined the induction of SCE in bystander cells in parallel with chromosomal aberrations in mouse knockout cells for both the p53 and NHEJ pathways. The results 1) confirm our early findings of a bystander effect for SCE induced by very low fluences of alpha particles; 2) indicate a lack of dependence on p53 status for this effect, and 3) indicate that cells deficient in non-homologous end joining show an enhanced bystander response for SCE as observed for mutations and chromosomal aberrations (16, 17).