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REQUIREMENTS FOR IDENTIFICATION OF LOW DOSE AND NON-LINEAR MUTAGENIC RESPONSES TO IONISING RADIATION

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□ Cancer results from multiple changes in gene expression that can occur both genetically and epigenetically. High doses of radiation can lead to mutations and cancer. At high doses the number of mutations caused by radiation is essentially linear with dose. Low dose radiation induced protective responses observed for cancer *in vivo* and cellular transformation *in vitro* would predict that hormetic responses would also be observed in mutation assays. Although there are a large number of different mutation assays available, very few are able to detect changes in mutation frequency in response to very low doses of DNA damaging agents. The easiest way to cope with this lack of data in the low dose range is to invoke a linear-no-threshold model for risk assessment. The reasons for the lack of data are discussed. In order to identify hormetic mutation responses, assays need to have a spontaneous frequency that is high enough to enable a reduction below spontaneous frequency to be detected in a feasible number of scored cells and also need to be able to identify both genetic and epigenetic changes. The pKZ1 chromosomal inversion assay fits the criteria for detecting hormetic responses to low dose radiation.

Keywords: pKZ1 inversion assay, chromosomal inversion, low dose ionising radiation, mutation assays, hormesis

INTRODUCTION

High doses of radiation can lead to mutations and an accumulation of mutations can lead to cancer. There is evidence from epidemiological studies that doses of radiation above 100 mSv cause cancer. However, epidemiological studies have not been able to provide convincing evidence for increased cancer risk for effective doses of a few tens of mSv per year above the background level of exposure. At very low doses it is not possible to perform classical epidemiology studies because of the very large number of people that would need to be included in such studies, the high frequency of cancer in the general population, the difficulty of measuring the true individual radiation exposure and identification of life-style and genetic confounders. In mouse studies, radiation doses and exposure conditions, together with diet and environmental conditions, can be precisely controlled., However the number of mice required to determine

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altered cancer risk at doses as low as, for example 1 mSv, can be in the millions. Such studies are both logistically and economically unfeasible.

Presently risk assessment for radiation exposure is based on the linear no-threshold (LNT) hypothesis that predicts that even the smallest amount of radiation increases cancer risk. The related phenomena of adaptive response, bystander effects and genomic instability all suggest that the LNT model is not accurate for low dose radiation exposure (reviewed in Morgan (2003)). There are studies that have shown that low doses of radiation may be protective. Increased survival of normal and cancer prone animals has been described after low dose radiation exposure, either as a low dose alone or prior to a subsequent high radiation dose (Mitchel *et al*, 1999; 2003; Ina *et al*, 2005). Transformation assays have been used for some time as a surrogate marker for tumorigenic potential, and similar protective effects have been identified (Azzam *et a*l, 1996; Redpath *et al*, 2003). However, transformation assays are performed *in vitro* and may not be indicative of the effect that is occurring post-irradiation in a whole animal. *In vivo* changes in mutation frequency after radiation exposure reflect DNA changes and can also act as a surrogate marker for tumorigenic potential. At high doses the number of mutations caused by radiation essentially follows a linear response. After extremely high doses of radiation the cell will die. However, as the radiation dose decreases there is a chance that the cell will survive either with radiation damage that has been fully repaired or radiation damage that has not been repaired correctly resulting in some mutations that are passed on to daughter cells. If those daughter cells continue to accumulate mutations then a cancer may result. Many mutation studies have been performed *in vitro* or after *in vivo* exposure to provide a surrogate measure for carcinogenesis and also for monitoring of radiation damage. Although there are a large number of mutation assays available, very few are able to detect a change in mutation frequency after very low doses of DNA damaging agents. The protective effects observed in tumour and transformation assays after low dose radiation exposure are examples of a hormetic response. Given the close relationship between mutation frequency and cancer it is expected that such protective effects should be accompanied by a reduction in mutation frequency. However, there is a paucity of data in the literature demonstrating a reduction to below the spontaneous mutation frequency after low dose radiation. Such protective effects may be happening but it is unlikely that they will be observed using the majority of popularly used mutation assay systems, largely because most mutagenic studies that have been performed for low doses of agents have not conformed to the criteria required for detection of hormetic responses. Calabrese and Baldwin (2001) have defined the general requirements in order to be able to detect a hormetic type response for biological endpoints of any type. The criteria are equally valid for mutation studies. The *a priori* study criteria include the need for:

TABLE 1. Lowest doses at which chromosomal aberrations and mutations have been detected in experimental systems in mammalian cells exposed to acute low-LET radiation exposure

System	Endpoint	Radiation	Lowest dose (mGy)	Reference
Human lymphocytes	Unstable chromosomal aberrations	X-rays	20	Lloyd *et al* (1992)
Human lymphocytes	Stable chromosomal aberrations	Gamma rays	250	Lloyd (1998)
Mouse	Pink-eye mutation	X-rays	10	Schiestl *et al* (1994)
TK_6 cells	Hprt and tk mutation	X-rays	250	Konig and Kiefer (1988)

adapted from Sources and Effects of Ionising Radiation 2000 UNSCEAR report, Vol II

- A well-defined NOAEL (no observed adverse effect)
- At least 2 doses below the NOAEL and at least one dose above the NOAEL
- The assay to have the capacity to display a stimulatory and an inhibitory response.
- Analyses that cover doses ranging over 3-4 orders of magnitude.
- Determination of the appropriate time of analysis as the responses can vary with time (Calabrese and Baldwin, 2002).

The criteria related to study design can be managed reasonably easily, but there are few mutation assays capable of feasibly detecting both stimulatory and inhibitory responses. We have demonstrated that the *in vivo* pKZ1 transgenic mouse assay is a very sensitive assay for detection of chromosomal inversions in response to extremely low doses of X-radiation exposure. Here we describe the reasons why the pKZ1 assay is so sensitive and why it is capable of detecting both stimulatory and inhibitory responses, compared with other currently used mutation assays.

SENSITIVITY OF MUTATION ASSAYS TO RADIATION EXPOSURE

The lowest doses at which alterations in chromosomal aberrations and mutations have been detected in mammalian experimental systems exposed to acute low LET radiation are outlined in Table 1. In mammalian cells the lowest dose causing an increase in mutations was 10 mGy (Schiestl *et al*, 1994). Using the γ-H2AX assay in mammalian cell lines, Rothkamm and Lobrich (2003) demonstrated that this assay could be used to detect double strand DNA breaks in response to doses as low as 1 mGy X-radiation.

Most mutation assays are performed *in vitro* because of the ease of artificial selection systems for mutant detection and lower cost than *in vivo* studies. The most commonly used assays have included chromosome aberrations in metaphase spreads, the micronucleus assay, the HPRT assay, and the comet assay. *In vivo* assays include analysis of blood samples

from animals, animal models harbouring mutations that enable a method of mutant selection such as the mouse pink-eye model, or transgenic animals with artificial selection systems. In almost all cases, the studies using these assays have been performed at doses greater than 100 mGy, doses which are not relevant to a likely human exposure in an occupational or public exposure situation.

The pKZ1 *in vivo* assay is a transgenic mouse assay. The transgene is comprised of an *E. coli lacZ* gene in an inverse transcriptional orientation relative to a chicken β-actin enhancer-promoter (EP) complex. Inversions in the transgene are facilitated by mouse recombination signal sequences flanking the *lacZ* gene, placing the *lacZ* gene in the correct transcriptional orientation to the EP complex, resulting in expression of the *lacZ* gene product, β-galactosidase (β-gal). The *E.coli* β-gal can then be detected histochemically in tissue sections using the chromogenic substrate, X-gal (Sykes *et al*, 1998). Using the pKZ1 assay we have demonstrated a statistically significant induction of chromosomal inversions after a single X-radiation dose of 5 μGy in both spleen and prostate (Hooker *et al*. 2004; Zeng *et al*. 2006), lowering the dose range of observable mutagenic radiation effects by three orders of magnitude.

SPONTANEOUS MUTATION FREQUENCY

A number of mutation assays have demonstrated that a low dose of radiation can result in the production of responses that can protect against the induction of a portion of mutations from a subsequent high dose exposure. However, using these same assays, no significant response has been observed after exposure to a single low radiation dose. Therefore the mutation assays are capable of detecting the effects of low doses of radiation but the number of mutant events that are scored are too low to detect after low doses of radiation exposure. Cancer studies that have shown that a low dose of radiation can result in fewer cancers compared to animals that did not receive radiation also suggest that in these cases fewer mutations are occurring in the treated animals than in the untreated animals. Historically, investigators have strived to ensure low spontaneous frequencies in mutation assays in order to detect rare events after DNA damaging agent exposure. If the spontaneous frequency were higher in these assays, then a larger number of induced mutations would need to be detected in order to see a statistically significant increase in mutations. In order to determine if there are less mutations occurring compared with the normal spontaneous mutation frequency, mutation assays must be capable of detecting mutation frequencies below the normal spontaneous mutation frequency. This is extremely difficult with a large number of mutation assays as the spontaneous frequency is of the order of 10^{-5} – 10^{-6}. In order to detect a mutation frequency below

these spontaneous frequencies with any statistical confidence, a very large number of cells would need to be screened. The feasibility of doing such studies is low for logistical reasons. Assays with higher spontaneous frequencies enable feasible detection below spontaneous frequencies. The commonly used cytokinesis-block micronucleus assay has a high spontaneous frequency (approximately 5×10^{-2}) and should therefore be capable of detecting a reduction below spontaneous frequency. The spontaneous inversion frequency in pKZ1 spleen cells is approx 10^{-4} and in pKZ1 prostate luminal epithelial cells is 3×10^{-3}. Using the pKZ1 assay we have demonstrated a statistically significant reduction to below spontaneous frequency in spleen (by analysing 2×10^5 cells) and prostate tissue (by analysing 6×10^3 cells) after a single acute X-radiation exposure of 1 or 10 mGy (Hooker *et al*, 2004, Zeng *et al*, 2006). High spontaneous mutation frequencies allow detection of a reduction in inversion frequency below the spontaneous frequency without the need to count a prohibitive number of cells.

MEASUREMENT OF DIRECT AND INDIRECT DNA DAMAGE

Mutation assays detect the response to DNA damage, either as DNA damage itself, which may or may not have been the initial lesion, or the result of repair of the DNA damage. It is usually assumed that the initial DNA damage that was produced by the DNA damaging agent occurs in the region of DNA that is analysed as the endpoint of the mutation assay. The dose of radiation administered to an organism enables estimates of the number of ionisation tracks deposited in individual cells. At extremely low doses of radiation very few cells will sustain a direct hit. It is known that cells do not need to sustain a direct hit in order to respond to radiation. This phenomenon is called the bystander effect (reviewed in Mothersill and Seymour, 2005). The signals that pass from the hit cell to the unhit bystander cell are likely to cause many effects, including mutations or protection from mutations. These mutations are not caused directly by the radiation and may involve epigenetic intermediates. Therefore, in order to detect a protective effect against mutations from low dose radiation, a sensitive assay is required which has both a high spontaneous frequency and the ability to respond to bystander signals. At doses of 1 mGy or less the bystander effect is playing a role as not all cells will receive an energy deposition (Bond *et al*, 1988). It is hypothesised that the chromosomal inversion in the pKZ1 transgene occurs as a result of the formation of a stem and loop structure, which is subsequently recognised by recombination enzymes that resolve the stem and loop structure into an inversion. Therefore any biological response to radiation exposure, either in a cell that is directly hit by radiation or any bystander cells, which affects chromatin structure or recombination

enzyme activity on stem and loop structures could be detected as a change in the inversion frequency. Although the radiation dose may be small, the overall biological response to the low dose might be quite large, which would explain the high sensitivity of the pKZ1 assay.

CONCLUSION

The paucity of hormetic responses for mutation end-points in the radiation literature is possibly due to the technical difficulty of detecting such responses in the majority of mutation assays. Mutation assays with the ability to detect both stimulatory and inhibitory responses are required. Detection of a reduction below spontaneous mutation frequency will be largely dependent on a relatively high spontaneous frequency. The overall sensitivity of the mutation assay is also likely to be dependent on the assay being capable of detecting bystander responses. It is also preferable that the assays be *in vivo* assays in order to incorporate normal metabolic and physiological processes. The pKZ1 *in vivo* assay fits all of these criteria. Even so, hormetic responses will only be detected with such an assay if the experimental design spans dose ranges that fit the *a priori* criteria for detection of hormetic responses (Calabrese and Baldwin, 2001).

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