Low Doses and Radiation Risk *in vivo*

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Abstract.

The "Linear No Threshold" hypothesis, used in all radiation protection practices, assumes that all doses, no matter how low, increase risk. However, in human and other mammalian cells, low doses of low LET radiation such as gamma and X-rays induce an adaptive response that protects against both radiation-induced and spontaneous risk. An *in vivo* test of the hypothesis in mice showed that a 100-mGy dose of γ-radiation protected the mice by increasing latency for acute myeloid leukemia initiated by a subsequent large dose. A similar result was observed in cancer prone mice, where a 10-mGy adapting exposure prior to a large acute dose increased latency for lymphomas without altering frequency. Increasing the adapting dose to 100-mGy eliminated the protective effect. In the cancer prone mice, a 10-mGy dose alone, without a subsequent high dose, increased latency for spontaneous osteosarcomas and lymphomas without altering frequency. Increasing the dose to 100-mGy decreased latency for spontaneous osteosarcomas but still increased latency for lymphomas, indicating that this higher dose was in a transition zone between reduced and increased risk, and that the transition dose from protective to detrimental effects is tumor type specific. In genetically normal fetal mice, prior low doses also protected against radiation-induced teratogenic effects. In genetically normal adult male mice, high doses induce mutations in sperm stem cells, detectable as heritable mutations in the offspring of these mice. A prior 100-mGy dose protected the male mice from induction of these heritable mutations by the large dose. We conclude that adaptive responses are induced by low doses in normal or cancer prone mice, and that these responses can reduce the risk of cancer, teratogenesis and heritable mutations. At low doses *in vivo*, the relationship between radiation dose and risk is not linear, and low radiation doses can reduce risk.

INTRODUCTION

Current national and international radiation risk estimates and all radiation-protection standards and practices are based on the so-called "Linear No-Threshold Hypothesis" that states that risk is directly proportional to dose without a threshold. This LNT hypothesis is in turn, based mainly on epidemiological data of humans exposed to high doses and dose rates but is considered to also apply at low doses and dose rates, with a two-fold reduction in risk. The LNT hypothesis is attractive because it allows radiation dose to be used as a surrogate for radiation risk. At low doses the LNT hypothesis is acknowledged to be an assumption, and other dose responses are also possible, including supralinear, sublinear or threshold/hormetic responses. In humans, risk is measured in three ways. The most important radiation-induced health risk is considered to be cancer, but the risks of birth defects and heritable mutations are also considered. This paper reviews data testing the validity of low dose risk estimates that are based on the LNT hypothesis.

While ultimately the influence of low doses on risk must be measured *in vivo*, it is important to understand the mechanisms underlying any such effects. Experiments conducted in human and other mammalian cells grown in tissue culture can provide such information. Therefore, in addition to *in vivo* data, this paper additionally reviews the results of some *in vitro* cellular

experiments that indicate the mechanisms that may be involved.

Considering the potential biological consequences of a radiation exposure to a normal cell, there are three general biological outcomes of DNA damage (Mitchel and Boreham 2000) as shown in Figure 1. When DNA damage is created as a result of one or more tracks of radiation through a normal cell, the cell will attempt to repair that damage. If the repair is successful and the DNA restored to its original state, i.e., an error-free repair, then the cell is also restored to normal. In this case, there is no resulting consequence to the cell and hence no resulting risk. A second possibility is that the cell recognizes that it cannot properly repair the damage, and as a consequence activates its genetically encoded cell death process, called apoptosis. Again, in this case, no risk of carcinogenesis results, since dead cells do not produce cancer. However, cell death during embryonic development can lead to teratogenic effects, and so this response is particularly important for that measure of risk. The third possible outcome of the DNA damage is repair that avoids cell death but which is error-prone, resulting in a mutation.

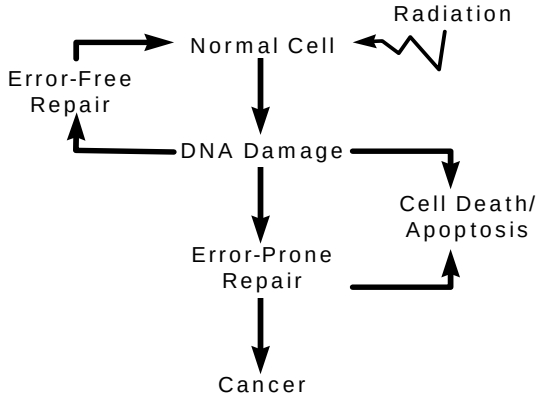


Figure 1. Possible outcomes of a cellular radiation exposure in a normal cell.

At this point, the cell may still activate its apoptotic cell death program but could also simply resume dividing. Creation of these genetic errors is part of a carcinogenic process called genomic instability, which can ultimately lead to cancer. Errors in the repair of germline cells can lead to heritable mutations, the other measure of radiation risk in humans. It is useful to note that the LNT hypothesis implies that risk is influenced only by dose, and hence predicts that the relative proportions of these three biological possibilities must be constant with dose and time. If they were not constant, then risk would vary with their relative proportions, and not strictly as a function of dose. Therefore, any variation in the relative proportions of these processes would indicate that the LNT hypothesis is invalid.

EXPERIMENTAL RESULTS AND DISCUSSION

Cellular Studies

A common result in cells exposed to radiation, particularly to a high dose, is a break in one or more chromosomes, which indicate DNA double strand breaks. If cells divide before repairing those breaks, the remaining pieces of chromosomes are packaged into micronuclei (MN). Measuring the frequency of MN in cells that have been exposed and allowed to repair therefore represents a measure of the competence of the cells at repairing such chromosomal breaks (and therefore DNA double strand breaks) in response to radiation damage. We have tested the influence of low doses and low dose rate exposures on the ability of human skin cells to repair

radiation breaks in chromosomes (Broome et al. 2002). Figure 2 shows the MN frequency in cells exposed to a variety of doses (1-500 mGy) delivered at a low dose rate (3 mGy/min) 3h before exposure to a high dose (4 Gy) delivered at a high dose rate (1.8 Gy/min). The LNT hypothesis predicts that the consequences of the two doses would be additive and yet the experiment shows that they are not. The combined exposure resulted in fewer broken chromosomes than the single acute 4 Gy exposure alone. The figure also shows that enhanced repair occurs after 1 mGy, which represents, on average, a single track per cell. The figure also shows that higher doses, representing multiple tracks/cell, produce the same result as one track/cell when those tracks from the higher doses are spaced out in time (3 mGy/min).

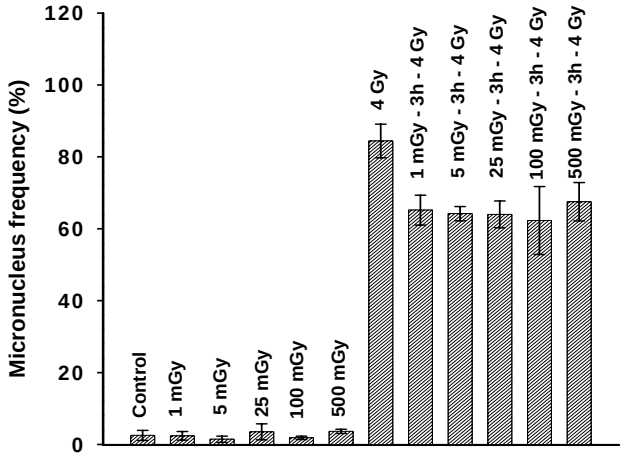


Figure 2. Low doses enhance the repair of broken chromosomes in human cells.

The data shown in Figure 2 indicates that one of the processes important in controlling risk, the repair of DNA (Fig. 1), is enhanced by a low dose exposure. By itself, however, this data does not indicate whether risk is increased or decreased, since the increased repair may be error-free or error prone.

Besides DNA repair, the other major process controlling risk shown in Figure 1 is apoptosis. Figure 3 shows that low doses prior to a high dose increase the probability that cells which have not properly repaired their DNA will die by apoptosis (Cregan et al. 1999). Since dead cells cannot become cancer cells or constitute a mutation, this process must reduce cancer risk and reduce the risk of heritable mutations. The influence on teratogenic effects is less certain. Enhancement of apoptosis late in gestation could increase teratogenic effects, but earlier in gestation may increase fetal death. If however, the elevated DNA repair were error free, neither effect would increase.

The ultimate test of the enhanced DNA repair for its relative error-free or error prone properties, and of the influence of increased apoptosis after low doses must be a test that directly measures risk. Evidence that induction of these cellular defence mechanisms after low dose exposures is actually reducing cancer risk is shown in Tables 1 and 2.

Table 1 shows the results of an experiment (Azzam et al. 1994) using rodent cells and examining the influence of a prior low dose, given at low dose rate, on the risk of malignant transformation resulting from a subsequent high radiation dose. The risk associated with the high radiation dose was reduced by a factor of 2-3 by the prior low dose, a result consistent with the evidence for improved repair of radiation damage shown in Figure 2. It clearly demonstrates that the elevated DNA repair capacity induced by a low dose must be relatively error-free.

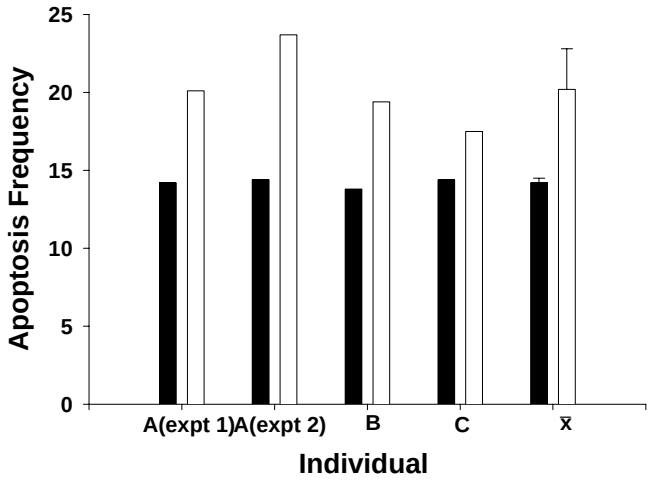


Figure 3. Low doses increase the probability of radiation-induced apoptosis in non-dividing human lymphocytes. The figure shows the results from 3 individuals (one individual repeated) and the average. Closed bars, 2 Gy; Open bars, 100 mGy + 6h + 2 Gy.

TABLE 1. Low doses reduce the risk of malignant transformation from high dose exposure

Treatment	Transformation Frequency ($X10^{-4}$)
Control	3.7
4 Gy (high dose rate)	41
100 mGy (low dose rate) + 24h + 4 Gy (high dose rate)	16

The ability of a prior low dose to reduce the risk of a subsequent high dose has importance for medical types of exposures, such as those used in cancer therapy. However, the effect of the low dose alone is more relevant for human exposures that are of a concern in radiation protection. Table 2 shows an experiment testing the effects of low gamma-radiation doses alone (without a second high dose) on malignant transformation in rodent C3H 10T1/2 cells (Azzam et al. 1996). All the doses tested, between 1 and 100 mGy, given at low dose rate, reduced the risk of spontaneous malignant transformation 3-4 fold, and all doses were equally effective (statistically not different from each other). The lowest dose tested, 1 mGy of ^{60}Co γ radiation, represents an average of 1 ionization track per cell. Since radiation tracks are random, at 1 mGy most cells

receive one track but some receive none or more than one. However all respond to the same extent as they did when they certainly received one or more tracks at the higher (10-100 mGy) doses. This maximum effect at 1 mGy parallels the rests on induced DNA repair shown in Figure 2. This is evidence therefore, that not all cells are actually required to be exposed (hit) by radiation in order to enhance their defences and reduce their risk. Such distributed effects are known as bystander effects and result from inter-cell signalling, and an example is shown in Table 3.

TABLE 2. Single Low doses reduce the risk of spontaneous malignant transformation

Treatment	Transformation Frequency ($X10^{-3}$)
Control	1.8
1 mGy	0.53
10 mGy	0.42
100 mGy	0.53

Although DNA repair and apoptosis are key processes controlling the risk of a cell becoming malignant, in the whole organism, risk of cancer is also controlled by the ability of the immune system to recognize and respond to the presence of a malignant cell. Table 3 shows the influence of a low dose on the ability of human lymphocytes to express a cell surface receptor (IL-2R) after a low dose of radiation (Xu et al. 1996). The ability of immune cells to receive signals is a measure of their immune responsiveness. The table shows that a low dose enhanced receptor expression in irradiated human lymphocytes, and that cellular signals for this enhanced expression were transmitted from exposed cells to unexposed cells, amplifying this response. These data suggest that low doses may enhance immune competence and reduce risk in animals or people who already have malignant cells.

TABLE 3. The percentage of human lymphocytes expressing IL-2 receptors after mitogen stimulation

Time (h) after Con A Stimulation	Control Cells	Irradiated Cells (10 mGy)	50% Control Cells + 50% Irradiated Cells
24	7.7 ± 4.1	17.8 ± 3.3	22.6 ± 4.8

The cellular data strongly suggests that the biological processes important in radiation risk are not constant, as required by the LNT hypothesis. Instead, low doses appear to alter their relative importance in ways that are likely to impact on risk in whole organisms, and in ways that would preclude a linear increase in risk with increasing dose.

Studies in animals

Cancer Risk in Animals

While experiments in cells provide important supporting information about the actual molecular and cellular responses to low doses, ultimately experiments testing the effect of low doses on measures of risk such as cancer must be conducted in whole mammals. Figure 4 show a test (Mitchel et al. 1999) of the influence of a low dose exposure on radiation-induced myeloid leukemia in genetically normal mice. The figure shows that exposure to a high dose of 1 Gy induces myeloid leukemia in mice, and as a result, those mice with the disease lose a substantial portion of their normal lifespan. However, increasing the total exposure, by exposing the mice to 100 mGy, at low dose rate, the day before the 1 Gy exposure delayed the onset of those cancers, effectively restoring a portion of the lifespan that would otherwise have been lost in those mice that developed the disease. It is important to note that the low "adapting" exposure did not significantly affect the frequency of the disease induced by the high radiation dose, only the latency. The carcinogenic process is thought to involve an initiating event, which subsequently triggers an accelerating process of genomic instability leading to multiple genomic rearrangements, ultimately producing a cancer cell. The frequency of cancer is thought to reflect the number of initiating events while the latency of the disease reflects the rate at which the genomic instability process proceeds. The results shown in Fig. 4 indicate that low doses delivered at low dose rate slow the rate of progression of the genomic instability process but do not change the frequency of the cancer initiating events.

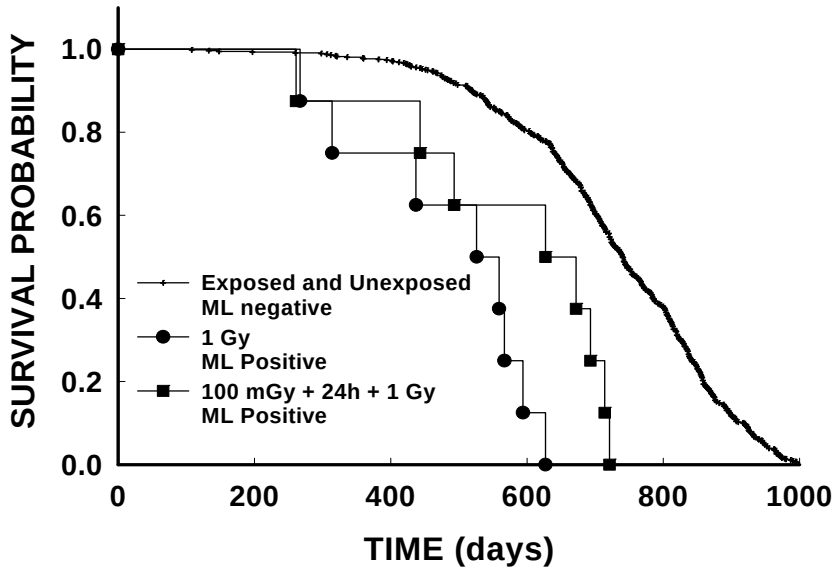


Figure 4. Delayed appearance (P<0.001) of radiation-induced myeloid leukemia (ML) in genetically normal mice by exposure to 100 mGy at low dose rate 24h before the carcinogenic 1 Gy exposure.

Radiation protection standards and practices applied to humans must also consider the possibility that some individuals may be more radiation-sensitive and cancer-prone than others, for genetic reasons. This raises the possibility that low doses may produce different, and potentially more harmful effects in such individuals, than those seen in genetically normal individuals. Figure 5 shows a test (Mitchel et al. 2004 *in press*) of this "worst case scenario". Mice that are heterozygous for the *Trp53* gene (*Trp53* +/-) are compromised in their ability to repair DNA damage and in their ability to initiate cell death in improperly repaired cells, two of

the processes most important for controlling cancer risk (Fig. 1). Consequently, such mice are cancer prone. Figure 5 shows that these mice spontaneously develop lymphomas, and a high 4 Gy dose of radiation increases the frequency and dramatically accelerates the appearance of these tumours.

A dose of 10 mGy, given at low dose rate the day before the 4 Gy exposure delayed the onset of these lymphomas ($p<10^{-4}$), but did not significantly change their frequency. Correcting animal survival for competing causes of death did not change this conclusion. This effect of increasing latency was also seen in the genetically normal (*Trp53* +/+) mice in Fig. 4. Increasing the low adapting dose to 100 mGy caused this protective effect to disappear in the *Trp53* +/- mice. While not increasing harm, 100 mGy apparently represents an upper threshold for doses that are protective against radiation-induced lymphomas in the cancer prone mice. In the normal mice (Fig. 4) protection was still seen at 100 mGy indicating a higher upper threshold for protective effects in the *Trp53* +/+ mice.

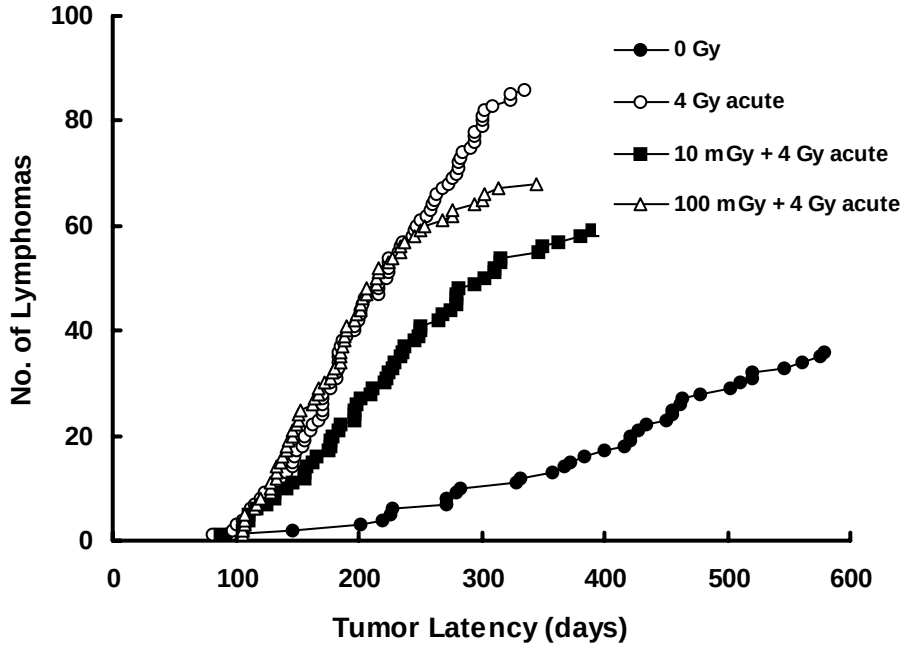
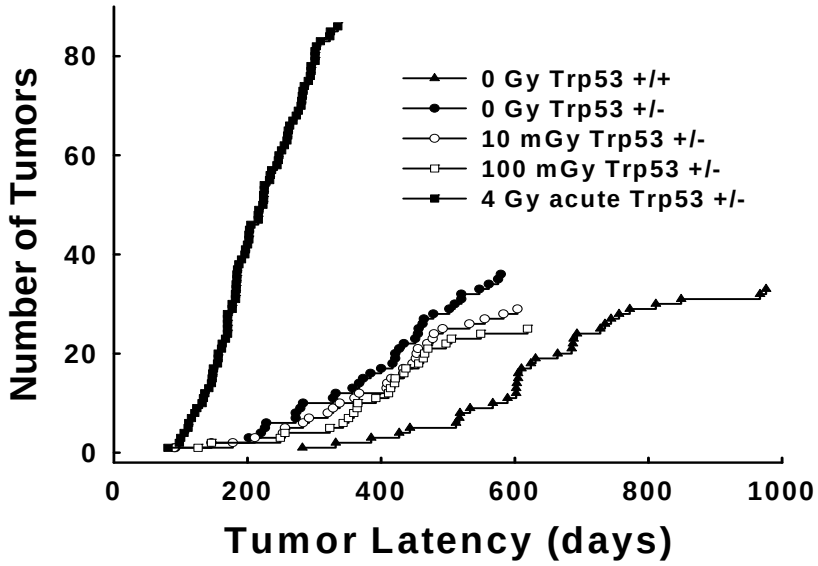


Figure 5. Appearance of lymphomas in unexposed cancer prone mice (*Trp53* +/-) and in mice exposed to 4 Gy with or without a prior low dose and dose rate exposure.

Experiments testing the *in vivo* effect of low doses on cancer risk produced by high dose exposure are important for improving our understanding of the dominant biological outcome of such exposures, and are potentially useful concepts for medical radiotherapy procedures. However, for the radiation protection standards and practices important in public and occupational health, it is more important to understand the influence of low doses on spontaneous cancer risk. Figure 6 shows the results of such a test (Mitchel et al. 2003) in the cancer prone *Trp53* heterozygous mice that represent the "worst case scenario". In these mice, the tumor types and frequencies did not change, indicating that the tumors appearing after irradiation remained the same spontaneous tumors seen in the absence of radiation. About 80% of the mice spontaneously develop malignant tumors. The figure shows that unexposed, genetically normal mice (*Trp53* +/+) of this strain also spontaneously develop lymphomas but that these cancers

appear much earlier in the unexposed, cancer prone (*Trp53*+/-) mice. Exposure to an acute 4 Gy dose of radiation dramatically accelerated this appearance. The figure also shows that a single exposure of either 10 or 100 mGy, given at low dose rate to young mice, restores a portion of the lifespan lost due to the disease in the unexposed, cancer-prone mice ($P<10^{-4}$). Unlike the result in Fig. 5, where the lymphomas developed in mice that had subsequently received a high dose, the protective effect against these spontaneously appearing cancers was not lost when the dose was increased to 100 mGy. This result suggests that the upper dose threshold for protective effects varies with the severity or nature of the cancer-inducing event, with the threshold being higher for less severe inducing events such as spontaneous occurrences.

Other tumours also appear spontaneously in these cancer-prone mice. Osteosarcomas develop in the spine and grow to the point where they create paralysis in the mice. Figure 7 shows the time that these spontaneous cancers create paralysis in the mice, with and without a single exposure to 10 mGy given at low dose rate when the mice were 8 weeks old (Mitchel et al.



2003).

Figure 6. The appearance of lymphomas in unexposed or radiation exposed genetically normal (*Trp53* +/+, lower curve only) or cancer prone (*Trp53* +/-) mice.

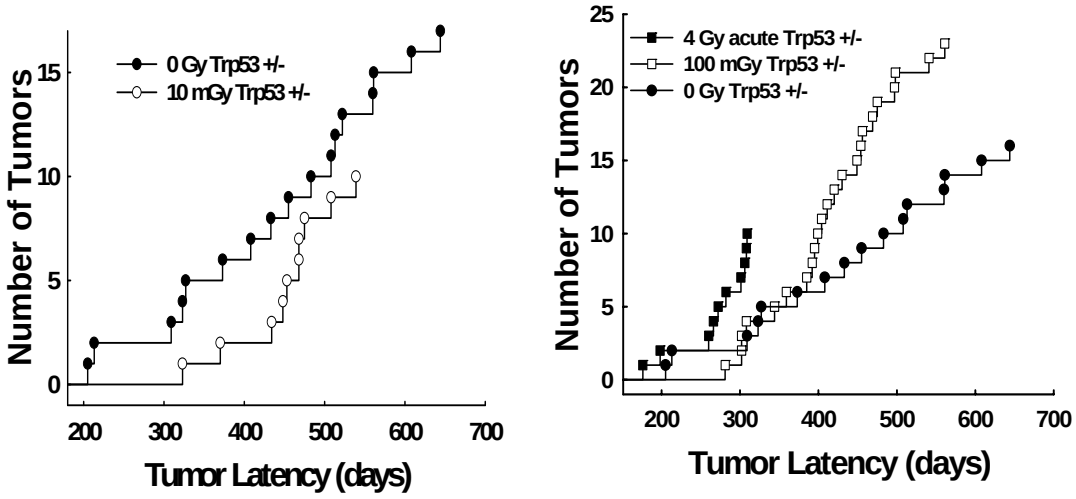


Figure 7. The appearance of spontaneous osteosarcomas in unexposed cancer prone mice (*Trp53* +/-), or exposed to 10 or 100 mGy at low dose rate. Exposure to 4 Gy is shown for comparison.

Compared to the mice not receiving the low dose, the appearance of the first spinal osteosarcoma was delayed by more than 100 days, and that delay persisted for all of the tumours that appeared, i.e. for the entire lifespan of the mice (P=0.005). This lifetime protection was also apparent for the spontaneous lymphomas shown in Fig. 6. However, unlike the case for spontaneous lymphomas (Fig. 6), increasing the low dose to 100 mGy resulted in a general acceleration of the appearance of the spontaneous spinal osteosarcomas (P<0.04, Fig. 7). This decrease in cancer latency clearly represents an increase in risk, rather than the risk decrease seen at 10 mGy. For this tissue type therefore, the upper threshold for protective effects of a low dose must lie between 10 and 100 mGy. Since, in the same animals, the upper dose threshold for protection against lymphomas exceeded 100 mGy, the dose threshold, where protective effects give way to detrimental effects, must be tissue-type specific.

Teratogenic risk in animals

Exposure of the murine fetus to large doses of radiation during the *in utero* period of organogenesis results in malformations. Wang et al. (1999) have linked these malformations to radiation-induced apoptosis. They have reported that mice exposed to a large doses of X rays *in utero* on day 11 of gestation showed an increase in the number of apoptotic cells in the predigital regions in the forelimb buds. That apoptotic response correlated with teratogenic anomalies in the limbs of the fetuses subsequently observed on gestational day 19. Susceptibility to radiation-induced apoptosis in the predigital regions and the resulting digital defects depended on *Trp53* status, with *Trp53* (+/+) mice the most sensitive, *Trp53* (+/-) intermediate, and *Trp53* (-/-) the most resistant (Wang et al. 1999).

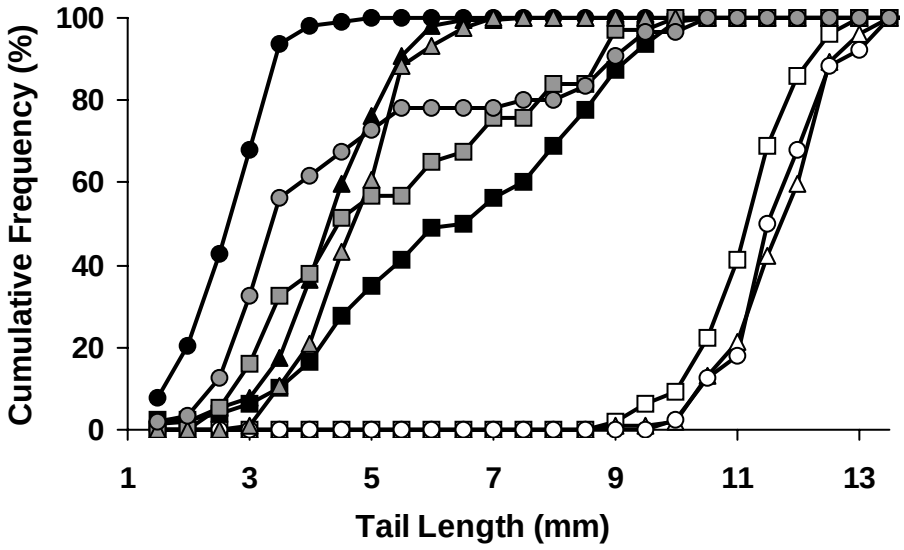


Figure 8. The influence of a low dose given on gestational day 10, on the tail length of fetal mice exposed to 4 Gy on gestational day 11. The figure shows the influence of *Trp53* genotype on the cumulative frequency of fetuses with the indicated tail length as measured on day 18 of gestation. Open symbols, unirradiated control fetuses; Closed symbols, fetuses exposed to 4 Gy on day 11 of gestation; Shaded symbols, fetuses exposed to 300 mGy, 24 h prior to 4 Gy irradiation on day 11 of gestation. Circles, *Trp53* +/+; Triangles, *Trp53* +/-; Squares, *Trp53* -/-.

Figure 8 shows that low doses can protect against the teratogenic effects of high doses (Mitchel et al. 2002). In those experiments tail shortening was used as a quantitative measure of the extent of the teratogenic effect. The influence of *Trp53* on susceptibility to radiation-induced tail shortening by high gamma doses was as reported by Wang et al. (1999). The figure also shows, however that low doses can protect against this teratogenic effect. In a similar way, the ability of a low dose to protect against these effects also depended on *Trp53* status. *Trp53* (+/+) fetal mice showed the most protection, *Trp53* (+/-) fetal mice showed a small but significant protection, while *Trp53* (-/-) fetal mice were sensitized to increased teratogenesis.

Heritable mutation risk in animals

The final measure of risk that must be considered in tests of the validity of the LNT hypothesis is the risk of heritable mutations. Boreham et al. (2004) have examined the risk of heritable mutations from radiation exposure in mice. In their experiments, male mice were exposed to 1 Gy of gamma radiation, with or without an exposure to 100 mGy 24h prior to the 1 Gy. The exposed mice were held for 10 weeks to remove all mature sperm and then bred to unexposed females. The offspring were examined for mutations received from the exposed male parent germline stem cells. They observed that the 1 Gy exposure approximately doubled the spontaneous rate of mutation in the offspring but that the prior exposure to 100 mGy reduced the

effect of the 1 Gy such that the resulting mutation rate was not different from the spontaneous rate in the absence of any exposure.

CONCLUSIONS

This paper has described experimental tests, at the molecular, cellular and whole animal levels, of the validity of the Linear No-Threshold Hypothesis as it applies to low doses and dose rates of low LET radiation. Biological processes though to control risk, including DNA repair, apoptosis, and immune competence, as well as a direct measure of risk, malignant transformation, were examined in cells. In animals, the three important measures of radiation risk, carcinogenesis, teratogenesis and heritable mutations were examined. At all levels of test, including direct measures of cancer risk *in vitro* and *in vivo*, the hypothesis has failed. The results *in vitro* and *in vivo* show that low doses decrease, rather than increase risk. This reduction in risk below the radiation-induced or spontaneous risk levels is not linear with dose. The decrease appears to reach a maximum with the first track of radiation through the cells, and stays at that level until the dose reaches at least 100 mGy in genetically normal animals. The upper dose threshold for protective effects therefore appears to be above 100 mGy for all three measures of risk, carcinogenesis, teratogenesis and heritable mutations. These results indicate that at low dose rate, the assumption of linearity may be valid only at doses above about 100 mGy (with some variation in different tissue types), and below this level, level radiation-induced protective effects dominate risk. In mice that are cancer prone for genetic reasons, this upper threshold appears to be reduced and may be around 10 mGy. Since these are direct measures of risk *in vivo*, any influence of bystander effects is included. The results in human and other mammalian cells, and in whole animals, described here parallel earlier observations in lower organisms (Mitchel and Morrison 1984, Boreham and Mitchel 1991, Boreham and Mitchel 1993, Dolling et al. 2000), indicating that these adaptive responses to low doses are not unique to mammals but are part of an evolutionarily conserved response.

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