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RADIATION-INDUCED NEOPLASTIC TRANSFORMATION *IN VITRO*, HORMESIS AND RISK ASSESSMENT

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□ Dose-response curves for various low-LET radiation sources have consistently been demonstrated to be J-shaped for the cancer-relevant endpoint of neoplastic transformation *in vitro*. Most of these studies have been performed where the radiation has been delivered at intermediate to high dose-rates (30-3000 mGy/min), where the threshold dose for induction of neoplastic transformation is around 100-200 mGy. Below these doses, the transformation frequency is less than that seen spontaneously, indicative of a hormetic effect. More recently, data have been obtained for low dose rates (<0.5 mGy/min) of low-LET radiation, and again hormetic effects are apparent but with threshold doses now being >1000 mGy. Similar trends have been reported in animal experiments as well as in human epidemiologic studies. Indeed, the relative risks for induction of neoplastic transformation *in vitro* in the dose range 1 to 1000 mGy agree well with those for incidence of radiation-induced breast cancer and leukemia in humans. These findings support the notion that the endpoint of neoplastic transformation *in vitro* is a plausible endpoint to not only study mechanisms involved in response to low doses of radiation, but also to provide information of potential importance to risk assessment.

Keywords: low dose, radiation, neoplastic transformation, hormesis, risk assessment

RELEVANCE OF *IN VITRO* ASSAYS

The *in vitro* assay of neoplastic transformation has a long history of providing quantitative and mechanistic data that parallels that found *in vivo* (Little 1989). Such findings relate to the effects of dose, dose-rate, radiation quality, and effects of chemical modifiers and as such are regarded as being of value to the study of radiation carcinogenesis. This is despite the obvious microenvironmental limitations and lack of an immune response compared to the *in vivo* situation. Another perceived limitation is the refractory nature of primary human cell cultures to radiation-induced transformation to the neoplastic phenotype. Even following immortalization, such cells are difficult to neoplastically transform with radiation and usually require repeated high radiation doses over a period of time. Such systems are of value in assessing molecular and cellular characteristics of the radiation-induced tumorigenic cells but are not suitable for quantitative studies (e.g. Hei et al. 2001). The major workhorse assay in the field of quantitation of radiation-

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induced neoplastic transformation *in vitro* has been the C3H 10T1/2 mouse embryo fibroblast system. Another assay that has recently been gaining wider acceptance is the HeLa x skin fibroblast human hybrid cell assay (Redpath et al. 1987, Mendonca et al. 1992). This assay has some advantages, both practical and mechanistically, over the C3H 10T1/2 system. Firstly, the assay takes 21-25 days to go to completion compared to up to 60 days for the C3H 10T1/2 assay. Secondly, the neoplastic phenotype has a cell surface molecular marker that can be used to identify foci of tumorigenic cells, as compared to the morphologic changes used with C3H 10T1/2 cells. Thirdly, the mechanism involved in the neoplastic transformation is the loss of putative tumor suppressor loci located on chromosomes 11 and 14 (Mendonca et al. 1998). Both the C3H 10T1/2 and the human hybrid cells should be considered as partway down the pathway from normal to tumorigenic, i.e. they are preneoplastic. The relevance of use of such cells could also be questioned. However, since most humans have burdens of preneoplastic cells in their body, they could be considered to be a particularly relevant target for carcinogens, including radiation.

ADAPTIVE RESPONSE

The ability of a low dose of radiation, say 1 to 100 mGy, to ameliorate the effect of a subsequent high radiation dose, say several Gy, is well described for a variety of *in vitro* and *in vivo* endpoints and has historically been described as an adaptive response. However, an adaptive response can also be observed following a low dose exposure alone, without using a high dose challenge to allow its detection. For example, it is conceivable that such low-dose responses could impact the shape of dose response curves at low doses, including for the endpoint of neoplastic transformation *in vitro*, where, like for human cancer, there is a certain background incidence. In addition, such low adapting doses can alter the the latency period for the appearance of spontaneous tumors *in vivo* (Mitchel et al. 2003). Mechanistically, adaptive responses have been linked to radiation-induced protein synthesis, including the upregulation of DNA repair and cellular antioxidants, (Wolff 1998 and references therein), the promotion of apoptosis (Cregan et al. 1999), and the activation of immunological networks (Ina and Sakai 2005a).

SUPPRESSIVE EFFECTS OF LOW DOSES OF LOW-LET RADIATION FOR NEOPLASTIC TRANSFORMATION *IN VITRO*

Azzam et al. (1996) demonstrated that low doses of 1, 10, and 100 mGy of Co-60 gamma irradiation suppressed the transformation frequency of C3H 10T1/2 cells to levels less than that seen spontaneously. This was subsequently verified for a dose of 10 mGy of Cs-137 gamma radiation in the human hybrid cell assay (Redpath and Antoniono 1998). Full dose-response curves were then developed for a series of low-LET

sources including Cs-137 gamma rays, 60 kVp x-rays, 28 kVp x-rays and 232 MeV protons (Redpath et al. 2001, Redpath et al. 2003b, Ko et al. 2004, Elmore et al. 2005, Ko et al. 2006). These dose-response curves were J-shaped, and consistently demonstrated suppression at low doses with thresholds between 100 and 200 mGy. These data have now been combined for analysis. It is realized that while all the data are for low-LET radiation sources, they are for sources of different energy that potentially have different biological effectiveness. However, even given this limitation, it was felt worthwhile to do a combined analysis to see if the low dose-suppression seen in individual studies still holds when that data are combined. Since these experiments were performed over a period of years using different batches of serum, and since serum batch is well known to influence background frequency, this combination had to be done for two groups separated by level ('low' and 'high') of spontaneous background frequencies. Details of these analyses are shown in Tables 1

TABLE 1. Summary of low background experiments to date on the effect of low doses of low-LET radiation on the neoplastic transformation of HeLa x skin fibroblast hybrid cells.

Dose Cohort, mGy	Paper No.*	Mean Dose (mGy)	No. of Surviving Cells S($\times 10^5$)	No. of Foci	No. of Flasks, N	No. of Flasks without Foci, n	Transformation Frequency ($\times 10^{-5}$)	± SE ($\times 10^{-5}$)	95% Confidence Intervals ($\times 10^{-5}$)
Control	1	0	15.66	73	376	315	4.25	0.54	
	2		29.32	65	762	699	2.24	0.28	
	3		35.07	106	1297	1198	2.94	0.30	
	4		25.44	92	669	582	3.66	0.39	
		sum	105.49	336.00	3104.00	2794.00	3.10	0.18	2.74, 3.45
1–10	1	5.1	15.41	36	377	346	2.10	0.38	
	2		25.28	37	708	672	1.46	0.24	
	3		31.23	28	1173	1148	0.81	0.16	
	4		11.5	39	287	249	3.54	0.58	
		sum	83.42	140	2545	2415	1.60	0.14	1.32, 1.88
11–100	2	69.2	16.08	25	426	401	1.60	0.32	
	3		28.67	63	960	900	2.16	0.28	
	4		29.64	99	879	781	3.51	0.35	
		sum	74.39	187	2265	2082	2.57	0.19	2.19, 2.94
101–300	2	226.7	8.62	33	216	188	3.48	0.66	
	3		8.38	27	288	262	3.25	0.64	
	4		13.16	54	347	300	3.84	0.56	
		sum	30.16	114	851	750	3.56	0.35	2.85, 4.27
301–500	2	397.7	11.76	45	288	248	3.66	0.58	
	3		8.81	44	288	248	4.89	0.77	
	4		15.71	67	479	421	3.94	0.52	
		sum	36.28	156	1055	917	4.08	0.35	3.38, 4.77
501–1000	2	850	7.34	47	192	151	6.28	0.98	
	4		11.8	77	320	260	5.63	0.73	
		sum	32.48	357	956	713	8.63	0.56	7.52, 9.74

*Paper 1 = Redpath and Antoniono, 1998; Paper 2 = Redpath et al., 2001; Paper 3 = Redpath et al., 2003; Paper 4 = Ko et al., 2006.

TABLE 2. Summary of high backround experiments to date on the effect of low doses of low-LET radiation on the neoplastic transformation of HeLa x skin fibroblast hybrid cells.

Dose Cohort	Paper No.*	Mean Dose (mGy)	No. of Surviving Cells S($\times 10^5$)	No. of Foci	No. of Flasks, N	No. of Flasks without Foci, n	Transformation Frequency ($\times 10^{-5}$)	± SE ($\times 10^{-5}$)	95% Confidence Intervals ($\times 10^{-5}$)
Control	5	0	25.13	229	672	497	8.07	0.61	
	6		14.99	152	442	333	8.35	0.80	
		sum	40.12	381.00	1114.00	830.00	8.17	0.49	7.20, 9.14
	5	2.7	28.88	223	900	734	6.35	0.49	
	6		28.73	253	882	702	7.01	0.52	
		sum	57.61	476	1782	1436	6.68	0.36	5.96, 7.40
11–100 mGy	5	53.7	33.48	243	912	728	6.14	0.45	
	6		14.7	164	444	325	9.42	0.87	
		sum	48.180	407	1356	1053	7.12	0.41	6.30, 7.94
101–300 mGy	5	208	37.79	387	978	686	9.18	0.54	
	6		13.83	160	444	330	9.53	0.90	
		sum	51.620	547	1422	1016	9.26	0.46	8.34, 10.18
501–1000 cGy	6	600	13.34	233	444	302	12.83	1.08	
		sum	13.340	233	444	302	12.83	1.08	10.66, 14.99

*Paper 5 = Ko et al., 2004; Paper 6 = Elmore et al., 2005.

and 2 and the corresponding dose-response curves are shown in Figure 1. As is shown, the combined data clearly show a hormetic effect with J-shaped curves for both 'low' and 'high' background frequencies. The most significant suppression was apparent for the lowest dose cohort (<10 mGy) for both groups and the threshold was between 100 and 200 mGy for both groups. It is of importance to note that the suppressive effect of low doses of low-LET radiation on neoplastic transformation *in vitro* has been demonstrated over a wide range of background frequencies from 1.8×10^{-3} (Azzam et al. 1996) to 2.2×10^{-5} (Redpath et al. 2001) and indicates that the hormetic effect is independent of genetic stability of the target cell population.

Mechanistic studies indicate that multiple mechanisms are likely involved in a dose-dependent fashion. These include the upregulation of DNA repair, and the hyper-radiosensitivity to radiation-induced cell death of a transformation prone subpopulation (Pant et al. 2003; Redpath, Short et al. 2003a).

Mathematical modeling of our neoplastic transformation data has invoked the concept of protective apoptosis-mediated (PAM) death of cells destined to become neoplastically transformed to account for the protective effect against neoplastic transformation seen at low doses (Schoellenberger et al. 2002, Scott et al. 2003, Scott 2005)

Bystander effects due to factors excreted into the extracellular medium do not appear to play a role in this assay although bystander effects as

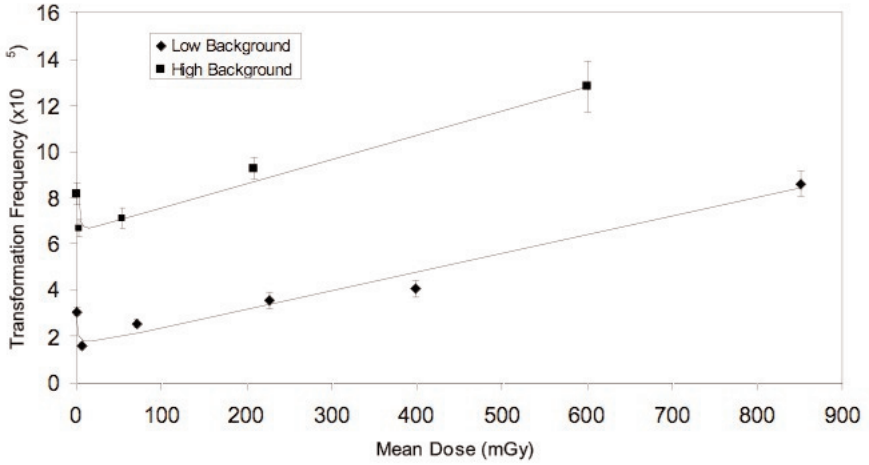

FIGURE 1. Neoplastic transformation frequency as a function of low-LET radiation dose: An analysis of combined data from several independent studies using the human hybrid cell assay. The analyses are stratified by level of spontaneous background into two groups ("low" and high"). For further detail see Tables 1 and 2.

a consequence of gap junction intracellular communication may do so in a way which partially offsets effects due to an adaptive response (Ko et al. 2006). Recent low dose-rate studies (Elmore et al 2006) have shown that the suppressive effects still exist at low doses and the threshold dose is increased as the dose-rate is decreased.

IN VIVO RESPONSES AT LOW DOSES

Dose-response curves consistent with a threshold effect have also been found in animal studies (Ullrich and Storer 1979) and low doses have been found to increase the latency period for tumor formation in both normal and cancer prone mice (Mitchel et al. 1999; Mitchel et al. 2003). Low dose-rate studies in animals also strongly support the possibility of dose thresholds (Ullrich and Storer 1979). Recent studies have implicated immune activation in the suppression of cancer induction in mice by chronic low dose-rate irradiation (Ina and Sakai 2005a, Ina et al. 2005). In addition, chronic low dose-rate irradiation has been shown to prolong the lifespan of mice genetically engineered to be susceptible to multiple severe diseases, again through immunological activation (Ina and Sakai 2005b).

COMPARISON WITH EPIDEMIOLOGIC FINDINGS

Dose-response curves for radiation induced cancer in adult humans can often be equally well fitted with a threshold, a linear-quadratic or a linear model emphasizing the difficulty in assessing what is really happening at low doses (≤ 100 mSv) from epidemiologic data. Furthermore,

when analyses are performed on incidence of (or mortality from) all solid cancers, as is often the case for A-bomb survivors, the variability of response in terms of individual tumor types is obscured. For example, Preston et al. (2003) have shown differences in mortality risk estimates for a variety of radiation-induced cancers. While the data for radiation-induced leukemia in the A-bomb survivors can be fit with a no-threshold model, it is also not possible to rule out the existence of a threshold in the range of 100 mSv (Little and Muirhead 1998). Indeed, the low-dose data from this study are very suggestive of a J-shaped, hormetic-type, dose-response (see UNSCEAR 2000). The same is true for radiation-induced breast cancer in humans. The paper on the pooled analysis of eight cohorts for radiation effects on breast cancer risks represents a valuable compilation of data for high and low dose-rate exposure (Preston et al. 2002). For doses <100 mGy delivered at high dose-rates, the relative risks trend, although not significantly so, to values <1. This is even more apparent for low dose-rate exposures where the relative risks are <1 even for exposures in dose cohorts up to 1000 mGy. Indeed, in one of the original papers on the low-dose cohorts the authors state that "it was the contribution of subjects with breast doses >1 Gy that produced a positive association between dose and subsequent breast cancer risk" (Lundell et al. 1996). This is but one illustration of a general concern that while epidemiologic data can often be fit to a linear no-threshold (LNT) model, such fitting is often heavily driven by the high dose data points and this can tend to obscure what is happening at low doses.

We have previously observed that relative risks for leukemia and breast cancer incidence and mortality agree well with those determined by our *in vitro* transformation assay for intermediate to high dose-rate low-LET radiation (Redpath et al. 2001). We now have data showing a similar excellent agreement for low dose-rate low-LET radiation when comparing relative risk for breast cancer incidence with that for neoplastic transformation *in vitro* (Elmore et al. 2006). The data indicate that for low to high dose-rates (1 to 2000 mGy/min) a threshold dose of 100 mGy cannot be ruled out and that for very low dose-rates (< 0.5 mGy/min) this threshold could well exceed 500 to 1000 mGy Furthermore, for both endpoints the existence of a hormetic effect cannot be statistically ruled out. A recent joint report of the French Academie des Sciences and Academie Nationale de Medecine also concluded that the LNT model could greatly overestimate the carcinogenic risks associated with low doses (< 100 mGy) of low LET radiation (Tubiana and Aurengo 2006). On the other hand the recent report from the U.S. National Academy of Sciences (BEIR VII PHASE II 2006) concluded that there is no compelling evidence to indicate a dose threshold below which the risk of tumor induction is zero. The fact that two distinguished committees came to opposite conclusions is largely a reflection of their differing interpretations of recent biological

data, including some of that referred to in this paper. Hopefully, further research into low-dose effects will eventually resolve this difference.

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