

Genomic Instability, Bystander Effect, Cytoplasmic Irradiation and other phenomena that may achieve fame without fortune

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Abstract

The possible risk of induced malignancies in astronauts, as a consequence of the radiation environment in space, is a factor of concern for long term missions.

Cancer risk estimates for high doses of low LET radiation are available from the epidemiological studies of the A-bomb survivors. Cancer risks at lower doses cannot be detected in epidemiological studies and must be inferred by extrapolation from the high dose risks. The standard setting bodies, such as the ICRP recommend a linear, no-threshold extrapolation of risks from high to low doses, but this is controversial.

A study of mechanisms of carcinogenesis may shed some light on the validity of a linear extrapolation. The multi-step nature of carcinogenesis suggests that the role of radiation may be to induce a mutation leading to a mutator phenotype. High energy Fe ions, such as those encountered in space are highly effective in inducing genomic instability. Experiments involving the single particle microbeam have demonstrated a "bystander effect", ie a biological effect in cells not themselves hit, but in close proximity to those that are, as well as the induction of mutations in cells where only the cytoplasm, and not the nucleus, have been traversed by a charged particle. These recent experiments cast doubt on the validity of a simple linear extrapolation, but the data are so far fragmentary and conflicting. More studies are necessary.

While mechanistic studies cannot replace epidemiology as a source of quantitative risk estimates, they may shed some light on the shape of the dose response relationship and therefore on the limitations of a linear extrapolation to low doses.

KEYWORDS: Cancer Risks, Instability, Bystander Effect, Microbeam.

1. Introduction

The possible risk of cancer in astronauts as a consequence of exposure to the elevated levels of radiation in space has been recognized from the inception of the manned space program. The problem is the *magnitude* of the risk. The standard approach is to say that good estimates of cancer risk from low LET radiation doses of 0.2 to 2.5 Gy are available from epidemiological studies of the A-bomb survivors, while reasonable radiation weighting factors to convert risks from low to high LET, are available from animal studies and *in vitro* experimentation. There is always the nagging doubt that the high energy heavy ions unique to the space environment may be unusually effective at producing genomic instability in a cell population, so that the radiation weighting factors derived largely from neutron exposures may be inadequate when applied to these special radiations.

The real problem surfaces when low doses and low dose-rates are involved, for which there are no credible epidemiological data. The ICRP recommends a linear extrapolation from the risks at high doses with no threshold, an approach that is described as "prudent and conservative". This view is not universally accepted. Research into mechanisms of radiation carcinogenesis may shed some light on the nature of the dose response relationship and therefore on the validity of a linear extrapolation to low doses.

2. Quantitative Risk Estimates

The study of the Japanese survivors of the A-bombs provides the best data for a quantitative estimate of the risks of radiation-induced cancer [1]. The International Commission on Radiological Protection (ICRP) suggests a figure of 8% per Sv for fatal cancer attributable to acute exposures of low LET radiation. This estimate is good over the dose range from about 0.2 to 2.5 Sv; some would argue that the estimate holds good to lower doses, but the uncertainties becomes large below about 0.2 Sv. As the data from Japan mature, it is evident that these estimates are very much an average for the whole population. In the first place there is a dramatic dependence on age, with the very young being much more sensitive than the old. In the second place, there is a gender difference with females being more radiosensitive than males, particularly at early ages [2].

There has been a controversy for many years concerning the possibility that *in utero* irradiation leads to childhood cancer and leukemia [3]. The association between obstetric x-ray examination and childhood malignancies has never been in doubt; the question has been whether radiation is the causative agent or whether there are other selective factors in operation. In a definitive study in 1997 Doll and Wakefield examined all the confounding factors in the several studies of *in utero* irradiation and came to the conclusion that a single obstetric x-ray exa-

mination, especially in the last trimester, significantly enhances the risk of a childhood malignancy [4]. A 40% increase in relative risk is associated with a dose of a few cGy. If this relative risk is translated into an absolute risk, it amounts to about 6%/Sv – a figure remarkably similar to that derived from the Japanese survivors. The effects of lower doses are discernible in children because, of course, the spontaneous background level of cancer is so much lower. If these data are accepted, then the dose range over which cancer risk estimates are available extends down to about 1cG. Below this, there are no reliable data demonstrating an association between radiation exposure and an excess incidence of cancer. Both ICRP and NCRP recommend that cancer risks at lower doses be estimated by using a linear no threshold extrapolation from risks at higher doses [5, 6]. This is described as “prudent and conservative”, but not everyone agrees with this judgment. Some argue that valuable resources are wasted on radiation protection at dose levels where no human detriment is actually observable. On the other hand, some would argue that low doses may be more hazardous than a linear extrapolation implies. Solving this dilemma is a matter of enormous societal importance. Epidemiological studies can never provide an answer; the only hope is that studies of the mechanisms of radiation carcinogenesis may shed some light on the nature of the dose response relationship at low doses, and therefore on the validity of the linear extrapolation.

3. Potential Mechanisms of Radiation Carcinogenesis

The truth is that we do not know the mechanism of one single radiation-induced tumor. From the wider world of biology, we know that loss of control of proliferation can be by the activation on an oncogene (usually a dominant process) or by the loss of a tumor suppressor gene (usually a recessive process) [7-9]. In the case of some leukemias and lymphomas, the malignancy appears to be associated with a single cytogenetic change. From example, in the case of Chronic Myeloid Leukemia, (CML) a symmetrical reciprocal translocation brings together the bcr and abl genes to form a fusion gene which is the hallmark of this malignancy. Inasmuch as radiation produces translocations efficiently (both symmetrical and asymmetrical) it is tempting to speculate that when CML is attributed to a radiation exposure, the mechanism involves the production by the radiation of this characteristic translocation. However, this has never been proved in any specific instance.

In the case of solid cancers, the situation is even less straightforward. Tumor suppressor genes are recessive acting, so that both copies must be inactivated or deleted for the malignant phenotype to be expressed, and it is difficult to imagine that a modest dose of radiation could delete the same gene on two separate chromosomes with a probability anywhere

near sufficient to account for the frequency of radiation-induced cancer. Many spontaneous solid tumors are characterized by loss of heterozygosity. In this process, one chromosome of a pair is lost, a deletion occurs in the other chromosome (including a tumor suppressor gene) and then the remaining chromosomes replicate. Of this series of errors, radiation may be responsible for the deletion, but it is not likely to be involved in the other steps.

One of the most pervasive dogmas in cancer research is that carcinogenesis is a multi-step process. This idea originated with the skin-painting experiments in mice over 60 years ago. The most recent reincarnation of this idea is the model for colon cancer proposed by Vogelstein and colleagues in which multiple changes involving oncogenes, tumor suppressor genes and chromosomal aberrations occur for a normal epithelium to become a metastatic tumor [10]. The problem for the radiation biologist is to explain how a modest dose of radiation can produce mutations in so many different genes and breaks in so many separate chromosomes; and yet the survivors of the A-bomb exposures express a whole spectrum of solid cancers, including malignancies of the gastrointestinal tract. The most likely reason is that the radiation produces a mutation in a gene (as yet unidentified) responsible for the stability of the genome, or for the fidelity of replication, leading to a mutation phenotype, in the same way that a mutation in a mismatch repair gene leads to the heritable form of colon cancer (HNPCC) studied by Vogelstein [11]. An attractive hypothesis, but as yet unproven. Many studies have shown that radiation induces an instability that is expressed in terms of chromosomal aberrations and mutations or transformation occurring many generations after irradiation.

In particular, Piao et al. [12] have examined the malignant transforming potential of human epithelial cells exposed to 1 GeV/nucleon ^{56}Fe ions accelerated at the Alternating Gradient Synchrotron at the Brookhaven National Laboratory. After irradiation with high doses and over a period of several months, cells showed a step-wise transformation including resistance to TGF- β induced growth inhibition in about 2 months post-irradiation, anchorage independent growth after about 3 months of irradiation, and finally tumorigenicity in nude mice. A total of 10 tumor cell lines were established from nude mice. Of more interest, it was found that at lower doses of ^{56}Fe ions, corresponding to an average of one particle per cell nucleus, no transformed cells were produced and no tumors formed in nude mice. However, cells exposed to such doses of ^{56}Fe ions were found to be susceptible to tumorigenesis many months later by a subsequent challenge dose of low LET radiation that alone is ineffective in inducing tumors. This indicates a heritable instability induced by heavy ions that persists for many generations. The instability induced by radiation is very similar in many ways to that characteristic of spontaneous cancer, but the link has yet to be proven [13-15].

4. Single Particle Microbeam Experiments

A number of ingenious experiments have been devised to address the question of the validity of a linear extrapolation from high to low doses. Many of these involve the single particle microbeam, a device capable of putting a pre-defined exact number of α -particles (including one) through a particular cell, and even choosing whether that particle traverses the nucleus or the cytoplasm.

A detailed description of the Columbia University microbeam has appeared elsewhere [16]. Briefly, each cell attached in a monolayer to a thin polypropylene base of a cell culture dish is identified and located by using an image analysis system, and its coordinates stored in a computer. The cell dish is then moved under computer control such that the centroid of each cell nucleus, (or a region of the cytoplasm remote from the nucleus, according to the plan of the particular experiment) is in turn positioned over a highly collimated shuttered beam of α -particles generated by a Van de Graaff accelerator. Each cell is exposed to a predetermined exact number of α -particles and a detector positioned above the cells signals to close the shutter when the desired number of particles (e.g. 1) are recorded, after which the next cell is moved over the beam. What is special about the Columbia microbeam is that developments in hardware and software have increased the microbeam throughput so that individual cells can be irradiated, one at a time, in about 1 second; This permits sufficient cells to be exposed for mutation and oncogenic transformation studies. Earlier microbeam systems were much slower so that biological studies were limited to chromosomal aberrations which can be scored in a smaller number of cells. Three types of experiments have been performed with the microbeam that address the question of biological effects at low doses; these will be described briefly.

5. Oncogenic Transformation Potential of a single α -particle

The goal of this study was to investigate whether the oncogenic effect of exactly one α -particle differs from normal broad beam conditions where a mean of one particle involves a Poisson distribution [17]. The data from microbeam and broad beam irradiations coincide for multiple traversals (2, 4, 8 particles), but exactly one particle is much less effective than a mean of one, and indeed does not differ from controls, even though 50,000 cells were exposed. The conclusion drawn from this experiment is that, since single α -particle traversals produce either zero or only a small risk of oncogenic transformation, the majority of the yield of transformed cells obtained after traversal by a (Poisson) mean of one α -particle must come from the minority of cells subject to multiple α -particle traversals. This result has important implications inasmuch as it casts doubt on the

validity of a linear extrapolation of cancer risks from high doses, involving multiple α -particle traversals, to very low doses, involving a single traversal.

5.1. Mutations by Cytoplasmic Irradiation

The development of the single particle microbeam allows the study of effects of radiation localized to specific parts of the cell. In particular it is possible to irradiate the cytoplasm, with no particle traversing the nucleus. Wu et al. [18] showed that cytoplasmic irradiation with one or more α -particle significantly increased the frequency of mutations in a human-hamster hybrid cell. Traversal of the cytoplasm by a single α -particle doubled the spontaneous mutation frequency, while a 2 to 3 fold enhancement was observed with four α -particle traversals of the cytoplasm. Of course the frequency of mutation induction was much less than when the nucleus was traversed, but still highly significant. The mechanism of this effect is hypothesized to involve the production of reactive oxygen species, based on two pieces of evidence. First, the mutation frequency was reduced by the addition of a radical scavenger, such as DMSO, but enhanced if the glutathione levels in the cell were depleted by the drug BSO. Second, the spectrum of mutations obtained with cytoplasmic radiation differed from that obtained with nuclear irradiation. Direct nuclear hits yielded primarily large scale deletions, while cytoplasmic traversals resulted mainly in point mutations, similar to those arising spontaneously. These experiments clearly demonstrate that the target for heritable effects of radiation is larger than the nucleus.

5.2. The Bystander Effect

The Bystander Effect refers to the observation of biological effects in cells not themselves traversed by a charged particle, but that in close proximity to cells that are.

The Bystander Effect goes one step further than cytoplasmic irradiation in that it implies that heritable changes can occur in cells that receive no direct damage at all, not just to their DNA. Rather, damage signals (as yet unidentified) are transmitted to these affected cells from neighboring traversed cells. Experiments will be described that include the scoring of micronuclei, mutation and oncogenic transformation.

(a) Micronuclei In Normal Human Fibroblasts

Perhaps the most direct and most dramatic demonstration of the bystander effect involves the observation of micronuclei in irradiated human fibroblasts [19]. Cells of one population were lightly stained with cyto-orange, a cytoplasmic vital dye, while cells

of another population were lightly stained blue with a nuclear vital dye. The two cell populations were mixed and allowed to attach to the culture dish and the computer programmed to irradiate all blue stained cells with 10 α -particles directed at the centroid of the nucleus. The cells were fixed and stained 48 hours later, at which time micronuclei and chromosome bridges were visible in a proportion of the non-hit (i.e. orange-stained) cells. This is an astonishing demonstration of the bystander effect, since the development of micronuclei implies significant chromosome damage and rearrangement, which is clearly visible in non-hit cells that have been fixed *in situ*.

(b) *Mutagenic Effects In Human-Hamster Hybrid Cells*

Zhou et al. [20] reported a study in which human-hamster hybrid (A_1) cells were exposed to α -particles using the Columbia microbeam. After identifying and locating all cells on the dish, the computer was programmed to expose 20% of the cells, randomly selected, to 20 α -particles directed through the centroid of the nucleus. This irradiation allows less than 1% of the cells to survive, and yet when assayed for mutations in the human chromosome 11, the mutation yield was four times background. These mutations must clearly arise from neighbor cells, not directly exposed, but in close proximity to irradiated cells. Unlike cytoplasmic irradiation the addition of a scavenger (DMSO) had no influence on the bystander effect. By contrast, the addition of Lindane, a drug that inhibits cell-to-cell communication significantly reduced, but did not eliminate the bystander effect.

(c) *Oncogenic Transformation In Mouse Fibroblasts*

Mouse fibroblast (C3H 10T1/2) cells were plated in a sparse monolayer and the computer programmed to irradiate every tenth cell, selected at random, with four α -particles directed at the centroid of the cell nucleus [21]. The cells were subsequently removed by trypsinization, replated at low density and transformed foci identified 6 weeks later by their morphological appearance. When 10% of the cells were exposed to four alpha particles, the oncogenic transformation rate for the whole population was not one tenth of that observed when all the cells were hit – in fact there was no significant difference in the oncogenic transformation rate when all cells were irradiated compared with when only 10% of the cells were irradiated.

That the oncogenic transformation rate would be unchanged irrespective of whether all or only a fraction of the cells were subject to the same level of damage is clear evidence for a ‘Bystander’ Effect, i.e. that unirradiated cells are responding to damage induced in irradiated cells. It should be noted that the cells were exposed at a very low density, with

essentially no overlap, and with typical nearest-neighbor separations of tens of microns, so a free-radical based mechanism for the transfer of information is unlikely.

These results, if applicable *in vivo*, would have significant consequences in terms of radiation risk extrapolation to low doses, implying that the relevant target for radiation oncogenesis is larger than an individual cell, and that the risk of carcinogenesis would increase more slowly, if at all, at higher doses. Thus a simple linear extrapolation of radiation risk from high doses (where they can be measured) to lower doses (where they must be inferred) would be of questionable validity. As with the mutation assay previously described, the presence of the drug Lindane during radiation, which inhibits cell-cell communication through gap junctions, greatly reduced but did not eliminate the bystander effect.

6. Conclusions

Data based on the Japanese A-bomb survivors provide good quantitative risk estimates for radiation induced cancer, at least for acute exposures to low LET radiation over the dose range from about 0.2 to 2.5 Sv. Animal and *in vitro* experiments provide data upon which tissue weighting factors are based, extending the risk estimates to include high LET radiations. *In utero* exposures to diagnostic x-rays have been shown to induce an excess incidence of leukemia and childhood cancers. These data extend the dose range over which radiation induced cancer has been observed down to about 1cGy. However below about 1cGy there is not direct observation of cancer induced in human populations by radiation. The International Commission of Radiological Protection (ICRP) recommends that risks at lower doses be calculated by a linear extrapolation of the risks at higher doses, with no threshold in dose assumed. This is described as “prudent and conservative”, but not everyone would agree with this assessment.

Quantitative risk estimates will always be based on epidemiological studies, and therefore restricted to high doses. Mechanistic studies may shed light on the shape of the dose response relationship and therefore on the validity of the linear extrapolation. An example of the type of experiment needed are those involving the single particle microbeam.

Data have been obtained that show that exactly one α -particle is less effective than a poisson distribution with a mean of one; this casts doubt on the validity of a linear extrapolation from high to low doses, even for densely ionizing radiations.

The bystander effect, as well as the observation that irradiation of the cytoplasm can produce heritable effects, implies that the target for radiation damage is bigger than the nucleus, and may even be bigger than the cell. This casts doubt on the size of the target and therefore on the dose at which low dose linearity would be expected. These experiments

provide compelling evidence that important heritable changes can arise in cells that receive no direct hit by a charged particle.

The data are fragmentary and even conflicting at the present time, but this must surely change as more effort is directed to address the problem.

Radiation has been shown to produce a genomic instability that is similar in many ways to that seen in spontaneous cancers; it is an attractive hypothesis that the production of genomic instability represents a mechanism of radiation carcinogenesis. It should be noted in this context that high energy heavy ions, such as ^{59}Fe , are particularly efficient at inducing instability. It may be, therefore, that they are not adequately represented by tissue weighting factors derived largely from experiments with neutrons.

Acknowledgments

The author takes pleasure in acknowledging much useful discussion with his colleagues at Columbia, especially Drs. Tom Hei, Charles Geard, Gerhard Randers-Pehrson and David Brenner. The research described was supported by Grants from the National Institutes of Health, No. CA 49062, NIH/NRRC P41 RR11623-03, NIH/NASA CA 73946 and NASA NAG 9-1148.

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