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GENOMIC INSTABILITY AND TUMORIGENIC INDUCTION IN IMMORTALIZED HUMAN BRONCHIAL EPITHELIAL CELLS BY HEAVY IONS

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ABSTRACT

Carcinogenesis is postulated to be a progressive multistage process characterized by an increase in genomic instability and clonal selection with each mutational event endowing a selective growth advantage. Genomic instability as manifested by the amplification of specific gene fragments is common among tumor and transformed cells. In the present study, immortalized human bronchial (BEP2D) cells were irradiated with graded doses of either 1GeV/nucleon ^{56}Fe ions or 150 keV/μm alpha particles. Transformed cells developed through a series of successive steps before becoming tumorigenic in nude mice. Tumorigenic cells showed neither *ras* mutations nor deletion in the p16 tumor suppressor gene. In contrast, they harbored mutations in the p53 gene and over-expressed cyclin D1. Genomic instability among transformed cells at various stage of the carcinogenic process was examined based on frequencies of PALA resistance. Incidence of genomic instability was highest among established tumor cell lines relative to transformed, non-tumorigenic and control cell lines. Treatment of BEP2D cells with a 4 mM dose of the aminothiol WR-1065 significantly reduced their neoplastic transforming response to ^{56}Fe particles. This model provides an opportunity to study the cellular and molecular mechanisms involved in malignant transformation of human epithelial cells by heavy ions.

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Introduction

Carcinogenesis is a multi-stage process with sequences of genetic events governing the phenotypic expression of a series of transformation events leading to the development of metastatic cancer. An understanding of the carcinogenic mechanisms of high LET radiation is essential for human risk estimation and radiation protection. The carcinogenic risk for human epithelial cells after exposure to high LET radiation has been estimated to be in the range of 10^{-12}/cell/Gy based on the breast cancer incidence among Japanese A-bomb survivors (Hei *et al.*, 1996a). However, the mechanisms for radiation carcinogenesis by high LET radiation such as alpha particles and heavy ions are not clear. The use of lung tumor tissues from underground miners exposed to radon in identifying consistent cellular and molecular alterations, as in the case of human colorectal cancers, is complicated by the findings that the majority of miners are also cigarette smokers. It will be ideal to use a human bronchial epithelial cell line that has been malignantly transformed by radiation to assess the various changes leading to malignancies. *In vitro* oncogenic transformation studies using rodent cell systems have shown that high LET radiation is more efficient in transforming cells than X or γ-rays at equivalent doses with a relative biological effectiveness ranging from 2.2 to 10 (Hei *et al.*, 1988, Hall and Hei, 1985). Since human

epithelial cells rarely undergo spontaneous immortalization and are extremely refractory to *in vitro* neoplastic transformation by carcinogens (Rhim, 1991 for review), model systems based on immortalized human epithelial cells are extremely valuable to provide qualitative data on mechanisms of radiation-induced carcinogenesis. This is particularly true when using ionizing radiation where large, multiple doses are often required either to immortalize cells (Namba *et al.*, 1986) or to convert previously immortalized cells to malignant cells (Yang *et al.*, 1991, Thraves *et al.*, 1990).

Immortalized Human Bronchial Epithelial Cell Model

To better understand the cellular and molecular mechanisms involved in human bronchial carcinogenesis induced by either heavy ions or environmental carcinogens such as asbestos fibers, we have developed a transformation model based on human papillomavirus immortalized human bronchial epithelial (BEP2D) cells as shown in Figure 1. BEP2D cells are initiated by lipofectin transfection of cloned full

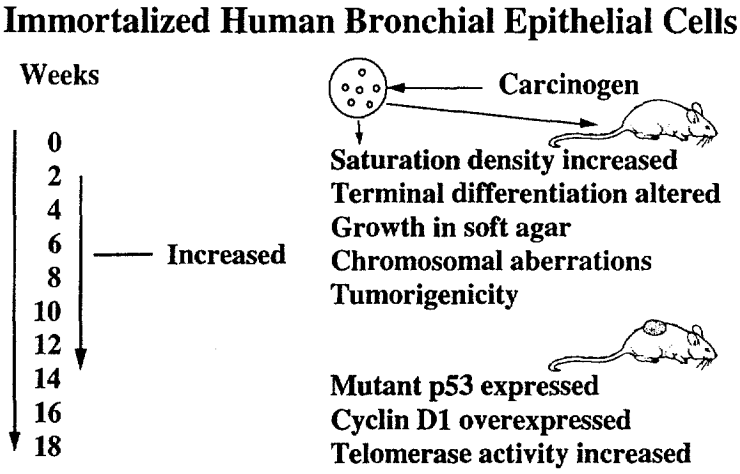

Figure 1. Schematic diagram illustrating the multistep process in the neoplastic transformation of immortalized human bronchial epithelial cells irradiated with a single 60 Gy dose of 1 GeV/nucleon ^{56}Fe ions. Irradiated cells need to undergo successive passages and the accumulation of additional phenotypic/ mutagenic changes before tumorigenicity can be demonstrated.

length HPV18 into normal human bronchial epithelial cells obtained as an outgrowth of bronchial explant (Willey *et al.*, 1991). Although these bronchial epithelial cells are immortal, they are anchorage dependent and do not form tumors in immunosuppressed host animals. After carcinogen treatment, transformed cells arise through a series of sequential stages including altered growth pattern, resistance to serum-induced terminal differentiation, agar-positive growth, tumorigenicity, and metastasis (Hei *et al.*, 1994a, 1996a & b, 1997). It should be noted that, while the majority of agar-positive BEP2D clones are non-tumorigenic, they all demonstrated the propensity to resist serum-induced terminal differentiation. In addition, each preceding stage represents a necessary, yet insufficient step towards the later, more malignant phase. Northern and Western blot analyses showed an over-expression of cyclin D1 (Hei *et al.*, 1996b) and mutated p53 oncoproteins (Hei *et al.*, 1996a) among the tumorigenic BEP2D cells. Since chromosome end to end associations and telomerase activity are often associated with

genomic instability and cellular immortality respectively, our recent data which show an overabundant increase in telomerase activity together with the highest frequency of chromosome end associations among the metastatic cell lines, suggest that they may be useful indicators of metastatic potential in radiation induced lung cancers (Pandita *et al.*, 1996).

Exponentially growing BEP2D cells plated in T25 tissue culture flasks were irradiated with graded doses of 1 GeV/nucleon ^{56}Fe ions accelerated with the Alternating Gradient Synchrotron at the Brookhaven National Laboratory. After irradiation, cells were trypsinized, counted, and replated for both survival and the expression of transformed phenotypes as described previously (Hei *et al.*, 1994a, 1996a & b). Irradiated cells demonstrated a dose dependent cytotoxicity with a mean lethal dose of 0.7 Gy as shown in Figure 2. Compared to the 0.2 Gy dose value obtained with 150 keV/μm ^{4}He ions, these very high energy particles were less effective in killing BEP2D cells under comparable culture conditions. The RBE for cell lethality at the D_0 dose was 6.0 and 3.4 for α-particles and ^{56}Fe ions respectively. Cells irradiated with either a 0.3 or 1 Gy dose of ^{56}Fe ions developed anchorage independent clones at frequencies ranging from 0.04 to 0.4% (Table I) similar to those previously reported after alpha particle irradiation (Hei *et al.*, 1996a). Upon inoculation into nude mice, only cells irradiated with the higher dose groups produced tumors (2/4 and 3/4 animals for the 0.6 and 1 Gy doses respectively).

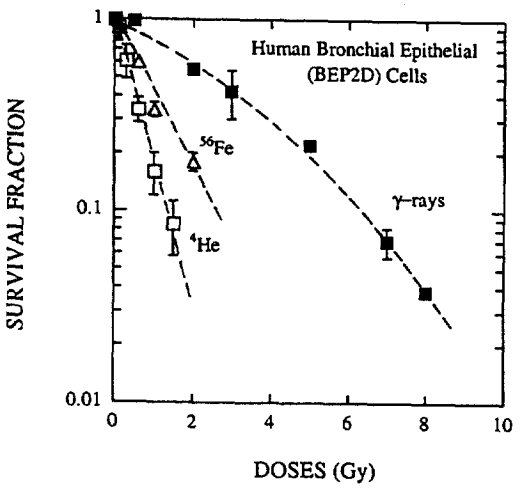

Figure 2. Survival fraction of BEP2D cells irradiated with graded doses of either 1 GeV/nucleon ^{56}Fe ions, 150 keV/μ alpha particles, or γ-rays. Data are pooled from 3-7 experiments. Bars represent ± SEM.

TABLE I

Neoplastic Transformation Incidence in BEP2D cells Irradiated with 1 GeV/nucleon ^{56}Fe Ions[1]

Dose (cGy)	Time in Culture (week)	Growth in Agar[2]	Tumorigenic incidence[3]
0	12	-	0/4[4]
30	12	+	0/4
60	12	+++	2/4
100	12	+++	3/4

[1] Total number of cells irradiated per group ranged from 2.3 to 12x10^6 cells

[2] Soft agar colonies ranged from 0.02 to 0.4%

[3] Animals with palpable nodules > 0.5 cm/ total number of animals injected

[4] Control BEP2D cells have not produced a single tumor among 47 animals injected including historical controls.

Evidence That Loss of Suppressor Gene Function Mediates Radiation-Induced Transformation of BEP2D cells

The development of recombinant DNA technology during the past decade has led to the recognition that cancer may be a result of either the activation of oncogene(s) or the loss of tumor suppressor genes. So far, no oncogene has been identified as the causal step in radiation induced tumor *in vivo*, or with radiation induced transformation *in vitro*. Although *ras* oncogenes were shown to be activated in certain X-ray induced lymphomas in mice (Guerrero *et al.*, 1984), this alteration was found in only a fraction of the tumors and might not represent the causal event. Likewise, consistent with our data with the BEP2D cells, no mutations in any of the *ras* oncogenes has been identified among radiation-induced tumorigenic human keratinocytes (Thraves *et al.*, 1990, 1995). The fact that heavy ions and other high LET radiation are efficient inducer of large chromosomal deletions (Evans *et al.*, 1991, 1998, Hei *et al.*, 1994b, Zhu *et al.*, 1996), provides a mechanism for the loss of suppressor functions. Studies with somatic cell hybrids have shown that tumor suppression occurs in neoplastic cells and can be corrected with cell fusion with normal human chromosomes (Sager, 1985, Saxon *et al.*, 1986). To understand the mechanism of radiation carcinogenesis, we carried out cell fusion studies to determine whether tumorigenicity of BEP2D cells behaves as a dominant or recessive trait as shown in Figure 3.

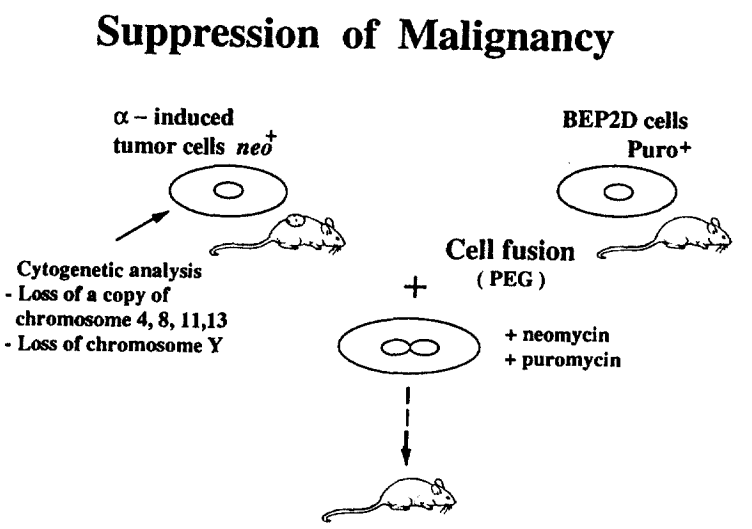

Figure 3. Cell fusion approach to examine the role of suppressor functions among tumorigenic BEP2D cells induced by alpha particles.

Exponential phase cultures of secondary BEP2D tumor cells induced by a single 60 cGy dose of alpha particles were transfected with the pRV/CMV expression vector containing a *neo* gene whereas control cells were stably transfected with the pBabe plasmid containing a puromycin resistant gene. 5 x10^6 tumorigenic cells were fused with an equal number of control BEP2D cells using polyethylene glycol 1500 applied in a drop-wise fashion. Resultant fusion cells were then selected in medium containing both G418 and puromycin over a 12 day period, expanded in culture, and re-inoculated into nude mice for tumorigenic expression. Results of these fusion experiments demonstrated that radiation-induced tumorigenic phenotype in BEP2D cells could be completely suppressed by fusion with non-tumorigenic

BEP2D cells. Furthermore, concurrent fusion of tumor cells to tumor cells resulted in tumorigenic hybrids whereas fusion among wild type BEP2D cells resulted in non-tumorigenic hybrid clones. These data indicate that non-tumorigenic BEP2D cells complement the loss of putative suppressor elements among tumorigenic cells and suggest that loss of suppressor gene(s) as a likely mechanism of radiation carcinogenesis.

Tumor Suppressor Gene and Genomic Instability

Studies on the functions of tumor suppressor genes have revealed that many of these gene products play a crucial role in the control of cellular growth and differentiation as well as in cell cycle control (Knudson, 1993, Muller *et al.*, 1993). Table II listed the many possible functions of tumor suppressor genes in mammalian cells including maintenance of genomic instability. A possibility that now seems more likely in cancer development is that tumor progression is a consequence of genomic instability and clonal selection, each mutation endowing a selective growth advantage. The notion is that a mutation may occur in a gene responsible for the stability of the genome and the fidelity of replication, resulting in what has been referred to as mutator phenotype, i.e. a single induced mutation followed by a cascade of further mutations. Support of this concept comes from the observation of microsatellite instability in a wide range of human tumors (Cheng & Loeb, 1993). The discovery of mutations in one of the five mismatch repair genes in cases of hereditary non-polyposis colorectal cancer, also support the idea that an induced mutation can result in instability and a mutator phenotype (Loeb, 1991). Although there is no clear cut evidence that genomic instability actually occurs among radiation induced cancer, the notion of global chromosomal instability arising from an initial event, possibly a mutation, makes it possible to understand conceptually how a single low dose of radiation can lead to a cancer many years after the initial exposure and seemingly involves multiple steps.

Table II
Possible Functions of Tumor Suppression Genes

Maintenance of genomic stability
Induce apoptosis
Induce differentiation and trigger cellular senescence
Regulate cell growth as a negative growth factor
Increase cellular communication
Inhibit proteolytic degradation of gene products involve in growth regulation

There is evidence that genomic instability contributes to the progression of tumorigenesis (Lengauer *et al.*, 1997). One aspect of genomic instability is gene amplification which is frequently observed in tumors and transformed cell lines. Amplification in the CAD gene which results in the acquired resistance to the chemotherapeutic agent PALA (N-phosphonacetyl-L-aspartate) has previously been demonstrated in rodent tumor cell lines (Tlsty *et al.*, 1989) but not in normal human fibroblasts (Wright *et al.*, 1990). Figure 4 shows the frequencies of PALA resistance among control, transformed but not yet tumorigenic, and tumorigenic BEP2D cells induced by a single 60 Gy dose of ^{56}Fe ions. CAD is a multifunctional protein that catalyzes the first three steps in the *de novo* biosynthesis of uridine monophosphate. PALA is a competitive inhibitor of the enzyme aspartate transcarbamylase (ATCase). In the present study, cultures were exposed to 9x LD_{50} concentration of PALA (180-200 μM) for 3 weeks to assess the frequency of drug-resistant clones. The frequency of PALA resistance among the

immortalized, control BEP2D cells was less than 10^{-7}. In contrast, the frequency of gene amplification in the five tumor cell lines ranged from 1-3 $x10^{-3}$ and between 7-9 $x10^{-5}$ among the four transformed cell lines examined. The results demonstrate that the step-wise neoplastic transformation process induced

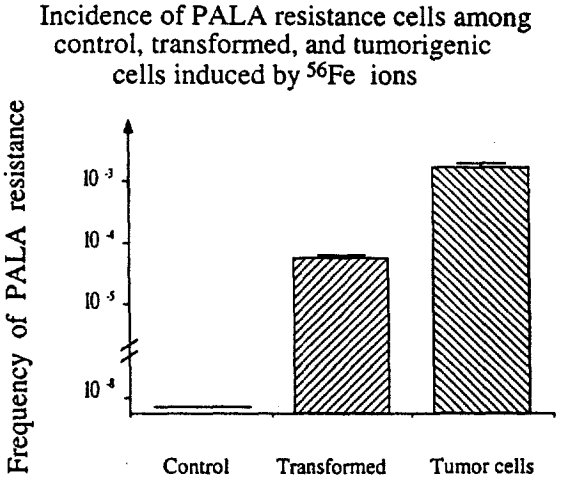

Figure 4. Frequency of PALA resistance among control, transformed, and tumorigenic BEP2D cells induced by heavy ions. Data are pooled from 3-5 experiments. Bars represent ± SEM.

by heavy ions is clearly associated with a gradual increase in genomic instability as determined by CAD gene amplification. Although the initial molecular events leading to gene amplification are not known, there is evidence to suggest that chromosomal breakage followed by formation of acentric fragments which harbor the target gene may play a role (Biedler *et al.*, 1988, Windle and Wahl, 1992). The significant increase in PALA resistance among the tumorigenic compared to control BEP2D cells may be useful as a predictive assay for tumorigenicity in BEP2D cells transformed by heavy ions.

Radiation Protection Studies with WR-1065 using BEP2D Cell Assay

Since the beginning of manned flight into space officially commenced in the sixties, the potential health hazard from exposure to the natural radiation environment outside the magnetic shielding of the earth has been a major concern of various space agencies including NASA. Although heavy ions constitute only a small percentage of the radiation field in outer space, they are thought to have a significant impact on the perceived risk. The ICRP has assigned a weighting factor of 20 for heavy ions, i.e. the risk of induced cancer from a given dose of heavy ions is 20x the risk from equivalent dose of low LET radiation (Sinclair, 1994). With the planned international space station program underway, information on realistic risk assessment and radiation protection is urgently needed.

2-(aminopropyl)-aminoethanethiol (WR1065) is the corresponding free thiol of the well-characterized radioprotector, Amifostine (WR-2721). Amifostine is a pro-drug which needs to be metabolized to its thiol form, WR-1065, before it is functionally active as a radioprotector (Grdina *et al.*, 1995). There is

evidence from both *in vitro* and *in vivo* studies that WR-2721 and WR-1065 are anticarcinogenic, antimutagenic, and protect against radiation and cis-platinum induced mutagenesis in mammalian cells (Hill *et al.*, 1986, Milas *et al.*, 1984, Grdina *et al.*, 1992). These studies form the basis for the current interest in Amifostine as a chemopreventive agent to be used as an adjuvant in chemotherapy and radiotherapy. Figure 5 shows the effects of a 4 mM dose of WR-1065 given 2 hr before and 2 hr after radiation on the malignant transformation incidence of BEP2D cells irradiated with a single 60 cGy dose of 1 GeV/nucleon ^{56}Fe ions. Control and irradiated cells with or without drug treatment were subcultured for a period of 10-12 weeks before being inoculated into nude mice for assessment of their tumorigenic potential in nude mice. Although pre-treatment with WR-1065 had no protective effect on clonogenic survival of ^{56}Fe ion-irradiated BEP2D cells (data not shown), it obliterated the tumorigenic potential of these cells upon inoculation in nude mice.

Successful chemopreventive drugs can target specific phase of the cancer developmental process such as enzymatic activation of chemical carcinogens, induce programmed cell death to functionally-altered cells, restore normal cell differentiation and tumor suppressor gene function. As such, chemopreventive drugs can be classified into several categories: anti-mutagenic (Phase II enzyme inducer oltipraz, polyphenols); anti-proliferative (retinoid, difluoromethylornithine); anti-inflammatory (aspirin, sulindac), and antioxidants (thiols, Amifostin). The precise mechanism for the anti-tumorigenic effects of WR-1065 in heavy ion-irradiated BEP2D cells is not clear. There is evidence that an increase in intracellular glutathione content following WR-1065 treatment may be important in mediating its anti-carcinogenic, anti-mutagenic effect *in vitro* (Grdina *et al.*, 1995).

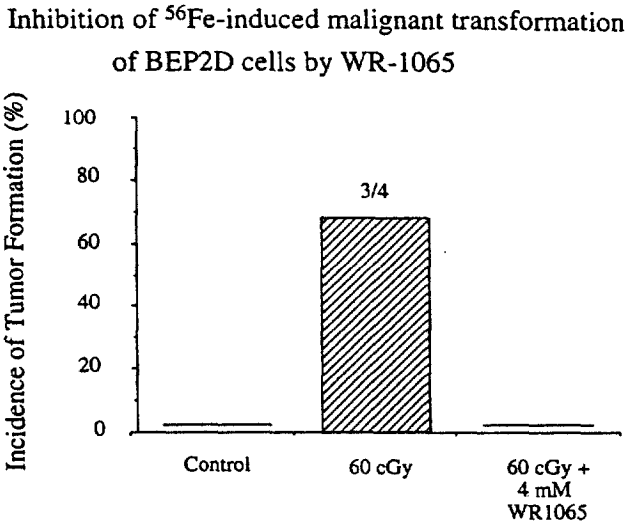

Figure 5. Tumorigenic incidence in nude mice injected with either control or ^{56}Fe ions irradiated BEP2D cells with or without pretreatment with a 4 mM dose of WR-1065 given 2 hr before and 2 hr after irradiation. 4-6 x10^6 cells in 0.2 ml phosphate buffered saline were injected per animal. Latency period averaged 8-10 weeks for irradiated BEP2D cells without pre-treatment with WR-1065. Animals injected with control and irradiated BEP2D cells pre-treated with drug were followed for period up to 8 months post-inoculation.

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