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Induction of genomic instability in normal human bronchial epithelial cells by ^{238}Pu α-particles

Christopher H.Kennedy[1], Charles E.Mitchell[1], Noelle H.Fukushima[2], Robin E.Neft and John F.Lechner[3]

Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, PO Box 5890, Albuquerque, NM 87185, USA

[1]These authors contributed equally to this study.
[2]Department of Energy/Associated Western Universities Summer Student Research Participant.

[3]To whom correspondence should be addressed

Pulmonary deposition of α-particle-emitting radon daughters is estimated to account for 10% of all lung cancer deaths in the USA. However, the nature and timing of early (preneoplastic) genetic alterations in radon-associated lung cancer are still relatively uncertain. The purpose of this investigation was to determine whether genomic instability occurs after exposure of cultured normal human bronchial epithelial cells to six equal, fractionated doses of α-particles (total doses 2–4 Gy). Two weeks after the final exposure, foci of phenotypically altered cells (PACs) were detected in 0, 63 and 77% of control, low and high dose cultures respectively. Of these, 18% exhibited extended life spans relative to unexposed controls. Elevated frequencies of binucleated cells (BNCs), a marker of genomic instability, were observed in 60 and 38% of the PAC cultures from the low and high dose groups respectively. The micronucleus assay also showed evidence of genomic instability in 40 and 38% of PAC cultures from the low dose and high dose groups respectively. No changes in microsatellite length, another marker of genomic instability, were detected in any of the PAC samples with the 28 markers used for this assay. However, one PAC (L2) showed a hemizygous deletion at 9p13.3. Another PAC (H9), which exhibited the highest frequency of cells containing micronuclei (MN), exhibited a hemizygous deletion at 7q31.3. Each loss may represent a stable mutation that resulted either directly from irradiation or later in progeny of exposed cells because of α-particle-induced genomic instability. The fact that elevated levels of BNCs and MN were present in the progeny many generations after irradiation indicates that the genetic alterations detected with these two markers were not a direct consequence of radiation exposure, but of resulting genomic instability, which may be an early change after exposure to α-particles.

Introduction

Human exposure to radon gas (^{222}Rn) occurs because of the diffusion of this inert gas from rocks and soils into enclosed areas (e.g. homes and underground mines). Data from both uranium miner epidemiological and experimental animal studies suggest a positive correlation between radon exposure and the relative risk (RR*) of developing lung cancer (1–3). Uranium miners constitute the population at highest risk for radon-associated lung cancer. In a recent epidemiological analysis, wherein original data from 11 studies were pooled, Lubin *et al.* (4) reported RRs for lung cancer at 100 working level months (WLM, 1 WLM = 3.5×10^{-3} J-h/m^3 or 2.7 mGy) as ranging from 1.2 to 6.1 (4). They extrapolated these data using the linear no-threshold theory of radiation carcinogenesis to predict RR values from indoor exposure to ^{222}Rn. They concluded that, in the USA, exposure to radon progeny may account for 10% of all lung cancer deaths and 30% of lung cancer deaths in never-smokers. Therefore, indoor radon exposure represents a significant health concern.

Although exposure to ^{222}Rn causes many genetic changes in lung epithelial cells, we have focused on the induction of genomic instability, a common feature of tumor cells (5). This instability has also been detected as an early change in progeny of normal human and murine hemopoietic cells exposed *in vitro* to α-particles (6,7). Genomic instability is caused by mutational or exogenous interference with pathways governing the accurate duplication and distribution of DNA to progeny cells and/or with pathways controlling regulatory modifications of DNA during normal development (8). The purpose of this investigation was to determine if genomic instability is also an early feature of human lung epithelial cells after exposure to ^{238}Pu α-particles.

Materials and methods

Cell culture

Normal human bronchial epithelial (NHBE) cells from a 15 year old male never-smoker (strain 2129) and bronchial epithelial cell growth medium (BEGM™) were purchased from Clonetics (San Diego, CA). These cells were determined by the supplier to be negative for the human immunodeficiency virus, hepatitis type B virus and mycoplasma. The Pathogene® DNA Probe Assay Kit (Enzo Diagnostics, Farmingdale, NY), an *in situ* hybridization assay (9), was used to analyze these cells for the presence of adenovirus types 4, 5, 7, 11, 20, 40 and 41. The cells were also analyzed by Digene Diagnostics (Silver Spring, MD) for the presence of HPV types 6, 11, 16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52 and 56 using the HPV Profile™ Kit, a dot blot assay (10). None of these viral types was detected. The cryopreserved cells (passage 1) were cultured and expanded in plastic flasks (Corning, Corning, NY) precoated with FNC Coating Mix (BRFF, Ijamsville, MD) (11).

Irradiation

NHBE cells (two vials of strain 2129, passage 1) were expanded for 7 days and passaged (1.4×10^4 cells/dish) into plastic rings with a 1.5 μm thick Mylar® film bottom and a growth area of 8 cm^2 to facilitate exposure to α-particles emitted from ^{238}Pu. These particles have a track length of ~35 μm in soft tissue (12) and their kinetic energy under these exposure conditions is comparable to radon decay products (6,13). After attaining confluence on the Mylar® film (3 days after innoculation), the cells were exposed over a 17 day period to a total dose of either 0 (unexposed controls, 20 dishes), 2 (low dose, 68 dishes) or 4 Gy (high dose, 64 dishes) of α-particles delivered in six equal, fractionated doses by using a stainless steel disk that was electroplated with sufficient ^{238}Pu to provide 0.857 Gy/min of α-particle energy. The average number of particle traversals was 1 particle/nucleus for the low fractionated dose (0.33 Gy) and 2 particles/nucleus for the high fractionated

*Abbreviations: RR, relative risk; WLM, working level months; NHBE, normal human bronchial epithelial; PACs, phenotypically altered cells; BNCs, binucleated cells; MN, micronuclei; DPBS, Dulbecco's phosphate-buffered saline; LOH, loss of heterozygosity.

Table I. Microsatellite markers used for analysis of chromosomal LOH

Locus	Map location	Locus	Map location
D1S252	1p13.1	D9S171	9p12-p13.1
D2S162	2p25.1	HBB	11p15.5
D3S1038	3p25	D11S875	11p15
D3S647	3p23	D13S131	13q14.1
D3S966	3p21 3	D13S133	13q
D3S1289	3p21.1	D15S97	15p13
D3S659	3p13	D15S165	15p11.2
D5S350	5p13.1-p14	ACTC	15q11-qter
D7S507	7p15.3-p21	CHRNB	17pter-p12
D7S495	7q31.3	TP53	17p13.1
INFα	9p22	D17S786	17p13
D9S144	9p21.3	D17S122	17p12-cen
D9S162	9p13.3	UT762	21
D9S200	9p13	AR	Xq11-q12

dose (0.67 Gy). Medium was replenished three times per week. The exposed cells capable of proliferation were allowed to grow to confluence after each exposure (48–72 h) before initiating the next irradiation. However, reproductively dead cells constituted a major fraction of the cell population on each confluent dish. The number of cells at risk (i.e. total number of cells at the time of the first radiation exposure) per dish was determined by fixing two cultures with neutral buffered formalin, staining with crystal violet (14) and counting cells within three 1.44 mm^2 areas on each dish.

Post-irradiation cell culture

Starting 1 week after the final exposure, cultures of unexposed controls and of samples exhibiting foci of phenotypically altered cells (PACs) were subcultured to plastic tissue culture dishes (Corning) pre-coated with FNC Coating Mix (11). Medium was replenished three times per week. Each culture was passaged to either a single 60 or 100 mm dish. Passaging all cells on each dish instead of just foci yielded a better recovery of viable cells and avoided the potential pitfalls associated with using a cloning ring on the Mylar® film (e.g. puncturing the film). Cells were then passaged and split into several dishes as the cultures became confluent. This was repeated until the cultures underwent senescence. Enumeration of the cells during passage revealed that a minimum of four to six population doublings transpired with each sequential subculturing. Samples of cells from passages 4–6 were fixed in Shandon's Cytospin Collection Fluid® (Shandon, Pittsburgh, PA) and cytospun onto microscope slides for detection of binucleated cells (BNCs) and cells containing micronuclei (MN).

Binucleated cell and micronuclei assays

Fixed cells were washed in Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, containing 0.5% Brij 35 (Sigma, St Louis, MO) and treated with 11 U RNase A (Sigma) in DPBS for 20 min at 37°C in a humidified chamber. The cells were then stained with 1.0 μg/ml propidium iodide (Sigma) in DPBS for 1 min at 37°C in a humidified chamber, washed twice with deionized water, air dried and coverslipped using 1 drop of Citifluor glycerol (Ted Pella, Redding, CA). Five hundred cells per sample and 1000 cells per sample were scored blind using a light microscope (40× magnification) to determine the number of BNCs and the number of cells containing MN respectively. PACs that continued to proliferate beyond passage 3 were analyzed in one or more passages between 4 and 6. The criteria described by Tolbert *et al.* (15) for cells to be included in the total cell count and for a cell to be scored positive for MN were adopted for these analyses. PAC cultures were considered to have significantly elevated levels ($P < 0.05$) of BNCs or cells containing MN if the following criteria were met: (i) the number of BNCs per 500 cells was greater than the mean number of BNCs per 500 cells + 2 SD for unexposed NHBE cells; (ii) the number of MN per 1000 cells was greater than the mean number of MN per 1000 cells + 2 SD for unexposed NHBE cells.

Microsatellite analysis

DNA was isolated from 10 PAC cultures and three unexposed NHBE cultures using a Blood & Cell Culture DNA Kit (Qiagen, Chatsworth, CA). Twenty-seven DNA microsatellite markers were obtained as MAPPAIRS (Research Genetics, Huntsville, AL). UT762(21) primers (16) were synthesized by National Biosciences (Plymouth, MN). The locus and map location of these markers are shown in Table I. These markers were chosen because they have been shown to be informative with lung tumor samples (16–19). PCR amplifications were performed essentially as described by Weber and May (20). Each PCR sample contained 25 ng template DNA, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 1.0 μCi [α-^{32}P]dCTP (3000 Ci/mmol), 1 μM each primer and 0.5 U AmpliTaq® DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a total volume of 50 μl. Samples were denatured for 5 min at 94°C and subjected to 27 cycles of amplification consisting of 1 min denaturation at 94°C, 1 min primer annealing at 54–60°C, 1 min elongation at 72°C, followed by a final 6 min extension step at 72°C. Samples were amplified in a microtiter dish using a thermal cycler (MJ Research, Watertown, MA). After PCR the samples were mixed with 10 volumes of a denaturing loading dye solution (0.3% bromophenol blue, 0.3% xylene cyanol, 10 mM EDTA, pH 8.0, and 90% v/v formamide) and subjected to electrophoresis on a 0.4 mm thick 6.5% polyacrylamide gel containing 7 M urea. After drying the gel was exposed to X-ray film at −72°C for 24–72 h. To score for loss of heterozygosity (LOH), a laser densitometer was used to determine the ratios of DNA fragments obtained from control and exposed cells and loss was scored when the ratio of the two alleles in the DNA from the exposed cells was significantly different from that seen in DNA from control cells (i.e. a minimum of a 35% decrease in intensity by densitometric tracing, a value commonly used in analysis of LOH). The ratios were also used to obtain a factor to correct for differences in DNA loading.

Results

Effect of irradiation on cell morphology and proliferation

NHBE cells (passage 2) were exposed to a total dose of 0, 2 or 4 Gy α-particles delivered in six equal, fractionated doses over a 17 day period. The mean number of cells per dish was determined to be $4.7\times10^4 \pm 8.9\times10^3$ cells/dish (mean ± SD) at confluence. Thus the total numbers of cells at risk were: low dose (68 dishes), 3.2×10^6 cells; high dose (64 dishes), 3.0×10^6 cells. Based on clonal growth assays (data not shown), 53% of the cells at risk (~1.2×10^6 cells) survived the first low dose (0.33 Gy) exposure and 23% (~3.9×10^5 cells) survived the first high dose (0.67 Gy) exposure. One week after the final exposure foci of PACs, which are characterized by a colony of morphologically distinct cells with a high mitotic index, were observed in 14 of 68 (21%) low dose group cultures and in 40 of 64 (63%) high dose group cultures at an incidence of 1 focus/dish. In no case was more than one focus present per dish. Figure 1 shows a typical unexposed culture and a typical PAC culture. The number of cells in each focus constituted >90% of the total number of replicating cells per dish based on microscopic examination; cells outside the focus exhibited squamous morphology, indicative of squamous terminal differentiation. The unexposed cultures were ~70% confluent 1 week after the final exposure. Cellular foci were not present in these cultures and the cells on these dishes were a mixed population that exhibited both replicative (prolate spheroid) and terminally differentiated (squamous) morphologies. Further, the number of cells on these dishes was significantly lower than that of the exposed cultures. Over the subsequent 2 weeks, additional foci developed in both exposed groups; the final number of foci was 43 of 68 (63%) in low dose group cultures and 49 of 64 (77%) in high dose group cultures. Thus, no significant dose–response effect was seen. However, as indicated by the higher percentage of foci in the high dose group 1 week after the final exposure compared with the low dose group, foci developed more rapidly after exposure to 4 Gy.

When the unexposed control cultures were passaged from the Mylar® film into FNC-coated plastic dishes (passage 3) the average number of cells recovered per culture was 3×10^4. These cells were not of clonal origin, as they were distributed evenly on the Mylar® surface at ~70% confluence and no colonies were present. When the PACs were passaged the average number of cells recovered per culture was 2×10^5. The majority of the cells (>90%) from each dish were within

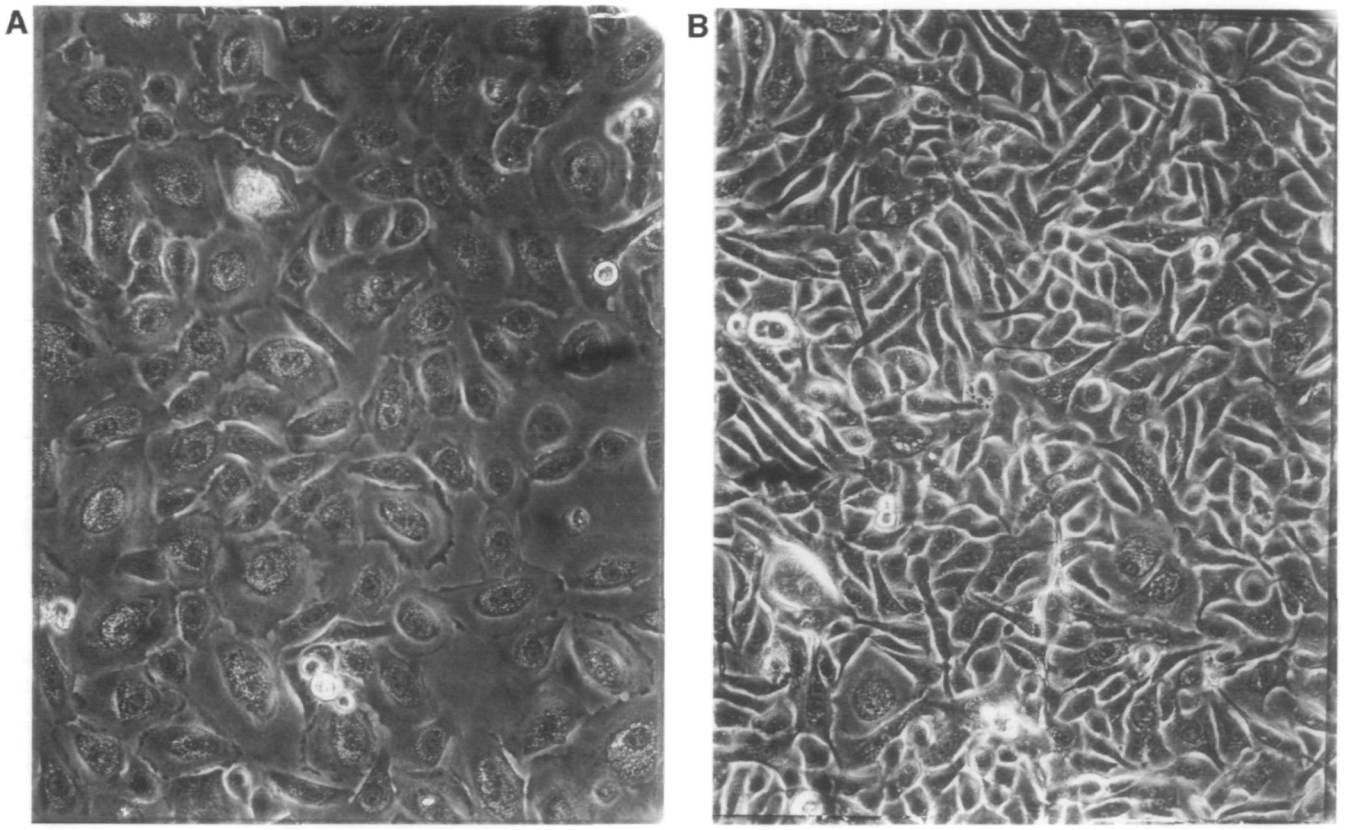

Fig. 1. Photomicrographs of (**A**) unexposed NHBE cells (U10) and (**B**) phenotypically altered HBE cells (H9), both in passage 4. The PACs received a total dose of 4 Gy α-particles.

Table II. Maximal passage numbers for NHBE cells exposed to α-particles and unexposed controls

Passage	Exposure group		
	Unexposed	Low dose	High dose
2[a]	5	25	15
3	9	30	34
4	2	3	3
5	2	4	6
6	0	2	4
7	0	1	1
8	0	1	1
9	2	1	0
Total	20	68	64

[a]The number of cells remaining on the Mylar® surface of these dishes was not sufficient for passage. No foci of cells were present on any of these dishes.

a single symmetrical focus (diameter ~2 cm), indicating that they were of clonal origin. The remaining cells (<10%) were distributed evenly on the Mylar® surface at ~50% confluence. If each focus originated from one cell, then ~17 population doublings occurred prior to passage of the average sized focus. Therefore, unexposed controls were at a significantly lower generation number than PACs in passage 3. The majority (71%) of PAC cultures underwent senescence in passage 3; however, one PAC culture from the low dose group (L2) did not cease to proliferate until passage 9 (Table II) and is estimated to have undergone a minimum of 62 population doublings (Table III). Cultures that continued to proliferate were passaged at confluence until they reached senescence.

Table III. Estimation of cumulative population doubling values as a function of passage number

Exposure group	Passage number							
	P2	P3	P4	P5	P6	P7	P8	P9
Unexposed	5	9	14	19	24	29	34	39
Low dose	5	32	37	42	47	52	57	62
High dose	5	38	43	48	54	59	64	[a]

[a]Eight was the highest passage number attained by any PAC culture from the high dose group.

Although two unexposed cultures (U10 and U14) did not undergo senescence until passage 9, it can be seen (Table III) that these cells were at a significantly lower generation number than the corresponding PAC (L2) when they ceased to proliferate. Based on the data in Table II and estimates in Table III, 13 (9/68) and 23% (15/64) of PAC cultures from the low and high dose groups respectively exhibited an extended lifespan (i.e. a higher number of population doublings) relative to the longest lived unexposed NHBE cultures; however, all of these PACs ultimately underwent senescence.

Effect of irradiation on BNC frequency

Unexposed controls and PACs fixed in passages 4–6, estimated to have undergone between 14–24 and 37–54 population doublings respectively (Table III), were stained with propidium iodide and assayed for the presence of BNCs. The background level was 16 ± 5 BNC/500 cells (mean ± SD, $n = 4$) in unexposed controls, so the significance level (i.e. mean + 2 SD, $P < 0.05$) was established as >26 BNC/500 cells. Neither

Table IV. Frequency of BNCs and/or cells containing MN following exposure to α-particles

Culture[a]	BNCs[b]	MN[c]
U2	22	2
U10	16	4
U11	10	4
U14	16	5
L2	35*	7*
L5	7	2
L12	36*	6
L32	24	6
L41	36*	16*
H6	17	9*
H9	35*	22*
H17	39*	8*
H20	18	2
H27	25	3
H29	27*	6
H37	13	6
H46	6	1

[a]Culture designations: U, unexposed control group; L, low dose exposed group; H, high dose exposed group.
[b]Number of BNCs per 500 cells. The value shown is the highest number scored for cells fixed in either passage 4, 5 or 6. Values significantly different from controls ($P < 0.05$) are indicated by an asterisk.
[c]MN-containing cells/1000 cells. The value shown is the highest number scored for cells fixed in either passage 4, 5 or 6. Values significantly different from controls ($P < 0.05$) are indicated by an asterisk.

control cells nor PACs exhibited an increase in the number of BNCs as a function of passage number (data not shown). Significantly elevated frequencies of BNCs were detected in 60 (3/5) and 38% (3/8) of the PAC cultures analyzed for this marker from the low and high dose groups respectively (Table IV).

Effect of irradiation on the frequency of cells containing MN

MN formation is a commonly used measure of radiation-induced chromosome aberrations (21). Unexposed controls and PACs described in the previous section were assayed for the presence of cells containing MN. Neither control cells nor PACs exhibited an increase in the number of cells containing MN as a function of passage number (data not shown). The background level in unexposed controls was 4 ± 1 MN-containing cells/1000 cells (mean ± SD, $n = 4$), so the significance level (i.e. mean + 2 SDs, $P < 0.05$) was established as >6 MN-containing cells/1000 cells. Significantly elevated frequencies of cells containing MN were detected in 40 (2/5) and 38% (3/8) of PAC cultures analyzed from the low and high dose groups respectively (Table IV). The highest frequency of cells containing MN (22 MN-containing cells/1000 cells) was detected in PAC culture H9 (passage 4). Of these five PACs with elevated frequencies of cells containing MN four also exhibited elevated frequencies of BNCs relative to unexposed controls (Table IV).

Effect of irradiation on microsatellite stability

DNA was extracted from six PAC cultures that exhibited significantly elevated levels of BNCs and/or MN, three that did not and three unexposed controls for microsatellite analysis. Figure 2 shows alterations in two markers in two PAC cultures (L2 and H9) relative to an unexposed NHBE culture (U10). In Figure 2A the smaller allele of marker D9S162 (9p13.3) was lost in PAC culture L2. In Figure 2B the smaller allele of marker D7S495 (7q31.3) was lost in PAC culture H9. Neither

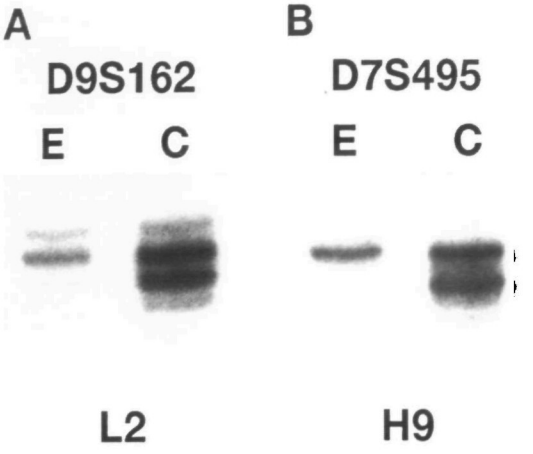

Fig. 2. Autoradiographs from LOH analysis with microsatellite markers. Control cells (C) and exposed cells (E) are shown with microsatellite markers on top.

loss was detected in unexposed controls nor in the other seven PAC cultures analyzed by this technique. These same alterations were also found when the analysis was repeated using independently extracted DNA samples (data not shown). No deletions or alterations in microsatellite length were detected with any other microsatellite marker (Table I).

Discussion

In this study NHBE cells were exposed to fractionated doses of α-particles to achieve a total dose (2–4 Gy, 730–1460 WLMs) that is comparable to the total occupational exposure received by uranium miners. The detection of elevated frequencies of BNCs and cells containing MN in the extended progeny of these cells is the first evidence that genomic instability is an early change in bronchial epithelial cells exposed to α-particles. These results support the findings of Kadhim *et al.* (6,7), who detected chromosome aberrations in the progeny of both human and murine hemopoietic cells 1 week after exposure of the parental cells to α-particles. These authors proposed that α-particles induce non-lethal lesions in stem cells that are transmitted to their progeny, resulting in genomic instability.

Microsatellite analysis was used in an attempt to identify specific genetic alterations in PAC cultures exhibiting elevated levels of BNCs and/or MN. Hemizygous deletions (i.e. LOH) of a 9p13.3 microsatellite marker (D9S162) and of a 7q31.3 marker (D7S495) were detected in two different PACs (i.e. one deletion per PAC). These two PACs exhibited elevated levels of BNCs (Table IV); however, elevated levels of cells containing MN were only observed in one PAC culture. Therefore, using only one molecular or cytogenetic marker may not always be effective for the detection of phenotypic changes after carcinogen exposure.

These hemizygous deletions may stem from direct damage to chromosomes 7 and 9 by α-particles during irradiation. Alternatively, these changes may have occurred after irradiation due to mutations in stability genes (22), although this is unlikely because microsatellite instability was not substantiated.

The 9p deletion maps to the chromosomal region adjacent to 9p21. This region contains the cyclin-dependent kinase-4 inhibitor $p16^{INK4A}$, a putative tumor suppressor gene that is frequently inactivated in lung cancer (23–27). Results of

preliminary fine mapping experiments (C.E.Mitchell, unpublished results) suggest that one allele of the $p16^{INK4A}$ gene is deleted in the PAC (data not shown). This gene can be inactivated by either homozygous deletion of 9p21 or by hemizygous deletion of this region followed by either methylation or mutation of the remaining copy of the $p16^{INK4A}$ gene (23–25,28).

The 7q deletion in PAC H9 maps to the vicinity of chromosome region 7q31.1. This region contains a putative tumor suppresser gene that is frequently lost in primary squamous cell carcinomas and in breast, colon and prostate cancers (29–31). Preliminary fine mapping results (C.E. Mitchell, unpublished results) suggest that practically the whole 7q arm is lost from one allele in this PAC (data not shown). Thus LOH of this putative tumor suppressor gene may have contributed to the extended lifespan (i.e. increased number of population doublings) of this PAC culture relative to unexposed NHBE cells.

The doses of radiation used in this study were sufficient to cause phenotypic changes in 92 cells (based on the number of foci present after irradiation and assuming that each focus arose from one cell) out of 6.2×10^6 cells at risk, or 1.5 cells/100 000 cells at risk. This efficiency is comparable with that achieved by Lechner *et al.* (32; 1 cell/100 000 cells at risk) after exposure of NHBE cells to nickel sulfate. In both the nickel study and the present study carcinogen-induced genetic changes resulted in the development of PAC cultures that exhibited an extended lifespan relative to unexposed controls. Because it is generally recognized that several mutations are necessary to convert a normal cell into a tumorigenic cell with an indefinite population doubling potential (i.e. immortalized cell), an extended cellular lifespan increases the odds that additional mutations will occur resulting in escape from senescence. However, resistance to immortalization has been observed in many *in vitro* carcinogenesis studies using normal human cells (33). Therefore, it is not surprising that at the radiation doses used in this study 0 of 6.2×10^6 NHBE cells at risk acquired an indefinite population doubling potential.

The fact that elevated levels of both BNCs and cells containing MN were detected in the extended progeny of NHBE cells exposed to α-particles indicates that these genetic aberrations were not a direct consequence of damage induced by ionizing radiation, but the result of genomic instability in progeny cells. Results of this preliminary study support the establishment of an *in vitro* model to identify the molecular basis of early phenotypic changes in NHBE cells caused by exposure to α-particles. Ultimately this information will be used to design chemopreventative agents to protect individuals at risk for radon-caused lung cancer due to either occupational or indoor exposure to ^{222}Rn.

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