Loss-of-heterozygosity analysis of 6-thioguanine-resistant mutants induced by radon exposure in mouse FM3A cells

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Summary Radon is an inert gas that can migrate from soils and rocks and accumulate in enclosed areas such as buildings and underground mines. The ubiquitous occurrence of radon in the environment is the primary cause of harmful radiation exposure to the public. To investigate the mutagenic effect of radon, mouse FM3A cells growing on soft agarose plates were exposed to alpha particles disintegrated from radon-222 and daughter elements. Mutation induction at the hypoxanthine phosphoribosyl transferase (Hprt) allele was examined at radon concentrations of 10, 230, 1,100, 6,500, 200,000, 1,000,000, and 10,000,000 Bq/m³ for an exposure period of 1 week. A typical inverse dose-rate effect was observed in the frequencies of 6-thioguanine-resistant (6TG-R) mutations, and lower mutation frequencies were exhibited at 230, 1,100, 6,500, and 200,000 Bq/m³ than at 10, 1,000,000, and 10,000,000 Bq/m³. Loss-of-heterozygosity (LOH) analysis at the Hprt locus revealed that deletion mutations were dominant at radon concentrations of 230, 1,100, 6,500, and 10,000,000 Bq/m³, but not at 10, 200,000, and 1,000,000 Bq/m³. These results suggested that alpha particles released from radon in the normal atmosphere did not exhibit the measured mutagenic effect in mouse FM3A cells, but that increased concentrations of radon led to a significant increase in the mutagenic effect of radon. At 6,500 Bq/m³, radon exposure induced the least number of 6TG-R mutants but all had LOH deletion mutations, which is the typically observed type of mutation in radiation carcinogenesis. Our results suggest that certain concentrations of environmental radon may have specific carcinogenic potential, and it should be avoided by proper ventilation wherever possible.

Keywords: Radon, ionizing radiation, mouse FM3A cells, mutation, hypoxanthine phosphoribosyl transferase (Hprt)

1. Introduction

Radon is a ubiquitous noble gas generated by the disintegration of uranium and radium in the earth’s crust. Radon itself disintegrates and generates daughter elements (I). For example, radon-222 disintegrates and generates the following cascade of daughter elements: polonium-218, lead-214, polonium-214, lead-210, bismuth-210, polonium-210, and lead-206. Alpha particles are released at relatively high energy levels throughout the disintegration of radon and its daughter polonium elements and this process is known as alpha decay. Alpha particles are identical to the helium nucleus, which is composed of 2 protons and 2 neutrons. The large mass of typical alpha particles allows them to travel only about a few centimeters in the atmosphere and 40-80 micrometers in human tissue. Because alpha particles cannot penetrate skin, external exposure to radon is not problematic for the general public. However, inhalation of radon releasing
alpha particles may cause serious health problems. The frequent occurrence of lung cancer in underground miners (2-4), as well as in the general public (5-7) has been reported. Additionally, radon exposure is suspected to be one of the major causes of lung cancer in non-smokers (8-10). Radon exposure also has a suspected role in the initiation of leukemia (11-13). Thus, the omnipresence of radon is a primary source of harmful radiation exposure for the general public.

Radon is an established carcinogen, but the molecular basis of carcinogenesis caused by radon has not been properly elucidated. High-energy alpha particles are estimated to kill at least 3 cells along their path, and the by-stander effect could cause mutations in surrounding cells (14-16). The by-stander effect caused by the cytoplasmic crossing of alpha particles and the subsequent transmission of chemical signals were suggested to play major roles in mutagenesis and carcinogenesis in cell populations hit by alpha particles (17-19). However, other reports voice the opposite position and claim that the proposed by-stander effect of alpha particles could be within experimental error (20-22).

We established a radon exposure apparatus and examined the mutagenic activity of alpha particles released during the alpha decay process of radon-222 using mouse FM3A cells. Mouse FM3A cells were derived from a mammary tumor in the C3H He/N mouse and grown on soft agar plates in an atmosphere containing 100% humidity. The ability of mouse FM3A cells to grow on these plates allowed us to investigate the mutagenic potential of radon gas by directly exposing cells growing on soft-agar plates to the atmosphere. Here we report the isolation of 6-thioguanine (6TG) resistant mutants induced by various concentrations of radon and the results of loss-of-heterozygosity (LOH) analysis of these mutants. Our results imply that there is a specific radon concentration range that has a greater carcinogenic potential.

2. Materials and Methods

2.1. Cell culture

Mouse FM3A cells were maintained in ES liquid medium (Nissui, Tokyo, Japan) containing 2% fetal bovine serum (FBS) (Nichirei, Tokyo, Japan) in a CO₂ incubator with an atmosphere containing 5% CO₂ and 100% humidity, as described previously (23,24). Prior to radon exposure, cells were cultured for 48 h in HAT medium containing 10⁻⁴ M hypoxanthine, 10⁻⁶ M aminopterin [also known as methotrexate (MTX)], and 10⁻⁵ M thymidine, and for 24 h in HT medium containing 10⁻⁴ M hypoxanthine and 10⁻⁷ M thymidine, as described previously (24). For radon exposure, cells were cultured on ES agarose plates containing 5% FBS and 0.5% agarose.

2.2. Radon exposure

Radon gas was generated from ceramic plates coated with radium-226. Various amounts of a ceramic radon source were stored in individually sealed lead chambers that were connected to the CO₂ incubator. Different radon concentrations were achieved using an appropriate radon chamber containing different amounts of ceramic plates coated with radium. Radon gas was injected into the CO₂ incubator through a sterilizing filter at a flow rate of approximately 0.1 liter per minute. For a natural background control, the room atmosphere was injected into the incubator through the filter at the same flow rate. Radon concentration was monitored using the AlphaGUARD radon monitor (Genitron, Frankfurt, Germany), and an average concentration was calculated.

Cells grown on ES agarose plates were exposed to an atmosphere containing radon gas at a concentration of 10, 230, 1,100, 6,500, 200,000, 1,000,000, and 10,000,000 Becquerel per cubic meter (Bq/m³) for 1 week. After radon exposure, cells were allowed to recover for 48 h prior to further treatment.

2.3. Isolation of 6TG™ mutants

For selection of 6TG™ clones, cells were plated onto ES plates containing 5% FBS, 0.5% agarose, and 10⁻⁵ M 6TG, as described previously (24). The number of cells used in the drug selection experiment was estimated from the number of colonies formed on ES plates without 6TG using an appropriate dilution of the cell suspension. Colonies that formed on the selection plates containing 6TG were independently isolated and cultured for further analysis. All chemicals were obtained from Wako Chemical (Osaka, Japan), unless otherwise specified.

2.4. LOH analysis

Genomic DNA was extracted from cells by proteinase K-sodium dodecyl sulfate (SDS) treatment and purified using the phenol-chloroform extraction method, as described previously (23,24). LOH at the hypoxanthine phosphoribosyl transferase (Hprt) locus coding for the Hprt gene was examined using the polymerase chain reaction (PCR) with the following oligonucleotide primers UniSTS178186 Hprt-F 5’-GAAATGTCA GTTGCTGCGTC-3’ and UniSTS178186 Hprt-R 5’-GCAAACACTGCTGAAACATG-3’ (25). The reaction mixture was prepared as recommended by the manufacturer (Takara, Shiga, Japan). The reaction was initiated for 5 min at 94°C, and followed by 40 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C using the GeneAmp PCR System 9700 (Applied Biosystems Inc., Carlsbad, CA, USA). PCR products were analyzed using 3% agarose gel electrophoresis (24).
3. Results

3.1. Induction of mutation by radon exposure

The apparatus for radon gas exposure was installed as described in Figure 1. Before cell culture, the CO₂ incubator was equilibrated for 24 h with a sterile atmosphere containing radon using a specific radon chamber.

Prior to radon exposure, mouse FM3A cells were cultured in HAT medium for 48 h to eliminate naturally occurring Hprt-deficient cells that were resistant to 6TG, as described previously (24). HAT medium was used because Hprt activity is not an absolute requirement for survival, and cells that lack Hprt activity will grow in normal growth medium, which can affect the results of experiments that examine mutation frequency.

Cells were allowed to recover from the toxic effects of MTX in HT medium for 24 h. Approximately 1.0 × 10⁴ cells were plated onto ES agarose plates and cultured for 7 days without lids in a sterile atmosphere containing various concentrations of radon, 5% CO₂, and 100% humidity. The radon concentrations used in our experiments were as follows: 10 (ordinary atmosphere natural background control), 230, 1,100, 6,500, 200,000, 1,000,000, and 10,000,000 Bq/m³.

Following radon exposure, cells were suspended in ES medium by rinsing the surface of agarose plates with the medium, recovered in normal growth medium (ES + 2% FBS), and used for selection experiments to isolate drug-resistant mutants.

3.2. Selection and isolation of 6TG₈ mutants

Cells were plated on ES agarose plates containing 5% FBS and 10⁻⁵ M 6TG. 6TG₈ colonies appeared after 1 week. Each colony was isolated independently and grown for further analysis. The number of colonies isolated and their mutation frequencies are summarized in Table 1. The control experiment with natural background (10 Bq/m³) showed 43 6TG₈ colonies at a mutation frequency of 5.0 × 10⁻⁶. The mutation frequency was similar to the background level at 230 Bq/m³ (34 colonies at a mutation frequency of 4.0 × 10⁻⁶), increased at 1,100 and 6,500 Bq/m³ (22 at 2.6 × 10⁻⁶ and 10 at 1.2 × 10⁻⁶, respectively), dropped to the background level at 200,000 Bq/m³ (37 at 4.3 × 10⁻⁶), and became enhanced again at 1,000,000 and 10,000,000 Bq/m³ (138 at 1.6 × 10⁻⁵ and 91 at 1.0 × 10⁻⁵, respectively). These mutation results exhibited a typical inverse dose-rate effect (Figure 2).

![Radon exposure apparatus](image)

Figure 1. Radon exposure apparatus. Radon gas was generated from ceramic plates coated with radium-226 and stored in a sealed lead chamber that was connected to the CO₂ incubator. Different radon concentrations were achieved by using an appropriate radon chamber containing appropriate amounts of ceramic plates coated with radium. The sealed lead chamber was prepared for radon gas, which was injected into the CO₂ incubator through a sterilizing filter at a flow rate of 0.1 liter per minute. For the background control, room atmosphere was injected into the incubator through the filter at the same flow rate. Radon concentration was measured using the AlphaGUARD radon monitor (Genitron), and an average concentration was calculated.

<table>
<thead>
<tr>
<th>Radon concentration (Bq/m³)</th>
<th>Number of 6TG₈ colonies</th>
<th>Mutation frequency</th>
<th>Number of LOH mutants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>43</td>
<td>5.0 × 10⁻⁶</td>
<td>13 (30.2)</td>
</tr>
<tr>
<td>230</td>
<td>34</td>
<td>4.0 × 10⁻⁶</td>
<td>23 (67.6)</td>
</tr>
<tr>
<td>1,100</td>
<td>22</td>
<td>2.6 × 10⁻⁶</td>
<td>21 (95.5)</td>
</tr>
<tr>
<td>6,500</td>
<td>10</td>
<td>1.2 × 10⁻⁶</td>
<td>10 (100)</td>
</tr>
<tr>
<td>200,000</td>
<td>37</td>
<td>4.3 × 10⁻⁶</td>
<td>8 (21.6)</td>
</tr>
<tr>
<td>1,000,000</td>
<td>138</td>
<td>1.6 × 10⁻⁵</td>
<td>51 (37.0)</td>
</tr>
<tr>
<td>10,000,000</td>
<td>91</td>
<td>1.0 × 10⁻⁵</td>
<td>69 (75.8)</td>
</tr>
</tbody>
</table>
3.3. LOH analysis at the Hprt locus

The genomic structure of the Hprt locus in 6TG<sup>R</sup> cells was examined with PCR using a set of UniSTS primers (24,25). The results of the LOH analysis are summarized in Table 1.

LOH at the Hprt locus was detected in 13 out of 43 6TG<sup>R</sup> colonies isolated from the control group. LOH increased at 230, 1,100, and 6,500 Bq/m<sup>3</sup> (23 out of 34, 21 out of 22, and 10 out of 10, respectively), returned to the background level at 200,000 and 1,000,000 Bq/m<sup>3</sup> (8 out of 37, and 51 out of 138, respectively), and again increased at 10,000,000 Bq/m<sup>3</sup> (69 out of 91) (Figure 3). DNA sequence analysis of the genomic Hprt gene was not performed.

4. Discussion

In this report, we established a radon exposure experimental system using mouse FM3A cells. A CO<sub>2</sub> incubator was equilibrated with an atmosphere containing various concentrations of radon gas.

Figure 2. Inverse dose-rate effect on mutation frequencies induced by radon exposure. An inverse dose-rate effect was observed in the mutation frequencies of mouse FM3A cells induced by radon exposure at various radon concentrations for 1 week. The mutation frequencies are represented on a logarithmic scale. The radon concentration in the background control atmosphere was 10 Bq/m<sup>3</sup>.

Figure 3. Ratio of LOH mutants induced by radon exposure. LOH analysis was performed on 6TG<sup>R</sup> mutants induced by various concentrations of radon exposure for 1 week. Each bar represents the number of mutant cells and the shaded bottom area represents the number of LOH mutants at the specified radon concentration. The radon concentration in the background control atmosphere was 10 Bq/m<sup>3</sup>.
Since the 6TG\textsuperscript{R} mutant phenotype can only be manifested by a lack of Hprt activity in mammalian cells, a mutation detection system using a combination of 6TG\textsuperscript{R} and Hprt-deficiency has been widely used in experiments examining mutagenic activity of various mutagens, including chemicals (26,27) and radiation (28,29), on mammalian cells.

6TG\textsuperscript{R} mutants were isolated at a frequency of \(5.0 \times 10^{-8}\) in our control experiments with a natural background radon concentration of 10 Bq/m\(^3\), consistent with our previous observations (24). The mutation frequency was \(4.0 \times 10^{-8}\) at 230 Bq/m\(^3\), showing that radon exposure did not significantly enhance mutation induction at the Hprt locus.

At 1,100 Bq/m\(^3\) and 6,500 Bq/m\(^3\), mutation induction was enhanced 2- and 4-fold, respectively. At 200,000 Bq/m\(^3\), mutation induction returned to the control level, exhibiting a typical inverse dose-rate effect (Figure 2). Mutation induction was again enhanced at the higher concentrations of 1,000,000 Bq/m\(^3\) and 10,000,000 Bq/m\(^3\), resulting in 3- and 5-fold enhancements, respectively.

Molecular examination of the Hprt locus revealed that the ratio of LOH mutants in all mutant clones, which presumably occurred by deletion mutations, did not correlate with mutation frequency. The ratio of LOH mutants was 13/43 (30.2%) in the mutant cells induced in ordinary atmosphere, consistent with our previous observations (24). However, the ratio of LOH mutants drastically increased as radon concentration increased, as shown in Table 1. The ratio of LOH mutants was 23/34 (67.6%) at 230 Bq/m\(^3\), 21/22 (95.5%) at 1,100 Bq/m\(^3\), and reached a maximum of 10/10 (100%) at 6,500 Bq/m\(^3\).

Surprisingly, the ratio of LOH mutants was reduced to 8/37 (21.6%) at 200,000 Bq/m\(^3\), which was lower than the background level, and 51/138 (37.0%) at 1,000,000 Bq/m\(^3\), which was close to the control level. We cannot explain these observations because the ratio of deletion mutants generally increases as the dose increases in radiation mutagenesis.

The ratio of LOH mutation was again increased at 10\(^7\) Bq/m\(^3\) to 69/91(75.8%). Although the ratio was lower at 10\(^3\) Bq/m\(^3\) than the induction level at 1,100 and 6,500 Bq/m\(^3\), 7 times more mutants were isolated at 10\(^3\) Bq/m\(^3\). The increases in both the number of mutants and the ratio of LOH mutants at the highest exposure concentration used in our experiments may be explained by the dose-effect of ionizing irradiation. However, this result sheds light on the unusually high proportion of LOH mutants at 1,100 and 6,500 Bq/m\(^3\).

Our observations may have resulted from a combination of different mechanisms such as direct hit by alpha particles and bystander effects.

Recently, there has been considerable concern about the potential carcinogenic effects of very low doses of alpha particle radiation. The ubiquitous existence of radon contributes to approximately half of the radiation exposure of the general public, suggesting that the deleterious mutagenic effects of radon are one of the greatest threats to the health of the general public. It has been estimated that as many as 15% of all lung cancer cases may be caused by exposure to residential radon (1,7). Our examination of the mutagenic effect of radon using mouse FM3A cells provides a molecular basis for these statistics. Detailed molecular analysis of the Hprt locus in the mutant cells obtained in our experiments may provide useful information that will reveal the underlying mechanisms that induce mutations. Further experiments with various exposure periods should be performed to understand the molecular basis of the health effects of radon.

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References


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