ANL/CP--73935

DE91 017964

**ALTERATION OF THE RETINOBLASTOMA GENE LOCUS
IN RADIUM-EXPOSED INDIVIDUALS** **Received**

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SEP 06 1991

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INTRODUCTION

The retinoblastoma gene (Rb) product is a nuclear protein which functions as a tumor suppressor or anti-oncogene (1). This protein functions in the cell cycle and therefore is an important gene in growth control. Inactivation of the Rb gene by mutations or hypermethylation would result in the loss of the Rb protein inhibitory effect on cell growth and thus contribute towards the development of retinoblastomas, osteosarcomas, and other malignant tumors (2,3).

Radium, a radioactive element emitting alpha-particle radiation was used during the early part of this century as a therapeutic agent and in the luminous paint on watch dials. People who painted the dials, refined radium from ore, or received it medicinally developed side effects that included anemia, sarcomas of the bone, and carcinomas of the paranasal sinuses and mastoid air cells (4,5). The radiation emitted by radium and its daughter products was recognized as the agent responsible for the development of these types of cancers (4). Although radium has been shown to induce DNA damage (6), primarily DNA deletions, the mechanism and extent to which this element induces damage to growth control genes that participate in the oncogenic process are unclear. Because radium induces osteosarcomas and fibrosarcomas, we studied bone and soft tissues for evidence of deletions or alteration to the retinoblastoma gene. We analyzed the Rb gene locus in 30 individuals, 17 of whom were exposed to radium either occupationally or iatrogenically.

The patients and their body burden of radium has been described previously (7). DNA and RNA were prepared from autopsy samples (7,8). Southern and Northern blot analysis was performed as described previously (1,2,7,8).

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RESULTS

Initially we analyzed the Rb-1 gene structure in DNA samples from normal and malignant bone tissues because radium deposits in the bone. Using a 5'-end probe of the Rb-1 cDNA, we detected in femur DNA samples from two age-matched control (C) individuals five *Hind* III restriction fragments that were 19, 6.0, 3.0, 1.5 and 1.2 kb in length (Fig. 1). The bone DNA samples from five of six radium-exposed (R) individuals and bone tumor DNA from three individuals contained 5'-end Rb DNA fragment patterns that differed from those observed in control femur DNA.

Figure 1. Alteration of the 5' end of the Rb-1 gene in femur and bone tumor DNA of radium-exposed individuals. DNA from the femur of patients R5, R15, R13, R8, R14, R12, C11, and C12 and bone tumors from patients R10, R16, and R2 were digested with *Hind* III, separated in an 0.8% agarose gel, and blotted to Nytran filter. The blot was hybridized to the 5' end of the Rb-1 cDNA.

To determine whether the 3' end of the Rb-1 gene was also altered in radium-exposed individuals, we analyzed this portion of the gene by means of Southern blotting with a 3.8-kb *Eco*R1 insert of the Rb-1 cDNA. The femurs from C11 and C13 had the characteristic *Hind* III digest pattern (fragments of 9.8, 7.5, 6.2, 5.3, 4.5, and 2.1 kb). Bone DNA from a number of radium-exposed individuals did not show this normal DNA pattern, which indicates that major rearrangements and/or deletions had occurred. The bone DNA from R8 had a normal *Hind* III restriction pattern. The femurs from R5 and R6 also showed a normal pattern, but only after overexposing the

blot. This suggests that a majority of cells in these tissues had a deletion in the Rb gene.

To further characterize this abnormal *Hind* III restriction pattern, we established a detailed restriction map of bone and bone tumor DNA. Neither R2 (a mastoid bone tumor), R10 (a fibrosarcoma), nor R15 (a pathologically normal femur) had the normal *Hind* III digestion pattern found in control femur bone samples. Each of these individuals had a body burden of radium greater than 30 kBq. The mastoid bone tumor of R2 had a deletion of the Rb gene from exon 8 to 12, and the fibrosarcoma DNA of R10 had a deletion from exon 6 to 12. The DNA from femur R15 had a deletion from exon 3 to exon 12. In 12 bone samples analyzed from radium-exposed individuals, eight samples had rearrangements or deletion in the Rb gene. These results indicate the presence of major changes in the structure of the 5' end and 3' end of the Rb gene in bone tissue from radium-exposed individuals.

In addition to bone tissue, we analyzed kidney tissue DNA from nine radium-exposed individuals and from six controls. In the six controls, no alteration in the Rb gene was found, but in four of nine individuals exposed to radium, the Rb gene was altered. In patient R14's kidney DNA, a deletion from exon 6 to 27 was observed, and patient R11 showed a deletion from exon 8 to 12. In the DNA from the kidney of patient R17, only one band of 3.8 kb was found, which suggests that both of the Rb alleles had been deleted. This implies that in a majority of cells in this kidney, the Rb gene had been deleted. Both patients R17 and R14 had body burdens of radium in excess of 20 kBq. In patient R8, a small deletion including exons 1 and 2 of the 5' end of the Rb gene had occurred. Unlike the femur, in which a selective group of cell types are being analyzed, the kidney represents a collection of different cell types, some of which may be more susceptible to radiation damage. These results suggest that a majority of the cells in the kidney of patients with high body burdens of radium show alterations in the Rb gene.

We performed Northern blot analyses to determine if genomic alteration in the Rb gene structure resulted in a loss of its expression (Fig 2). In the kidney tissue samples from a number of radium-exposed individuals, Rb mRNA was detected at a reduced amount when compared to the amount from either kidney or liver tissue from control individuals. The presence of Rb mRNA in kidney tissue from R8, who showed a deletion of the 5' end of the Rb gene, suggests that a fraction of the cells in the kidney which did not have this deletion were actively expressing the Rb gene.

Even though the quality of RNA from many of the bone samples was unsatisfactory for analysis, those few samples with intact ribosomal bands

Figure 2. Expression of the RB gene in kidney and bone tumor cell lines. Northern blot analysis of RNA extracted from the kidney of C1, the kidney and liver of R12, the kidney of C2, R6, C3, R8, and R2, the liver of R12, R6, and the osteosarcoma cell lines Saos2, T85, SKS, and H-3. Total RNA (10 μ g) was electrophoretically separated in a 1.5% agarose formaldehyde gel, blotted, and hybridized to 3.8-kb Rb cDNA (A) or human actin (B).

were analyzed for the level of Rb expression. Rb mRNA was detected in bone tissue from R11, R14, R8, and R5 but not from R15, R12, or R16. No Rb mRNA was found in the bone tumor tissue from R8 or R6. It is unclear why a substantial amount of Rb mRNA is detected in the majority of bone tissues which had rearrangements and/or alterations of the Rb gene. In a cell line derived from the fibrosarcoma of patient R10 (H-3), a deletion of the 3' end of the Rb gene was detected. This resulted in the expression of a 4.7-kb mRNA as well as an altered mRNA of approximately 4.2 kb. A shortened Rb mRNA was found in the Saos2 cell line, which is also due to a 3' end deletion of the Rb gene (9). Perhaps a minority of cells in these tissues express elevated levels of Rb mRNA.

DISCUSSION

This study was performed to determine if the retinoblastoma suppressor gene was altered in individuals exposed to radium. We analyzed the Rb gene in 30 individuals, 17 of whom were exposed to radium either occupationally or iatrogenically. In the kidney DNA from four of nine radium-exposed individuals, the Rb gene was deleted. Three of these alterations in the Rb gene were internal deletions, which resulted in the absence of Rb mRNA accumulation. In one of these individuals who had a

high body burden of radium (58 kBq), both alleles of the Rb gene were deleted. The fourth kidney sample had a 5'-end deletion of the Rb gene but still accumulated Rb mRNA, which suggests that a minor fraction of cells in this kidney were transcribing the Rb gene.

Because hereditary retinoblastoma patients are at a high risk of developing secondary neoplasms, osteosarcomas, and fibrosarcomas, we analyzed the distal head of normal femurs from radium-exposed individuals. Four of nine of these femurs had an internal deletion in the Rb gene. In two patients with a high body burden of radium (greater than 38 kBq), similar areas of the Rb gene were deleted. In six of the femurs analyzed for Rb mRNA, four actively transcribed the Rb gene, while in two femurs with gene deletions no Rb mRNA was detected. In two samples, R19 and R14, Rb mRNA was present even though each DNA had both a rearrangement and a deletion in the Rb gene. Two possibilities can explain this inconsistency. Either the internal Rb gene deletion does not affect transcription or a minor fraction of cells in these femurs are actively transcribing a normal Rb gene. A comparison of Rb transcripts from these femurs indicates that additional mRNA sizes of 3.8, 3.2, and 2.2 kb are evident besides the normal 4.7-kb transcript. We are uncertain whether these mRNAs represent products of altered RB genes or degradation products. However, we were able to show that two different Rb mRNAs are produced from a fibrosarcoma cell line H-3 derived from a tumor of patient R10.

These results imply that the Rb gene is susceptible to radium-induced damage and confirm previous studies showing that radiation preferentially causes genomic deletions (10). In three radium-induced tumors analyzed, we found that the 7.8-kb *Hind* III fragment that contains exons 13-17 was a common site for deletion in the Rb gene and may represent a breakpoint cluster region (11). The pronounced alterations in the non-tumorigenic femurs from radium-exposed individuals suggests that in the many years of exposure there was a selection of cells with alterations, presumably because of their growth advantage. Also it implies that deletions of one of the Rb alleles can be one of the events (perhaps an initial one) in the progression of radium-induced sarcomas. Similar to other types of tumorigenic processes, radium-induced cancer probably proceeds through a series of genetic alterations involving tumor suppressor genes and dominant oncogenes.

ACKNOWLEDGMENT

This work was supported by the United States Department of Energy, Office of Health and Environmental Research, under Contract No. W-31-109-Eng-38.

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