CONF-9304154--1

ANL/BIM/CP--79174

DE93 010659

PCR Detection of Retinoblastoma Gene Deletions in Radiation-induced Mouse Lung Adenocarcinomas[1]

M.E. Churchill, M.A. Gemmell and G.E. Woloschak
Biological and Medical Research Division, Argonne National Laboratory, 9700 South Cass Ave., Argonne, IL 60439, USA

ABSTRACT: From 1971 to 1986, Argonne National Laboratory conducted a series of large-scale studies of tumor incidence in 40,000 BCF_1 mice irradiated with ^{60}Co γ rays or JANUS fission-spectrum neutrons; normal and tumor tissues from mice in these studies were preserved in paraffin blocks. A polymerase chain reaction (PCR) technique has been developed to detect deletions in the mouse retinoblastoma (*mRb*) gene in the paraffin-embedded tissues. Microtomed sections were used as the DNA source in PCR reaction mixtures. Six *mRb* gene exon fragments were amplified in a 40-cycle, 3-temperature PCR protocol. The absence of any of these fragments (relative to control PCR products) on a Southern blot indicated a deletion of that portion of the *mRb* gene. The tumors chosen for analysis were lung adenocarcinomas that were judged to be the cause of death in post-mortem analyses. Spontaneous tumors as well as those from irradiated mice (569 cGy of ^{60}Co γ rays or 60 cGy of JANUS neutrons, doses that have been found to have approximately equal biological effectiveness in the BCF_1 mouse) were analyzed for *mRb* deletions. In all normal mouse tissues studied (18 total), all six *mRb* exon fragments were present on Southern blots. Tumors in six neutron-irradiated mice also had no *mRb* deletions. However, 1 of 6 tumors from γ-irradiated mice (17%) and 6 of 18 spontaneous tumors from unirradiated mice (33%) had a deletion in one or both *mRb* alleles. All deletions detected were in the 5′ region of the *mRb* gene.

1. INTRODUCTION

Ionizing radiation is known to be both mutagenic and carcinogenic (Upton 1984; Shigematsu and Kagan 1986). Many studies have focused *in vitro* on radiation-induced transformation in cultured cells (Borek 1979; Elkind and Han 1979; Kennedy *et al.* 1984) and on *in vivo* malignant tumor formation in whole animals (United Nations Scientific Committee on Effects of Atomic Radiation 1977, 1982; Grahn *et al.* 1990, 1992). It is widely accepted that radiation-induced cell killing and mutations are the result of DNA damage, including DNA strand breaks, base modifications, insertions, and deletions (Yunis 1983; Hutchinson 1985). However, little is known about the specific molecular mechanisms by which ionizing radiation induces cellular transformation. We report the development of a PCR technique which allows detection of large DNA deletions in paraffin-embedded lung adenocarcinomas from mice irradiated with ^{60}Co γ rays or JANUS fission-spectrum neutrons.

From 1971 to 1986, Argonne National Laboratory conducted a large-scale study of tumor incidence in approximately 40,000 BCF_1 mice irradiated with ^{60}Co γ rays or JANUS fission-spectrum neutrons (Grahn *et al.* 1990, 1992). Pathologic examinations of the mice were conducted

[1]The authors thank Dr. Bruce Carnes and Ms. Betty Jean Wright for their expert assistance in selecting tumors for study, Ms. Kay Bexson for excellent secretarial assistance, and Drs. Carol Giometti, Radoje Drmanac, Lydia Ling-Indeck, and Frank Collart for reviewing this manuscript. Work supported by the U.S. Department of Energy, Office of Health and Environmental Research, under Contract No. W-31-109-ENG-38.

to determine the effects of varying radiation doses and dose schedules on the formation of neoplasias classified as "lethal" or "contributing to death." The tumors and normal tissues were fixed in buffered formalin and preserved in paraffin blocks for later analyses. Although the quality of DNA in these paraffin-preserved tissues was generally not sufficient for ordinary Southern blot studies, specific sequences from the DNA can be amplified and detected using PCR technology (Goeltz *et al.* 1985; Impraim *et al.* 1987; Shibata *et al.* 1988). The preserved tissues from this BCF_1 mouse study thus have the potential to provide a wealth of information about the molecular genetics of radiation-induced tumor formation in mice.

2. MATERIALS AND METHODS

cDNA Clones. We thank the following individuls for generously providing cDNA clones: Dr. Dryja (Harvard Univ., Boston, MA) for Rb cDNA and Dr. David Baltimore (Rockefeller Univ., New York, NY) for *Rag-1* cDNA.

Tissue Samples. During the Argonne mouse irradiation study, a database containing information on each mouse involved in the study was established. We selected paraffin blocks of mouse tissues for our study based on information from this database. Control mice and mice that had been irradiated with known doses of radiation were identified, and paraffin blocks containing normal lungs or lung adenocarcinomas from these mice were retrieved from the Argonne Tissue Repository.

BCF_1 mice (C57BL/6 x BALB/c) were bred in the Argonne animal facility. Mice entered the experiment at 16 weeks of age. Radiation facilities, dosimetry, and methods of exposure have been reported in detail by Grahn *et al.* (1972) and Williamson and Frigerio (1972). The lung tissues used in this study were derived from male mice irradiated with a single dose of either 569 cGy of ^{60}Co γ rays or 60 cGy of JANUS fission-spectrum neutrons (JANUS neutrons have a mean energy of 0.85 MeV and a γ-ray contamination level of only 2.5%). Control mice were not irradiated. The γ-ray and neutron doses chosen have approximately equal biological effectiveness in the BCF_1 mouse (Grahn *et al.*, 1990, 1992).

DNA Sample Preparation and PCR Protocol. Paraffin tissues were prepared for PCR by the method of Wright and Manos (1990). Primers (20-mers) for amplification of six segments of the *mRb* gene were designed according to the cDNA sequence (from 5′ to 3′) published in Bernards *et al.* (1989); see Table 1. Segments 1–5 are contained in the coding region, and segment 6 is contained in the 3′ untranslated region. Sequences of the primers (5′ to 3′) were:
Sense 1: TGACACAACCCAGCAGTGCG; Anti 1: TTACCAGGTCATCTTCCATC;
Sense 2: TCGAGGTTCTCTGTAAAGAA; Anti 2: GGATCAGTCTGAAGTGTTTT;
Sense 3: GCAAACTCCTAAATGACAAC; Anti 3: TCTGCTATACGTAGCCATTA;
Sense 4: TTGAAATCTACCTCCCTTGC; Anti 4: TCTCATGAGCTCATACTCAT;
Sense 5: GGTAACATCTATATATCACC; Anti 5: TGATTCACCAATTGAGACCA;
Sense 6: ACAGATTTCATACCTCAGAC; Anti 6: CAACCTTGGAGTATTATCAA.
Primers (20-mers) for amplification of the *Rag-1* gene standard were based on the cDNA sequence published by Schatz *et al.* 1989. The sense primer (1063–1082) was 5′-CTATTGTCCCTCTTGCCGAT-3′ and the antisense primer (1571–1590) was 5′-GGTTGAAAAATCTGCCTCCC-3′, giving a 528-bp fragment.

PCR reaction mixtures (50 μL) were run in a Perkin Elmer Cetus DNA Thermal Cycler and contained 10 mM Tris·HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM each dATP, dCTP, dGTP, dTTP; 0.3 μM each primer; 5.0 μL of tissue digest; and a 45-μL mineral oil overlay. This reaction mixture was held at 94 °C for 5 min, 1.25 units of *Taq* polymerase was added (hot start), and the following protocol was used for amplification: 1 min at 94 °C/1 min at 42 °C/2 min at 65 °C: 40 cycles; 7 min at 65 °C: 1 cycle. Amplified reaction mixtures were stored at –20 °C

until being used for electrophoresis. Amplifications of the *mRb* and *Rag-1* genes were done under the same conditions.

Southern Blot Procedure. PCR reaction mixtures (18 μL) of the *mRb* gene were run on a 3.2% NuSieve 3:1 Agarose (FMC) gel at 90 mA for 4 h. The gel was denatured two times for 30 min in 0.6 M NaOH/1.5 M NaCl and neutralized two times for 30 min in 1.5 M Tris·HCl/1.5 M NaCl, pH 8.0. The DNA was transferred to an MSI neutral nylon membrane with 10× SSC transfer buffer. The membrane was rinsed, dried, and UV-crosslinked. Membranes were hybridized to ^{32}P-random-hexanucleotide-labeled *mRb*-containing plasmid in BIOS Speed-Hyb solution at 53 °C. Membranes were washed four times in 2× SSC, 0.5% SDS at 53 °C, dried, and autoradiographed. ' PCR reaction mixtures of the *Rag-1* gene were run similarly except for the following: (a) 1.5% ME agarose gel (FMC) was run at 80 V for 4 h; the gel was denatured and neutralized only once each; the *Rag-1* plasmid was used for probing; and washing was in 0.5× SSC, 0.5% SDS, three times at 53 °C.

Method of Quantitating *mRb* Deletions. The basic idea in quantitating deletions is to amplify the six *mRb* PCR segments from control and tumor tissues and quantitate the intensity of autoradiograph bands by microdensitometry. Because it was impossible to eliminate all normal tissue from the tumor samples, bands from a tumor tissue with a deletion are lighter than bands from control tissues, but may not be completely absent. Furthermore, the intensity of bands will be different on different blots due to differences in amount of probe used, efficiency of crosslinking, etc. Thus, PCR fragment #4 was amplified using *mRb* cDNA plasmid. When a Southern blot containing PCR amplifications of tumor or normal paraffin tissues was run, a known amount of the standard band #4 was included on the gel. The intensity of each band on the gel was compared to the intensity of this standard band, giving a mathematical ratio (R1) that allows standardization of band intensities from gel to gel and sample to sample: R1 = intensity of paraffin *mRb* band/intensity of standard *mRb* band. Because the efficiency of amplification and the amount of tissue varied from sample to sample, a standard gene that was not deleted in tumors (*Rag-1*) was amplified, and the intensity of this band was used to correct for amplification efficiency and the amount of tissue used. The intensity of the band produced (528 bp) was compared to the intensity of a standard *Rag-1* band prepared by PCR of a *Rag-1* cDNA plasmid. A ratio R2 was designated: R2 = intensity of paraffin *Rag*-1 band/intensity of standard *Rag*-1 band. In order to evaluate whether one *mRb* band was less abundant than another, each band was assigned a ratio R, where R = R1/R2. We have determined that bands from control tissues had R values that were greater than 0.2, often as large as 5.0, while bands from tumor tissues with *mRb* deletions had R values that were small (usually 0 to 0.1).

3. RESULTS

Table 2 presents results of *mRb* amplifications. No normal lung tissues of the 18 examined showed deletion of the six *mRb* fragments tested. This was true of normal lungs from both unirradiated and irradiated animals. The R values for all bands amplified from normal lung tissues were 0.2 or greater, indicating the presence of the band. For tumor tissues, only bands with R values less than 0.2 (usually less than 0.1) were considered to be deleted. One paraffin block containing normal lung tissue consistently failed to amplify either *mRb* or *Rag-1* fragments because of extensive necrosis of the tissue before preservation. This tissue, which was treated the same as all others, served as a negative PCR control.

Normal lung tissue from unirradiated mice (12 samples), γ-irradiated mice (6 samples), and neutron-irradiated mice (6 samples) showed the presence of all *mRb* bands in all blots (R > 0.2). All adenocarcinomas studied were determined by postmortem analysis to be the cause of death of the animals. In 12 of 18 adenocarcinomas that developed in unirradiated mice, no *mRb* deletions

were detected. In 6 of 18 (33%) spontaneous adenocarcinomas, *mRb* deletions were detected. Four of the tumors had a deletion of fragments 1 and 2, while two of the tumors had a deletion of fragments 1, 2, and 3. In 5 of 6 adenocarcinomas from γ-irradiated animals, no *mRb* deletions were detected. One of the tumors had a deletion of fragments 2 and 3. In 6 adenocarcinomas that developed in neutron-irradiated mice, none showed an *mRb* deletion. These data are summarized in Table 2.

4. DISCUSSION

It was not practical to amplify the entire *mRb* gene for two reasons: (1) a large number of fragments sized 250 bp or smaller would be necessary to amplify a 4.7-kb gene and (2) the intron structure for the entire *mRb* gene is not known. The technique of detecting *Rb* deletions used here has several inherent limitations. First, if the fragment being amplified is present in one allele but deleted in the other, a band will still appear on the Southern blot. Since both alleles of the *Rb* gene are usually mutated in tumors (Dunn *et al.* 1989; Stanbridge 1990), this limitation is reasonable. Second, deletions in the *mRb* gene that fall between PCR fragments in the gene sequence will not be detected. Because this study focused exclusively on *mRb* coding regions, deletions in introns or in non-amplified coding regions of the *mRb* gene would be missed using this approach. However, large deletions, sometimes of the entire gene, are often found in retinoblastomas (Dunn *et al.* 1989), so it is reasonable to expect that tumors will be found having large *mRb* deletions that can be detected by our technique. Third, point mutations or small deletions and insertions will not be detected. A point mutation in the primer sequence might prevent primer binding during the PCR annealing step. However, our annealing temperature of 42 °C (necessary because of the low GC content of the *mRb* primers) is not sufficiently stringent to detect point mutations in the primer sequence. Future experiments aimed at increasing the resolution of the technique are being planned.

All the *mRb* deletions found in BCF_1 lung adenocarcinomas were located at the 5′ end of the gene, involving fragments 1, 2, and 3, thus generating alterations in the N-terminus of the Rb protein. This result is in contrast to results obtained in human retinoblastomas, in which deletions have been found throughout the entire gene (Dunn *et al.* 1989; Kloss *et al.* 1991). The functional significance of 5′ deletions in the mouse adenocarcinomas is not known. The Rb protein is a DNA-binding protein (Stanbridge 1990), and association of Rb protein with viral transforming proteins, such as SV40 large-T antigen or adenovirus E1A protein, inactivates it (Kaelin *et al.* 1990). The *mRb* deletions observed in the tumors examined in this report could cause a total absence of *mRb* protein in cells or deletion of a portion of the protein required for a critical cellular function. A region of the amino acid sequence in human Rb protein from residues 379 to 792 has been identified as being required for binding of Rb protein to SV40 large-T antigen and E1A protein (Kaelin *et al.* 1990). However, the deletion of *mRb* gene fragments 1 and 2 in mice is not in this region (they would cover amino acid residues 169 to 347). The presence of so many deletions at the N-terminal end of the Rb protein is highly suggestive that the presence of this region is essential for the normal function of Rb protein in mice.

Contrary to our expectation in designing this study, all but one *mRb* deletion were found in spontaneous tumors rather than in tumors from irradiated mice. It is obvious that deletions in the *mRb* gene occur commonly in the absence of irradiation. Our data suggest that γ- or neutron-irradiation does not cause an increased frequency of *mRb* deletions. The observed increase in frequency of lung adenocarcinomas upon γ- or neutron-irradiation of mice (Grahn *et al.* 1990, 1992) is apparently the result of a mechanism other than *mRb* deletion. Experiments are underway to examine these tissues for lesions in other dominant and suppressor oncogenes.

5. REFERENCES

Bernards, R., G.M. Schackleford, M.R. Gerber, J.M. Horowitz, S.H. Friend, M. Schartl, E. Bogenmann, J.M. Rapaport, T. McGee, T.P. Dryja and R.A. Weinberg (1989) Proc. Natl. Acad. Sci. USA **86**, 6474–6478.

Borek, C. (1979) Radiat. Res. **70**, 209–232.

Dunn, J.M., X. Zhu, B.L. Gallie and R.A. Phillips (1989) in: Current Communications in Molecular Biology, Recessive Oncogenes and Tumor Suppression, W. Cavanee, N. Hastie and E. Stanbridge (Eds.) Cold Spring Harbor Laboratory Press, New York, pp 93-100.

Elkind, M. M. and A. Han (1979) Radiat. Res. **79**, 233–240.

Goeltz, S.E., S.R. Hamilton and B. Vogelstein (1985) Biochem. Biophys. Res. Comm. **130**, 118–126.

Grahn, C., J.F. Thomson and B. Carnes (1990) U. S. Nuclear Regulatory Commission publication NUREG/CR-4704 (ANL-86–33), Vol. 3.

Grahn, D., L.S. Lombard and B.A. Carnes (1992) Radiat. Res. **129**, 19–36.

Grahn, D., E.J. Ainsworth, F.S. Williamson and R.J.M. Fry (1972) in: Radiobiological Applications of Neutron Irradiation, International Atomic Energy Agency, Vienna, pp 211–228.

Hutchinson, F. (1985) Progr. Nucleic Acid Res. Mol. Biol. **32**, 115–125.

Impraim, C.C., R.K. Saiki, H.A. Erlich and R.L. Teplitz (1987) Biochem. Biophys. Res. Comm. **142**, 710–716.

Kaelin, W.G., M.E. Ewen, and D.M. Livingston (1990) Mol. Cell. Biol. **10**, 3761–3769.

Kennedy, A.R., J. Cairns and J.B. Little (1984) Nature **307**, 85–86.

Kloss, K., P. Wahrisch, V. Greger, E. Messmer, H. Fritze, W. Hopping, E. Passarge and B. Horsthemke (1991) Amer. J. Med. Genet. **39**, 196–200.

Schatz, D.G., M.A. Oettinger and D. Baltimore (1989) Cell **59**, 1035–1048.

Shibata, D.K., N. Arnheim and W.J. Martin (1988) J. Exp. Med. **167**, 225-230.

Shigematsu, I. and A. Kagan (1986) GANN Monograph on Cancer Research, **32**, Scientific Societies Press, Tokyo, Japan, and Plenum Press, New York.

Stanbridge, E.J. (1990) Ann. Rev. Genet. **24**, 615–657.

United Nations Scientific Committee on the Effects of Atomic Radiation. (1982) Report to the General Assembly, with annexes, United Nations, New York.

United Nations Scientific Committee on the Effects of Atomic Radiation. (1977) Report to the General Assembly, with annexes. United Nations, New York.

Upton, A.C. (1984) in: Radiation Carcinogenesis: Epidemiology and Biological Significance, J.D. Boice and J.F. Fraumeni (Eds.) Raven Press, New York, p 9.

Williamson, F.S. and N.A. Frigerio (1972) in: Proceedings of the First Symposium on Neutron Dosimetry in Biology and Medicine, G. Burger, M. Schraube and H.G. Ebert (Eds.) Commission of the European Communities, Luxembourg, pp 743–755.

Wright, D.K. and M.M. Manos (1990) in: PCR Protocols: a Guide to Methods and Applications, M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.) Academic Press, San Diego, pp 153–158.

Yunis, J.J. (1983) Science **221**, 227–236.

DISCLAIMER

Table 1. Amplified segments in *mRb* gene

Segment #	Sense Primer	Antisense Primer	Length of Segment Amplified (bp)
1	601–620	701–720	120
2	901–920	1101–1120	220
3	1501–1520	1561–1580	80
4	2001–2020	2146–2165	165
5	2481–2500	2572–2591	111
6	3501–3520	3575–3594	94

Table 2. *mRb* deletions detected in BCF_1 mouse lung adenocarcinomas

Irradiation condition	Adenocarcinomas tested[a]	*mRb* deletions found [number of (%)]
Unirradiated	18	6 (33)
γ-irradiated[b]	6	1 (17)
Neutron-irradiated[c]	6	0 (0)

[a]Deletions were not found in 12 normal lung tissues from irradiated and 6 normal lung tissues from untreated mice.

[b]Animals received a single 569-cGy dose of ^{60}Co γ rays in 20 min.

[c]Animals received a single 60-cGy dose of JANUS fission-spectrum neutrons in 20 min.

END

DATE
FILMED
6/11/93