Radiation and Chemical Activation of ras Oncogenes in Different Mouse Strains

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A survey of a large series of radiation- or chemically induced thymic lymphomas in (AKR × RF)F₁, RF/J, 129/J, and C57BL/6J mouse strains for activated ras oncogenes showed that of the tumors containing transforming activity, in more than 75% of the cases this activity segregated with either K-ras or the N-ras gene. H-ras activity was never detected. The genetic background of the host influenced susceptibility to tumor induction and oncogene activation. The K-ras gene was preferentially activated over the N-ras gene (approximately 2:1) whether the inducing agent was radiation or the chemical N-nitrosoethylurea. The activating mutation for the K-ras gene was consistently identified as a GGT to GAT transition in codon 12. In contrast, several different mutations of the N-ras gene were identified and localized to codons 12, 13, or 61. Assessment of the allelic composition of the ras locus shows that some proportion of the tumors lost the normal ras allele.

Introduction

Induction of genetic alterations in DNA of mammals by carcinogenic agents has long been a useful model system for understanding the events in cancer development. Somatic mutations of specific cellular genes are known to occur in human tumors and in a variety of experimental animal tumors. Cellular genes belonging to the ras gene family have been repeatedly identified in a significant proportion of these human and animal tumors (1,2).

The ras gene family is comprised of three genes that have homology in their DNA sequences. Two members were identified in the oncogenic Harvey (H-ras) and Kirsten (K-ras) rat sarcoma viruses. The third member of the family, N-ras, was isolated from a human neuroblastoma. This family of genes encodes a protein known as p21 due to its mobility upon gel electrophoresis. It is highly conserved in evolution and is normally expressed in mammals in a tissue-specific manner (3,4).

The function of the p21 proteins is largely unknown. However, the fact that they are so highly conserved in evolution implies an important role in cell growth or differentiation or both. The present evidence is for a role in signal transduction (1). The ras proteins are located at the inner surface of the cell membrane. Here they may act as messengers to transmit signals received from outside the cell from hormones and growth factors to inside the cell where the signal is translated as a message for cell division.

The mechanism of carcinogen-induced oncogene activation is not understood. The genetic lesions that result in activation of the ras cellular gene are single point mutations. The presence of the mutation leads to a substitution of amino acids that have been identified at positions 12, 13, 61, or 117 of the p21 protein (1,5,6).

Experimental Animal Model of Tumor Induction

In an attempt to understand some of the events involved in carcinogen-induced tumors in man, we have chosen to study tumor induction in inbred strains of mice. Ionizing radiation, neutron radiation, and the DNA alkylating agent N-nitrosomethylurea (NMU), all known to produce tumors in experimental animals, were chosen for study. The inbred strains RF/J, C57BL/6, 129J, BALB/c, and the F₁ hybrid between AKR/J and RF/J used in these studies each provide different genetic back-
grounds to determine the influence of the host on tumor induction by the different agents.

Thymic lymphomas were induced in these strains of mice with high frequency using well-defined treatment protocols. Ionizing gamma radiation was given according to the Kaplan protocol of 4 weekly doses of 150 to 175 rads/dose beginning at 4 weeks of age (7). High LET ionizing neutron radiation was used to treat 4-week-old RF/J female mice once with 100 rads of 0.4 MeV neutrons. NMU treatment consisted of 5 weekly injections IP (30 mg/kg) of 4-week-old animals (8).

Several questions concerning tumor initiation and progression can be addressed using animal model systems. Use of genetically identical mice of different strains treated with the same carcinogenic agents will allow one to determine whether the genetic background of the host influences oncogene activation. A second question that is of interest is whether treatment of the same strain of mice with different carcinogenic agents produces activating mutations that are carcinogen specific. Last, since cancer development is believed to be a multistage process, we would like to determine at what stage of tumor development ras genes are involved.

Methods for Detecting Transforming DNA Sequences

The ability to detect transforming DNA sequences or activated oncogenes is essential to the success of studying their role(s) in tumor development. The biological activity of oncogenes is measured in assays where tumor-derived DNA is introduced into mouse fibroblasts. The presence of a transforming DNA sequence is scored by a change in morphology of the mouse fibroblast after gene transfer by focus formation in 5% calf serum. Under these conditions of selection, transformed cells have a growth advantage over normal cells.

Another method that is able to detect transforming genes that lack the ability to induce focus formation in mouse fibroblasts is outlined in Figure 1. Tumor DNA is cotransfected with a dominant selectable biochemical marker: Cells that take up the foreign DNA will survive in the presence of the antibiotic G418 and form colonies. After 2 weeks, the colonies are harvested for inoculation into nude mice. Tumor formation is associated with the presence of transforming DNA sequences (9).

The transformation arising in either the focus assay or the tumorigenicity assay are then subjected to molecular analysis to characterize the oncogene (ras or non-ras) and its activating mutation. Because the DNA gene transfers were intraspecific, positive 3T3 transformants were screened by Southern blot analysis for the presence of DNA rearrangements which occur when transfected DNA sequences integrate into the NIH 3T3 genome. The ras genes are detected in high frequency in these 3T3 transformants as additional DNA bands over the endogenous ras germline pattern. Amplification of the acquired ras sequences is frequently observed in positive transformants.

Methods for Identifying Activating ras Mutations

The genetic lesions that activate ras genes have been identified as single point mutations in each of the ras genes at similar sites. The codons 12, 13, or 61 are most frequently involved in this family of oncogenes. Three methods have been used in these studies to identify the activating mutations in the K- and N-ras genes characterized by Southern blot analysis. Two N-ras positive transformants were cloned, and the ras DNA fragments were sequenced to determine the activating mutations (10). The presence of certain mutations is known to change a restriction enzyme site, thus creating DNA fragment polymorphisms that are easily detected in
Southern blots. The most widely used method has been oligonucleotide mismatch hybridization (11). This method is based on the difference in melting temperature of a perfectly matched DNA—DNA hybrid relative to that of a hybrid with a single base pair mismatch. Synthetic oligonucleotides of 19 bases complementary to the normal 12th, 13th, or 61st codon of the N-ras gene and to the normal 12th or 61st codon of the K-ras gene were prepared. In addition, oligomers of 19 bases were prepared for all possible mutations of these codons, each with a base substitution at the middle position #10 of the oligomer. The mutations were first identified in the NIH 3T3 transformants and then in the primary lymphoma which served as the original source of the transforming DNA.

Results
Tumor Induction and Detection of Transforming Activity

The inbred strains RF/J, 129/J, C57BL/6J, and the F1 hybrids of (AKR/J × RF/J) mice were treated with the Kaplan protocol of four fractionated doses of gamma radiation or with five NMU injections at weekly intervals. Thymic lymphomas were induced with high frequency in all groups of mice by both carcinogenic treatments with only one exception. 129/J mice were resistant to tumor induction by gamma radiation but highly susceptible to thymic lymphoma induction by NMU treatment.

Thymic lymphomas have also been induced in RF/J mice by neutron irradiation. The frequency (65%) was somewhat lower than that observed for radiation-induced tumors (> 90%). The latent period of 6 months, however, was similar for both radiation-induced tumors in this strain.

Tumor DNAs were screened for transforming activity in NIH 3T3 cells using the calcium-phosphate method of DNA-mediated gene transfer (12). Either the focus assay or the nude mouse tumorigenicity assay (Fig. 1) was used. DNAs from carcinogen-induced thymic lymphomas of different mouse strains varied in overall transforming activity. Approximately 85% of the thymic lymphoma DNAs from RF/J and 129/J mice were active in the focus-forming assay (10). With the same assay, 60 to 80% of carcinogen-induced tumors of (AKR times RF)F1 mice produced 3T3 transformants (13). Tumors induced in C57BL/6J mice were analyzed in parallel in both assays. The nude mouse assay detected transforming activity in 80% of both groups of carcinogen-induced tumors, whereas the focus assay was less sensitive. None of the DNAs tested from radiation-induced thymic lymphomas of C57BL/6J mice produced foci whereas 50% of the DNAs from NMU-induced tumors were active (14).

Identification of the Carcinogen-Activated Oncogene and Its Distribution

Initially the DNAs from the 3T3 transformants were screened for ras sequences (H-, N-, and K-ras), as these oncogenes are most frequently detected in this assay. A total of 58 tumors from all four strains of mice produced 3T3 transformants. Of these, 78% had transforming activity that segregated with either the K-ras (28/58) or the N-ras (17/58) gene. In no instance has H-ras activity been detected in any carcinogen-induced thymic lymphomas.

The remaining 22% (13/58) of the transformants did not appear to be positive for any activated ras gene by Southern blot analysis and p21 protein analysis. Preliminary screening of these transformants with a panel of known oncogene probes shows that they are negative for myb, myc, neu, and raf.

The frequency distribution of activated ras oncogenes in DNAs from NMU- or radiation-induced thymic lymphomas of different mouse strains is summarized in Table 1. The K-ras gene was preferentially activated, being present in 50% of the DNAs from carcinogen-induced tumors. Activation of the N-ras oncogene was observed in 29% of the tumor DNAs. The non-ras transforming activity present in the last group of transformants remains to be identified.

The results of this survey would suggest that host genes can influence the frequency of ras gene activation as well as the type of ras gene, which becomes activated as a result of carcinogen treatment. NMU treatment of 129/J or RF/J mice produced activation of K- and N-ras genes with similar frequencies. In contrast, C57BL/6J or (AKR × RF)F1 mice treated similarly with NMU show preferential activation of K-ras or N-ras genes, respectively. Likewise, radiation treatment of RF/J and C57BL/6J mice produces tumors in which the K- and N-ras genes are activated in equal proportions. On the other hand, the K-ras gene is preferentially activated in (AKR × RF)F1 mice following radiation treatment.

Table 1. Frequency distribution of ras oncogene activation in carcinogen-induced thymic lymphomas in different mouse strains.

| Strain of mouse | Treatment of animal | Characterization of oncogenes* |
|-----------------|---------------------|-------------------------------|
|                 |                     | K-ras | N-ras | Non-ras | Non-Mu | H-ras | N-Mu | K-ras | N-ras | non-ras |
| (AKR × RF)F1    | γ-rays              | + + + + |      | -      | -      |       |       |       |       |       |
|                 | N-methylnitrosourea|       | + + + +| -      | -      |       |       |       |       |       |
| RF/J            | γ-rays              | + + | + | + | + + | + | + | + | + | + |
|                 | N-methylnitrosourea|       | + + + +| + + | + | + | + | + | + | + |
| C57BL/6J        | γ-rays              | + | + | + | + | + | + | + | + | + |
|                 | N-methylnitrosourea|       | + + + +| - | - | - | - | - | - | - |
| 129/J           | γ-rays              | NA | NA | NA | NA | NA | NA | NA | NA | NA |
|                 | N-methylnitrosourea| + + | + + | + | + | + | + | + | + | + |

Number analyzed, 58 tumors 28 17 13

*NIH 3T3 transformants were obtained in the focus forming assay or the nude mouse assay. DNAs from transformants were screened for specific K- and N-ras sequences by Southern blot analysis and by oligonucleotide mismatch hybridization.

**NIH 3T3 transformants negative for ras transforming sequences were screened with a panel of known oncogene probes.

†Frequency distribution of ras and non-ras transforming sequences in NIH 3T3 transformants. Number of transformants identified per total number of positive transformants analyzed: (+) 0–25%; (+ +) 26–50%; (+ + +) 51–75%; (+ + + +) 76–100%.

‡NA, not applicable. 129/J mice are resistant to tumor induction.
Analysis of Genetic Mutations in Activated ras Oncogenes

The ras gene point mutations were identified by screening the DNA from the 3T3 transformants using the oligonucleotide mismatch hybridization method. A total of 45 ras positive 3T3 transformants were analyzed and point mutations were identified in 30. Table 2 summarizes these results. Of 28 K-ras positive 3T3 transformants, 21 (75%) contained the G to A transition previously identified in codon 12 of the K-ras gene (15). This mutation substitutes aspartate (GAT) for glycine (GGT). The remaining 7 K-ras positive transformants contain a different mutation(s) which has yet to be identified.

The mutations occurring in the N-ras gene, although less frequently activated than the K-ras gene, were much more heterogeneous in their nature. A total of 17 N-ras positive transformants were analyzed. Mutations were identified in 9 (53%) transformants, which affected all three codons (12, 13, and 61).

Previously we have identified a CAA to AAA transition in the 61st codon of the N-ras gene that substitutes glutamine for lysine (16). One-third of the N-ras positive transformants contained this same mutation. An additional mutation was localized to codon 61 by cloning and sequencing (10). This was a CAA to CTA mutation changing glutamine for leucine.

Approximately half of the mutations occurred as GGT to GAT transitions in codon 12 (4 transformants) and in codon 13 (1 transformant). These mutations result in the substitution of aspartate (GAT) for glycine (GGT).

Unknown genetic factors appear to influence which ras gene becomes mutated, as well as the frequency with which specific codons are mutated. NMU treatment of C57BL/6 or (AKR × RF)F1, mice results in preferential activation of the K-ras and N-ras genes respectively. As shown in Table 2, the treatment of RF/J strain mice with two different agents, γ radiation and the chemical NMU, results in identical point mutations in the 12th codon of the K-ras gene or the N-ras gene, arguing against carcinogen specific mutations in this mouse strain.

The K-ras gene is most frequently identified as the transforming sequence in carcinogen-induced tumors of all the strains of mice tested so far. The overwhelming majority of the transformants contain the identical mutation in codon 12, a GGT to GAT transition, irrespective of the inducing agent used.

Somatic Loss of Normal ras Allele in Carcinogen-Induced Thymic Lymphomas

Homozygosity of abnormal genes is a frequently observed phenomenon in several human malignancies (17-19). We have reported loss of the normal N-ras allele in one (AKR × RF)F1, NMU-induced thymic lymphoma (20).

We therefore examined the allelic composition of some of the DNAs from carcinogen-induced thymic lymphomas that transferred activated K-ras genes. Using oligonucleotide mismatch hybridization with oligomers that recognize the normal and the mutated DNA sequences, primary tumors can be screened for the dosage of the allele variants. Four tumors analyzed to date show homozygosity for the abnormal K-ras allele (10). Thus, it appears that tumors with activated ras genes have a tendency to lose the normal copy and/or increase the number of copies of the mutant allele (Fig. 2). This might be one of the steps involved in tumor progression.

A working model of the changes that may occur in vivo in ras genes following carcinogen exposure is shown in Figure 2. Initiation, the exposure to carcinogenic agents, may result in mutation of the K- or N-ras genes directly or indirectly through the action of other genes. Mutations that are relevant biologically, i.e., confer a growth advantage to a cell, are selected in vivo. During tumor progression the mutated allele undergoes duplication. Subsequently, in some percentage of the tumors, the normal ras allele is lost.

Conclusions and Perspectives

Inbred strains of mice have been treated with different carcinogenic agents, gamma or neutron radiation, and N-nitrosomethylurea. These agents induce thymic lymphomas with high frequency. Activation of oncogenes of

Table 2. Frequency distribution of point mutations identified in activated ras oncogenes by mismatch hybridization.

| Strain of mouse | Treatment of animal | K-ras codon 12 | Unknown | N-ras 12 | 13 | 61 | Unknown |
|----------------|---------------------|---------------|---------|---------|---|---|---------|
| (AKR × RF)F1   | γ-rays              | + + + b       | +       | -       | - | - | -       |
| RF/J           | N-methylnitrosourea| -             | -       | +       | + | + | + +     |
| C57BL/6J       | γ-rays              | + + +         | +       | -       | - | - | + +     |
| 129/J          | γ-rays              | + + +         | +       | -       | - | + | + +     |

Characterization of mutation

| Number analyzed, 45 tumors | 21 | 7 | 4 | 1 | 4 | 8 |

*Unknown mutation.

bFrequency distribution of ras mutations in ras-positive NIH 3T3 transformants. Number of K- or N-ras mutations identified per total number of K- or N-ras positive transformants analyzed: (+) 0-25%; (+ +) 26-50%; (+ ++) 51-75%; (+ +++ ) 76-100%.

cNA, not applicable. 129J mice are resistant to tumor induction.
the ras gene family have been systematically surveyed in these tumors. The main conclusions from these studies are:

a) The genetic background of the host modifies the susceptibility of a particular mouse strain to the action of a particular carcinogen (i.e., 129/J mice are resistant to γ irradiation).

b) The only ras oncogenes detected are K-ras and N-ras.

c) Oncogene activation is not carcinogen specific (i.e., the same carcinogen activates different ras genes in different mouse strains) but is modified by unknown host genes.

d) Activation of the K-ras gene occurs predominantly by a point mutation in codon 12 resulting in a GGT to GAT transition.

e) Activation of the N-ras gene occurs by point mutations in codons 12, 13, or 61.

f) Somatic loss of the normal N-ras or K-ras gene together with duplication of the mutated ras gene occurs during tumor progression.

Our previous studies have used protocols requiring multiple exposure of the animals to the carcinogen. More recently, C57BL/6J, RF/J and 129/J mice were treated with a single exposure to NMU (80 mg/kg). Similarly, RF/J mice received a single exposure to neutron radiation. Thymic lymphomas were induced in NMU-treated C57BL/6J mice and neutron-irradiated RF/J mice. We want to examine the frequency of oncogene activation in these tumors and identify the mutations. In addition, we hope to be able to determine more precisely the timing of the events outlined in Figure 2 that occur in high frequency in ras genes in this model system of carcinogen-induced murine thymic lymphoma.

This research work was supported by Public Health Service grants CA 40583 and CA 36327 and ES 03847 from the National Institutes of Health. A. P. is a Leukemia Society of America Scholar.