Analysis of Transforming Genes in Indirectly Induced Radiogenic Thymomas in Mice

OHTSURA NIWA1), MASAHIRO MUTO2), FUMIO SUZUKI3), RYO KOMINAMI4) AND KENJIRO YOKORO1)

1 Department of Pathology, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Minami-ku, Hiroshima 734, Japan
2 Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa, Chiba 260, Japan
3 Division of Radiation Biology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920, Japan
4 Department of Biochemistry, Faculty of Medicine, Niigata University, Niigata 951, Japan

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The expression of oncogenes was studied in 12 types of 178 mouse tumors induced by radiations and chemicals. DNA was analyzed in tumors in which the overexpression of oncogenes was noted. Amplification of the myc oncogene was found in chemically induced sarcomas, but not in sarcomas induced by radiation. Activation of oncogenes by small mutations and the inactivation of tumor suppressor genes has to be taken in account in the radiation induction of mouse tumors. We therefore made further analyses of radiogenic thymomas. Loss of heterozygocity was revealed in directly induced thymomas by the deletions of allele specific minisatellite bands. Analysis of a hypervariable minisatellite locus also revealed that these thymoma cells suffered high recombinogenic activity during tumorigenesis. In addition, transfection of cellular DNA to normal Golden hamster cells identified the activated K-ras oncogene in the directly induced radiogenic thymomas. Indirectly induced radiogenic thymomas were tested similarly. Transformed cells from secondary transfection experiment were positive for the mouse-specific repetitious sequences, but devoid of mouse ras oncogenes. Indirectly induced radiogenic thymomas originate from unirradiated normal thymus cells transplanted in irradiated hosts. The spontaneous activation of oncogenes yet to be identified may therefore be involved in the development of this tumor.

INTRODUCTION

The activation of oncogenes has been shown to function in the development of human tumors as well as in the chemically induced tumors of laboratory animals. Moreover, inactivation of tumor suppressor genes, as revealed by the loss of heterozygocity, is now well documented for human tumors. Tumorigenesis triggered by ionizing radiation has been an ongoing subject of intensive study in humans and experimental animals. Most of
the human studies, however, have been on epidemiological analyses of exposed populations. Experimental studies of radiation carcinogenesis in animals and in tissue culture cells have relied mainly on kinetic analyses of the process of tumorigenesis and cellular transformation\(^1\). Therefore, the molecular mechanism by which radiation induces tumors is still largely unknown.

Radiation is an efficient inducer of DNA breaks which lead to the rearrangement and deletion of genes rather than point mutation\(^2\). Interestingly, the ras oncogene activated by point mutations has been demonstrated in tumors induced by radiation\(^3-6\). These mutations may be the direct result of DNA damage caused by radiation, or they may have arisen independent of DNA damage during the development of radiation-initiated tumors. Analysis of molecular changes in the various types of radiation-induced tumors is necessary if we are to understand the role of radiation in carcinogenesis.

We surveyed the oncogenes of various types of mouse tumors induced by radiation and chemicals. The amplification and rearrangement of these oncogenes were rare in radiogenic mouse tumors. We also analyzed the loss of heterozygocity, the mutations at a minisatellite locus and the activation of oncogenes in thymomas induced directly or indirectly by X-rays, the results of which analyses are reported here.

**MATERIALS AND METHODS**

**Mice**

C57BL/6N x C3H/He F\(_1\) (BCF\(_1\)) mice and Balb/c mice were purchased from Charles River Japan, Inc., Atsugi, Kanagawa. Balb/c nu/nu mice were the gift of Dr. A. Matsuzawa, Institute of Medical Science, University of Tokyo. NFS mice and C3H/He x C57BL/6N F\(_1\) (CBF\(_1\)) mice were bred in the animal facility of our institute.

**Cells and cell culture**

GHE cells, from a normal fibroblastic cell line established from Golden hamster embryos, have been described elsewhere\(^7\). These cells were cultured in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum. They were transfected with pSV2neo and cellular DNA isolated from mouse thymomas as described elsewhere\(^8\).

**Induction of solid tumors**

Seven- to eight-week-old mice were treated with a variety of chemicals and radiations. Mice were autopsied when moribund. Tumors were excised, a part being processed for histological examination and the rest stored at \(-70^\circ\)C for further analysis.

The chemicals used and modes of application have been reported elsewhere\(^9\). Briefly, methylcholanthrene (MCA) was dissolved in olive oil, then injected subcutaneously into mice\(^10\). Sarcomas were induced with alpha-tocopherol (TP) using to the procedure described elsewhere\(^11,12\). Nitrosoethylurea was dissolved in 10% ethanol and administered orally through polyethylene tubing\(^13,14\). Mice were fed a basal diet containing 0.05% pheno-
barbital. Diethylene glycol was added at 0.5% and diethylenenitrosoamine at 0.01% to the drinking water. All the tumors induced by these agents were categorized as chemically induced tumors (chem).

Tumors also were induced by a variety of radiations (rad). Mice were irradiated with $^{252}$Cf neutrons, X-rays and $^{60}$Co gamma rays in the irradiation units at our institute. Tritiated water was injected intraperitoneally into the mice.

Radiogenic Thymomas

Directly induced thymomas were those that occurred in mice irradiated four times with 1.7 Gy of X-rays at weekly intervals. Indirectly induced thymomas were those derived from normal thymocytes grafted to thymectomized and irradiated thy-1 congenic B10 mice. The indirect tymomas used had been serially transplanted in syngenic mice.

Purification of DNA and RNA

Tumor tissues were rapidly frozen by immersing them in liquid nitrogen, after which they were ground to a fine powder in a mortar. DNA and RNA were purified from these ground tissues as described elsewhere.

DNA and RNA hybridization

Cellular DNA was digested with the appropriate restriction enzymes then processed for Southern blotting hybridization. The total cellular RNA was denatured in 10% formaldehyde then layered on membrane filters for dot blot analysis. $^{32}$P-labelled probes were made by the random primer method.

Probes

The probes used in the analysis of the oncogenes were v-H-ras, v-K-ras, mouse c-myc, v-myb, v-fos, v-sis, v-abl, v-erbB, human erbB2, v-fps, v-fes, v-src, v-yes, human syn, v-fms, v-erbA, mouse pim-1 and human ret. A mouse minisatellite clone, Mo-2L, was cloned from a mouse genomic library (R. Kominami unpublished). The hypervariable minisatellite locus, Pc-1, has been described elsewhere. A mouse-specific probe, 7014, containing a mouse B1 repeat and a mouse L1 sequence was used to detect the mouse sequences in transfected GHE cells.

RESULTS

Expression and amplification of oncogenes in mouse tumors

The 177 tumors examined could be classified into 12 types (Table 1). They were derived mainly from BCF1 mice, but other strains are included; 105 of these tumors were induced by radiations (rad) and 72 by chemicals (chem). The expression of 18 oncogenes were studied by dot blot hybridization of the total cellular RNA from these tumors. RNA from mouse embryo fibroblasts was used as the standard, and overexpression was scored when the expression of an oncogene was higher in the tumor cells than in the fibroblasts.
Table 1. Overexpression of oncogenes in mouse tumors

| tumor      | inducer | no. cases | Hras | Kras | myc | myb | fos | sis | abl | erbB | erbB2 | fps | fes | src | yes | syn | fms | erbA | piml | ret | MuLV |
|------------|---------|-----------|------|------|-----|-----|-----|-----|-----|------|-------|-----|-----|-----|-----|-----|-----|------|------|-----|------|
| sarcoma    | chem    | 31        | 0    | 2    | 16  | 0   | 4   | 0   | 5   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 4    |
|            | rad     | 8         | 1    | 0    | 4   | 0   | 0   | 0   | 1   | 1    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
| ovarian    | rad     | 28        | 0    | 0    | 7   | 0   | 6   | 0   | 2   | 0    | 1     | 0   | 0   | 0   | 8   | 0   | 0   | 0    | 0    | 0   | 0    |
| hepatoma   | chem    | 21        | 0    | 0    | 1   | 0   | 2   | 0   | 0   | 2    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
|            | rad     | 7         | 0    | 0    | 0   | 0   | 0   | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
| mammary    | chem    | 1         | 0    | 0    | 0   | 0   | 0   | 0   | 1   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
|            | rad     | 11        | 0    | 0    | 4   | 0   | 1   | 0   | 1   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 2    |
| lung       | chem    | 1         | 0    | 0    | 0   | 0   | 0   | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
|            | rad     | 3         | 0    | 0    | 0   | 0   | 1   | 0   | 0   | 1    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
| thyroid    | rad     | 14        | 2    | 0    | 0   | 0   | 2   | 0   | 0   | 0    | 2     | 0   | 0   | 0   | 3   | 0   | 0   | 0    | 0    | 0   | 0    |
| lymphosarcoma | rad | 4         | 0    | 0    | 3   | 0   | 0   | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
| pituitary  | rad     | 4         | 0    | 0    | 2   | 0   | 2   | 0   | 0   | 0    | 3     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
| stomach    | chem    | 3         | 1    | 2    | 0   | 0   | 0   | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
| adrenal    | chem    | 1         | 0    | 0    | 1   | 0   | 0   | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 1   | 0   | 0   | 0    | 0    | 0   | 0    |
|            | rad     | 1         | 0    | 0    | 1   | 0   | 1   | 0   | 1   | 1    | 0     | 0   | 0   | 0   | 1   | 0   | 0   | 0    | 0    | 0   | 0    |
| thymoma    | chem    | 14        | 0    | 0    | 9   | 2   | 0   | 0   | 3   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 3    |
|            | rad     | 17        | 0    | 0    | 11  | 1   | 0   | 0   | 4   | 0    | 0     | 0   | 0   | 0   | 1   | 0   | 0   | 0    | 0    | 0   | 2    |
| leukemia   | rad     | 8         | 0    | 0    | 5   | 2   | 0   | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0    | 0    | 0   | 0    |
| subtotal   | chem    | 72        | 1    | 4    | 27  | 2   | 6   | 0   | 9   | 2    | 0     | 0   | 0   | 0   | 1   | 0   | 0   | 0    | 0    | 0   | 7    |
|            | rad     | 105       | 3    | 0    | 37  | 3   | 13  | 0   | 9   | 3    | 6     | 0   | 0   | 12  | 0   | 0   | 0   | 0    | 0    | 0   | 4    |
| total      |         | 177       | 4    | 2    | 64  | 5   | 19  | 0   | 18  | 5    | 6     | 0   | 0   | 13  | 0   | 0   | 0   | 0    | 0    | 0   | 11   |
Table 1 clearly shows that overexpression of the myc oncogene was frequent in a variety of tumors (64 of the 177) as were the fos (19 of the 177) and abl (18 of the 177) oncogenes. We therefore analyzed tumor DNA for the possible rearrangement and amplification of these three oncogenes. There were no changes in the fos and abl oncogenes in tumors which had elevated levels of expression; whereas, amplification of the c-myc gene was detected in 7 of 31 chemically induced sarcomas, but not in 8 sarcomas induced by radiations (Table 2). More cases of radiation-induced sarcoma must be investigated in order to verify the significance of these findings. Nevertheless, these results indicate that gross changes in known oncogenes are not common in radiation-induced mouse tumors. These tumors may well have loss of function of tumor suppressor genes or carry oncogenes activated by small mutations. We therefore made a more detailed analysis of the radiogenic thymomas.

**Analysis of radiogenic thymomas with minisatellite probes**

Two types of radiation-induced mouse thymomas were given further analysis. Nine directly induced thymomas from BCF1 mice and 2 from B10 mice that had formed in mice irradiated with 4 times of 1.7 Gy X-rays were analyzed. Indirectly induced thymomas from B10 mice which originated from normal thymocytes grafted in the irradiated hosts also were analyzed. Both types of thymoma expressed the c-myc gene at a high level without changes to the gene structure (data not shown).

To study large mutations in thymomas, we used two types of minisatellite probe. The first, Mo-2L, detected multiple bands specific to each strain of mice, but the somatic and germline mutation rates was not high; therefore, this probe was suitable for detecting the deletion of chromosome segments. The second, Pc-1, was a locus-specific probe that detected germline and somatic mutations of the recombination type.

| Inducer        | Strain | Total cases | Number overexpressed | Number amplified |
|----------------|--------|-------------|-----------------------|-----------------|
| Chemically induced |        |             |                       |                 |
| MCA            | NFS    | 6           | 3                     | 1               |
|                | Balb nu/nu | 7           | 2                     | 2               |
|                | Balb/c | 4           | 1                     | 0               |
|                | CBF₁   | 7           | 6                     | 1               |
| TP             | NFS    | 1           | 1                     | 1               |
|                | BCF₁   | 6           | 3                     | 3               |
| Radiation induced |        |             |                       |                 |
|                | BCF₁   | 8           | 4                     | 0               |

Table 2. Amplification of the c-myc gene in sarcomas
When the DNA from directly induced thymomas of BCF1 mice was analyzed with the Mo-2L probe, the deletion of C3H/He-specific bands (designated T1 and T2) was frequently noted (Fig. 1, panel b and Table 3). A similar analysis of BCF1 sarcomas did not show these deletions (Fig. 1, panel c, and in a larger number of sarcomas analyzed in different experiments).

Results of analysis with the Pc-1 probe are shown in Fig. 2. When cut with the Hae III restriction enzyme, the germline Pc-1 locus gave a 9-kb band for C57BL/6N and a 3.5-kb one for C3H/He mice. In the directly induced thymomas, the band lengths of both alleles changed frequently. These changes in the Pc-1 locus indicate that the genomes of thymoma cells experienced a high recombinogenic activity during tumorigenesis.

This analysis was not made for indirectly induced thymomas because they were derived from homozygous B10 mice, and the loss of one of the two alleles in homozygote cells was difficult to identify under this condition.

Table 3. Analysis of ras oncogenes and minisatellite loci in radiogenic thymomas

| Thymomas       | c-myc overexpressiona) | Minisatellite locusb) | Activated K-rasc) |
|----------------|-------------------------|-----------------------|-------------------|
|                |                         | Mo-2L-T1 | Mo-2L-T2 | Pe-1 |
| directly induced |                         |            |          |      |
| 1 BC2729       | +                       | nt        | nt       | r    | –    |
| 2 BC2736       | +                       | nt        | nt       | r    | –    |
| 3 BC3826       | +                       | –         | –        | r    | –    |
| 4 BC3851       | +                       | nt        | nt       | –    | –    |
| 5 BC3878       | +                       | –         | d        | r    | –    |
| 6 BC3879       | +                       | –         | d        | d    | +    |
| 7 BC3910       | +                       | –         | d        | d    | –    |
| 8 BC3966       | +                       | d         | –        | d    | –    |
| 9 BC3971       | +                       | d         | –        | d    | –    |
| 10 B10-9B10    | +                       | nt        | nt       | nt   | –    |
| 11 B10-14B10   | +                       | nt        | nt       | nt   | +    |
| indirectly induced |                         |            |          |      |
| 1 B10-3D       | +                       | nt        | nt       | nt   | –    |
| 2 B10-5D       | +                       | nt        | nt       | nt   | –    |
| 3 B10-7D       | +                       | nt        | nt       | nt   | –    |
| 4 B10-20D      | +                       | nt        | nt       | nt   | –    |
| 5 B10-22D      | +                       | nt        | nt       | nt   | –    |
| 6 B10-23D      | +                       | nt        | nt       | nt   | –    |
| 7 B10-25D      | +                       | nt        | nt       | nt   | –    |
| 8 B10-26D      | +                       | nt        | nt       | nt   | –    |

a) +: overexpression observed.
b) nt: not tested, d: deletion and r: recombination.
c) +: activated K-ras of mouse origin present in transfected GHE cells.
Fig. 1. Analysis of Mo-2L-related minisatellite bands. DNA was digested with Hae III and analysed by Southern blotting with the Mo-2L probe. A: Analysis of DNAs from normal spleens of C57BL/6N (B6), C3H/He (C3), and BCF1 and CBF1 (F1) mice. B: Analysis of BCF1 thymomas of cases 3, 5, 6, 7, 8 and 9. C: Four cases of MCA-induced sarcomas. Open triangles indicate the T1 and T2 bands derived from C3H/He parents.

Transfection study

DNA was isolated from radiogenic thymomas and transfected onto normal GHE cells. The DNAs from both the directly and indirectly induced thymomas induced foci in transfected GHE cell cultures; whereas the DNA isolated from normal thymus did not. Foci were tested for the presence of mouse repetitious sequences and ras oncogenes. Some of the primary foci induced by DNA from the directly induced thymomas had the mouse K-ras oncogene, which suggests that this gene was activated by point mutation (Fig. 3).

Although cells from the primary foci induced by the DNA from indirectly induced thymomas carried mouse repetitious sequences, the mouse ras gene was not present in the transformed cells. Secondary transfection experiments were done on foci induced by the DNA of indirectly induced thymomas. During the study, transformed foci which did not carry mouse repetitious sequences frequently were encountered. These foci might have been induced spontaneously, or by mouse sequences in which mouse B1 repeats and L1
elements were absent. To assure isolation of foci which had received exogenous transfecting DNA, we co-transfected the pSV2neo gene together with cellular DNA of the primary foci and selected the resulting secondary foci in the presence of G418 sulfate. Independently isolated secondary foci carried a mouse-specific sequence that migrated as a 2.5-kb long band after digestion with Eco R1 restriction enzyme (Fig. 4).

Results of these analyses are summarized in Table 3.

DISCUSSION

Amplification of the c-myc oncogene was found in chemically induced sarcomas but not in those induced by radiations. Although DNA damage has been proposed as the
trigger for the over-replication and amplification of genes\textsuperscript{20}, the amplification of oncogenes has been thought to occur in the later stages of human malignancies\textsuperscript{21}. Therefore, lack of amplification of the c-myc gene in radiation-induced sarcomas may be the result of differences in the progression of tumors initiated by radiation and chemicals. Overexpression of c-myc, fos and abl oncogenes was frequent in radiation-induced tumors that showed no changes in gene structure. The elevated expression of these genes must therefore be controlled at the transcriptional level.

Fig. 3. Presence of the activated K-ras oncogene in directly induced radiogenic thymomas. DNA was isolated from normal GHE cells (GHE). Primary foci were induced by BCF1 thymoma 3971 (3971) and B10 thymoma 14B10 (14B10). DNA was cut with Eco R1 and hybridized with the v-K-ras probe, HiHi3. The open triangle shows the position of the major band for the mouse-specific K-ras oncogene.
Fig. 4. Presence of a mouse repetitive sequence in secondary transfected foci. DNAs from a primary focus (1) and four secondary foci derived from it (1-1 to 1-4) were digested with Eco RI and hybridized with a mixture of the mouse-specific 7014 probe and pSV2neo. The small triangles indicate the hybridization to pSV2neo. The large triangle indicates a mouse-specific band which present in the DNAs of 1-2 and 1-4.

The presence of the activated K-ras oncogene in directly induced mouse thymomas is consistent with previous reports \(^3,^4\). The induction of small mutation does take place after exposure to ionizing radiations\(^2^2\). The greatest numbers of mutations induced by ionizing radiations, however, are deletions and rearrangements\(^2^3\). Therefore we speculate that the mutation responsible for the activation of the K-ras oncogene in thymomas may have been induced spontaneously independent of the damage induced by irradiation.

In addition to activated ras oncogene, we found deletions of the specific restriction fragments, T1 and T2 in directly induced thymomas. Interestingly, these bands are derived from C3H/He mice that were resistant to radiation induction of thymomas. Deletion of the
T1 and T2 bands may indicate that these bands naturally tend to be spontaneously deleted. But, BCF1 sarcomas were found to have deletion of Mo-2L-related bands that differ from T1 and T2, indicative of tumor type specificity in the deleted bands. Therefore, deletion of the DNA regions near T1 and T2 probably has a role in the development of thymomas in irradiated mice. DNA regions corresponding to the T1 and T2 bands must be cloned in order to clarify the significance of these findings.

A hypervariable minisatellite probe, Pc-1, revealed that thymoma cells have high recombinogenic activity during transformation, the reason for which is unknown at present. But, because such changes are never observed in normal cells, this activity indicates that thymoma cells must be genetically unstable. The Pc-1 locus has been reported to have a high rate of germline and somatic mutations\(^{18,24}\); nevertheless, the frequency of mutation found in the experiments reported here is far higher than that of normal cells.

Minisatellite sequences\(^{25}\) are thought to be distributed on a variety of mouse chromosomes and show strain-specific variations\(^{18}\). Our study has demonstrated the usefulness of minisatellite probes for detecting the loss of heterozygocity and genetic instability of tumors in interstrain F1 mice.

Because the activated ras oncogene was present in the directly induced thymomas and radiation tends to cause large mutation, we speculate that the point mutation of the ras gene might have occurred spontaneously during the development of the thymomas. Indirectly induced thymomas are indistinguishable in their histology and biological characteristics from directly induced ones. If activation of the ras oncogene is spontaneous in directly induced thymomas, a similar situation would be expected for indirectly induced thymomas. We did not detect any activated ras oncogenes in the transformants produced by the DNA from indirectly induced thymomas; but, the presence of mouse-specific repetitious sequences in the secondary transformants of GHE cells indicates that activated oncogenes of an unidentified nature might function in the formation of indirectly induced thymomas which arise from unirradiated thymus grafted in the irradiated host. Whatever the mutation and the oncogene responsible for development of indirectly induced thymomas might be, activation would have to occur during the conversion of normal thymocytes to thymomas; therefore, the host environment of irradiated mice is mutagenic to grafted thymocytes. It was once thought that mutation in the host is caused by retroviruses activated by radiations\(^{26}\); but, retroviral involvement in the formation of radiogenic thymomas can be excluded on the basis of recent experiments\(^{15}\). The possibility, however, remains that there are transmissible agents distinct from retroviruses\(^{27,28}\). The cloning of the mouse-specific sequence in the secondary transformant of GHE cells should reveal the nature of the mutation responsible for the puzzle of indirectly induced thymomas in mice.

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