Validation of Transgenic Mice Carrying the Human Prototype c-Ha-ras Gene as a Bioassay Model for Rapid Carcinogenicity Testing

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Carcinogenicity testing is indispensable for identifying environmental carcinogens and for evaluating the safety of drugs in the process of development. Conventional 2-year rodent bioassays are one of the most resource-consuming tests in terms of animals, time, and costs. Development of rapid carcinogenicity testing systems that can assess carcinogenicity within a short period has become a social demand and is essential to improve efficacy in the identification of environmental carcinogens as well as in the development of new drugs. In this review we introduce the rapid carcinogenicity testing system using transgenic (Tg) mice carrying the human prototype c-Ha-ras gene, namely rasH2 mouse (CB6F1-TgHras2 mouse is the same mouse). The studies have been conducted to validate the rasH2 mouse as a model for the rapid carcinogenicity testing system. Our current validation studies revealed that rasH2 mice are able to detect various types of mutagenic carcinogens within 6 months. The rasH2 mice may also be able to detect various nonmutagenic carcinogens. The validation studies also revealed that rasH2 mice are generally much more susceptible to both mutagenic and nonmutagenic carcinogens than control non-Tg mice. No significant tumor induction has been observed in rasH2 mice with either mutagenic or nonmutagenic noncarcinogens. More rapid onset and higher incidence of more malignant tumors can be expected with a high probability after treatment with various carcinogens in the rasH2 mice than in control non-Tg mice. The rasH2 mouse appears to be a promising candidate as an animal model for development of a rapid carcinogenicity testing system. — Environ Health Perspect 106(Suppl 1):57–69 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl1/57-69yamamoto/abstract.html

Key words: rasH2 mouse, CB6F1–TgHras2 mouse, human c-Ha-ras, short-term carcinogenicity test, transgenic mouse, chemical carcinogenesis

Introduction

Continuous endeavors to conquer cancer have been made through approaches from basic and clinical medicine as well as through approaches from public health. However, in many countries cancer remains the top-ranking cause of death. Many human cancers are believed to be caused by environmental carcinogens. To reduce risk, it is first necessary to identify environmental carcinogens and second to prevent people from being exposed to such carcinogens. Epidemiologic studies are probably the only way to confirm human carcinogens; however, this approach is so retrospective that carcinogens can be identified only after many victims have appeared. In vitro tests, i.e., mutagenicity tests, transformation assays, etc., may be helpful to predict carcinogenicity; but such in vitro testing is still subsidiary. Carcinogenicity testing using experimental animals is the only way for the prospective identification of possible human carcinogens. Therefore, carcinogenicity testing is indispensable for identifying environmental carcinogens and also for evaluating the safety of drugs in the process of development. Current carcinogenicity testing using experimental animals is not always relevant to human risk assessment; mice and rats are generally used because of their relatively short life spans and small body sizes. Conventional rodent carcinogenicity testing extends for more than 2 years and requires a large number of animals, which entails an enormous cost for both animal experiments or pathology studies. Moreover, thousands of new chemicals are synthesized every year for potential use. All of these chemicals do not require 2-year rodent bioassays, but there are many suspected chemicals in the environment that need to be subjected to bioassays but have not been tested. Clearly, there is a need to improve the process of carcinogen identification so more chemicals can be evaluated. From both animal welfare and ethical viewpoints as well, reducing the number of animals used in testing should be encouraged. Developing rapid carcinogenicity testing systems that can evaluate carcinogenicity within short periods and that require smaller numbers of animals compared to conventional 2-year bioassays is essential to improve the efficiency of environmental carcinogen identification and drug development.

To develop rapid carcinogenicity testing systems, animals susceptible to carcinogens are indispensable. Transgenic (Tg) animals carrying a protooncogene and/or animals lacking a tumor-suppressor gene are expected to be more susceptible to various carcinogens than normal animals, because carcinogenesis is a multistage process driven by genetic and epigenetic damage in susceptible cells that gain a selective growth advantage and undergo clonal expansion, probably as a result of activation of protooncogenes and/or inactivation of tumor-suppressor genes. Recently, studies have reported on validation of the use of either p53-knockout (+/−) mice (1), v-Ha-ras transgenic mice (TG.AC mice) (1,2),
pim-1 transgenic mice (3), XPA-deficient (−/−) mice (4), and human c-Ha-ras transgenic mice (5–8) as short-term bioassay models for carcinogen identification. ras family genes are involved in regulating cell proliferation and are activated by somatic point mutations in various human tumors (9–11) as well as in experimental animal models (11,12). Activation of the ras family genes by point mutation has been detected in various human tumors at the highest frequency for any oncogene (11). Therefore, the Tg mouse carrying the human c-Ha-ras gene may be a potential candidate as an animal model for rapid carcinogenicity testing.

Validation of Tg mouse carrying the human c-Ha-ras gene, namely rasH2 mouse (CB6F1-TgHa-ras2 mouse is the same mouse), as an animal model for rapid carcinogenicity testing in which carcinogenic potential is determined within 26 weeks (approximately 6 months) is now under way in the Central Institute for Experimental Animals (CIEA, Kanagawa, Japan) the National Institute of Health Sciences (NIHS, Tokyo, Japan), and several Japanese pharmaceutical companies, i.e., Sankyo Co. Ltd. (Tokyo, Japan), Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan), Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan), and Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). We are also conducting interlaboratory validation studies between the United States and Japan based on close mutual collaboration and harmonization (6).

In this review, current validation studies investigating the carcinogenic response of rasH2 mice to various carcinogens are introduced and compared with that of control nontransgenic (non-Tg) mice and the results of the 2-year bioassay.

**Tg Mice Carrying Prototype Human c-Ha-ras Gene Used for Rapid Carcinogenicity Testing (rasH2 Mice)**

The strain of Tg mice carrying the prototype human c-Ha-ras gene, namely rasH2 mice, was originally established by Saitoh et al. at CIEA (13). The Tg mouse carries the human c-Ha-ras gene with its own promoter region that encodes the prototype c-Ha-ras gene product, i.e., p21, which has no capacity for transforming NIH3T3 cells (13). Approximately five to six copies of human c-Ha-ras gene are integrated into the genome of each Tg mouse in a tandem array (13). Transgenes are expressed in both tumors and normal tissues, and the total amount of p21 that is detected by immunoblot analysis is 2 to 3 times higher in Tg mice than in non-Tg mice (13). No mutations of the transgenes have been detected in normal tissues of the Tg mice (13).

The genetic background of the Tg mice used in our validation studies is F1 of transgenic male C57BL/6J and normal female BALB/cByJ. C57BL/6J males carrying the transgene were crossed with BALB/cByJ females. The F1 offspring were screened by the polymerase chain reaction or Southern blot analysis for the presence of the human prototype c-Ha-ras gene.

Body weights of both male and female rasH2 mice are 80 to 90% of those of corresponding non-Tg mice (Figure 1) (6). The organ/body weight ratios of brain, thyroid gland, heart, lung, liver, spleen, kidney, adrenal glands, testes, and ovaries in the Tg mice are similar to those of non-Tg mice (6). Data revealed that there are no significant differences in blood chemistry and hematology of the Tg and non-Tg mice (6). Figure 2 shows the survival rate of rasH2 mice. The survival rate of male and female rasH2 mice at 77 weeks was 53 and 32%, respectively (Figure 2). Approximately 30 and 20% of...
rasiH2 mice developed spontaneous hemangiosarcomas/hemangiomas and lung adenomas/adenocarcinomas, respectively, within 82 weeks after birth (Table 1). Hepatocellular carcinomas, forestomach papillomas, skin papillomas, Harderian gland adenomas, and lymphomas were also observed by 82 weeks (Table 1). These results are consistent with previous observations (13). Incidences of spontaneous tumors generally were very low in the rasiH2 mice during the 6-month carcinogenicity experiments, which were terminated at the latest by 35 weeks (survival rate of rasiH2 mice at 35 weeks: 95–100%) (5–8). A few spontaneous lung adenomas and spleen hemangiosarcomas were occasionally observed, and forestomach papillomas and skin papillomas were rarely observed (5–8).

Seven- to nine-week-old rasiH2 mice were used for carcinogenicity testing. Among the littermates, mice (CB6F1) not carrying the human c-Ha-ras gene were used for non-Tg controls. Duration of the carcinogenicity tests was set for 26 weeks (approximately 6 months) or less except in the case of ethylene thiourea, which was 28 weeks (5–8). Doses of chemicals were determined according to the doses used for conventional 2-year bioassays or from the literature-cited studies (5–8). In most cases low- and high-dose groups for each chemical tested were established. Although the numbers of animals used varied, each dose group in our recent validation studies generally consisted of 15 male and 15 female Tg and non-Tg mice (7). Control vehicle-treated groups consisted of 10 male and 10 female Tg and non-Tg mice each (7).

Rapid Carcinogenicity Testing Using rasiH2 Mice

Table 2 lists chemicals for which rapid carcinogenicity testing either has been completed or is now under way in Japan. Eighteen Salmonella mutagenesis assay-positive trans-species carcinogens, seven Salmonella mutagenesis assay-negative trans-species carcinogens, two Salmonella mutagenesis assay-negative single-species (mouse only) carcinogens, one Salmonella mutagenesis assay-negative single-species (mouse only) carcinogen, four Salmonella mutagenesis assay-negative noncarcinogens, and four Salmonella mutagenesis assay-negative noncarcinogens were subjected to the rapid carcinogenicity testing.

Although mutagenicity is a major mechanistic determinant of carcinogenicity, it is neither sufficient nor necessary for carcinogenicity. Approximately one-third of nonmutagenic chemicals are carcinogenic, and approximately one-third of the mutagenic chemicals are noncarcinogenic in the 2-year rodent bioassay (14, 15). It has been proposed that chemicals that induce tumors in two rodent species are less influenced by the genetic variability among different species than chemicals that induce tumors in only one species (16). Thus, trans-species carcinogens appear to be more hazardous for humans than single-species carcinogens.

**Table 1.** Comparison of spontaneous tumor incidences (%) between rasiH2 mice and non-Tg mice by 82 weeks of age.

| Animals   | Sex | No. of mice | Lung Adenoma/adenocarcinoma | Forestomach Papilloma | Skin Papilloma | Liver Hepatoma, etc. | Harderian gland Adenoma | Hemangiom/Hemangiosarcoma | Others |
|-----------|-----|-------------|-----------------------------|-----------------------|---------------|---------------------|------------------------|--------------------------|--------|
| Tg mice   | Male | 35          | 8 (23)                      | 1 (3)                 | 1 (3)         | 9 (25)*             | 0 (0)                  | 9 (26)*                  | 2 (6)  |
|           | Female| 40         | 7 (18)                      | 4 (10)                | 2 (5)         | 1 (3)               | 2 (5)                  | 13 (33)*                 | 0 (0)  |
| Non-Tg mice | Male | 52         | 17 (33)                     | 0 (0)                 | 0 (0)         | 0 (0)               | 0 (0)                  | 0 (0)                    | 3 (7)  |
|           | Female| 42         | 2 (5)                       | 0 (0)                 | 0 (0)         | 0 (0)               | 0 (0)                  | 0 (0)                    | 0 (0)  |

*p < 0.01 versus corresponding non-Tg mice.

**Table 2.** List of chemicals for rapid carcinogenicity testing using rasiH2 mice.

| Chemical Name                        | Species | Refs |
|--------------------------------------|---------|------|
| 3-(N-methyl-4-nitro-2-nitrosoaniline) |         |      |
| 4-Nitro-o-phenylenediamine            |         |      |
| N,N-Diethylnitrosamine               |         |      |
| Melphanal                             |         |      |
| N-Methyl-N-nitro-N-nitrosoguanidine   |         |      |
| N,N-Dimethylhydrazine                 |         |      |
| N-Methyl-Nitrosourea                  |         |      |
| N,N-Dityrosamine                      |         |      |
| Phenacine                             |         |      |
| Procarbazine                          |         |      |
| 4-Acetoxyacrylamide                   |         |      |
| 4-Vinyl-1-cyclohexene diopside        |         |      |
| 4-Vinyl-1-cyclohexene diopside        |         |      |
| Benzene                              |         |      |
| Cyclophosphamide                      |         |      |
| Diethylstilbestrol                    |         |      |
| Ethyl acrylate                        |         |      |
| Ethylene thiourea                     |         |      |
| Nitro-toluene                         |         |      |
| Ethyl nitrosourea                     |         |      |
| Saltnella mutagenesis - assay-positive carcinogens | | |
| Salmonella mutagenesis - assay-negative noncarcinogens | | |
| Resorcinol                           |         |      |
| Rotenone                             |         |      |
| N-Butyl- and N-nitroso-2-nitroso-1-2-dioxane | | |
| N-Acetoxy-1-cyclohexene diopside      |         |      |
| Xylenes                               |         |      |
test of cupferon is now under way (Table 2). Among these mutagenic carcinogens, cyclophosphamide (19), melphalan (27), MNNG (28), phenacetin (36), procarbazine (38), and thiopeta (41) are classified as human carcinogens (Group 1) or are probably carcinogenic in humans (Group 2A).

*p-Cresidine* has been shown to be a bladder carcinogen in both mice and rats (17). *p-Cresidine* was mixed in the feed at a concentration of 0.25 or 0.5% and administered for 26 weeks except for the first 2 weeks of treatment when all *p-cresidine*-fed mice were exposed to a 0.25% concentration to acclimatize the animals to this agent. *p-Cresidine* induced hyperplasia significantly and transitional cell tumors nonsignificantly in the urinary bladder in both Tg and non-Tg mice. Incidences of both hyperplasia and transitional cell tumors were dose related. Diffuse hyperplasia was observed in 100% of 0.5% *p-cresidine*-treated Tg and non-Tg mice. Incidence of transitional cell tumors in *p-cresidine*-treated Tg mice was also similar to that in corresponding non-Tg mice.

Cyclophosphamide, an antineoplastic agent, is carcinogenic not only in rodents but also in humans (19). Gavage administration of either 10 or 30 mg/kg of cyclophosphamide twice a week for 25 weeks induced lung tumors both in the Tg and non-Tg mice; however, the tumor incidence was relatively low (5). Incidence of lung adenoma in cyclophosphamide-treated Tg mice was not significantly different from that of corresponding non-Tg mice (5). Cyclophosphamide did not induce mammary tumors, lymphomas, and urinary bladder tumors [target in rat bioassay (19)] in the Tg mice (5). These equivocal results may be attributable to inappropriate doses of cyclophosphamide, since dose range-finding studies were not performed before carcinogenicity testing, and no toxic effects such as hematuria, were observed in the treated mice. Carcinogenicity testing of cyclophosphamide will be conducted again in the International Life Sciences Institute (ILSI)/Health and Environmental Sciences Institute (HESI) project on alternatives to carcinogenicity testing, which is mentioned later in this review article.

DEN is carcinogenic in various animal species (20). The major target organs of DEN are the liver, lung, forestomach, and hematopoietic system (20). Single intraperitoneal injections of 90 mg/kg of DEN induced forestomach squamous cell carcinomas and lung adenocarcinomas only in the Tg mice as early as 3 months after administration (5). Six months after DEN administration, incidence of both types of malignant tumors in the Tg mice increased substantially (5). These malignant tumors were not observed in DEN-treated non-Tg mice during the 6-month observation period (5).

1,2-Dimethylhydrazine is known to induce colorectal tumors in mice (21). Subcutaneous injection of 20 mg/kg of 1,2-dimethylhydrazine once a week for 20 weeks induced colorectal adenocarcinomas in 100% of the male Tg mice, but corresponding non-Tg mice and control vehicle-treated Tg mice never developed tumors (K i toh et al., unpublished data).

4HAQO is mutagenic and known to produce pancreatic acinar cell carcinoma in mice (22). A single intravenous injection of 20 mg/kg 4HAQO significantly induced forestomach papillomas in the Tg mice but not in the corresponding non-Tg mice within 26 weeks (7). Incidence of forestomach papilloma was dose dependent in the Tg mice. 4HAQO-treated Tg mice also developed skin papillomas around the injection site at a higher rate than corresponding non-Tg mice (7). Although tumor incidence was low, papillomas in oral cavities, subcutaneous trichoeplithelioma, thymic lymphoma, and mammary gland adenocarcinoma were also observed in 4HAQO-treated Tg mice but not in corresponding non-Tg mice or in vehicle-treated Tg mice. Although 4HAQO has been reported to lead to pancreatic acinar cell carcinoma in mice (22), no pancreatic lesions were observed in either the Tg or non-Tg mice (7).

MAM is carcinogenic in rodents and induces colon tumors (24,25), lung tumors (24), and perianal squamous cell carcinomas (26). Subcutaneous injection of 20 mg/kg of MAM once a week for 6 weeks caused skin (restricted to anus and scrotum) papillomas, colon adenomatous polyps, squamous cell carcinomas of the rectum, and forestomach papillomas in the Tg mice but not in non-Tg mice 24 weeks after the first MAM administration (5). Similar lung adenoma incidence was observed in the Tg and non-Tg mice treated with MAM (5).

Intraperitoneal injection of 0.3 or 1.5 mg/kg melphalan, a chemotherapeutic agent for multiple myeloma, once a week for 26 weeks induced forestomach squamous cell tumors in the Tg mice, but the incidence was not statistically significant from that of vehicle-treated Tg mice. Incidence of forestomach squamous cell tumors was higher in Tg mice than in non-Tg mice. Incidence of forestomach squamous cell tumors were dose dependent in the Tg mice. Lung adenomas were also developed in melphalan-treated Tg and non-Tg mice. Although melphalan has been reported to induce lung tumors and lymphosarcomas in mice (27), lymphosarcomas were not observed in melphalan-treated Tg and non-Tg mice.

MNNG is an alkylating agent and is carcinogenic in various species of animals including the mouse (28). Forestomach and esophagus are target organs of MNNG following its oral administration (28). Single gavage administration of 2.5 mg/mouse of MNNG induced forestomach papillomas in 100% of male and female Tg mice, whereas only 11% of female and 0% of male non-Tg mice developed papillomas 13 weeks after MNNG treatment (5). Even at 26 weeks after MNNG administration, squamous cell carcinomas were observed only in MNNG-treated Tg mice but not in corresponding non-Tg mice (5).

MNUG is carcinogenic in various species of animals and produces skin, forestomach, lymphatic systems, and lung tumors (29). Intrapertitoneal injection of MNU, either once (75 mg/kg) or 5 times (once a day for consecutive 5 days; 15 mg/kg), induced various types of tumors in the Tg mice (5). MNUG significantly induced skin papillomas, forestomach papillomas, and lymphomas in the Tg mice 14 weeks after the start of MNU administration (5). Neither skin papillomas nor forestomach papillomas developed in MNU-treated non-TG mice (5). Forestomach squamous cell carcinoma was also observed in MNU-treated Tg mice but not in corresponding non-Tg mice (5). Ando et al. (46) also reported higher incidences of forestomach and skin papillomas in rats (46) mice after single intraperitoneal injection of MNU than in corresponding non-Tg mice. Incidence of lymphoma was also higher in male Tg mice treated with 75 mg/kg × 10 of MNU than in corresponding non-Tg mice (5).

NNK is known as a mutagenic tobacco-specific carcinogen and induces lung tumors in rats (30,31), mice (30–32), and hamsters (30,31). NNK was administered intraperitoneally once a week for 26 weeks at a dose of 3 or 6 mg/kg; mice were sacrificed 26 weeks after the start of the experiment. The incidences and multiplicity of lung adenoma in 6 mg/kg NNK-treated mice (both Tg and non-Tg mice) and
3 mg/kg NNK-treated female non-Tg mice were significantly higher than those in corresponding vehicle-treated control mice (7). Incidence of lung adenoma was dose dependent in both Tg and non-Tg mice. Unexpectedly, the incidence of lung adenoma in 3 mg/kg NNK-treated female Tg mice was significantly lower than that in corresponding non-Tg Tg mice (7). Multiplicities of lung adenoma in 3 mg/kg NNK-treated female Tg mice and 6 mg/kg NNK-treated Tg mice (both male and female) were also significantly lower than those in corresponding non-Tg mice (7). However, this is a very rare and exceptional case. Even in this case, as described above, significant tumor induction was clearly observed in the Tg mice (7). At present, it is not known why lung tumor response to NNK is higher in non-Tg mice than in the Tg mice.

4NQO is known to produce squamous cell carcinomas of the skin (33) and oral cavity (34), and lung tumors (35) in mice. Approximately 90% of 4NQO-treated Tg mice (both male and female) developed skin papillomas 16 weeks after a single subcutaneous injection of 15 mg/kg of 4NQO (5). Squamous cell carcinomas of skin were also observed in 4NQO-treated Tg mice (5). No skin tumors were observed in 4NQO-treated non-Tg mice and in vehicle-treated animals (5). Incidence of lung adenoma in 4NQO-treated Tg mice was also higher than that in corresponding non-Tg mice (5). Lung adenocarcinomas were observed in 4NQO-treated Tg mice but not in corresponding non-Tg Tg mice (5).

Phenacetin, used as an antipyretic analgesic, has been reported to be mutagenic and carcinogenic for rats (36), mice (36,37), and humans (36). Phenacetin was mixed in the feed at a concentration of 0.7 or 1.4% and administered for 24 weeks; mice were sacrificed 26 weeks after the start of the experiment. Phenacetin induced spleen hemangiosarcomas and forestomach papillomas in the Tg mice but not in non-Tg mice (7). Incidences of lung adenoma and spleen hemangiosarcoma in 1.4% phenacetin-treated male Tg mice were significantly higher than those in corresponding non-Tg mice (7). Incidences of lung adenoma/adenocarcinoma and spleen hemangiosarcoma were dose dependent in male Tg mice. Although phenacetin has been reported to produce tumors in the kidney and urinary bladder in experimental animals (36,37), we observed no lesions in the kidneys and urinary bladder of either the Tg or non-Tg mice (7).

Procarbazine has an antineoplastic activity in advanced Hodgkin's disease, and also is known to be a mutagenic carcinogen not only in rodents (38,39) but also in humans (38). Procarbazine has been reported to induce malignant lymphoma, leukemia, lung adenoma, etc., in mice (38,39). Intraperitoneal injection of either 6 or 12 mg/kg procarbazine 3 times per week for 24 weeks induced lung adenomas in both Tg and non-Tg mice 26 weeks after the start of the experiment (7). Lung adenocarcinomas and spleen hemangiosarcomas developed in Tg mice but not in corresponding non-Tg mice (7). Procarbazine did not induce malignant lymphoma or leukemia in the Tg mice (7).

4,4'-Thiodianiline, an intermediate in the manufacture of several diazo dyes, has been reported to be mutagenic and carcinogenic in rodents (40,41). Thiodianiline was mixed in the feed at a concentration of 2000 or 4000 ppm and administered for 24 weeks; mice were sacrificed at 26 weeks after the start of the experiment. 4,4'-Thiodianiline has been reported to induce tumors in the liver and thyroid in rodents (40). Very high incidences of thyroid follicular cell hyperplasia were observed in both 4,4'-thiodianiline-treated Tg and non-Tg mice (7). Incidences of thyroid follicular cell hyperplasia in 4,4'-thiodianiline-treated Tg and non-Tg mice were significantly higher than those in vehicle-treated control mice (7). Thyroid adenomas were also induced in 4,4'-thiodianiline-treated Tg and non-Tg mice (7). Incidences of hyperplasia and adenoma in 4,4'-thiodianiline-treated Tg mice were not significantly different from those in carcinogen-treated non-Tg mice. 4,4'-Thiodianiline also significantly induced lung adenomas in 2000 ppm-treated female Tg mice (7). Incidences of lung adenoma in 4000 ppm-treated male and female Tg mice were lower than those of corresponding 2000 ppm-treated Tg mice, indicating that 4000 ppm 4,4'-thiodianiline is toxic. The incidence of lung adenoma in 4,4'-thiodianiline-treated Tg mice was significantly higher than that in corresponding non-Tg mice (7). Although statistically not significant, lung adenocarcinomas, spleen hemangiosarcomas, forestomach papillomas, altered liver foci, and hepatocellular carcinomas were produced in the Tg mice, but not in non-Tg mice (7).

Thiotepa is an ethylenamine alkylating agent and is used clinically for cancer chemotherapy (41,42). Thiotepa is mutagenic and carcinogenic not only in rodents (41,42) but also in humans (41). Thiotepa has been reported to induce lymphoma, lymphocytic leukemia, skin squamous cell carcinoma, and lung adenoma in mice (41,42). Intraperitoneal injection of either 1 or 2 mg/kg thiotepa 3 times a week for 24 weeks induced forestomach papillomas, lung adenomas, and thymic lymphomas in both Tg and non-Tg mice 26 weeks after the start of administration (7). The incidence of forestomach papilloma in 2 mg/kg thiotepa-treated male Tg mice was significantly higher than those in corresponding non-Tg mice and vehicle-treated Tg mice (7). Incidence of forestomach papilloma was dose dependent in the Tg mice. Lung adenocarcinomas were observed only in thiotepa-treated Tg mice (7). Thiotepa also induced thymic lymphomas in both Tg and non-Tg mice (7).

Vinyl carbamate, an active metabolite of urethan, produces lung and liver neoplasms (43,44). Single intraperitoneal injections of 60 mg/kg vinyl carbamate induced lung adenomas and adenocarcinomas in 100 and 50% of the Tg mice, respectively, 16 weeks after carcinogen administration (8). Although vinyl carbamate-treated non-Tg mice also developed lung adenomas with more than 90% incidence, tumor multiplicity was much lower than that in corresponding Tg mice (8). Incidence of lung adenocarcinoma was much higher in vinyl carbamate-treated Tg mice than in corresponding non-Tg mice (8). Approximately 90% of the vinyl carbamate-treated Tg mice had spleen hemangiosarcomas but none occurred in corresponding non-Tg mice (8).

4-Vinyl-1-cyclohexene diepoxide has been reported to induce skin squamous cell carcinoma in mice (45). Dorsal skin application of 4-vinyl-1-cyclohexene diepoxide at a dose of either 5 or 10 mg/kg 5 times per week for 24 weeks caused skin papillomas around the application sites 26 weeks after the start of carcinogen administration (7). Forestomach papillomas, thymic lymphomas, and lung adenomas were also induced in both Tg and non-Tg mice (7). The incidence of skin papillomas in the 10 mg/kg 4-vinyl-1-cyclohexene diepoxide-treated female Tg mice was significantly higher than that in the vehicle-treated Tg mice (7). Dose-dependent induction of skin papilloma was observed in Tg mice. The incidence of skin papilloma in 10 mg/kg 4-vinyl-1-cyclohexene diepoxide-treated Tg mice (both male and female) was significantly higher than that in corresponding non-Tg mice (7). Although not
statistically significant, the incidences of forestomach papilloma, thymic lymphoma, and lung adenoma in the Tg mice were also higher than those in corresponding non-Tg mice (7). Skin squamous cell carcinomas and spleen hemangiosarcomas were observed in 4- vinyl-1-cyclohexene diepoxide-treated Tg mice but not in corresponding non-Tg mice (7). Skin papillomas were observed as early as 13 weeks after the start of application in Tg mice but at 16 weeks after the start of application in non-Tg mice (7).

Benzene (47,48), cyclosporin (49), diethylstilbestrol (50), 1,4-dioxane (51), ethyl acrylate (52), ethylene thiourea (53), and furfural (54) are known Salmonella mutagenesis assay-negative trans-species carcinogens. Rapid carcinogenicity testing of benzene, cyclosporin, 1,4-dioxane, ethyl acrylate, and ethylene thiourea has already been completed (Table 2). Carcinogenicity tests of diethylstilbestrol and furfural are now under way (Table 2). Among these carcinogens, benzene (47), cyclosporin (49), and diethylstilbestrol (50) are classified as human carcinogens.

According to Salmonella mutagenesis assay, benzene is nonmutagenic. However, benzene induces chromosomal aberrations, sister chromatid exchange, and DNA damage in both rodent cells and human cells in vitro (47); therefore benzene is classified as a genotoxic carcinogen. Benzene is carcinogenic not only in rodents (47,48) but also in humans (47). It has been reported to induce malignant lymphoma, Zymbal gland carcinoma, lung tumors, Harderian gland tumors, ovarian tumors, and mammary gland tumors in mice (47,48).

Gavage administration of benzene at a dose of 100 mg/kg 5 times a week for 24 weeks significantly induced forestomach papillomas in female Tg mice and lung adenomas in male Tg mice 26 weeks after the start of administration (7). Dose-dependent inductions of forestomach squamous cell tumors and lung adenoma/adenocarcinoma were observed in Tg mice. Incidences of forestomach papilloma and lung adenoma in 100 mg/kg benzene-treated Tg mice (both male and female) were significantly higher than those in corresponding non-Tg mice (7). The incidence of forestomach squamous cell carcinoma in 50 mg/kg benzene-treated male Tg mice was significantly higher than that in corresponding non-Tg mice (7).

Cyclosporin is widely used as an immunosuppressive agent for humans and also is known to be nonmutagenic but carcinogenic in humans (49). Cyclosporin has been reported to produce leukemia in mice (49). Gavage administration of cyclosporin at a dose of 10 or 25 mg/kg 5 times a week for 24 weeks induced skin papillomas, skin squamous cell carcinoma, and forestomach papillomas in Tg mice but not in non-Tg mice 26 weeks after the start of administration (7). The incidences of these tumors in cyclosporin-treated Tg mice were not significantly higher than those in vehicle-treated controls (7). Lung adenomas but not leukemia were observed in both Tg and non-Tg mice (7).

1,4-Dioxane, a dimer of ethylene oxide, is used extensively as an industrial solvent and is known to be nonmutagenic but carcinogenic in rats and mice (51). Administration of 1,4-dioxane in drinking water at a concentration of 0.5 or 1% for 24 weeks induced lung adenomas in Tg mice but not in non-Tg mice 26 weeks after the start of administration (7). The tumor incidence of 1,4-dioxane-treated Tg mice, however, was not significantly higher than that of vehicle-treated controls. Incidence of lung adenoma in 1% 1,4-dioxane-treated male Tg mice was significantly higher than that in corresponding non-Tg mice (7). Although 1,4-dioxane has been reported to induce hepatoellular carcinoma in B6C3F1 mice (51), no significant liver lesions were observed in either Tg or non-Tg mice (7).

Ethyl acrylate is known as a nonmutagenic carcinogen in rats and mice and has been reported to induce forestomach tumors in rodents (52). Gavage administration of ethyl acrylate at a dose of 100 or 200 mg/kg 5 times a week for 24 weeks induced forestomach papillomas in Tg mice but not in corresponding non-Tg mice 26 weeks after the start of administration (7). Forestomach squamous cell carcinomas were also observed in 200 mg/kg ethyl acrylate-treated male Tg mice but not in corresponding non-Tg mice (7). When the numbers of mice having forestomach squamous cell tumors (papilloma and squamous cell carcinoma) were combined, the incidence of forestomach squamous cell tumors in 200 mg/kg ethyl acrylate-treated male Tg mice was significantly higher than that in corresponding non-Tg mice or vehicle-treated control mice (7). The incidence of forestomach papilloma was dose dependent in the Tg mice.

Ethylene thiourea is known to produce thyroid neoplasms both in rats and mice (53). Only female mice were used for carcinogenicity tests. Mice were fed diets containing 0.1 or 0.3% of ethylene thiourea for 28 weeks. Ethylene thiourea at the dose of 0.1% did not lead to development of either thyroid tumors or hyperplasia in Tg and non-Tg mice (6), whereas 0.3% ethylene thiourea induced thyroid adenomas in 26 and 20% of Tg and non-Tg mice, respectively. Ethylene thiourea at a dose of 0.3% also induced thyroid follicular cell hyperplasia in 64 and 76% (statistically significant vs corresponding vehicle-treated groups) of Tg and non-Tg mice, respectively. Results indicate that the carcinogenic effect of ethylene thiourea is dose related. Incidence of thyroid adenocarcinoma was also similar, i.e., 9% in Tg and 4% in non-Tg mice, and no significant differences were observed between the Tg and non-Tg mice (6).

Mouse-only single-species carcinogens, especially nonmutagenic carcinogens that induce liver tumors, may not be relevant to carcinogenic risk to humans (55). To investigate whether rasH2 mice respond to mouse-only carcinogens, rapid carcinogenicity testing of two Salmonella mutagenesis assay-positive carcinogens, i.e., 6-nitrobenzimidazole (56) and 5-nitro-o-toluidine (57), and one Salmonella mutagenesis assay-negative carcinogen, i.e., 1,1,2-trichloroethane (58), has been conducted (Table 2). Histopathologic examination of the 1,1,2-trichloroethane study was completed recently.

1,1,2-Trichloroethane induces hepatocellular carcinoma and adrenal pheochromocytoma in mice but not in rats (58). In the NTP 2-year bioassay B6C3F1, mice were treated with 1,1,2-trichloroethane by gavage at a dose of 195 or 390 mg/kg 5 times a week for 78 weeks (58). Since substantial numbers of 400 mg 1,1,2-trichloroethane-treated Tg mice died within 1 week after the start of administration, the dose of the carcinogen was reduced. 1,1,2-Trichloroethane was administered by gavage at a dose of 100 or 200 mg/kg 5 times a week for 24 weeks, and mice were sacrificed 26 weeks after the start of administration. Although 200 mg of 1,1,2-trichloroethane induced calcified foci in the liver of both Tg and non-Tg mice, it induced neither liver tumors nor any other tumor-types in Tg and non-Tg mice (Urano et al., unpublished data).

High sensitivity to various carcinogens is essential in any animal model used for rapid carcinogenicity testing, but it is also important to clarify whether the rasH2 mice respond negatively to noncarcinogens. p-Anisidine (59), 2-chloromethylpyridine...
CARCINOGENICITY TESTS IN c-Ha-ras TRANSGENIC MICE

hydrochloride (60), 8-hydroxyquinoline (61), and 4-nitro-o-phenylenediamine (62) are classified as Salmonella mutagenesis assay-positive noncarcinogens (Table 2). Resorcinol (63), rotenone (64), xylene (mixed) (65), and tetraethylthiuram disulfide (66) are classified as Salmonella mutagenesis assay-negative noncarcinogens (Table 2).

p-Anisidine was mixed in the feed at a concentration of 0.225 or 0.45% and administered for 26 weeks. p-Anisidine did not induce tumors in either Tg or non-Tg mice.

8-Hydroxyquinoline, an antimicrobial agent, is reported to be mutagenic but not carcinogenic in rodents (61). It was mixed in the feed at a concentration of 1500 or 3000 ppm and administered for 24 weeks, and mice were sacrificed 26 weeks after the start of administration. No significant tumor induction was observed in either Tg non-Tg mice (7).

4-Nitro-o-phenylenediamine, a component of hair dyes, is a mutagenic noncarcinogen (62). 4-Nitro-o-phenylenediamine was mixed in the feed at a concentration of 3750 or 7500 ppm and administered for 24 weeks; mice were sacrificed 26 weeks after the start of administration. Lung adenomas were induced in 3 of 15 mice in 7500 ppm 4-nitro-o-phenylenediamine-treated male Tg mice. The incidence of lung adenoma was slightly higher than the tumor incidences observed in other noncarcinogen-treated Tg mice; however, the incidence of lung adenoma was not statistically significant (7).

Resorcinol, a nonmutagenic noncarcinogen (63), was administered by gavage at a dose of 225 mg/kg 5 times a week for 26 weeks. It did not induce tumors in either Tg or in non-Tg mice.

Rotenone is used as a pesticide and was a nonmutagenic noncarcinogen in a 2-year rodent bioassay (64). Rotenone was mixed in the feed at a concentration of 600 or 1200 ppm and administered for 24 weeks, and mice were sacrificed 26 weeks after the start of rotenone administration. No tumor induction was observed in either Tg or non-Tg mice (7).

Xylenes (mixed) are a nonmutagenic noncarcinogen tested in a 2-year rodent bioassay (65). Xylenes (mixed) were administered by gavage at a dose of 500 or 1000 mg/kg 5 times per week for 24 weeks; mice were sacrificed 26 weeks after the start of xylene (mixed) administration. Tumor induction was not observed in either Tg or non-Tg mice (7).

Carcinogenicity tests of 2-chloro- methylpyridine and tetraethylthiuram disulfide are currently under way (Table 2).

Spleen hemangiosarcomas and lung adenomas are occasionally observed as spontaneous tumors in the Tg mice. Forestomach papillomas and skin papillomas are rarely observed, however, so the incidences of these spontaneous tumors are relatively low (5–8).

Assay Validation

Test results obtained to date are summarized in Tables 3 and 4. Table 3 lists the names of the tested chemicals, dose, route of administration, and presence of rapid tumor response in Tg mice. Rapid tumor response means that tumors actually develop within 26 weeks (28 weeks for ethylene thiourea) in rasH2 mice in response to tested chemicals. Positive tumor response (+ in Table 3) means that the incidence of at least one type of tumor that developed in chemical-treated Tg mice is significantly higher (p < 0.05, Fisher’s exact test) than that in control vehicle-treated Tg mice. Negative tumor response (− in Table 3) means that the incidence of any type of tumor developed in chemical-treated Tg mice is ≤ 13% (< 2 of 15), or that tumor incidence is similar for chemical-treated and vehicle-treated groups. Certain types of spontaneous tumors are observed from time to time at a rate of 1 in 10, and very rarely at a rate of 2 in 10 in Tg mice; therefore, tumor incidence of ≤ 13% was defined as negative. If the numbers of animals used are 15 for each chemical-treated group and 10 for each vehicle-treated group, tumor incidence of ≥ 47% (≥ 2 of 15) is required for statistical significance (i.e., positive tumor response) even when the tumor incidence of vehicle-treated controls is 0%. Therefore, responses classified as neither positive nor negative were divided into the following two categories: one that is designated as (+) in Table 3, i.e., the incidence of at least one type of tumor that developed in chemical-treated Tg mice, is ≥ 25% (≥ 4 of 15) but not statistically significant compared to the control group. It seems possible that chemicals classified in this category may have the potential to be carcinogenic. The other category, which is designated (+) in Table 3, i.e., the incidence of at least one type of tumor that developed in chemical-treated Tg mice, is more than 13% but less than 25% (3 of 15). Chemicals in this category may not have carcinogenic potential. Table 3 also compares tumor incidence between Tg and non-Tg mice as well as comparing the incidence of malignant tumors in the Tg and non-Tg mice. Table 4 compares tumor spectra observed in Tg mice treated with tested chemicals and tumor spectra observed in 2-year mouse bioassays or in the literature-cited studies (based on mouse data) for the same chemicals.

As summarized in Tables 3 and 4, most of the tested mutagenic (trans-species) carcinogens induced at least one type of tumor in carcinogen-treated Tg mice within 26 weeks with a significantly higher incidence than that in control vehicle-treated Tg mice. Dose-dependent tumor induction was observed with most of the chemicals tested. The results obtained to date clearly demonstrate that in most cases Tg mice are much more susceptible to various mutagenic carcinogens than non-Tg mice. Tumors develop much sooner in Tg mice than in non-Tg mice (Table 3). Moreover, most of the malignant tumors are observed in the carcinogen-treated Tg mice and only a few or none in the corresponding non-Tg mice. All of these results indicate that rasH2 mice are useful for detecting mutagenic (trans-species) carcinogens.

Integration of information from chemical structure, mutagenicity, and toxicity studies may, to a certain extent, be useful in predicting the carcinogenic potential of relatively potent genotoxic carcinogens (67). Detectability of relatively weak carcinogens including nonmutagenic (trans-species) carcinogens is important in validating the usefulness of rasH2 mice in carcinogen testing procedures. Whether the Tg mouse can be used to detect nonmutagenic carcinogens is especially important for pharmaceutical companies because if they are developing products found to be genotoxic, development may be abandoned. Although all studies on nonmutagenic carcinogens are not yet completed, results obtained to date indicate that nonmutagenic carcinogens may also be detectable with a high probability by rasH2 mice (Table 3). Dose-dependent tumor induction was observed with three of five chemicals tested. It has been demonstrated that nonmutagenic carcinogens having single-site carcinogenic effects, for example, ethyl acrylate, cannot be detected in p53-knockout (+/−) and TG.AC models (7). Although at present it is not known whether ethyl acrylate is carcinogenic in humans, our results suggest that rasH2 mice can be used to detect such carcinogens (7). Further validation studies are required, however, before reaching a final
Table 3. Results of rapid carcinogenicity testing using rasH2 mice.

| Chemicals tested | Dose | Route of administration | Rapid tumor responsea in Tg mice | Tumor incidenceb | Malignant tumors |
|------------------|------|-------------------------|----------------------------------|----------------|-----------------|
| Mutagenic (Salmonella) carcinogens | | | | | |
| p-Cresidine | 0.25 or 0.5% for 26 wk | Gavage | +* | Tg = Non-Tg | + |
| Cyclophosphamide<sup>f</sup> | 30 mg/kg × 2/wk for 25 wk | Gavage | ± | Tg = Non-Tg | + |
| DEN<sup>e</sup> | 90 mg/kg × 1 | Subcutaneous | +* | Tg = Non-Tg | + |
| 1,2-Dimethylhydrazined<sup>d</sup> | 20 mg/kg × 1 for 20 wk | Intraperitoneal | * | Tg = Non-Tg | + |
| 4HAAO<sup>e</sup> | 10 or 20 mg/kg × 1 | Subcutaneous | +* | Tg = Non-Tg | + |
| MAM<sup>e</sup> | 20 mg/kg × 1/ wk for 6 wk | Intraperitoneal | +* | Tg = Non-Tg | + |
| Melphalan | 0.3 or 1.5 mg/kg × 1/ wk for 26 wk | Intraperitoneal | +* | Tg = Non-Tg | + |
| MNNG<sup>e</sup> | 2.5 mg × 1 | Subcutaneous | +* | Tg = Non-Tg | + |
| NNK<sup>e</sup> | 75 mg/kg × 1 or 15 mg/kg × 5 | Gavage | +* | Tg = Non-Tg | + |
| 4NO<sup>e</sup> | 15 mg/kg × 1 | Subcutaneous | +* | Tg = Non-Tg | + |
| Phenacetin<sup>e</sup> | 0.7 or 1.4% for 24 wk | Gavage | +* | Tg = Non-Tg | + |
| Procarbazine<sup>e</sup> | 6 or 12 mg/kg × 3/wk for 24 wk | Intraperitoneal | +* | Tg = Non-Tg | + |
| 4,4'-Thiodianiline<sup>e</sup> | 2000 or 4000 ppm for 24 wk | Intraperitoneal | +* | Tg = Non-Tg | + |
| Thioacetamide<sup>e</sup> | 1 or 2 mg/kg × 3/wk for 24 wk | Intraperitoneal | +* | Tg = Non-Tg | + |
| Vinyl carbamate<sup>e</sup> | 60 mg/kg × 1 | Intraperitoneal | +* | Tg = Non-Tg | + |
| 4-Vinyl-1-cyclohexene diepoxide<sup>e</sup> | 5 or 10 mg × 5/ wk for 24 wk | Subcutaneous | +* | Tg = Non-Tg | + |
| Benzene<sup>e</sup> | 50 or 100 mg/kg × 5/wk for 24 wk | Gavage | +* | Tg = Non-Tg | + |
| Cyclosporine<sup>e</sup> | 10 or 25 mg/kg × 5/wk for 24 wk | Gavage | +* | Tg = Non-Tg | + |
| 1,4-Dioxane<sup>e</sup> | 0.5 or 1% for 24 wk | Water | +* | Tg = Non-Tg | + |
| Ethyl acrylate<sup>e</sup> | 100 or 200 mg/kg × 5/wk for 24 wk | Gavage | +* | Tg = Non-Tg | + |
| Ethylene thiourea<sup>e</sup> | 0.3% for 26 wk | Feed | +* | Tg = Non-Tg | + |
| 1,1,2-Trichloroethane | 100 or 200 mg/kg | Gavage | +* | Tg = Non-Tg | + |
| Mutagenic (Salmonella) noncarcinogens | | | | | |
| N-Acetilneuraminic<sup>e</sup> | 0.225 or 0.45% for 26 wk | Gavage | +* | Tg = Non-Tg | + |
| 8-Hydroxyquinolines<sup>e</sup> | 1500 or 3000 ppm for 24 wk | Gavage | +* | Tg = Non-Tg | + |
| 4-Nitro-0-phenylenediamine<sup>e</sup> | 3750 or 7500 ppm for 24 wk | Gavage | ± | Tg = Non-Tg | + |
| Nonmutagenic (Salmonella) noncarcinogens | | | | | |
| Resorcinol | 225 mg/kg × 5/wk for 26 wk | Gavage | + | Tg = Non-Tg | + |
| Rotenone<sup>e</sup> | 600 or 1200 ppm for 24 wk | Gavage | + | Tg = Non-Tg | + |
| Xylene (mixed)<sup>e</sup> | 500 or 1000 ppm × 5/wk for 24 wk | Gavage | + | Tg = Non-Tg | + |

*rapid tumor response means that tumors actually developed within 26 weeks (28 weeks for ethylen thiourea) in rasH2 (Tg) mice in response to tested chemicals. (+) "Positive" indicates that the incidence of at least one type of tumor developed in chemical-treated Tg mice was significantly (p<0.05, Fisher's exact test) higher than that in control vehicle-treated Tg mice. (+) indicates that the incidence of at least one type of tumor developed in chemical-treated Tg mice was ≥25% but not statistically significant versus control. (±) "Positive-negative" indicates that the incidence of at least one type of tumor developed in chemical-treated Tg mice was more than 13% but less than 25%. (−) "Negative" indicates that the incidence of any type of tumor developed in chemical-treated Tg mice ≤13% or that tumor incidence was similar between the vehicle-treated control and dosed groups. (−) Tumor incidence: Incidence of at least one type of tumor (in cases of p-cresidine and ethylene thiourea, incidences of diffuse hyperplasia of urinary bladder and thyroid follicular cell hyperplasia, respectively) developed in chemical-treated Tg mice was statistically different from that of corresponding non-Tg mice (p<0.05; Fisher's exact test). *Data from Yamamoto et al. (5). †Data from Urano et al. (6). ‡Data from Mitsuoki et al. (8). ††Data from Itoh et al. (unpublished data). Conclusion on the usefulness of rasH2 mice for detecting nonmutagenic carcinogens.

Nonmutagenic mouse-only carcinogens may not be relevant to the problem of carcinogenic risk to humans (55). It has been suggested that liver damage caused by a nonmutagenic mouse-only carcinogens, such as 1,1,2-trichloroethane, is related to carcinogenic activity of this type of agent (55). Although 1,1,2-trichloroethane caused hepatotoxicity in both Tg and non-Tg mice, it did not induce hepatocellular carcinomas in either Tg or non-Tg mice. 1,1,2-Trichloroethane did not induce any other types of tumors in Tg and non-Tg mice within 26 weeks, suggesting that rasH2 mice may not respond to nonmutagenic mouse-only carcinogens. If that is true, the potential usefulness of this Tg mouse further increases. Additional validation studies are required to clarify this point. As shown in Table 4, most of the carcinogens tested induced some of the target organ tumors in the 2-year bioassay or in the literature-cited studies as well as certain types of tumors specific to Tg mice, e.g., lung alveolar epithelial tumors, spleen hemangiosarcoma, forestomach squamous cell tumors, and skin squamous cell tumors. Because these tumors develop spontaneously in aged rasH2 mice, it seems likely that carcinogens enhance spontaneous tumorigenesis by providing a certain step for carcinogenesis. Moreover, because there are strain differences in target organs and tumor spectra in carcinogen-induced tumors (68,69), it is not necessarily true that rasH2 mice (genetic background: CB6F<sub>1</sub>) induce so-called target organ tumors as observed in B6C3F<sub>1</sub> mice (used for the 2-year mouse bioassay). Therefore, chemicals that induce tumors in the lung, forestomach, spleen, and skin in rasH2 mice should be considered carcinogens.

It has been suggested that rasH2 mice have a low susceptibility to hepatocarcinogenesis, probably because of their genetic background (5–8). C57BL/6 mice have low susceptibility to chemically induced hepatocarcinogenesis (70,71), whereas C3H mice are highly susceptible to hepatocarcinogenesis (69,70). BALB/c mice are very resistant to hepatocarcinogenesis (69), and the F<sub>1</sub> hybrid of female C57BL/6 and male BALB/c has low sensitivity to hepatocarcinogenesis (44,71). Therefore, it seems highly possible that CB6F<sub>1</sub> mice, the F<sub>1</sub>
| Chemicals                              | Tumors observed* and reference, (incidence >13%) | Expected tumors* and reference |
|---------------------------------------|------------------------------------------------|--------------------------------|
| Mutagenic (Salmonella) carcinogens (trans-species) | | |
| p-Cresidine                           | Urinary bladder transitional cell tumors       | Urinary bladder tumors, liver tumors (17) |
| Capuron                               | Lungen adenoma (5)                             | Hemangiobroma, hepatocellular carcinoma, carcinoma of auditory and sebaceous glands, Harderian gland adenoma (18) |
| Cyclophosphamide                      | Lungen adenoma and adenocarcinoma              | Lungen tumors, lymphomas, mammary tumors, (lungen tumors in rats) (19) |
| DEN                                   | Forestomach squamous cell carcinoma (5)        | Lungen tumors, lungen tumors, leukemia, forestomach tumors (20) |
| 1,2-Dimethylhydrazine                 | Colorectal adenocarcinoma                      | Colorectal tumors (21) |
| 4-HAQ                                 | Forestomach papilloma, skin papilloma (7)      | Pancreatic acinar cell tumors (22) |
| MAM                                   | Skin papilloma (perianal and scrotum), lungen adenoma (5) | Colorectal tumors, lungen tumors, perianal squamous cell carcinoma (24–26) |
| Melphalan                             | Forestomach papilloma and squamous cell carcinoma, lungen adenoma (5) | Lungen tumors, lymphomas (27) |
| MNNG                                  | Forestomach papilloma, lymphoma                | Forestomach and esophagus tumors (28) |
| MNU                                   | Lungen adenoma                                 | Skin tumors, forestomach tumors, lymphomas, lungen tumors (29) |
| NNK                                   | Lungen adenoma and adenocarcinoma              | Lungen adenoma and adenocarcinoma (30–32) |
| 4NQ                                   | Lungen adenoma and adenocarcinoma              | Squamous cell carcinoma of skin and oral cavity, lungen tumors (33–35) |
| Phencetin                             | Forestomach papilloma, spleen                  | Kidney and urinary bladder tumors (36,37) |
| Procarbazine                          | Lungen adenoma and adenocarcinoma              | Malignant lymphoma, leukemias, olfactory neuroblastos, lungen adenoma, uterine adenocarcinoma (38,39) |
| 4,4'-Thiodianilone                    | Lungen adenoma and adenocarcinoma              | Hepatocellular carcinoma, thyroid follicular cell tumors (40) |
| Thiopeta                              | Forestomach papilloma, thymic lymphoma, lungen adenoma and adenocarcinoma (7) | Lymphoma, lymphocytic leukemia, skin squamous cell carcinoma, lungen adenoma (41,42) |
| Vinyl carbamate                       | Lungen adenoma and adenocarcinoma              | Liver tumors, lungen tumors, thymic lymphoma, Harderian gland tumors (43,44) |
| 4-Vinyl-1-cyclohexene diepoxide       | Skin papilloma, forestomach papilloma, lungen adenoma (7) | Skin squamous cell carcinoma, ovarian tumors, lungen tumors (45) |
| Nonmutagenic (Salmonella) carcinogens (trans-species) | | |
| Benzene                               | Forestomach papilloma and squamous cell carcinoma, lungen adenoma and adenocarcinoma (7) | Malignant lymphoma, Zymbal gland carcinoma, Harderian gland tumors, ovarian tumors, lungen tumors, mammary gland tumors (47,48) |
| Cyclosporin                           | Forestomach papilloma, skin papilloma/squamous cell carcinoma (7) | Mammary gland tumors, ovarian tumors, tumors of uterus and vagina (50) |
| Diethyliylstibestrol                  | Lungen adenoma (7)                             | Hepatocellular carcinoma (51) |
| 1,4-Dioxane                          | Forestomach papilloma/squamous cell carcinoma (7) | Forestomach papilloma and squamous cell carcinoma (52) |
| Ethyl acetate                         | Thyroid adenoma/adenocarcinoma (6)             | Thyroid tumors, hepatocellular tumors, pituitary adenoma (53) |
| Ethylene thiourea                    |                                             | Hepatocellular adenoma and carcinoma, renal cortical adenoma and carcinoma, forestomach papilloma (54) |
| Furural                               |                                              | Hepatocellular carcinoma (mouse only) (56) |
| Mutagenic (Salmonella) carcinogens (single-species: mouse only) | | |
| 6-Nitrobenzimidazole                  |                                              | Hepatocellular carcinoma, hemangiobroma, hemangioma (mouse only) (57) |
| 5-Nitro-o-toluidine                   |                                              | |
| Nonmutagenic (Salmonella) carcinogens (single-species: mouse only) | | |
| 1,1,1,2-Trichloroethane               |                                              | |
| Mutagenic (Salmonella) noncarcinogens |                                              | |
| p-Anisidine                           | Noncarcinogenic* (59)                         | |
| 2-Chloromethylpyridine                | Noncarcinogenic* (60)                         | Noncarcinogenic* (61) |
| 8-Hydroxyquinoline                    | Noncarcinogenic* (62)                         | Noncarcinogenic* (63) |
| 4-Nitro-o-phenylenediamine            | Noncarcinogenic* (64)                         | Noncarcinogenic* (66) |
| Nonmutagenic (Salmonella) noncarcinogens | Noncarcinogenic* (65)                         | Noncarcinogenic* (67) |
| Resorcinol                            |                                              | |
| Rotenone                              |                                              | |
| Tetraethylium disulfide               |                                              | |
| Xylenes (mixed)                       |                                              | |

*Tumors set in bold type are those for which incidence was significantly higher (p < 0.05; Fisher's exact test) than in vehicle-treated controls. Based on mouse bioassay (unless otherwise indicated). Unknown (carcinogenicity test is now underway). Itoh et al. (unpublished data). Negative. Noncarcinogenic in both rats and mice.
hybrid of male C57BL/6 and female BALB/c, are not susceptible to chemically induced hepatocarcinogenesis. Although the liver carcinogen 4,4'-thiodianiline induces hepatocellular carcinomas and altered foci in the liver (7), incidences of these lesions in the liver are low (7), reflecting the low susceptibility of this Tg mouse to liver carcinogens. Alternatively, liver tumors need more time (>6 months) to develop even in Tg mice because spontaneous liver tumors develop in male Tg mice with a high incidence until they were 82 weeks of age (Table 1).

Incidences of thyroid tumors in ethylene thiourea- and 4,4'-thiodianiline-treated Tg mice were similar to those of corresponding carcinogen-treated non-Tg mice (Table 3) (7). It is well known that thyroid carcinogenesis in rodents is a typical example of hormonal carcinogenesis (55). Serum levels of thyroxine and triiodothyronine are actually decreased and thyroid-stimulating hormone is increased after rodents are treated with ethylene thiourea (53). Hypersecretion of thyroid-stimulating hormone may promote hyperplasia and eventually thyroid adenomas and carcinomas (55). Similar incidences of thyroid tumors in treated Tg and corresponding non-Tg mice suggest that hormonal carcinogenesis may not be enhanced in rasH2 mice. Additional studies are required to clarify whether this peculiar outcome is thyroid specific. The study on diethylstilbestrol is currently under way.

Because false-positive errors in human carcinogen identification may hinder appropriate drug development, overprediction of carcinogenic potential should be avoided as much as possible. Therefore, it must be determined whether rasH2 mice respond negatively to noncarcinogens. The results obtained to date clearly show that rasH2 mice do not respond to nonmutagenic noncarcinogens. As for mutagenic noncarcinogens, one of three mutagenic noncarcinogens (e.g., 4-nitro-a-phenylene diamine) induced lung tumors in Tg mice with slightly higher incidence than other noncarcinogens (7). Although incidences of spontaneous tumors are generally low in this testing system, lung tumors are most frequently observed as spontaneous tumors (5–8). Therefore, a slight increase in lung tumor incidence may not have carcinogenic significance. On the other hand, however, a slight increase in incidences of spontaneous tumors may hinder assessment of the carcinogenic potential of borderline chemicals. Increasing the number of animals used in each test group may remedy this problem (this point is further discussed in the next paragraph). In any event, further validation studies are required to confirm the absence of false positive response of rasH2 mice to noncarcinogens, especially mutagenic noncarcinogens.

In the conventional 2-year bioassay, incidences of spontaneous tumors are high; thus the discrimination between chemically induced and spontaneously generated tumors may occasionally become difficult. Incidences of spontaneous tumors in rasH2 mice are low, at least within the time frame of carcinogenicity testing terminated at 33 to 35 weeks of age (5–8). Thus, in general, spontaneous tumors do not practically hinder assessment of the carcinogenic potential of tested chemicals. However, several mutagenic carcinogens, e.g., MAM, melphalan, procarbazine, and several nonmutagenic carcinogens, such as cyclosporin and 1,4-dioxane, did not show statistically significant differences from controls even though the tumor incidence was greater than or equal to 25% (≥4 of 15) in treated animals (Table 3). The main reason this problem arises is due to the insufficient number of animals used for the testing, especially for the vehicle-treated control groups (10 animals each). When this project was begun, numbers of animals available for each testing were limited. Thus, the number of vehicle-treated animals should have been reduced. Reducing the numbers of animals resulted in a difficult situation with statistical analyses of the borderline data. The incidence of such equivocal outcomes might possibly be reduced by increasing numbers of animals used (at least 15 to 20 animals are necessary for each group). If an equivocal result is obtained with a novel compound even when using a sufficient number of animals and appropriate doses, prolongation of the observation period to more than 6 months might provide more definitive results. A 2-year rat bioassay should be conducted in such cases. Further validation studies (such as the ILSI/HESI project in which 15 animals are used for each group) are required to clarify this point. Again the relatively low incidence of spontaneous tumors and the high sensitivity to carcinogens allow us to reduce the number of animals (compared with those in the 2-year bioassay) for testing. Reduction in the number of animals used and in duration of the experimental period (compared with those in the 2-year bioassay) markedly decrease overall costs of testing. The validation studies described here revealed that the rasH2 mouse appears to be promising as an animal model for a rapid carcinogenicity testing system.

To scientifically justify the usefulness of this rapid carcinogenicity testing system, it appears important to clarify the mechanism of rapid tumor induction in rasH2 mice on molecular bases. Lung alveolar epithelial tumors (adenomas/adeno carcinomas) were frequently induced in rasH2 mice after administration of various carcinogens. Incidences of transgene mutations (point mutation at codon 61) in vinyl carbamate-induced lung tumors were very low, i.e., approximately 7% (8). Lung tumors induced by other carcinogens also had a relatively low incidence of transgene mutations, i.e., 6% on average (0–24% depending on carcinogens tested) (S. Wakana et al., unpublished data). The Ki-ras gene has been proposed as a determinant of lung tumor susceptibility in mouse strains, and point mutations of the Ki-ras gene are actually detected with high frequency in lung tumors (72–74). Consistent with previous findings, point mutations of the murine Ki-ras gene either on codon 12 or 61 were observed in 70% of vinyl carbamate-induced lung tumors in non-Tg mice (8). However, Ki-ras mutations were never observed in lung tumors developed in vinyl carbamate-treated Tg mice (8). Moreover, Ki-ras mutations were never observed in lung tumors induced by either phenacetin, procarbazine, DEN, NNK, or benzene in rasH2 mice (S. Wakana et al., unpublished data). These results show that point mutations of the transgene and the murine Ki-ras gene do not play a major role in lung tumor induction by various carcinogens in rasH2 mice, indicating that the mechanism of lung tumor induction in this Tg mouse is different from that in non-Tg littermates. Our preliminary results suggest that the enhanced lung tumor induction in Tg mice is related neither to overexpression of transgene nor to microsatellite instability (S. Wakana et al., unpublished data). Further studies on molecular analyses such as detection of loss of heterozygosity are now under way to clarify the mechanism of rapid tumor induction in this Tg mouse.

Perspectives

The interlaboratory validation studies between the United States and Japan are continuing. RR Maronpot at the National Institute of Environmental Health Sciences (NIEHS) is in charge of the U.S. studies. Table 5 lists six chemicals—vinyl carbamate, p-cresidine, cyclosporin, resorcinol, melphalan, and p-anisidine—that have
been subjected to the interlaboratory validation studies. CIEA has supplied Tg mice to NIEHS. All chemicals have been tested under similar experimental protocols. Although the validation studies are not complete, similar results have been obtained in the United States and Japan. An intensive survey of databases of rodent carcinogenicity studies conducted over the past 25 years revealed that the rat is more sensitive in detecting carcinogens than the mouse; and mouse-only tumors, especially liver tumors, have almost no relevance to carcinogenic risk in humans (55). Current regulatory requirements for the assessment of the carcinogenic potential of chemicals in the European Union (EU), United States, and Japan stipulate long-term rodent carcinogenicity studies in two rodent species. Because of the cost of long-term bioassays, their extensive use of large numbers of animals, their poor mechanistic basis, and their relatively low relevance to human risk assessment, the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) is considering whether the use of 2-year carcinogenicity tests with two rodent species could be reduced without compromising human safety. Recent ICH-Expert Working Group draft guidelines proposed that the carcinogenic potential of pharmaceuticals could be evaluated from the data of one long-term conventional rodent (rat) carcinogenicity study plus data from one short- or medium-term testing system such as transgenic models, newborn models, or initiation/promotion models.

Studies on validation of the use of p53-knockout mice and/or TG.AC mice (v-Ha-ras transgenic mice) as short-term bioassay models for carcinogen identification have been conducted by R. Tennant and his colleagues at NIEHS (1,2). At present, among various genetically engineered animals, ratH2 mice, p53-knockout mice, TG.AC mice, and XPA-deficient (−/) mice, as animal models for short-term carcinogenicity testing, more than 20 chemicals will be tested in each model in the project.

Although the usefulness and the limitations of the rapid carcinogenicity testing system using rash2 mice have not yet been fully determined, a considerable amount of data that demonstrate potential usefulness have already been accumulated as described in this review.

### Table 5. Interlaboratory validation studies between the United States and Japan on rapid carcinogenicity testing using rash2 mice.

| Chemicals           | Instituteᵃ | United States | Japan | Status |
|---------------------|------------|---------------|-------|--------|
| Vinyl carbamate     | NIH        | NIEHS         |       | Completed |
| p-Cresidine         | NIH        | NIEHS         |       | Completed |
| Cyclosporin         | OEA        | NIEHS         |       | Completed |
| Resorcinol          | Industry¹ᵇ| NIEHS         |       | Completed |
| Melphalan           | Industry²ᶜ| NIEHS         |       | Completed |
| p-Anisidine         | NIH        | NIEHS         |       | Completed |

ᵃSites where carcinogenicity tests were conducted or are being conducted.ᵇYamanouchi Pharmaceutical Co. Ltd.ᶜKyowa Hakko Kogyo Co.

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