Evaluation of the wap-ras Transgenic Mouse as a Model System for Testing Anticancer Drugs

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ABSTRACT

Transgenic mouse models have provided many valuable insights into the molecular mechanisms of tumorigenesis; unfortunately, there is a paucity of published information on the utility of these models for evaluating potential anticancer therapeutics. Line 69 wap-ras transgenic mice have an activated, human c-Ha-ras gene on their Y chromosome. Adult males develop salivary and/or mammary adenocarcinomas. Both tumor types express high levels of human ras oncoprotein. Two new sublines, designated wap-ras/F, were created by selective breeding. Subline 69–2 wap-ras/F males developed multiple mammary tumors at puberty. Tumor onset was delayed by cyclophosphamide treatment prior to puberty. Mammary tumors from cyclophosphamide-treated mice weighed 0.57 ± 0.09 g/mouse (SD ± SEM; n = 8), while tumors from control mice weighed significantly more at 2.36 ± 0.25 g/mouse (n = 8; P < 0.001; SD ± SEM). These results suggest that subline 69–2F mice will be valuable for testing therapeutic regimes designed to interfere with processes occurring early in tumorigenesis, before palpable tumor presentation. Tumor sensitivity to several clinically relevant cytotoxins was also tested in adult wap-ras males with palpable tumors. Both salivary and mammary tumors were sensitive to cyclophosphamide and 5-fluorouracil, but not methotrexate. This suggests that wap-ras transgenic mice will indeed be useful in the discovery of novel therapeutics against neoplasia.

INTRODUCTION

Mutated ras genes are powerful transforming agents in vitro and are found in a wide variety of human tumors in vivo (1, 2). For this reason, the ras genes have been popular targets for generating transgenic mouse models of human cancer (3). These models have provided valuable insights into the molecular mechanisms of tumorigenesis. Unfortunately, there is a paucity of published information on the utility of these models as tools to evaluate potential anticancer therapeutics. Bell et al. (4) examined the effect of streptozotocin on a related transgenic tumor model, in which the mice express an elastase-I SV40 T-antigen fusion gene in pancreatic cells. At 4 weeks of age, prior to overt tumor presentation in the pancreas, mice were treated with streptozotocin or drug vehicle. When examined at 20 weeks of age, insulinomas and β-cell hyperplasia were significantly reduced in the streptozotocin-treated mice, however the protocol required extensive histological study of the entire pancreas in each mouse. The goal of our study was to develop a less labor-intensive, more generalized protocol for testing the effectiveness of potential anticancer agents in transgenic mice expressing activated human oncogenes.

Line 69 wap-ras transgenic mice have an activated, human c-Ha-ras gene on their Y chromosome and develop salivary and/or mammary tumors that express the human ras oncoprotein (5). The salivary tumors are adenocarcinomas arising from serous areas of the submandibular gland that characteristically have densely packed cords and sheets of moderately anaplastic cells. The mammary gland tumors are adenosquamous carcinomas with multiple foci of squamous differentiation or adenocarcinomas containing glandular tissue. Neoplastic tissue has a high mitotic index, and tumor-bearing animals have an ongoing inflammatory response as evidenced by inflammatory cell infiltration of affected tissue. Microscopic lung metastases were present in 14% of the tumor-bearing animals surveyed (5). These attributes suggested that line 69 wap-ras transgenic mice would provide a useful model for testing therapies directed against ras-associated tumorigenesis. In this paper, breeding improvements in the wap-ras mouse strain are described and the antitumor efficacy of 3 clinically utilized cytotoxins is examined using a general drug-testing protocol that should facilitate future drug studies with specific, anti-ras agents.

MATERIALS AND METHODS

Immature wap-ras/F males from subline 69–2F were given an i.p. injection of saline vehicle or cyclophosphamide at a dose of 50 mg/kg/day on days 0, 7, 14, and 21. The mice were 4–5 weeks old at the start of the experiment and had no palpable tumors. Four litters of mice were used, with males from each litter split evenly between control and treatment groups. On day 43, all mice were sacrificed (72–78 days old), and then the mammary tumors from each mouse were dissected free from the body wall and weighed. Tumor weights were compared using an unpaired Student’s t test.

Adult wap-ras males with tumors were enrolled in a treatment group on day 0. Tumor dimensions were easily measured on hand-held mice using a digital caliper. After 3 weeks, the mice were sacrificed by cervical dislocation and tissues collected for histology. Tumor volume was calculated as length × width × height. All drugs were purchased from Sigma Chemical Co., St. Louis, MO. All drugs or vehicle substances were given by i.p. injection once per day. Cyclophosphamide was dissolved in 0.9% saline and administered at a dose of 60 mg/kg on days 0–4 and 7–11. 5-Fluorouracil was sonicated in carboxymethylcellulose (0.4% carboxymethylcellulose, 0.5% Tween-80, 0.15 M NaCl) and given at 50 mg/kg on days 0–4 or days 0–3. Methotrexate was sonicated in 0.4% carboxymethylcellulose and given at 20 mg/kg on days 0–3. At 4–6 weeks of age, some male mice were anesthetized and castrated as described previously (6). All animal procedures were performed in accordance with the rules set forth in the NIH Guide for the Care and Use of Laboratory Animals. Tissue samples were fixed in 10% buffered formalin and processed overnight in a Miles VIP Tissue Processor. Five-μm tissue sections were cut with a Leitz microtome. The slides were stained with a routine Harris hematoxylin and eosin stain (7) and photographed using a Leitz Laborlux S photomicroscope.

To quench assay noise due to variability in tumor measurements from day to day (mostly human variability), tumor volumes from days 0, 1, and 2 were averaged for each individual tumor (mean volume0–2). For each tumor, volume measurements for days 3–18 were normalized to mean volume0–2, e.g., normalized volume = volume / mean volume0–2. Normalized tumor volumes for different treatment groups were compared by Student’s t test using a Macintosh IICl computer running Statview II software (Abacus Concepts, Berkeley, CA).
RESULTS AND DISCUSSION

General Characteristics of Tumorigenesis in wap-ras Males.
The original wap-ras transgenic mouse line 69 was a random-bred strain derived from 2 inbred lines, C57BL/6 and SJL. Males developed salivary gland tumors at approximately 9 months of age. By instituting a controlled breeding program in which offspring from males with early tumor onset were selected to sire the next generation and brother-sister matings were the norm, several sublines were created with differing profiles of tumor emergence. In general, the age at onset of salivary tumors decreased as the lines became more inbred, and a second tumor-type arising from mammary tissue appeared (5). A 1991 survey of 443 male wap-ras mice with single tumors revealed that 36% of the total population developed mammary tumors, 63% developed salivary tumors, and 2% presented with both tumors. It was not uncommon for a second tumor-type to arise within a few weeks after detection of the first tumor. One wap-ras subline (69-2) developed mammary tumors at a higher rate than the general population (50% versus 26% in other sublines) and mammary tumors developed at an earlier age (Fig. 1). This trend toward early tumor onset was also apparent for salivary tumors in this subline, although the difference from the general population was less dramatic (Fig. 2). In general, mammary tumors developed in younger mice than did salivary tumors. No tumorigenesis was observed in prepubital males, although orchiectomy did not prevent development of mammary (2 of 17) or salivary (6 of 17) tumors. This implies that high testosterone levels are not necessary for tumorigenesis in wap-ras mice.

Several wap-ras males were mated with inbred FVB/N females for 3 reasons: (a) to improve the low fertility rate of the strain; (b) to transfer the transgene into the same genetic background as other transgenics being produced in our laboratory and thereby simplify analysis of crosses between them; and (c) to discover whether a new genetic background would affect tumor pathology. To fix the transgene in the FVB/N genetic background, males from each successive generation were bred to FVB/N females. Two new sublines, designated wap-ras/F, were created. After 2 generations of FVB/N crosses, subline 69-2F males developed a more severe phenotype than the parent line. All males in the second generation had multiple mammary tumors (3-6 per mouse) that were palpable by 1.9-2.6 months (53-72 days; n = 8). Males in the third generation developed mammary tumors at 1.8-2.4 months (50-66 days; n = 9). This phenotype remained consistent in the fourth (n = 12) and fifth (n = 27) generations. Since all generations were produced by crosses with FVB/N females, the observed phenotype is probably not due to C57BL/6 or SJL genetic elements other than those on the Y chromosome. By contrast, subline 69-9F males had no detectable tumors at ages up to 8.9 months, indicating that the phenotype is not due to a simple synergistic effect between the transgenic Y-chromosome and genetic ele-

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Fig. 1. Age at onset of palpable mammary tumors in wap-ras males. Age subdivisions: 1, >0.5 to <1.5 months; 2, >1.5 to <2.5 months; 3, >2.5 to <3.5 months; 4, >3.5 to <4.5 months; 5, >4.5 to <5.5 months; 6, >5.5 to <6.5 months; 7, >6.5 to <7.5 months; 8, >7.5 to <8.5 months; 9, >8.5 to <9.5 months; 10, >9.5 to <10.5 months; 11, >10.5 to <11.5 months; 12, >11.5 to <12.5 months; 13, >12.5 to <13.5 months; 14, >13.5 to <14.5 months; 1 month = 4 weeks. Subline 69-2: n = 70 mice; not subline 69-2: n = 69 mice.

Fig. 2. Age at onset of palpable salivary tumors in wap-ras males. Age subdivisions: 1, >0.5 to <1.5 months; 2, >1.5 to <2.5 months; 3, >2.5 to <3.5 months; 4, >3.5 to <4.5 months; 5, >4.5 to <5.5 months; 6, >5.5 to <6.5 months; 7, >6.5 to <7.5 months; 8, >7.5 to <8.5 months; 9, >8.5 to <9.5 months; 10, >9.5 to <10.5 months; 11, >10.5 to <11.5 months; 12, >11.5 to <12.5 months; 13, >12.5 to <13.5 months; 14, >13.5 to <14.5 months; 1 month = 4 weeks. Subline 69-2: n = 88 mice; not subline 69-2: n = 200 mice.

Fig. 3. Effect of cyclophosphamide on tumor emergence in line 69-2F males.
DRUG TESTING IN wap-ras MICE

Fig. 4. Histopathology. a, wap-ras salivary adenocarcinoma (× 20). b, × 40. c, Lung metastasis in same mouse shown in a and b (× 20). d, × 40. e, Hepatic abscess with center of homogenous to granular eosinophilic necrotic material (N) surrounded by a zone of karyorrhectic debris and neutrophils (K), then a zone of macrophages, lymphocytes, and a few neutrophils (M) (× 4). f, × 10. g, wap-ras mammary adenocarcinoma (× 10). h, × 20.

Ten subline 69-2F males were orchiectomized at 1.5 months. All developed mammary tumors by 2.1 months, implying that high testosterone levels are not needed for tumor progression. The precise timing of microscopic tumor initiation is still under investigation. Sixteen males from subline 69-2F were treated with cyclophosphamide or vehicle control starting at 4–5 weeks of age, prior to palpable tumors. Cyclophosphamide delayed overt tumor onset by approximately 18 days (Fig. 3). Mammary tumors from cyclophosphamide-treated mice weighed 0.57 ± 0.09 g/mouse, while tumors from control mice weighed signif-
DRUG TESTING IN wap-ras MICE

Fig. 5. Effects of cytotoxins on salivary tumor growth in wap-ras males. a, cyclophosphamide. Dose = 60 mg/kg/day given on days 0–4 and 7–11. Range for mean volume: 476.60–3121.55 mm³ in the treatment group (n = 15 tumors/14 mice) and 168.51–4731.95 mm³ in the control group (n = 21 tumors/20 mice). b, 5-fluorouracil for 5 days. Dose = 50 mg/kg/day given on days 0–4. Range for mean volume: 1280.94–3127.61 mm³ in the treatment group (n = 5 tumors/5 mice) and 413.52–2051.51 mm³ in the control group (n = 9 tumors/9 mice). c, 5-fluorouracil for 4 days. Dose = 50 mg/kg/day given on days 0–3. Range for mean volume: 505.01–4346.78 mm³ in the treatment group (n = 13 tumors/11 mice) and 626.68–2886.98 mm³ in the control group (n = 13 tumors/13 mice). d, methotrexate. Dose = 20 mg/kg/day given on days 0–3. Range for mean volume: 874.94–2817.50 mm³ in the treatment group (n = 12 tumors/12 mice) and 626.68–2886.98 mm³ in the control group (n = 13 tumors/13 mice). *P < 0.05; **P < 0.01; ***P < 0.001.

Cytotoxin Sensitivity and Histopathology of wap-ras Tumors.

The experimental design of this drug study was similar to a human clinical drug study. Mice in the transgenic colony were checked for palpable tumors on Friday. Mice with tumors were enrolled in a treatment group on the following Monday (day 0). Each tumor on a mouse was treated as a separate entity. Tumors that appeared during the course of the study could not be evaluated, because their physiological status at the time of drug treatment was unknown.

In general, salivary tumor volumes doubled every 8 days. Salivary tumors contained islands, sheets, and columns of densely packed, moderately to markedly anaplastic, round-to-polygonal cells sometimes arranged in acinar or tubular patterns (Fig. 4, a and b). Generally, the neoplastic cells contained moderate-to-scanty, amphoteric-to-lightly basophilic cytoplasm, and medium-to-large, round nuclei with multiple, prominent, often irregularly shaped nucleoli. Mitotic figures were numerous and often abnormal. In most tumors, one or more internal areas were necrotic, suggesting that the tumors were outgrowing their blood supply. A minimal-to-moderate increase in neutrophils, lymphocytes, macrophages, eosinophils, and/or mast cells was often seen at periphery of the tumors. Lung metastases had similar morphology (Fig. 4, c and d). Cytotoxin treatment did not substantially affect these tumor characteristics, except to increase necrosis. Cyclophosphamide had a significant inhibitory effect on salivary tumor growth rates in wap-ras males. Dose = 60 mg/kg/day given on days 0–4 and 7–11. Range for mean volume: 476.60–3121.55 mm³ in the treatment group (n = 15 tumors/14 mice) and 168.51–4731.95 mm³ in the control group (n = 21 tumors/20 mice).
Fig. 6. Effects of cytotoxins on mammary tumor growth in wap-ras males. a, cyclophosphamide. Dose = 60 mg/kg/day given on days 0-4 and 7-11. Range for mean volume at Wk2 = 107.99-2834.11 mm³ in the treatment group (n = 15 tumors/11 mice) and 298.16-2955.43 mm³ in the control group (n = 16 tumors/16 mice). b, 5-fluorouracil for 5 days. Dose = 50 mg/kg/day given on days 0-4. Range for mean volume at Wk2 = 353.00-1335.28 mm³ in the treatment group (n = 9 tumors/6 mice) and 350.69-1696.42 mm³ in the control group (n = 6 tumors/6 mice). c, 5-fluorouracil for 4 days. Dose = 50 mg/kg/day given on days 0-3. Range for mean volume at Wk2 = 203.37-1645.19 mm³ in the treatment group (n = 8 tumors/8 mice) and 514.53-3261.86 mm³ in the control group (n = 6 tumors/6 mice). d, methotrexate. Dose = 20 mg/kg/day given on days 0-3. Range for mean volume at Wk2 = 279.29-1707.51 mm³ in the treatment group (n = 8 tumors/8 mice) and 514.53-3261.86 mm³ in the control group (n = 6 tumors/6 mice). *P < 0.05; **P < 0.01; ***P < 0.001.

ras male mice (Fig. 5a). Tumor regression was apparent in 7 salivary tumors treated with cyclophosphamide. FUra treatment for 5 days also had a significant inhibitory effect on salivary tumor growth (Fig. 5b), although none of the tumors regressed, and half of the mice initially enrolled in this treatment group were killed by the drug. FUra treatment for 4 days was also effective (Fig. 5c), with much lower toxicity. Two mice in the 5-day FUra group and 1 mouse in the 4-day FUra group had macroscopic liver lesions that were identified as abscesses by microscopic examination (Fig. 4, e and f). By contrast, methotrexate had no effect on salivary tumor growth (Fig. 5d).

Mammary tumors were usually composed of areas of densely packed sheets, clumps, or columns of cells and areas with glandular morphology, acinar, and/or tubular structures. However, some tumors also had areas of squamous differentiation that sometimes composed up to 75% of the tumor (Fig. 4, g and h). Generally, the neoplastic cells were medium-to-large and round-to-polygonal. They contained scanty-to-moderate amounts of basophilic cytoplasm and medium-to-large, round-to-oval nuclei with multiple, prominent, often irregularly shaped nucleoli. Mitotic figures were numerous and often abnormal. Many tumors contained one or more areas of necrosis.

### Table 1 Inhibitory effects of cytotoxins on wap-ras tumor growth

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Salivary tumors</th>
<th>Mammary tumors</th>
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<tbody>
<tr>
<td></td>
<td>Wk2</td>
<td>Wk3</td>
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<tr>
<td>Cyclophosphamide</td>
<td>53</td>
<td>73</td>
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<tr>
<td>5-Fluorouracil (4 days)</td>
<td>47</td>
<td>44</td>
</tr>
<tr>
<td>5-Fluorouracil (5 days)</td>
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<td>59</td>
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3 The abbreviation used is: FUra, 5-fluorouracil.
A minimal-to-mild increase in neutrophils, lymphocytes, macrophages, eosinophils, and/or mast cells was often seen at the periphery of the tumor. The order of frequency of occurrence of these cells was neutrophils > macrophages > lymphocytes > eosinophils > mast cells. Cytotoxin treatment did not substantially affect these tumor characteristics, except to increase necrosis. Mammary tumor growth was significantly inhibited by cyclophosphamide (Fig. 6a) and FUra (Fig. 6, b and c). Tumor regression was apparent in 2 tumors treated with cyclophosphamide, 4 tumors treated with FUra for 5 days, and 1 tumor treated with FUra for 4 days. Five of 6 mice in the 5-day FUra group and 1 mouse in the 4-day FUra group had macroscopic liver abscesses, although none of the control mice had this pathology. Methotrexate had no effect on mammary tumor growth rates (Fig. 6d).

The drug sensitivities of the 2 types of wap-ras tumors were remarkably similar (Table 1). The growth inhibitions effected by FUra were approximately 44% (4 day) and 62% (5 day) for both salivary and mammary tumors. Salivary tumors appeared to be slightly more sensitive to cyclophosphamide than mammary tumors, although more studies will be needed to confirm the significance of this observation. For unknown reasons, only mice treated with FUra developed macroscopic liver lesions. This might reflect a previously unsuspected defect in the livers of these particular mice that affects FUra metabolism, or it might be an indirect effect caused by FUra suppression of immune function, although the experiments were not of sufficient length for gross changes in immune function to appear.

A transgenic mouse model of ras-mediated oncogenesis offers several advantages for the development human disease of therapies. Unlike most animal models of tumorigenesis, the wap-ras mice have an intact immune system. This permits study of the role of immune system modulators in disease progression. Also, tumors arise spontaneously, without the administration of chemical agents or environmental perturbations, eliminating the possibility that nonspecific pharmacological side effects will complicate experimental data analysis. Both salivary and mammary tumors exhibited classic responses to clinically relevant, anticancer drugs. This suggests that wap-ras transgenic mice will indeed be useful in the discovery of novel therapeutics against neoplasia.

REFERENCES

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