Chemoprevention of Spontaneous Tumorigenesis in p53-Knockout Mice

Stephen D. Hursting, Susan N. Perkins, Diana C. Haines, Jerrold M. Ward, and James M. Phang

Laboratory of Nutritional and Molecular Regulation [S. D. H., S. N. P., J. M. P.], Pathology/Histotechnology Laboratory, Science Applications International Corporation [D. C. H.] and Veterinary and Tumor Pathology, Office of Laboratory Animal Science [J. M. W.], National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

Abstract

Spontaneous tumorigenesis was evaluated in male p53-knockout (p53−/−) mice treated with dehydroepiandrosterone (DHEA), quercetin, d-limonene, or all-trans retinoic acid to determine whether tumor development in these mice can be modulated by cancer-chemopreventive agents. DHEA-treated mice experienced a delay in tumorigenesis (particularly lymphomas) and subsequent mortality (P < 0.01) relative to untreated control mice. Quercetin, d-limonene, and all-trans retinoic acid each had no effect on spontaneous tumor development in p53−/− mice. These data demonstrate that tumor development in p53−/− mice can be delayed by DHEA and suggest that p53−/− mice provide a useful model for evaluating strategies to offset the increased risk of tumorigenesis resulting from loss of p53 tumor suppressor function.

Introduction

Chemoprevention has evolved over the past two decades as a viable strategy for cancer prevention with the aim of inhibiting the development of cancer through pharmacological or nutritional intervention before the appearance of a clinically detectable tumor. Currently, members of at least 25 different classes of chemicals have demonstrable chemopreventive activity (2), among them vitamins and their analogues, trace metals, nonnutritive dietary components, hormones, drugs, and many others. Several established carcinogen-induced rodent tumor models are being used to screen agents for chemopreventive activity (3), and efforts to identify promising chemopreventive agents using these models have been successful because efficacy testing for several compounds has moved out of the preclinical laboratory to human chemoprevention trials in populations at high risk for cancer development (4). However, carcinogen-induced models are limited by the fact that carcinogens generally produce DNA damage randomly throughout the genome and often cause biochemical effects that may confound the detection or interpretation of the activity of the chemopreventive compound being evaluated.

Future progress in cancer chemoprevention may be facilitated by the use of animals with specific genetic susceptibility for tumor development, such as p53−/− mice. p53−/− mice provide an attractive tumorigenesis model because tumor development in these animals is rapid and spontaneous (5), and because p53 mutations are the most commonly observed genetic lesions in human tumors (6). We have shown previously that CR delays the onset of spontaneous tumorigenesis in p53−/− mice (7). The purpose of the present study was to test the effects of several putative cancer chemopreventive agents, including DHEA, quercetin, d-limonene, and ATRA, on spontaneous tumorigenesis in male p53−/− mice.

Materials and Methods

Animals and Diets. One hundred thirty-nine male weanling p53−/− mice (genetic background: 94% C57BL/6 and 6% 129/Sv; GenPharm International, Mountain View, CA) were housed individually in polycarbonate cages on hardwood bedding and maintained on a 12-h light-dark cycle at 24°C. Following a 2-week acclimation period after receipt, during which all mice were fed ad libitum AIN-76A semipurified diet (7), the mice were randomized to one of five dietary treatment groups (27–28 mice/group): (a) control, which received AIN-76A diet with no added agents; (b) DHEA, which received AIN-76A diet containing 0.3% (w/w) DHEA; (c) quercetin, which received AIN-76A diet containing 2% quercetin; (d) d-limonene, which received AIN-76A diet containing 7.5% d-limonene; and (e) ATRA, which received AIN-76A diet containing 0.0012% ATRA. The rationale for the dose of chemopreventive agents used was based on literature reports of the most efficacious dose with the least amount of body weight loss for DHEA (8), quercetin (9), d-limonene (10), and ATRA (11). All diets were purchased from Research Diets, Inc. (New Brunswick, NJ) as 1-g tablets after addition of 0.5% (w/w) of both silicon dioxide and magnesium stearate. All chemopreventive agents were purchased from Sigma Chemical Co. (St. Louis, MO). Diet mixed with DHEA, quercetin, or ATRA in 10-kg lots were tableted, stored at 4°C, and administered ad libitum to all groups in standard feeders in weekly aliquots; diet mixed with d-limonene was vacuum packed in 100-g aliquots immediately after tableting, then stored frozen and administered ad libitum to the d-limonene group in daily aliquots. Any d-limonene diet remaining in a cage after 24 h was removed, weighed, and replaced with a fresh aliquot. All mice also received distilled water ad libitum. Food intakes and body weights were recorded weekly.

Aliquots of each diet were sampled at the beginning and end of the study and stored at −70°C until analyzed. Samples of test diet (or control diet spiked with a known amount of one of the chemopreventive agents being tested) were extracted with methanol, and DHEA, quercetin, and ATRA content was determined by reversed phase HPLC using an acetonitrile/water-based mobile phase. d-limonene content was determined by gas chromatography using a flame ionization detector. Measured concentrations of each chemopreventive agent in the spiked feed and test feed samples were all within 10% of expected values.

Spontaneous Tumorigenesis Study. All mice were observed daily for clinical signs of ill health. Moribund mice were killed with CO2. All animals that were killed or found dead were necropsied, and their tissues were fixed in 10% neutral buffered formalin. In addition, a portion of spleen, thymus, liver, and lymph nodes from some mice with lymphoma were fixed in Bouin’s fixative. Tissues were embedded in paraffin, and sections were prepared at 4–6 μm. Sections were stained with hematoxylin and eosin and histopathologically analyzed to determine cause of death and tumor types. Mean weekly body weights were compared by Student’s t test, and the percentage of animals within each group with different individual tumors and with multiple primary tumors were compared between treatment groups using Yates corrected χ² test (12). The mean number of days on study were compared between treatment groups using the Wilcoxon rank sum test (12). Differences in survival were compared using the Cox proportional hazards model (13).

Bouin’s-fixed tissues were stained with a rabbit antiserum raised against a
pant T-lymphocyte antigen (CD3; 1:100; DAKO Corp., Carpinteria, CA) or a biotinylated mAb raised against a B-lymphocyte antigen (B220, 1:50; Boehringer Mannheim, Indianapolis, IN), using Vectorstain Elite kits (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as the chromogen.

Results

The growth curves of the p53−/− mice on each diet treatment were not statistically different from each other, with the exception of the DHEA group, which grew more slowly than the control mice throughout the study (mean weights at week 15, when > 50% of controls were still alive: DHEA, 24.4 g; control, 41.2 g; P = 0.014). In addition, the ATRA group grew at a similar rate as the controls for the first 10 weeks, then gradually lost weight over the following 10 weeks before plateauing in weight for the remainder of the study (ATRA, 29.4 g; control, 41.2 g; P = 0.04 at week 15). The decreased body weight experienced by the DHEA group relative to controls may be partially explained by decreased food intake (on average, 82% of controls), although in terms of food intake/g of body weight, the DHEA-treated mice were similar to controls after the first week of study. The mid-study decrease in body weight observed in the ATRA group could not be explained by decreased food intake because those mice consumed comparable levels of food to those of the controls throughout the study.

Marked differences were seen in the Kaplan-Meier survival curves for the respective treatment groups (Fig. 1). All mice, regardless of treatment, died of spontaneous tumor development, with the exception of four DHEA-treated and three (/-limonene-treated mice that died of undetermined causes, and one quercetin-treated and one d-limonene-treated mouse that died of liver failure. DHEA-treated mice showed a statistically significant delay in tumor-related mortality relative to controls (P < 0.01). The mean time on study for the DHEA-treated mice was 174 days compared to 108 days for the control mice (P < 0.01). In contrast, treatment did not differ from controls.

DHEA-treated mice, relative to controls, demonstrated decreased development of hematopoietic malignancies (Fig. 2 and Table 1).

Thirty-nine % of DHEA-treated mice died from hematopoietic tumors compared to 89% of control mice (P < 0.01). The majority of hematopoietic tumors developed in each group were lymphomas, which were either lymphoblastic lymphomas typically of thymic origin and expressing the T-cell marker CD3 (Figs. 3, A and B) or nonlymphoblastic lymphomas (including immunoblastic, follicular center cell, or highly pleomorphic lymphomas) usually not of thymic origin and expressing CD3 (Fig. 3C) and less commonly the B-cell marker B220 (Fig. 3, D–F). DHEA specifically decreased lymphoblastic lymphoma development because only 7% of DHEA-treated mice died of lymphoblastic lymphoma compared to 43% of controls (P < 0.01). DHEA also appeared to slightly decrease the development of nonlymphoblastic lymphomas because 18% of DHEA-treated mice died of nonlymphoblastic lymphoma compared to 36% of controls (P = 0.13). In contrast, DHEA-treated mice relative to controls died more frequently from nonhematopoietic tumors (47 versus 11%; P < 0.01), particularly hemangiosarcomas (25 versus 4%; P = 0.025) or other tumors, most commonly testicular interstitial cell tumors or hepatoblastomas (22 versus 7%; P = 0.11). DHEA-treated mice, relative to controls, also more frequently developed two or more tumors (typically multiple sarcomas or a sarcoma and another tumor type such as a testicular interstitial cell tumor or hepatoblastoma) in the same animal (56 versus 17%; P < 0.01). Thus, DHEA delayed the onset of tumor development and death in p53-knockout mice, and altered the types of tumors developed in these mice, with an apparent shift in the DHEA-treated mice from the predominantly lymphomas found in controls to a more even mix of hematopoietic and other tumors, particularly hemangiosarcomas.

No statistically significant differences relative to controls in the types of tumors developed were observed for mice receiving quercetin (Table 1). However, (/-limonene and ATRA (relative to controls) appeared to marginally decrease lymphoma development (/-limonene: 53 versus 79%, P = 0.04; ATRA: 64 versus 79%, P = 0.21) but increase nonhematopoietic tumors (/-limonene: 25 versus 11%, P = 0.16; ATRA: 36 versus 11%, P = 0.03). The number of days on study for mice treated with quercetin, (/-limonene, or ATRA did not differ from controls.

Discussion

Numerous studies have shown that administration of DHEA, a steroid hormone produced endogenously by the adrenal gland, inhibits carcinogen-induced skin, mammary, colon, and lung tumorigenesis (8). The results reported here indicate that DHEA administered in the diet (but not quercetin, (/-limonene, or ATRA) significantly delays the development of spontaneous tumors in p53−/− mice, a mouse strain containing two germline p53-null alleles that is susceptible to spontaneous tumorigenesis at an early age (5). These findings suggest that the p53 gene product is not an absolute requirement for DHEA to exert its cancer chemopreventive effects, although these data do not rule out a possible role for the p53 gene in the cancer-chemopreventive effects of DHEA reported previously in other rodent strains. In addition, these results demonstrate that a chemopreventive agent (DHEA) can modulate tumorigenesis in mice with greatly increased genetic susceptibility to cancer, in this case due to the absence of the p53 tumor suppressor gene product.

Results from this study may provide important clues about potential cellular/molecular targets for chemoprevention. The DHEA-induced suppression of tumor formation has been attributed to its modulation of the pentose-phosphate shunt, specifically the inhibition of G6PD activity. This inhibition can lead to reduced NADPH and ribose-5 phosphate cellular pools and hence diminished synthesis of the deoxyribonucleotides required for cell replication (14). Decreased de-
Table 1 Effect of chemopreventive agents on cause of death in male p53-knockout mice

<table>
<thead>
<tr>
<th>No. of mice/group</th>
<th>Control</th>
<th>DHEA</th>
<th>Quercetin</th>
<th>d-Limonene</th>
<th>ATRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cause of death (% of total per treatment)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td>0</td>
<td>14</td>
<td>7</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Neoplastic</td>
<td>100</td>
<td>86</td>
<td>93</td>
<td>86</td>
<td>100</td>
</tr>
<tr>
<td>Hematopoietic</td>
<td>89</td>
<td>39*</td>
<td>78</td>
<td>61*</td>
<td>64*</td>
</tr>
<tr>
<td>Lymphomas</td>
<td>79</td>
<td>25*</td>
<td>74</td>
<td>53*</td>
<td>64</td>
</tr>
<tr>
<td>Lymphoblastic</td>
<td>43</td>
<td>7*</td>
<td>48</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>Neutrophiblastic</td>
<td>36</td>
<td>18</td>
<td>26</td>
<td>21</td>
<td>40</td>
</tr>
<tr>
<td>Histiocytic sarcoma</td>
<td>7</td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Mast cell tumor</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nonhematopoietic</td>
<td>11</td>
<td>47*</td>
<td>15</td>
<td>25</td>
<td>36*</td>
</tr>
<tr>
<td>Hemangiosarcoma</td>
<td>4</td>
<td>25*</td>
<td>4</td>
<td>11</td>
<td>14*</td>
</tr>
<tr>
<td>Other tumors</td>
<td>7</td>
<td>22</td>
<td>11</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>Multiple tumors</td>
<td>17</td>
<td>56*</td>
<td>37</td>
<td>28</td>
<td>37</td>
</tr>
<tr>
<td>Mean days to death due to neoplasia</td>
<td>108</td>
<td>174*</td>
<td>118</td>
<td>95</td>
<td>102</td>
</tr>
</tbody>
</table>

* Different from controls at p < 0.01.

Typically a lymphoma and a sarcoma in the same animal, although occasionally multiple sarcomas or a sarcoma and another tumor type (i.e., testicular tumor, hepatocellular carcinoma, neuroblastoma) were observed in the same animal.

Development of liver preneoplastic foci has been associated with an inhibition of liver G6PD activity in DHEA-treated mice (15). However, mice fed diets containing 0.45% DHEA had plasma levels of DHEA that may not be high enough to produce significant inhibition of G6PD (16). Thus, some of the in vivo effects of DHEA may be through mechanisms other than the direct inhibition of G6PD. An alternative mechanism for the tumor-modulating effects of DHEA is suggested by a report from an in vitro study that DHEA-sulfate can inhibit the enzyme farnesyltransferase and thus modulate ras isoprenylation and activation (17). In addition, DHEA has been shown to inhibit lymphopoiesis (but not myelopoiesis or erythropoiesis) by suppressing the production of lymphocytes from their progenitors through an unidentified mechanism without affecting mature lymphocytes (16). This specific effect of DHEA on lymphopoiesis may explain the decreased development of lymphomas in DHEA-treated p53-/- mice, relative to controls, in the present study.

Long-term administration of DHEA to rodents produces many of the same beneficial effects as CR on cell proliferation, tumorigenesis, inflammation, and autoimmunity, prompting the hypothesis that CR and DHEA exert their effects through similar mechanisms (18). We have shown that CR (60% of control calorie intake), which inhibits a variety of carcinogen-induced rodent tumors (19), also significantly delays spontaneous tumor development in male p53-/- mice (7). It is interesting to note that DHEA in the present study suppressed body weight gain to a similar extent as the CR regimen in our previous study (7), although food intake in the DHEA-treated mice was, on average, 822% of controls (thus 3% higher than for our CR mice). This degree of decreased food intake in the DHEA group appears to contribute very little to the DHEA-induced delay in spontaneous tumorigenesis, based on an ongoing study in our laboratory in which control and DHEA-treated p53-/- mice are being compared to a group that is pair-fed to the DHEA-treated mice. Results from this study indicate the DHEA group is experiencing a delay in spontaneous tumorigenesis relative to controls, whereas the pair-fed group does not differ from controls.

Consideration of the purported mechanisms of the agents that did not modulate spontaneous tumorigenesis in p53-knockout mice may also be informative. Quercetin can inhibit the activity of ultimate carcinogenic metabolites by directly reacting with and detoxifying these compounds (20). Quercetin can also down-regulate the expression of mutant p53 protein (21), which may explain the growth-inhibitory properties of this flavonoid in carcinogen-induced models and may also explain why quercetin had no effect in p53 mice. The monoterpene d-limonene, the major constituent of citrus fruit oils, has been shown to inhibit isoprenylation of small G-proteins, including the ras p21 oncprotein (22). d-Limonene also increases levels of the mannose 6-phosphate/insulin-like growth factor II receptor and transforming growth factor β levels in regressing mammary tumors (23). Recent studies have suggested that some retinoids may require wild-type p53 protein for their chemopreventive activity (24). Retinoids are thought to exert their anticancer effects by controlling cell proliferation and differentiation via regulation of gene expression. The
Fig. 3. A, lymphoblastic lymphoma of thymic origin from a male p53−/− mouse fed control diet. Shown is a homogeneous population of round lymphoblasts with scant cytoplasm (hematoxylin and eosin, × 750). B, lymphoblastic lymphoma from a male p53−/− mouse fed control diet showing immunoreactivity for the pan T-cell marker CD3 (ABC immunohistochemistry and hematoxylin, × 750). C, nonlymphoblastic lymphoma (CD3 immunoreactive, immunohistochemistry not shown) from a male p53−/− mouse fed control diet showing pleomorphic nuclei and abundant cytoplasm (hematoxylin and eosin, × 750). D, nonlymphoblastic lymphoma (B220 B-cell marker immunoreactive, see E and F) from a male p53−/− mouse fed control diet showing pleomorphic nuclei and abundant cytoplasm with plasmacytoid features (hematoxylin and eosin, × 750). E, nonlymphoblastic lymphoma (same as D) showing B220 immunoreactivity in the majority of cells (ABC immunohistochemistry and hematoxylin, × 300). F, nonlymphoblastic lymphoma (same as D) showing cell surface B220 immunoreactivity (ABC immunohistochemistry and hematoxylin, × 750).
critical genes have yet to be identified, although a relationship between retinoids, growth factors such as transforming growth factor β, and oncogenes/tumor suppressor genes has been suggested (25). Thus, it is possible that the antiproliferative effects of quercetin, d-limonene, or ATRA work through a p53-mediated pathway absent in the p53~'~ mice or, alternatively, these agents may lack chemopreventive activity for the particular types of tumors (primarily lymphomas and sarcomas) developing spontaneously in these mice.

Studies comparing the differential expression of genes in response to DHEA and CR (treatments that resulted in a delay of spontaneous tumorigenesis in p53~'~ mice) and in response to d-limonene, quercetin, and ATRA (treatments that did not effect survival) are under way in our laboratory to identify the molecular targets of chemopreventive activity. Elucidation of the mechanisms underlying the tumor-delaying effects of DHEA and CR in p53~'~ mice may facilitate new strategies for cancer chemoprevention.

Results from the present study provide validation of p53~'~ mice as a model for testing chemoprevention strategies to offset the increased cancer risk resulting from loss of p53 tumor suppressor function. The need for such targeted prevention approaches is becoming urgent as our ability to characterize genetic susceptibility advances. In addition, the carcinogen-induced tumor models primarily relied on in the chemoprevention field for efficacy testing may be limited by the fact that chemical carcinogens generally produce DNA damage randomly throughout the genome and often cause biochemical effects that may interfere with the detection or interpretation of the activity of the chemopreventive compound being evaluated. The relevance of high-dose carcinogen models to human cancer is often tenuous as well. Thus, future progress in cancer chemoprevention may be greatly facilitated by the use of animals such as p53~'~ mice that are developed to manifest specific and highly relevant genetic susceptibility for tumor development.

Acknowledgments

The authors thank Lisa Riffle, Darlene Reaver, Craig Driver, Aretha Smith, Christine Perella, Dan Logsdon, Jane Miller, Mark Shredar, Dee Green, Jo Ann May, Kay Schekles, Mary Milner, and Barbara Kasprzak for excellent technical assistance; Charles Riggs for statistical assistance; and Drs. Ed Ulman and Arthur Schwartz for helpful discussions.

References

Chemoprevention of Spontaneous Tumorigenesis in p53-Knockout Mice
