Genetic Variation in Liver Tumor Susceptibility, Plasma Testosterone Levels, and Androgen Receptor Binding in Six Inbred Strains of Mice

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ABSTRACT

We compared six inbred mouse strains for their relative susceptibilities to liver and lung tumor induction. Male and female mice were tested at 12 days of age with a single i.p. injection of N-ethyl-N-nitrosourea (ENU; 0.25 μmol/g), and tumor multiplicity was analyzed at 32 weeks of age (males) or 44 weeks of age (females). Male mice of the SWR/J and C57BL/6J strains were relatively resistant to hepatocarcinogenesis, averaging 0 and 0.3 tumors per animal, respectively. Male C57BR/cdJ, P/J, and SM/J mice had intermediate susceptibilities, averaging seven to 17 tumors per animal, and male CBA/J mice were the most susceptible, averaging 45 tumors per animal. Female mice were more resistant than male mice; no liver tumors were observed for SWR/J females; C57BL/6J, SM/J, P/J, and CBA/J females averaged less than one tumor per animal and C57BR/cdJ females averaged five tumors per animal. In contrast to the results for liver tumor induction, there was no difference between the sexes in lung tumor susceptibility. Male and female SWR/J mice were the most susceptible, averaging 14 lung tumors per animal; male and female CBA/J mice were moderately susceptible, averaging six tumors per animal and the C57BR/cdJ, C57BL/6J, P/J, and SM/J strains were relatively resistant, averaging less than three tumors per animal.

To determine if levels of testosterone, a potent liver tumor promoter in mice, or its receptor contribute to the strain variation in liver tumor susceptibility, we measured levels of plasma testosterone as well as binding properties of the hepatic androgen receptor for the six inbred strains. Testosterone in male mice ranged from 1.8 to 7.4 ng/ml and in females ranged from 0.21 to 0.42 ng/ml, which is consistent with the greater susceptibility of male mice to liver tumor development. However, variation in testosterone levels among the different strains of mice was not correlated with liver tumor susceptibility.

We also demonstrated the presence of high affinity androgen receptors in mouse hepatic cytosol. The amounts of this receptor for the six strains tested ranged from 24 to 34 fmol/mg cytosolic protein. The apparent Kd of the receptor for [3H]mibolerone (a synthetic androgen) differed between the strains: SWR/J, C57BL/6J, and C57BR/cdJ mice had the highest affinity (Kd = 0.22 nM), P/J and CBA/J strains had an intermediate affinity (Kd = 0.36 nM), and the SM/J strain had the lowest affinity receptor (Kd = 0.45 nM). The strain variation in the affinity or abundance of the androgen receptor was not related to the strain variation in liver tumor induction.

INTRODUCTION

Inbred strains of mice differ greatly in their susceptibilities to the development of both spontaneous and chemically induced liver tumors (1). This variation represents a valuable resource for understanding the genetic and biochemical mechanisms of hepatocarcinogenesis. Previous comparative studies have shown that C3H and CBA mice are among the most susceptible of all inbred mouse strains to hepatocarcinogenesis while the C57BL/6, A, BALB/c, and DBA/2 strains are relatively resistant (2–6). Direct comparisons between these studies are hampered by the variety of agents and protocols used for inducing tumors.

We have used a protocol in which preweanling mice are given a single injection of ENU1 as a means of comparing inbred mouse strains for their susceptibilities to hepatocarcinogenesis (7, 8). ENU is a direct-acting mutagen and has a very rapid biological half-life (9), which minimizes confounding effects due to differences which may exist in pathways of metabolic activation and in pharmacokinetics. Treatment of preweanling mice with ENU results in the efficient initiation of hepatocytes as a consequence of the rapid proliferation of the target cells (8, 10, 11). The observation that the time to appearance of liver tumors is related to both the strain and the sex of the animals indicates that the endogenous promoting environment has a major influence on the rate of liver tumor development. For example, we have previously demonstrated that the 20–40-fold difference in induced liver tumor multiplicity observed between C3H/HeJ and C57BL/6J male mice resulted from a greater degree of tumor promotion in the former strain (8). Vesselinovitch (12, 13) and Moore et al. (11) concluded that the greater susceptibility of B6C3F1, male mice relative to female mice resulted from the promotion of liver tumor development by testosterone. In both instances, promotion was characterized by an increase in the growth rate of preneoplastic lesions leading to an increase in the rate of appearance of liver tumors.

We have recently shown that the tumor-promoting effects of testosterone are mediated by the androgen receptor (14). Thus, male Tf(m) (testicular feminization) mutant mice, whose primary defect is the absence of functional androgen receptors, were much more resistant to the development of liver tumors than their normal male littermates.

In the present study, six genetically diverse (15) inbred mouse strains were compared for their sensitivities to liver tumor induction by ENU. Levels of plasma testosterone and binding properties of the hepatic androgen receptor were measured to determine if these parameters were correlated with tumor multiplicity.

MATERIALS AND METHODS

Mice and Tumor Induction. The mice used in these studies were bred in our laboratory from stocks of SWR/J, C57BL/6J, C57BR/cdJ, P/J, SM/J, and CBA/J strains obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in plastic cages on corn cob bedding (Anderssons, Maumee, OH) and acidified tap water ad libitum. Twelve-day-old male and female mice were given a single i.p. injection of ENU (0.25 μmol/g), which was synthesized as previously described (16) and dissolved in sterile triacetin (Pfaltz and Bauer, Inc., Stamford, CT). Male mice were killed at 32 weeks of age and females at 44 weeks of age by CO2 asphyxiation. All tumors >2 mm in diameter and visible on the surface of the lungs were enumerated. The lungs from each animal were fixed in 10% formalin and the number of tumors visible on the surface of the trunks was counted. The trunk blood was collected in tubes coated with a 5% solution of EDTA. The blood was centrifuged and the plasma from three to five animals was pooled and frozen at −20°C. Testosterone was extracted twice and the concentration was measured by radioimmunoassay.

The abbreviations used are: ENU, N-ethyl-N-nitrosourea; AR, androgen receptor.

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from 1 ml of thawed plasma with 3 ml of diethyl ether (17). The ether extracts were combined and evaporated to dryness and the residue was resuspended in 1 ml buffer (0.05 M Tris, 0.1% w/v gelatin, pH 8). For each pooled sample, testosterone was assayed on three separate occasions using a radioimmunoassay kit (Amersham Corp., Arlington Heights, IL). When samples of water containing known amounts of testosterone were extracted and assayed as above, recovery was approximately 90%.

Androgen Receptor Assay. Hepatic cytosol was prepared as described (18) with the modifications listed below. Female mice, which were used to minimize interference by endogenous androgens, were killed at 15 to 20 weeks of age by cervical dislocation. Livers were immediately perfused with 20 ml of 0.9% NaCl, removed and homogenized with five strokes of a Potter-Elvehjem homogenizer in 4 ml AR buffer [10 mM Tris-HCl, 1.5 mM EDTA, 1.0 mM 2-mercaptoethanol, 25 mM sodium molybdate, and 10% (v/v) glycerol, pH 7.4] (19). The homogenate was centrifuged at 1000 × g for 10 min at 4°C, the lipid layer was removed and the supernatant centrifuged at 216,000 × g for 45 min at 4°C. The cytosol was collected carefully to avoid lipid contamination and stored at −60°C. Preparations of cytosol from between five and eight individual mice were analyzed independently for each strain.

On the day of assay, cytosol was thawed and diluted with AR buffer to a protein concentration of 9–10 mg/ml. Protein concentration was determined by the Bradford method (20). Receptor binding was determined in duplicate 0.1 ml aliquots using 0.1–8.0 nM [3H]-mibolerone (7,17-dimethyl-19-nortestosterone; 70 Ci/mmol, New England Nuclear, Boston, MA). Nonspecific binding was determined by adding a 300-fold excess of unlabeled mibolerone to duplicate reaction tubes. After incubation for 24 h at 4°C, the cytosol was added to 0.2 ml hydroxylapatite columns (fast flow, Calbiochem, La Jolla, CA), allowed to bind for 5 min at room temperature, and washed four times (30 ml total) with 10 mM Tris-HCl, 1.5 mM EDTA, pH 7.4, to remove unbound steroid. The bound mibolerone was eluted with 1.0 ml of 95% ethanol and radioactivity was measured in a scintillation counter. Specific binding was calculated as (total dpm – nonspecific dpm)/mg cytosol protein. The binding data were analyzed by the EBDA program (Elsevier Biosoft, Cambridge, England) (21) according to the method of Scatchard (22).

RESULTS

Tumor Induction. The multiplicities of liver tumors induced by ENU, for both sexes of six inbred strains of mice, are shown in Table 1. Among the males, CBA/J mice were the most susceptible (45 tumors per animal), P/J and SM/J mice were moderately susceptible (16 tumors per animal), C57BR/cdJ mice were less susceptible (seven tumors per animal), and C57BL/6J and SWR/J mice were relatively resistant (<3 tumors per animal). The tumor multiplicities in these four groups differed from each other at the P < 0.05 level [Wilcoxon rank sum test, (23)]. Female mice were much more resistant to liver tumor induction than their male counterparts; this difference is greater than is apparent from Table 1 because the three groups differed from each other at the P < 0.05 level.

Androgen Receptor Binding. Radiolabeled mibolerone was used as the ligand for all androgen receptor binding studies because of its specificity for the androgen receptor and its resistance to metabolism (24). Kinetic analysis of the binding of [3H]-mibolerone to the hepatic androgen receptor (data not shown) demonstrated that equilibrium binding was attained by 24 h at 4°C; all subsequent binding assays were incubated under these conditions. The abilities of various steroids to compete with mibolerone for binding to the hepatic androgen receptor are shown in Table 2. The endogenous androgens dihydrotestosterone and testosterone competed well with mibolerone, estradiol competed slightly, and triamcinolone acetonide and progesterone did not compete for binding.

The affinity and amount of androgen receptors in liver cytosol from female mice of the six strains is shown in Table 3. Scatchard analysis of mibolerone binding yielded straight lines (correlation coefficients greater than 0.96) over a 10-fold range of bound/free values indicating the presence of a single high affinity binding site (Fig. 1). The Hill coefficients for binding ranged from 0.85 to 1.15, which indicates noncooperative binding (data not shown). The amount of androgen receptor in female livers varied from 24 to 34 fmol/mg cytosolic protein.

Table 2 Hepatic androgen receptor specificity

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Molar excess</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mibolerone</td>
<td>3 x</td>
<td>76</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>30 x</td>
<td>100</td>
</tr>
<tr>
<td>Testosterone</td>
<td>300 x</td>
<td>100</td>
</tr>
<tr>
<td>Estradiol</td>
<td>3 x</td>
<td>25</td>
</tr>
<tr>
<td>Triamcinolone acetonide</td>
<td>30 x</td>
<td>89</td>
</tr>
<tr>
<td>Progesterone</td>
<td>3 x</td>
<td>23</td>
</tr>
<tr>
<td>Testosterone</td>
<td>6.1 x</td>
<td>1.8 (0.4)</td>
</tr>
<tr>
<td>Triamcinolone acetonide</td>
<td>30 x</td>
<td>8.6 (3.0)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>30 x</td>
<td>5.3 (2.8)</td>
</tr>
</tbody>
</table>

Table 3 Androgen receptor binding and plasma testosterone levels

<table>
<thead>
<tr>
<th>Strain</th>
<th>Androgen receptor</th>
<th>Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KO (pmol/ml)</td>
<td>Bmax (fmol/ml)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>SWR/J</td>
<td>0.80 (0.03)²</td>
<td>5 (5)²</td>
</tr>
<tr>
<td>C57BR/cdJ</td>
<td>0.22 (0.03)²</td>
<td>0 (2)²</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>0.22 (0.03)²</td>
<td>0 (2)²</td>
</tr>
<tr>
<td>P/J</td>
<td>0.36 (0.05)²</td>
<td>28 (4)²</td>
</tr>
<tr>
<td>CBA/J</td>
<td>0.36 (0.06)²</td>
<td>34 (6)²</td>
</tr>
<tr>
<td>SM/J</td>
<td>0.45 (0.08)²</td>
<td>24 (2)²</td>
</tr>
</tbody>
</table>

KO and Bmax values were determined using the EBDA program (21). Five to eight mice were used per group.

² Testosterone was measured by radioimmunoassay on three separate occasions from plasma pooled from at least three mice per group.

Values are mean (standard deviation).
and CBA/J mice had significantly lower plasma testosterone levels (1.8 and 3.1 ng/ml) compared with males of the other four strains (5.5 to 7.4 ng/ml) (P < 0.05, Student's t test).

The abundance of androgen receptors in hepatic cytosol from male mice was also determined for four of the six inbred strains and the levels observed were 30-50% that seen in female mice (data not shown). These values are probably an underestimation of total cellular receptor, as androgen binding to its receptor is known to increase the concentration of nuclear androgen receptors (25) and this nuclear fraction would not have been detected by our assay. We have recently shown that castration of male mice increased the number of androgen receptors in hepatic cytosol two- to threefold, to a level similar to that seen in female mice (14).

Plasma Testosterone. Testosterone levels for the six inbred mouse strains. All aliquots (0.1 ml) of hepatic cytosol were incubated with 0.1-4 nM [3H]mibolerone with or without 300-fold excess of unlabeled mibolerone for 20 h at 4°C. Specific binding was calculated as total minus nonspecific binding. Bound/free (B/F) is plotted versus bound according to the method of Scatchard (22). Data for SM/J (O), CBA/J (x), and C57BR/cdJ (C) mice are shown.

The apparent K_d of the hepatic androgen receptor for mibolerone was quite low and differed between the strains. A representative Scatchard plot from each affinity group is shown in Fig. 1. Hepatic cytosol prepared from SWR/J, C57BL/6J, and C57BR/cdJ female mice had the highest affinity (K_d = 0.22 nm), P/J and CBA/J female mice had an intermediate affinity (K_d = 0.36 nm), and SM/J female mice had a K_d of 0.45 nm. These results were consistent between different cytosol preparations and the differences between groups were statistically significant (P < 0.05, Wilcoxon rank sum test). We could not detect differences between the strains in the rate of dissociation of [3H]mibolerone from the receptor nor in the thermal stability of the receptor at 37°C (data not shown).

The abundance of androgen receptors in hepatic cytosol from male mice was also determined for four of the six inbred strains and the levels observed were 30-50% that seen in female mice (data not shown). These values are probably an underestimation of total cellular receptor, as androgen binding to its receptor is known to increase the concentration of nuclear androgen receptors (25) and this nuclear fraction would not have been detected by our assay. We have recently shown that castration of male mice increased the number of androgen receptors in hepatic cytosol two- to threefold, to a level similar to that seen in female mice (14).

Plasma Testosterone. Testosterone levels for the six inbred strains ranged from 0.21 to 0.42 ng/ml for the female mice and from 1.8 to 7.4 ng/ml for the male mice (Table 3). Male P/J and CBA/J mice had significantly lower plasma testosterone levels (1.8 and 3.1 ng/ml) compared with males of the other four strains (5.5 to 7.4 ng/ml) (P < 0.05, Student's t test).

DISCUSSION

We observed a wide range of susceptibilities to both liver and lung tumor induction by ENU among the six inbred strains tested. Liver tumor multiplicities for male mice ranged from 0 (SWR/J) to 45 (CBA/J) tumors per animal and for females ranged from 0 (SWR/J) to 5 (C57BR/cdJ) tumors per animal. In contrast to the greater susceptibility of male mice relative to female mice for liver tumor induction there was little or no difference between the sexes in lung tumor susceptibility. Furthermore, SWR/J mice, which were the most resistant to liver tumor induction, were the most sensitive to lung tumor induction. These differences in the inbred strain distribution of liver and lung tumor susceptibilities indicate that the genes which influence tumor induction act in a tissue-specific manner (26).

Previous genetic studies of lung tumor induction among inbred strains of mice have indicated that at least three loci contribute to the strain variation in lung tumor susceptibility (27). The rank order for susceptibility to spontaneous lung tumor development among inbred mouse strains is A > SWR > CBA = C3H > C57BL > C57L = DBA, the latter strains being relatively resistant (26). Our data for ENU-induced lung tumors concur with this grouping for the SWR, CBA, and C57BL strains and would place the C57BR/cdJ, P, and SM strains in the resistant group.

CBA mice are closely related to C3H mice, as both strains were derived from the C/St line, which in turn was derived from a single cross between a male of the partially inbred DBA line and a female from the outbred Bagg albino stock (28, 29). Both CBA and C3H strains are highly susceptible to hepatocarcinogenesis (30). We have shown previously (7) that the greater susceptibility of the C3H/HeJ strain relative to the C57BL/6J strain may be attributed to a single locus, designated Hcs (Hepatocarcinogen sensitivity), which influences the rate of growth of preneoplastic lesions throughout the promotion phase of hepatocarcinogenesis (8). The close genetic relationship of CBA and C3H mice (28) and their similar susceptibilities to hepatocarcinogenesis indicate that these strains may possess the same Hcs allele derived from the C/St line.

The SM/J and P/J strains both displayed a moderate susceptibility to liver tumor induction. These mice shared a common outbred parent (29) which may have contributed to the similar susceptibilities of these mice. We are currently testing whether their susceptibility results from different alleles of the Hcs locus or from entirely different genes.

The SWR line of mice was derived from an outbred Swiss stock of mice (29) and these mice were the most resistant to liver tumor induction. The C57 family of inbred mice is also genetically quite distinct from the other inbred lines (15, 31). As discussed above, C57BL/6J mice are relatively resistant to liver tumor induction. C57BR/cdJ mice were unusual in that they are closely related to C57BL/6J mice yet the males were 20-fold more susceptible than the C57BL/6J males and the females had the highest tumor multiplicity among the strains tested. The genetic basis for this difference is not known, but it has been reported that a derivative of the C57BL line, C57L, is also susceptible to liver tumor induction (32).

The greater susceptibility of male mice to liver tumor induction relative to female mice reported here has also been observed in other inbred mouse strains and F1 hybrids. For example, C3H, CBA, B6C3F1, and (DBA/2 × CE/J)F1 mice developed a greater number of spontaneous hepatomas over their lifetime than did females of the same strains (30, 33-35). For two of these strains (C3H and B6C3F1, hybrid), testosterone has been shown to be primarily responsible for the sex difference in susceptibility. Thus, castration of male C3H and B6C3F1 mice decreased both the growth rate of preneoplastic lesions and the number of liver tumors (12, 13, 30) and testosterone administration to females increased the growth rate of preneoplastic lesions and increased tumor yield (11, 33). In the current study, testosterone levels among the six strains were 7- to 28-fold greater in male mice relative to female mice, which is consistent with the greater tumor multiplicity observed among the male mice. Within a given sex, testosterone levels varied 4-fold among the males and twofold among the females but these levels were not correlated to liver tumor susceptibility (Table 1 and 3). For example, C57BR/cdJ and CBA/J females had similar levels of plasma testosterone yet differed 7-fold in liver
tumor multiplicity and SWR/J males had high levels of testosterone (6.6 ng/ml) but were resistant to liver tumor induction. The binding properties (specificity, affinity, and amount) of androgen receptors from mouse hepatic cytosol reported here are similar to those observed for other androgen responsive tissues (36, 37). There was slight but reproducible strain-to-strain variation in the amount and affinity of the hepatic androgen receptor but this variation was not correlated with liver tumor susceptibility. The recent cloning of the androgen receptor (38, 39) should make it possible to determine if the strain-dependent differences in the apparent affinity for miibolorene represents polymorphism of the androgen receptor gene.

The greater susceptibility of male mice to hepatocarcinogenesis relative to female mice is a result of the tumor-promoting effects of testosterone in male mice and the tumor inhibiting effects of estrogens in female mice (11-14). However, the cause of the strain-to-strain variation in liver tumor susceptibility remains unknown. We have determined that for a set of six genetically diverse mouse strains, circulating levels of testosterone and binding properties of the hepatic androgen receptor do not explain the strain-dependent differences in tumor susceptibility. It is possible that polymorphism in other properties of the androgen receptor (e.g., efficiency of the hormone-receptor complex as a transcriptional transactivator) may contribute to tumor susceptibility. However, in at least one inbred strain, the C3H mouse, the high susceptibility phenotype involves a mechanism which is separate and independent of the androgen-receptor promotion pathway (8).

A more detailed genetic analysis of the C57BR/cdJ, P/J, and SM/J strains will be required to determine if the gene(s) which result in the moderate to high susceptibility of these strains are alleles of the Hcs locus or represent additional genetic mechanisms for the control of liver tumor development.

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