Effects of Neonatal and Adult Castration and of Testosterone Substitution in Male Rats on Growth of Enzyme-altered Hepatic Foci in the Resistant Hepatocyte Model

Barbro Lindhe, Inger Porsch-Hällström, Jan-Åke Gustafsson, and Agneta Blanck

ABSTRACT

Marked sex differences in the growth of enzyme-altered hepatic foci have been observed in rats treated according to the "resistant hepatocyte model." The present study was performed to investigate the effect of neonatal and adult castration of male rats, with or without testosterone substitution, on the growth rate of foci during selection of initiated cells with 2-acetylaminofluorene and partial hepatectomy. Neonatal castration of male rats decreased focal growth to the same level as in female rats. Castration of adult male rats 2 wk before initiation with diethylnitrosamine also decreased the growth rate of foci, but less markedly than in neonatally castrated rats. Testosterone substitution of male rats castrated as neonates or as adults, from 10 days after initiation with diethylnitrosamine, restored focal growth to that of sham-castrated controls. Previous investigations concerning the role of gonadal hormones in sex differentiation of various liver functions indicated a role of the hypothalamic-pituitary-liver axis in mediating the effects of androgens. It is therefore also suggested that the effects of androgens on early steps of hepatocarcinogenesis observed in the present study are mediated by similar mechanisms, possibly through an influence on the metabolism of 2-acetylaminofluorene.

INTRODUCTION

Sex-differentiated chemical hepatocarcinogenesis in the rat has been studied extensively (1-9). Male rats develop hepatocellular carcinomas both earlier and more frequently than female rats when treated with, e.g., 2-AAF (1) or aflatoxin B1 (2) in the diet. The role of gonadal as well as of pituitary hormones has been investigated by means of different hormonal manipulations (3-8). In the male rat neonatal and adult castration (3, 4), estrogen administration (5), hypophysectomy (6), and hypothalamic deafferentation (7) have been shown to decrease liver tumor formation in 2-AAF-treated rats. An increased sensitivity to 2-AAF has been observed in oophorectomized female rats receiving testosterone, when compared with intact rats (8).

In most of these studies it was impossible to discern at which stage of the hepatocarcinogenic process a sexually differentiated event, or an effect of a hormonal manipulation, had occurred. During the last decades several models for rat liver carcinogenesis have been presented, by which the liver cancer process can be separated into at least two distinct steps, initiation and promotion (9-11). These models offer unique possibilities to study the interaction between hormones and specific events in hepatocarcinogenesis.

Previous studies indicate that various sex-differentiated liver functions are regulated via a hypothalamic-pituitary-liver axis, due to the sexual dimorphism in pituitary secretion of GH (12-15). Hepatic metabolism of steroids was first used as a model to elucidate these mechanisms (13). Sex differences in both GH secretion and hepatic metabolism of steroids become apparent at puberty (17). Androgen-dependent GH secretion in male rats seems to be regulated at the hypothalamic level via an influence on secretion of somatostatin and of a GH-releasing factor (18). This hypothalamic influence is imprinted by testicular androgens in the neonatal male rat and has generally been considered as important for the capacity of the animal to respond to testosterone treatment as an adult (19, 20).

Marked sex differences in the size of enzyme-altered foci and in the latency time for development of hepatocellular carcinomas have been observed in the RH-model (21, 22). Continuous infusion of GH to male rats has been shown to "feminize" both hepatic metabolism of steroids and xenobiotics (14, 23) and the growth rate of enzyme-altered foci in the RH-model (24). The present study was designed in order to investigate the role of androgens in rat liver carcinogenesis as a further step to test the notion of the hypothalamic-pituitary-liver axis as a major determinant for the sexual dimorphism in susceptibility of rats to treatment with chemical hepatocarcinogens.

MATERIALS AND METHODS

Chemicals. DEN was obtained from Fluka AG, Buchs, Switzerland. The diet containing 0.02% 2-AAF was delivered from AB Ewos, Söderåsens, Sweden. (4,14C)Androstene-3,17-dione (androstenedione) (59 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England. Unlabeled androstenedione was purchased from The Upjohn Co., Kalamazoo, MI. All other chemicals and solvents were of reagent grade and were obtained from common commercial sources.

Animals and Hormone Treatment. Pregnant female Wistar rats were obtained from Möllegård Breeding Centre, Ltd., Stensved, Denmark. All rats in the experiment were born within a period of 4 days. They were weaned at the age of 28 days and maintained under standardized conditions of light (lights on from 6 a.m. to 6 p.m.) and temperature (21 ± 1°C). From weaning until carcinogen treatment was started, rats were housed (5-6 rats/cage) in cages with solid floors, covered with dust-free softwood carvings. From 8 wk of age until the end of the experiment, the animals were kept in wire-bottomed cages. Neonatal castration of male rats was performed within 36 h after birth, whereas adult male rats were castrated at 42 days of age. Sham operations of male rats were performed. Two groups of neonatally castrated rats received testosterone substitution from 10 days after initiation (see below and Fig. 1) with either 5- or 10-mm-long constant release capsules containing crystalline testosterone (Sigma), as previously described (25). The capsules were placed s.c. in the scapular region. One group of male rats castrated as adults received 5-mm implants at the same time. All groups of male rats not receiving testosterone, as well as the female rats, were implanted with 5-mm-long empty tubes.

The Resistant Hepatocyte Model. All rats were treated according to a slightly modified RH-model (Ref. 9; Fig. 1). Ten days after initiation with DEN (200 mg/kg of body weight), rats received empty or testos-
calculated (13; 9 > <J)./V.O-Sulfation of N-OH-2-AAF was measured
erin jelly. The number of foci per area unit and the size of foci were
cut, stained for -y-glutamyltransferase (31 ), and mounted in glyc
supernatant contained approximately 6 mg of protein/ml. The sulfo-
glutathione and other low-molecular-weight substances. Fifty mivi so
was performed on a Sephadex G-25 column (45 x 2.5 cm) to remove
partial purification of the 105,000 x g supernatant used for this assay
in and effects of hormonal manipulations on liver metabolism, the ratio
ured as previously described (21). In order to determine sex differences
say and interassay coefficients of variation were below 7 and 11%,
was performed according to the method of Ernster et al. (27) and storedat —70°C until used. In the experiment with only castrated/sham-
say and interassay coefficients of variation were below 7 and 11%,
Subcellular Preparation. Liver was collected at PH, and microsomes
were prepared according to the method of Ernster et al. (27) and stored at 
—70°C until used. In the experiment with only castrated/sham-
castrated rats, the 105,000 x g supernatant was collected also.
Assays. Protein was determined according to the method of Lowry
et al. (28). The microsomal metabolism of androstenedione was me-
measured as previously described (21). In order to determine sex differences in
and effects of hormonal manipulations on liver metabolism, the ratio
between 5a-reduction and 16a-hydroxylation of androstenedione was cal-
culated (13; 9 > <J)./V.O-Sulfation of N-OH-2-AAF was measured
by the indirect method described previously by Mulder et al. (29).
Partial purification of the 105,000 x g supernatant used for this assay
was performed on a Sephadex G-25 column (45 x 2.5 cm) to remove
glutathione and other low-molecular-weight substances. Fifty mm so-
dium phosphate buffer was used as eluent. The chromatographed
supernatant contained approximately 6 mg of protein/ml. The sulfo-
transferase assay was modified by measuring the reaction rate at 37°C
instead of at 31°C.
Morphometric Analysis of Foci Positive for γ-Glutamyltransferase.
At the time of sacrifice, 2- to 3-mm-thick liver slices were cut and
immediately fixed in cold acetone (0–4°C). The slices were embedded
in low-melting-point paraffin as previously described (30). Sections
were cut, stained for γ-glutamyltransferase (31), and mounted in gly-
cerin jelly. The number of foci per area unit and the size of foci were calculated using a semiautomatic image analyzer (MOP-Videoplan;
Kontron, Munich, Federal Republic of Germany).
Statistical Analysis. Results were expressed as the mean ± SD.
Statistical comparisons were performed using the Wilcoxon rank sum
test (32), and the level of significance was set at P < 0.05.

RESULTS
In the present experiment sex differences were observed in
both the number and size of enzyme-altered hepatic foci (Table
1). Whereas male rats, sham castrated as adults, exhibited 4.7-
fold larger foci than the female rats, the number of foci was only
1.7-fold higher in male than in female rat livers.
Neonatal castration of male rats decreased the size and
number of foci to the female level. Foci in neonatally castrated
rats were 2.7-fold smaller than in their sham-castrated controls.
Testosterone treatment with 5- or 10-mm-long implants re-
stored both the size and number of foci to the levels in neo-
natally sham-operated male rats. A less pronounced decrease of
focal size was observed following castration of rats at 42 days of
age (1.6-fold), whereas no effect on the number of foci was
observed. Also this effect was reversed by testosterone treat-
ment. Neonatal sham castration led to a slight decrease in focal
size compared with the corresponding group of males sham
operated as adults. On the other hand, no significant differences
were seen between neonatally castrated rats receiving testoster-
one compared with rats sham operated as adults or rats cas-
trated when adults and then substituted with testosterone. The
testosterone levels in sham-operated controls and castrated rats
receiving 5-mm-long implants were comparable, whereas neo-
natally castrated rats receiving 10-mm-long implants exhibited
higher serum levels (1.9-fold compared with neonatally cas-
trated rats with 5-mm implants).
As a measure of the sex differences in hepatic steroid metab-
olism and of the efficiency of the hormonal manipulations, the
ratio between the rate of formation of the 5α-reduced and 16α-
hydroxylated metabolites, respectively, of androstenedione was
measured. A high ratio corresponds to a female pattern of
androstenedione metabolism, whereas the male ratio is much
lower. A markedly higher 5a/16α ratio was found with micro-
somes from female rat liver than with microsomes from male
controls, sham-operated as neonates or as adults. Neonatal
castration of male rats increased the ratio to the female level,
whereas adult castration led to a partial feminization. Testos-
terone treatment of male rats castrated as neonates or at 42
days of age decreased the 5a/16α ratio to the level of sham-
operated controls.
In the separate experiment, where livers were collected at PH
in the RH-model, the rate of N,O-sulfation of N-OH-2-AAF
was significantly lower in preparations from castrated males
than in sham-castrated males (0.11 ± 0.02 nmol/min/mg of
protein; n = 10, compared with 0.23 ± 0.08 nmol/min/mg of
protein; n = 10).

DISCUSSION
Results obtained in the present study showed that both neo-
natual and adult castration of male rats decreased the growth
rate of enzyme-altered hepatic foci in the RH-model. In both
cases testosterone substitution restored focal growth to the
levels of sham-operated controls. The sex difference in the size
of foci was even more pronounced than that previously reported
(21, 24). It is also worth noting that sham castration of male
rats in the neonatal period led not only to a slightly higher 5α/
16α ratio, but also to a slower growth rate of foci, when
compared with male rats sham operated as adults.
Several investigators have shown that castration of male rats
before administration of 2-AAF inhibits formation of hepatic
tumors and that neonatal castration seems to be more efficient
than adult castration (3, 4). Also hypothalamic lesions in the
median eminence area of male rats decreased tumor formation
of male rats subsequently exposed to dietary 2-AAF (7). The
design of these studies, where 2-AAF was given in the diet for
long periods of time, did not allow any speculations as to
whether sex-differentiated hepatocarcinogenesis was due to a
sexual dimorphism at the initiation and/or promotion stages.
Nevertheless, these findings indicated that the regulation of
sex-differentiated liver carcinogenesis might be due to a neu-
roendocrine influence on some central hepatic function(s).
In the RH-model, sex differences have been shown to occur,
not during DEN initiation, but during selection/promotion with 2-AAF (21, 22, 24). As the low dose of 2-AAF used in this protocol (0.02%) does not cause initiation (9), it is reasonable to assume that the observed sex differentiation of focal growth (21, 24) and the latency time for cancer development (22) are due to an effect on promotion. The differences in the number of foci observed in the present study are probably due to the fact that 2-AAF/PH treatment of female and neonatally castrated male rats is less efficient in promoting initiated cells to foci of a size that can be detected by the method used than in the other groups of rats.

Early studies on hypothalamo-pituitary regulation of liver function focused on the metabolism of steroid hormones (19, 20). Neonatal castration of male rats was reported to lead to a “feminization” of hepatic steroid metabolism and to an unresponsiveness to testosterone treatment, measured as the capacity to restore a male pattern of steroid metabolizing enzymes (19). On the other hand the feminizing effect of adult castration was considered to be completely reversible by testosterone administration (19). Today it is well established that the sexual dimorphism in the secretory pattern of GH exerts a major influence on sex differentiated hepatic metabolism of steroids as well as of various xenobiotics (13, 14, 24). Feminization of GH secretion following neonatal castration was reported to be reversible by androgen treatment (33). These findings, as well as the data presented here, support the view that neonatal imprinting is not an absolute prerequisite for testosterone responsiveness, at least not with respect to GH-mediated regulation of liver metabolism. Interestingly, adult castration is much less efficient than neonatal castration in feminizing both the response to 2-AAF/PH selection in the RH-model and the metabolism of steroids and xenobiotics (34). This finding indicates that neonatal imprinting by androgens is important for sexual differentiation of hepatocarcinogenesis in the rat and that this phenomenon is not necessarily coupled with an influence on testosterone responsiveness.

It has previously been suggested that hepatic metabolism is an important determinant for sex-differentiated hepatocarcinogenesis where 2-AAF is involved. Several pathways of 2-AAF metabolism have been shown to be both sex differentiated (14, 24, 34) and GH regulated (14, 24, 35). These pathways include NO-sulfation of N-OH-2-AAF (\(\delta > 9\)), yielding a very reactive and toxic sulfate ester (36, 37). This metabolite of 2-AAF is only weakly mutagenic but seems to be important for the promotive effects of 2-AAF in different experimental systems (38, 39). The sulfotransferase level in neonatally castrated male rats has previously been reported to be comparable to that of female rats, whereas adult castration did not significantly alter the enzyme activity (34, 40). In view of the previously mentioned effect of neonatal sham castration on focal growth, it is of interest to note that a significant decrease in sulfotransferase activity has been reported in neonatally sham-castrated rats compared with intact males (34). These investigations were performed in rats not treated with carcinogens. In rats fed 2-AAF, a marked decrease in NO-sulfation has been observed (41). The significantly higher sulfotransferase activity in sham-castrated than in castrated male rats observed here, at the time of PH in the RH-model, indicates that the influence of testosterone/GH-secretion on this pathway of 2-AAF metabolism in this specific case becomes manifest during 2-AAF treatment.

Differential mitoinhibition of normal and initiated cells, respectively, has been suggested as a basis for the efficient selection of putatively preneoplastic lesions in the RH-model (9). As very early nodules from male rats, obtained 11 days after completed 2-AAF selection, show markedly lower sulfotransferase levels in vitro than liver from noninitiated male rats treated with 2-AAF/PH4, it is quite conceivable that the sulfotransferase levels are lower in foci than in surrounding liver already during 2-AAF selection. Based on the assumption that the sulfation pathway is important for the mitoinhibitory effects of 2-AAF, it is tempting to speculate that the differential inhibition might be more efficient in the liver from male rats, where the difference in sulfotransferase activity between the population of initiated cells and the surrounding liver is more pronounced, than in female or “feminized” male rat liver, where the sulfotransferase activity is low in the whole liver.

Other possible explanations of the sex-differentiated and androgen-dependent focal growth in the model used include a direct influence of GH secretion and/or androgens on foci during the period when a majority of the liver cells are unable to respond to growth stimulation. In fact, testosterone has been shown to be essential for the capacity of male rat liver to regenerate, when a 90% PH was performed (42). This effect might also be mediated via GH secretion, but a direct influence of androgens on male rat liver cannot be excluded.

In conclusion, the data obtained in the present study clearly show that androgens are important for sex differentiation of 2-AAF/PH promotion in the RH-model, probably due to an

### Table 1 Effects of neonatal and adult castration and of testosterone substitution (5- or 10-mm-long tubes) of male Wistar rats on the number and areas of enzyme-altered foci after treatment according to the resistant hepatocyte model

<table>
<thead>
<tr>
<th>Sex</th>
<th>No.</th>
<th>Treatment</th>
<th>No. of foci/cm²</th>
<th>Area/foci (mm²)</th>
<th>5α/16α ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂</td>
<td>10</td>
<td>Neonatal castration</td>
<td>16.2 ± 4.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26 ± 0.11</td>
<td>126.0 ± 41.8</td>
</tr>
<tr>
<td>♀</td>
<td>8</td>
<td>Neonatal castration</td>
<td>16.3 ± 5.8</td>
<td>0.27 ± 0.10</td>
<td>104.9 ± 48.9</td>
</tr>
<tr>
<td>♀</td>
<td>10</td>
<td>Neonatal castration + 5 mm testosterone</td>
<td>33.0 ± 7.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.71 ± 0.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.9 ± 10.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>♀</td>
<td>14</td>
<td>Neonatal sham castration</td>
<td>28.6 ± 4.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.76 ± 0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.7 ± 2.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>♀</td>
<td>14</td>
<td>Neonatal castration</td>
<td>30.9 ± 6.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.72 ± 0.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.8 ± 6.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>♀</td>
<td>14</td>
<td>Neonatal castration + 10 mm testosterone</td>
<td>31.0 ± 8.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.76 ± 0.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40.3 ± 23.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>♀</td>
<td>9</td>
<td>Adult castration</td>
<td>31.0 ± 11.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.11 ± 0.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.2 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>♀</td>
<td>7</td>
<td>Adult sham castration</td>
<td>28.2 ± 9.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.21 ± 0.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3 ± 1.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD.
<sup>b</sup> Significantly different from neonatally sham-castrated male rats.
<sup>c</sup> Significantly different from neonatally castrated male rats with 5-mm-long testosterone implants.
<sup>d</sup> Significantly different from neonatally castrated male rats with 10-mm-long testosterone implants.
<sup>e</sup> Significantly different from neonatally sham-castrated male rats.
<sup>f</sup> Significantly different from male rats castrated at 42 days of age.

A. Blanck. unpublished observations.
indirect effect on the rat liver via the sexually dimorphic secretory pattern of GH. This might constitute a general mechanism for sex differentiation of rat liver carcinogenesis in various models where 2-AAF or other hepatocarcinogens are used. Further studies are needed to identify the specific biochemical and biological events responsible for GH regulation of hepatocarcinogenesis.

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