Effects of 2-Acetylaminofluorene on Liver Cell Proliferation after Partial Hepatectomy of Female Rats

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SUMMARY

The effect of the hepatocarcinogen 2-acetylaminofluorene (AAF) on liver regeneration in female Sprague-Dawley rats was studied. While livers of normal female rats were highly resistant to this carcinogen, partial hepatectomy of female animals maintained on diet containing 0.04% AAF disclosed a marked hepatotoxicity which resulted in abnormal, nodular regeneration.

The incorporation of thymidine-3H into DNA and the mitotic index were markedly inhibited in livers of AAF-treated animals following partial hepatectomy. Cell proliferation in control rats began to increase at 18 hr after partial hepatectomy, reached a maximum at 48 hr, and returned to normal by the 7th day. In contrast, cell proliferation in animals maintained on AAF for 3 weeks increased at a much lower rate at 18 hr after partial hepatectomy and continued to increase slowly for 2 weeks. Regeneration in AAF-treated rats was not uniform but occurred at discrete foci which led to a nodular regeneration. Sham operation had no effect on liver cell proliferation in animals maintained on AAF.

Female rats were fed 0.02 or 0.04% AAF for 3 weeks, subjected to partial hepatectomy, and then maintained on AAF for an additional 28 weeks. These animals developed adenocarcinoma of the mammary gland and ear duct, but no hepatocellular carcinoma was observed. Thus, the failure of the hyperplastic liver nodules to progress to the stage of carcinoma indicates a requirement for some other factor in addition to cell division for tumor induction in livers of female rats.

INTRODUCTION

One of the more pronounced effects of p.o. administration of the hepatocarcinogen AAF3 to male rats is a marked increase in liver cell proliferation which leads to formation of hyperplastic nodules (2, 14). Merkow et al. (15) have presented evidence that the hyperplastic liver nodules contain the precursor cells for tumor formation. Although the majority of hyperplastic liver nodules induced by AAF do not become malignant tumors (21), the time required for development of hyperplasia is correlated with the minimum time for which the carcinogen must be fed to produce liver tumors (12). That cell proliferation plays an important role in tumor induction was demonstrated by Laws (13), who found that partial hepatectomy of male rats during p.o. administration of AAF leads to more rapid formation of hyperplastic nodules and accelerated the appearance of tumors. Partial hepatectomy of male rats during administration of AAF proved to be extremely toxic, however, with most of the animals dying within 2 weeks (13). In contrast, female rats are much more resistant than males to induction of liver tumors by AAF (20).

We have recently compared the proliferative response in livers of male and female Sprague-Dawley rats maintained on a diet containing 0.04% AAF (11). In contrast to the marked increase in liver cell proliferation observed in the males, the carcinogen produced no significant change in the rate of cell proliferation in livers of female animals. These results suggested that the resistance of female rats to induction of liver tumors by AAF may be due to failure of the carcinogen to initiate cell proliferation in females. It was of interest, therefore, to determine whether or not female rats would develop liver tumors if cell division were induced by partial hepatectomy during administration of AAF and to investigate the effects of the carcinogen on liver regeneration in female rats where the hepatotoxicity of the carcinogen might be minimized.

MATERIALS AND METHODS

Animals and Diet. Female Sprague-Dawley rats (180 to 200 g) were purchased from the Holtzman Company, Madison, Wis. To allow conditioning to the grain diet previously described (6), we maintained all animals on control diet for 1 week prior to the experimental period. Diets containing 0.02 or 0.04% AAF were prepared by addition of AAF dissolved in acetone (11). Partial hepatectomies were performed under ether anesthesia as described by Higgins and Anderson (4).

Compounds. AAF was synthesized by the method of Ray and Geiser (18). Thymidine-methyl-3H (6.7 Ci/m mole) was purchased from New England Nuclear, Boston, Mass. Each animal received 15 μCi thymidine-methyl-3H i.p. 1 hr before being killed.

Mitotic Index and Thymidine-3H Incorporation. The mitotic index of liver and the incorporation of thymidine-3H...
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into liver DNA were determined as previously described (11). Radioactivity was determined by liquid scintillation counting in Triton-X fluor. Counting efficiency was determined by addition of internal standard.

RESULTS

Formation of Hyperplastic Nodules. To establish whether AAF would have an effect on liver regeneration in female rats and to determine something of the toxicity of the carcinogen following partial hepatectomy, we maintained 6 female rats on 0.04% AAF for 3 weeks and then partially hepatectomized them. The animals were maintained on 0.04% AAF for an additional 5 weeks and sacrificed. The liver of a typical animal is shown in Fig. 1. Gross examination indicated that restoration of liver mass was suppressed. Numerous protruding hyperplastic nodules were visible over the entire surface of the liver. Histological examination (Fig. 2) showed nodular regeneration throughout the lobe with marked bile duct and venous dilation and extensive fibrous tissue formation.

Thymidine Incorporation and Mitotic Index. For investigation of the effects of AAF on cell proliferation during the early phase of regeneration, 3 groups of female rats containing 30 rats in each group were placed on: (a) control diet; (b) diet containing 0.02% AAF; and (c) diet containing 0.04% AAF. Three weeks later the animals were partially hepatectomized and returned to their respective diet. At various times following partial hepatectomy, 5 animals of each group were given 15 μCi of thymidine-3H i.p. and were sacrificed 1 hr later. The mitotic index and thymidine-3H incorporation into DNA were determined.

The results over the 1st 80 hr following partial hepatectomy are shown in Chart 1. Animals on control diet exhibited a marked increase in incorporation of thymidine-3H beginning 18 to 20 hr postoperatively and reaching maxima at 36 and 60 hr. In contrast, incorporation of thymidine-3H into liver DNA by animals on 0.02 or 0.04% AAF was markedly reduced. Both levels of the carcinogen were approximately equal in suppressing thymidine incorporation. Incorporation of thymidine-3H in AAF-treated animals increased at a very low rate.

The mitotic index of livers from control animals began to increase at about 20 hr following partial hepatectomy and reached a maximum at 48 hr or about 12 hr following the 1st peak in thymidine incorporation (Chart 1). The mitotic index of livers of animals fed AAF paralleled the thymidine incorporation. There was a low but constant increase in the mitotic index over the 80-hr period. Again, both levels of AAF had about equal effects in suppressing cell proliferation.

Fig. 1. Entire liver of female rat maintained on 0.04% AAF following partial hepatectomy. Partial hepatectomy was performed after 3 weeks of AAF administration, and the rat was killed 5 weeks after partial hepatectomy. Note the presence of multiple hyperplastic nodules.

Fig. 2. Photomicrograph of section of liver in Fig. 1. Note extensive nodule formation and cirrhosis. H & E, x 41.
AAF and Liver Regeneration

Chart 1. Effect of AAF on thymidine-³H incorporation and mitotic index of liver during the early period of liver regeneration in female rats. Incorporation of thymidine-³H (6.7 Ci/m mole; 15 μCi/rat) into liver DNA and liver mitotic index of animals maintained on control diet (•), 0.02% AAF (○), and 0.04% AAF (▪). Animals were maintained on their respective diets for 3 weeks prior to partial hepatectomy (0 hr) and during the period of regeneration. Values shown represent the mean of 5 animals ± S.E.

Neither thymidine incorporation into DNA nor the mitotic index of livers of sham-operated female rats on 0.04% AAF was affected (Chart 2). These results are in agreement with our previous report (11) that AAF had no effect on cell proliferation in normal female rat liver. As observed in the previous experiment (Chart 1), both thymidine-³H incorporation and mitotic index of livers of animals on control diet reached a maximum at approximately 48 hr and returned to normal values by the end of 1 week. Animals on 0.04% AAF, however, exhibited a markedly reduced rate of cell proliferation during the 1st week whether measured as thymidine-³H incorporation into DNA or mitotic index. By the end of 2 weeks, distinct hyperplastic nodules began to form in livers of animals on AAF. The mitotic indices of individual nodules were equal to that of regenerating liver at its maximum rate of cell proliferation (Chart 2).

Tumor Formation in Females. A chronic feeding experiment was performed to determine whether the hyperplastic nodules induced in females by partial hepatectomy during administration of AAF would progress to the stage of hepatomas. Female rats were maintained on control diet or 0.02 or 0.04% AAF for 3 weeks and were then partially hepatectomized or sham operated. The animals were returned to their respective diets for an additional 28 weeks. The 0.04% AAF was very toxic to partially hepatectomized rats, and a large number of animals died between 2 and 3 weeks following surgery. Consequently, the partially hepatectomized animals on 0.04% AAF, as well as their sham-operated control group, were returned to control diet throughout their 3rd postoperative week to allow recovery and were subsequently maintained on 0.02% AAF for the remainder of the 28-week period.

Although a considerable number of malignant tumors of...
both mammary gland and ear duct (Zymbal's gland) were observed in each group of animals receiving AAF, no liver cell carcinomas were found (Table 1). We did not determine the incidence of liver tumors in male rats on the dietary regimen followed in Table 1. However, one would expect the incidence of tumors in male rats fed 0.04% AAF for 31 weeks, with or without partial hepatectomy, to approach 100%. Feeding 0.03% AAF in the diet to male Sprague-Dawley rats for only 13 weeks gave a 70% incidence of liver tumors at 35 weeks, whereas the incidence of liver cancer in female rats was only 7% (6).

**DISCUSSION**

Numerous reports (5, 13, 17) have indicated that DNA replication and cell division play an important role in chemical carcinogenesis; this subject has been discussed at length by Warwick (23). That chemical carcinogenesis in liver is a multistep process is well established (15). The temporal sequence of events believed to occur in liver during the process of chemical carcinogenesis by AAF is shown schematically in Chart 3. While there are quantitative differences between the 2 sexes, considerable metabolism and binding of AAF to protein and nucleic acids occur in both male and female rats (8, 9). Although the rate of N-hydroxylation of AAF may be limiting in some species (16), both male (10) and female (3) Sprague-Dawley rats form considerable amounts of the N-hydroxy metabolite. On the other hand, the level of liver sulfotransferase, which forms the reactive sulfate derivative, is initially 5 to 10 times higher in male than female rats (1, 9, 11). The enzyme activity is markedly reduced in both sexes during the 1st 2 or 3 weeks of administration of AAF (9, 11). About 2 to 4 times as many fluorene residues are bound to liver rRNA and tRNA in male rats than in female animals after a single injected dose of AAF or N-hydroxy-2-acetylaminofluorene and binding to liver DNA is approximately equal in the 2 sexes (8). Differences in binding of AAF-9-14C to liver DNA and tRNA in the 2 sexes were minimal when the labeled carcinogen was administered p.o. over an 8-week period, whereas binding to liver rRNA was considerably lower in female rats (7, 9).

The cell proliferation that occurs during AAF administration is presumed to be the result of the hepatotoxicity of the compound or its metabolites (1, 11). The only tenable explanation now for the marked difference in hepatotoxicity and cell proliferation in livers of male and female Sprague-Dawley rats (1, 11) is the different levels of liver sulfotransferase activity in the 2 sexes (1, 9, 11). The fact that females do have significant amounts of the enzyme suggests the possibility of a threshold dose or rate of activation, below which the liver cells are able to eliminate the compound or repair the damage produced by the carcinogen. That feeding the carcinogen does produce biochemical changes in the liver of female Sprague-Dawley rats was demonstrated by a decreased response to enzyme induction (22).

![Diagram of carcinogenesis process](chart_3.png)

**Chart 3. Schematic comparison of the effects of continuous feeding of AAF on livers of male and female Sprague-Dawley rats.** X, point at which the process is terminated in normal and partially hepatectomized females. Further details are discussed in the text.

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>28-wk survivors&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ear duct gland</th>
<th>Mammary gland</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control diet: sham</td>
<td>17/20</td>
<td>0/17</td>
<td>0/17</td>
<td>0/17</td>
</tr>
<tr>
<td>2</td>
<td>Control diet: PH&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23/25</td>
<td>0/23</td>
<td>0/23</td>
<td>0/23</td>
</tr>
<tr>
<td>3</td>
<td>0.02% AAF: sham</td>
<td>11/16</td>
<td>3/11</td>
<td>8/11</td>
<td>0/11</td>
</tr>
<tr>
<td>4</td>
<td>0.02% AAF: PH</td>
<td>14/27</td>
<td>5/14</td>
<td>10/14</td>
<td>0/14</td>
</tr>
<tr>
<td>5</td>
<td>0.04% AAF: sham</td>
<td>19/25</td>
<td>9/19</td>
<td>13/19</td>
<td>0/19</td>
</tr>
<tr>
<td>6</td>
<td>0.04% AAF: PH</td>
<td>14/50</td>
<td>7/14</td>
<td>3/14</td>
<td>0/14</td>
</tr>
</tbody>
</table>

<sup>a</sup> No. of rats surviving at least 28 weeks postoperative/no. of animals started.
<sup>b</sup> No. of animals with 1 or more tumors at 28 weeks/no. of survivors at 28 weeks.
<sup>c</sup> PH, partial hepatectomy.
Our results indicate that partial hepatectomy of female rats during p.o. administration of AAF reveals a degree of hepatotoxicity not observed in livers of normal female animals. As indicated in Chart 3, partial hepatectomy after a period of AAF administration initiated a severe hepatotoxicity which was followed by cell proliferation. Although the rates of thymidine-3H incorporation and cell proliferation were inhibited initially in livers of AAF-treated animals compared to partially hepatectomized controls, the cell proliferation continued to increase long after the rate of cell proliferation had returned to normal in control animals (Chart 2). Furthermore, it was found that liver regeneration in the presence of AAF was not uniform but occurred at discrete loci, resulting in formation of hyperplastic nodules (Fig. 1).

Although formation of hyperplastic nodules may be a necessary condition for liver tumor formation (15), our results indicate that it is not necessarily a sufficient one. This has been demonstrated in male rats also (21). Although AAF produced hyperplastic nodules in females as early as 2 weeks following partial hepatectomy, none of the females maintained on AAF for 28 weeks postoperatively developed liver tumors (Table 1). Reuber (19) concluded that the progression of livers of AAF-treated animals compared to partially hepatectomized controls, the cell proliferation continued to increase long after the rate of cell proliferation had returned to normal in control animals (Chart 2).

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A comparison of each step of the process in male and female animals should be helpful in distinguishing the effects of the carcinogen which are required for tumor production from those that may be incidental.

REFERENCES

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