Increased Susceptibility of Aged Rats to Hepatocarcinogenesis by the Peroxisome Proliferator Nafenopin and the Possible Involvement of Altered Liver Foci Occurring Spontaneously

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ABSTRACT

We investigated the mechanism of the hepatocarcinogenic action of nafenopin (NAF), a nongenotoxic peroxisome proliferator. Groups of male rats aged 13 wk (designated "young") or 57 wk (designated "old") were fed NAF for 13 mo; additional groups received a basal diet or a phenobarbital (PB)-containing diet as positive control.

The following results were obtained. (a) NAF produced numerous hepatocellular adenomas and carcinomas in old animals but very few in young animals. A similar result, although less pronounced, was seen with PB. Adenomas of PB-treated groups mostly consisted of eosinophilic and glycogen-storing cells. However, adenomas and carcinomas of NAF-treated livers were composed of weakly basophilic cells. (b) Phenotypically altered foci, evaluated in hematoxylin:eosin-stained sections, appeared spontaneously in untreated livers. The majority of these foci was either of the eosinophilic-clear cell or the tigroid cell type. In addition, we identified foci which are characterized by weak, diffuse cytoplasmatic basophilia. Their phenotype was similar to that of adenomas and carcinomas in NAF-treated rats. The number and size of eosinophilic-clear cells and of tigroid cell foci increased considerably with the age of the animals. At the end of the experiment, approximately 2.4% of liver tissue was occupied by focal lesions. NAF, but not PB, treatment led to a selective increase in number and size of weakly basophilic foci. This subtype has previously been described as a likely precursor lesion for liver tumors induced by an aflatoxin B1-NAF initiation-promotion regimen (B. Kraupp-Grasl et al., Cancer Res., 50: 3701-3708, 1990).

These findings suggest that the peroxisome proliferator NAF leads to tumor development in aging rat liver by promotion of spontaneously occurring preneoplastic lesions. The type of lesion appears to differ from that promotable by PB.

INTRODUCTION

A great number of drugs that induce hepatic peroxisome proliferation cause the emergence of liver cell carcinoma in lifetime animal bioassays (1-6). This chemically heterogeneous group of compounds includes widespread environmental pollutants, such as phthalates (2, 5-8, 11, 12). Despite extensive investigations the precise mechanisms of the hepatocarcinogenic action of peroxisome proliferators have not been elucidated. For the assessment of health risks to exposed humans, it is most important to clarify these mechanisms.

The potent hepatomitogenic and enzyme-inducing effects of these nongenotoxic carcinogens led us and others to hypothesize that they may be tumor promoters. However, the promoting action has not been generally accepted. NAF (13) and the uricosuric benzbromarone (10) failed to enhance hepatocarcinogenesis initiated by N-2-acetylaminofluorene or N-nitrosomorpholine. In other studies the hypolipidemias WY-14.643 (14, 15), clofibrate (16), and NAF (11, 17) enhanced diethylnitrosamine-induced hepatocarcinogenesis.

Recently we described that NAF exerts a strong tumor-enhancing effect in aflatoxin B1-initiated rat livers, possibly through promotion via a hitherto neglected subpopulation of putative preneoplastic liver foci (18).

If peroxisome proliferators are tumor promoters, their hepatocarcinogenic action conceivably could be explained by promotion of preneoplastic foci occurring spontaneously in livers of rats and mice during aging (19-21). Indeed, aged rats and mice have been shown to be more susceptible than young animals to the hepatocarcinogenic effects of the tumor promoter PB (22, 23). Hypothetically, therefore, if NAF were a tumor promoter, it then should produce more tumors in livers of aged than of young rats. The present study was designed to evaluate this hypothesis. PB-treated young and aged rats served as positive controls.

Alternatively, excessive H2O2 production through enhanced peroxisomal enzyme activities has been suggested as the causal mechanism of hepatocarcinogenesis by peroxisome proliferators. In the subsequent paper, the possibilities of changes in parameters representative for the discussed mechanism are studied.

MATERIALS AND METHODS

Animals and Treatment. A total of 177 male specified pathogen-free Wistar rats were obtained from "Kleintierfarm Madorin AG," Fullsindorf, Switzerland, at the age of 8 to 10 wk. Animals were randomly assigned to experimental groups, kept under standardized conditions (macrolon cages, 12-h light phase, 23 ± 2°C room temperature, 40 to 70% relative humidity), and received water and food ad libitum. The pellet diet (produced by "Sandoz Forschungsinstitut GmbH," Vienna, Austria, according to Organization for Economic Co-operation and Development (OECD) guidelines) was free of fish flour, was poor in nitrosamines and toxic substances, 19% proteins, 4% fat, 50.5% carbohydrates, 3.4% crude fibers, 7.2% ash, and 11.3% moisture. Three wk before treatment was started, animals were changed to another pelleted diet of a nearly identical composition (fish flour free, poor in nitrosamines and toxic substances, and consisted of 20.9% protein, 5.3% fat, 51.9% carbohydrates, 3.4% crude fibers, 7.2% ash, and 11.3% moisture; Altromin 1324N, Lage, Germany). NAF, a kind gift of Ciba-Geigy, Basel, Switzerland, was dissolved in 100% acetone (analytical grade) and mixed into an unpelleted diet (Altromin 1321N) of the same composition as 1324N. Evaporation of acetone was achieved by agitation.

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1 To whom requests for reprints should be addressed.
ing the chow in intervals during at least 1 day. The NAF-containing diet was administered as powder. PB (Fluka AG, Buchs, Switzerland) was mixed into a powdered diet (Altromin 132IN), which was pelleted afterwards. Food consumption was determined by weighing food dishes, and dietary NAF and PB concentrations were adjusted to provide the planned dose (NAF, 100 mg/kg of body weight/day; PB, 50 mg/kg of body weight/day). Body weights were recorded at regular intervals. The animals were killed by decapitation under CO2 anesthesia. Body and liver weights were recorded, and relative liver weights were calculated.

Experimental Protocol. The experimental protocol is given in Fig. 1. One group of rats was 13 wk old at the start of the treatment and is designated “young” throughout the entire experimental period. Another group aged 57 wk at the beginning of the experiment is designated “aged” or “old.” From each group 10 animals were killed immediately. Three further subgroups were fed either a basal diet (Group 0 young and Group 0 old) or a PB-containing diet (Group PB young and Group PB old) or a NAF-containing diet (Group NAF young and Group NAF old). The experiment was scheduled such that the young and aged animals were treated in a parallel fashion at the same time for 55 to 59 wk until sacrifice.

Fig. 1. Experimental protocol. Young and old male rats were fed one of the following diets: basal diet; PB-containing diet; or NAF-containing diet. Numbers on the bars indicate sacrificed animals.

Table 1 Effect of PB and NAF on body and relative liver weights of young and old male rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Start of experiment</th>
<th>End of experiment</th>
<th>Relative liver wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start of experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O young</td>
<td>10</td>
<td>328.9 ± 16.4*</td>
<td>2.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>End of experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O young</td>
<td>18</td>
<td>305.6 ± 13.8</td>
<td>2.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>PB young</td>
<td>28</td>
<td>315.6 ± 21.3</td>
<td>2.9 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td>NAF young</td>
<td>17</td>
<td>318.8 ± 12.4</td>
<td>4.9 ± 0.5*</td>
<td></td>
</tr>
<tr>
<td>Start of experiment</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>O old</td>
<td>10</td>
<td>524.3 ± 62.5</td>
<td>2.6 ± 0.2</td>
<td></td>
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<tr>
<td>End of experiment</td>
<td></td>
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<tr>
<td>O old</td>
<td>29</td>
<td>542.2 ± 48.1</td>
<td>2.4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>PB old</td>
<td>20</td>
<td>544.5 ± 49.4</td>
<td>3.3 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td>NAF old</td>
<td>19</td>
<td>528.2 ± 47.7</td>
<td>5.5 ± 1.2*</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD (group).

The significance of differences among the O-, PB-, and NAF-treated groups of either young or old rats was determined by Dunnett’s t test. The Student’s t test was performed for analysis of differences between pairs of groups, i.e., young and aged animals treated with the same diet. The significance of differences in tumor incidences was checked by 95% and 99% confidence limits.

RESULTS

Effects of Treatment on Survival and on Body and Relative Liver Weights. Except for 10 young and 10 aged animals which were killed at the beginning of the experiment, 63 of the 65 animals treated with the same diet was determined by Student’s t test: (c), significant for 99% confidence limits; (b), significant for 95% confidence limits.* The significance of differences between all experimental groups of the same age was calculated by means of Dunnett’s t test. The Student’s t test was performed for analysis of differences between pairs of groups, i.e., young and aged animals treated with the same diet. The significance of differences in tumor incidences was checked by 95% and 99% confidence limits.

TABLE 2 Effect of PB and NAF on the incidence of tumors in livers of young and aged rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Start of experiment</th>
<th>End of experiment</th>
<th>Relative liver wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>O young</td>
<td>0</td>
<td>0</td>
<td>0 ± 0'</td>
</tr>
<tr>
<td>PB young</td>
<td>71</td>
<td>4</td>
<td>7 ± 3.6</td>
</tr>
<tr>
<td>NAF young</td>
<td>88</td>
<td>24</td>
<td>9.1 ± 6.7</td>
</tr>
<tr>
<td>Start of experiment</td>
<td></td>
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<tr>
<td>O old</td>
<td>0</td>
<td>0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>PB old</td>
<td>100</td>
<td>45</td>
<td>30.4 ± 3.59</td>
</tr>
<tr>
<td>NAF old</td>
<td>100</td>
<td>89</td>
<td>59.1 ± 4.84</td>
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</tbody>
</table>

* Significant for P< 0.01.

Statistics. There was no difference in the formation of preneoplastic or neoplastic lesions in the livers between 55 and 59 wk of treatment (data not shown). Therefore animals sacrificed at these time points were assigned to one group. Wherever indicated, standard deviations are given. The significance of differences between all experimental groups of the same age was calculated by means of Dunnett’s t test. The Student’s t test was performed for analysis of differences between pairs of groups, i.e., young and aged animals treated with the same diet. The significance of differences in tumor incidences was checked by 95% and 99% confidence limits.

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young and 68 of the 92 aged rats survived until termination (Fig. 1). The most common contributing causes of death included pituitary tumors and soft tissue tumors. No relation of premature deaths to any treatment could be observed (Fig. 1). In Tables 1 and 2 and Figs. 3 to 5, only effective numbers of animals are indicated.

The body weights and the liver weight/body weight ratios are shown in Table 1. While young rats showed less body weight gain than expected, old rats lost weight. As food consumption was not affected (data not shown), this may be explained by the change to a diet with a slightly lower amount in nutritive substances. An even more pronounced reduction of body weights was seen in the NAF-treated groups. Similar observations have been reported on NAF (13) and other peroxisome proliferators, such as di(2-ethylhexyl)phthalate (8), WY-14,643 (15), and clofibrate (16). Relative liver weights of rats from NAF- and PB-treated groups were significantly increased (Table 1).

Quantitative Macroscopical Evaluation of Liver Tumors. No major macroscopical abnormalities could be detected in livers of rats (13 and 57 wk of age) killed at the beginning of the experiment (Table 2). At the end of the experiment, however, livers carried grayish-white lesions which appeared as tumors. Their incidences and mean numbers per tumor-bearing liver

Fig. 2. Histology of prneoplastic and neoplastic hepatocytes in PB- and NAF-treated old rats. A (and detail in B), PB treatment, hepatocellular adenoma; C to F, NAF treatment; C and D, hepatocellular adenoma; E, hepatocellular carcinoma; F, weakly basophilic focus. Arrows, borders of the lesions. A, C, and F, ×125; B, D, and E, ×320.
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were small in controls, yet higher in old than in young rats (Table 2). Furthermore, all livers of the NAF- and PB-fed old animals showed numerous macroscopical lesions. The incidence of lesions with diameters larger than 3 mm was much higher than in treated young animals (Table 2). These differences in incidences, size, and multiplicity of macroscopical lesions between young and aged untreated, PB-treated, and NAF-treated animals were highly significant.

Histology and Incidences of Liver Tumors. In PB-treated animals adenoma cells showed intense acidophilia and ground glass appearance (Fig. 2, A and B) or had a "clear" cytoplasm. Most cells were enlarged. Mitotic figures were infrequent.

In NAF-treated animals hepatocellular adenoma (Fig. 2, C and D) and carcinoma (Fig. 2E) showed a distinct phenotype. They consisted predominantly of weakly basophilic cells or of a mixture of eosinophilic and basophilic cells which were arranged mostly in trabecular patterns. In some adenomas and in most carcinomas, mitotic figures were numerous and sometimes abnormal.

In NAF-treated, aged rats the incidence of adenoma and carcinoma was far above that in young rats (Table 2). Under PB application, exclusively benign liver tumors developed with a higher incidence in old animals. Table 2 gives the mean number of adenomas and carcinomas detected per tumor-bearing liver. In aged NAF-treated rats, each of the tumor-bearing livers carried an average of 6 adenomas and 6 carcinomas, while PB administration led to the development of approximately 3 adenomas/tumor-bearing aged rat. The means in all the other groups were much lower.

Effect of Age and Treatment on Phenotypes and Growth of Liver Foci. Recent reports indicate that many of the histological markers used to identify liver foci are poor markers in rats treated with peroxisome proliferators (8, 10, 11, 13-15, 17, 28). Therefore we evaluated liver foci in hematoxylin: eosin-stained sections. Eosinophilic-clear cell, tigroid, and weakly basophilic cell foci were identified as described previously (18, 24-26) and were all found in the present study. These weakly basophilic foci (Fig. 2F) resembled the phenotype of tumor cells (Fig. 2, C to E) in NAF-treated young and old animals. A similar phenotype was seen in foci of livers which were treated with NAF subsequent to initiation with aflatoxin B,

Livers from untreated rats contained almost no foci at Wk 13 (Fig. 3). The number and size of foci increased considerably until Wk 68 to 72 and further until Wk 112 to 116, so that the total area occupied by focal tissue expanded at least 600-fold within the observation period. Then approximately 2.4% of the section areas consisted of focal cells. These age-dependent increases were highly significant. It should be noted that number and size of foci were similar at Wk 68 to 72 in the group designated "young" and at Wk 57 in the group of animals designated "old."

The distribution of foci among the different phenotype and size classes is shown in Figs. 4 and 5. At Wk 68 to 72 and 112 to 116 the vast majority of spontaneous foci were of the eosinophilic-clear cell type. A smaller proportion was found to belong to the subtype of tigroid foci. Weakly basophilic cell foci were detected rarely. Small eosinophilic-clear cell and tigroid foci predominated at all age groups studied, but larger foci appeared with increasing age. This pattern is consistent with the concept that, while new, small foci steadily were formed, the older foci continuously grew. In PB-treated animals, foci were found to be mostly of the eosinophilic-clear cell or of the tigroid subtype. Somewhat unexpectedly, there was no clear-

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**Fig. 3.** Effect of age on liver foci in untreated control animals. Foci numbers and area per cm² of tissue section and foci numbers and volume per liver are given. Ten animals per age group were evaluated. Statistics were by the Student’s t test; points, mean; bars, SD. Young animals, controls at Wk 13 versus controls at Wk 68 to 72; old animals, controls at Wk 57 versus controls at Wk 110 to 114. a, P < 0.001; b, P < 0.01; c, P < 0.05.

**Fig. 4.** Effects of age, NAF, and PB on different subtypes of liver foci. Number and total area (mm²) of foci per cm² of tissue section and number and volume (mm³) per liver are shown. Ten animals per group were evaluated. Groups: young and old controls, young and old PB-fed, young and old NAF-fed. The significance of differences in Groups 0 (0) and young old (0), PB young and PB old (0), and NAF young and NAF old (0). The significance of differences in Groups 0, PB, and NAF for the young or old animals was determined by the means of Dunnett’s t test. Columns: mean; bars, SD. a, P < 0.01; b, P < 0.05.
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Fig. 5. Size distribution of foci of different subtypes. Ten animals per group were evaluated. Columns are group means and are given per cm$^2$ of tissue section. Size classes are the following: 1. $<$0.008 mm$^2$; 2. 0.008 to 0.016 mm$^2$; 3. 0.016 to 0.032 mm$^2$; 4. 0.032 to 0.064 mm$^2$; 5. 0.064 to 0.128 mm$^2$; 6. 0.128 to 0.25 mm$^2$; 7. 0.25 to 0.5 mm$^2$; 8. $>$0.5 mm$^2$.

A cut increase in the number and size of these foci in PB-treated rats of both age groups (Figs. 4 and 5).

NAF treatment in aged rats produced a slight, if any, increase in the number and size of eosinophilic-clear cell foci, while number and size of tigroid cell foci were definitely smaller than in the untreated group of the same age (Fig. 4). Weakly basophilic cell foci showed a pronounced, statistically significant increase in number and area in both age groups treated with NAF.

Transformation of the data to number and volume per total liver produced similar results (Fig. 4). NAF did not appear to accelerate growth of eosinophilic-clear and tigroid cell foci (Figs. 4 and 5); whether the dramatic expansion of weakly basophilic foci was due to an increase in size or an increase in number or both is hard to evaluate due to the small number in the control groups (Fig. 5).

DISCUSSION

In the present experiment aged rats were found to be considerably more susceptible than young rats to liver tumor formation by NAF. Similar observations have previously been made with the well-established liver tumor promoter PB (22, 23) and are confirmed in this experiment. Several explanations for the enhanced tumor yield by NAF in old rats are possible.

(a) The concentration of NAF could reach different levels in livers of young and old animals. However, we did not find any significant difference between these two NAF-treated age groups in any of the parameters studied so far: relative liver weight; content of DNA per unit of liver weight and per unit of liver protein; activity of induced peroxisomal $\beta$-oxidation; activity of glutathione peroxidase; and fatty acid profiles in liver homogenates. Therefore differences in effective doses of NAF between the livers of young and aged rats seem unlikely.

(b) Old rats could be more susceptible to the induction of $H_2O_2$-generating peroxisomal enzymes and subsequent lipid peroxidation according to the hypothesis raised by Reddy and coworkers (1, 2). Biochemical analysis, however, did not reveal significant age differences in parameters indicative for this mechanism. Therefore, this hypothesis does not seem applicable for the present experiment.

(c) NAF could possess weak initiating potential being below the detection limit of presently available tests. If the mechanisms for DNA repair were less effective in old than in young animals, then a faster accumulation of NAF-initiated lesions should be seen in aged animals. This possibility seems unlikely as the sum of all putative preneoplastic lesions did not differ between aged NAF-treated animals and their controls.

(d) NAF could promote tumor development in aged rats. Several observations in the present experiment support this assumption. At the start of treatment, there were almost no foci found in the young, but many in the old group. These "spontaneously" formed lesions might have served as targets for tumor promotion by NAF. Furthermore, NAF produced a dramatic expansion of the subtype of weakly basophilic foci, similar to a previous initiation-promotion study (18). The phenotype of these foci resembled that of tumors in NAF-treated livers. The present findings support our hypothesis that NAF leads to tumor development in rat liver by promotion of spontaneously appearing foci involving a specific subtype, the hitherto neglected weakly basophilic foci. These putative precursor lesions of NAF-induced tumors may be different from lesions promotable by PB.

Under the present experimental conditions NAF was more potent than PB in producing tumors. In addition, aged rats...
showed a low susceptibility to PB as a promoting agent for number and size of foci. A similar observation was reported previously by Xu et al. (29) in aged male F344 rats applying different histochemical markers. Ward (22), however, observed a clear-cut effect of PB on foci development in aged rats. In young rats PB was of the same or even higher effectiveness than NAF when promotion followed initiation with aflatoxin B (18). The reasons for these differences in the promotional activities of NAF and PB in various models are not known. There are at least two possibilities. (a) Targets for NAF and PB promotion may be different in type and in their occurrence after genotoxic or “spontaneous” initiation. (b) For tumor promotion NAF may require a further advanced stage of preneoplastic development.

In view of the ubiquitous presence of a vast number of carcinogenic factors in our environment, the formation of initiated cells may be a frequent event in the liver and other organs of aged organisms. In this context, and with respect to risk assessment, the present findings raise some concern as hypolipemic drugs are preferentially administered to older people. So far, however, no enhanced incidence of liver tumors has been observed in persons treated with hypolipidemics (30, 31).

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REFERENCES

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