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Toxicology Letters 149 (2004) 25–35
Toxicology Letters
www.elsevier.com/locate/toxlet

# Role of apoptosis for mouse liver growth regulation and tumor promotion: comparative analysis of mice with high (C3H/He) and low (C57Bl/6J) cancer susceptibility

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**Abstract**

Apoptosis constitutes one of the organisms defense lines against cancer. We investigated whether failure of apoptosis may be concurrently causative for the high cancer susceptibility in C3H/He as compared to C57BL/6J mice (low cancer susceptibility). First, in short-term in vivo experiments (7–21 days), mouse liver growth (C3H/He, C57BL/6J) was induced by administration of phenobarbital (PB; 2 days 500 ppm + 5 days 750 ppm via the food) or nafenopin (NAF; 7 days 500 ppm via the food), cessation of PB or NAF treatment served to initiate liver involution. Liver weight, DNA content, hepatocyte ploidy and apoptotic activity were studied as endpoints. Secondly, in a long-term study liver carcinogenesis was initiated by a single dose of *N*-nitrosodiethylamine (NDEA, 90 mg/kg b.w.) to 5-weeks-old C57Bl/6J and C3H/He mice. After 2 weeks, mice received either standard diet or a diet containing phenobarbital (PB, 90 mg/kg b.w.) for up to 90 weeks. Cell proliferation and apoptosis in normal liver tissue and (pre)neoplastic tissue was quantitatively analysed by histological means. The short term studies revealed that PB and NAF-induced mouse liver growth is essentially due to cell enlargement (hypertrophy). A moderate increase of liver DNA content was brought about by hepatocellular polyploidization; C3H/He mice exhibited the most pronounced ploidy shift, corresponding to their high cancer susceptibility. Upon cessation of PB or NAF treatment, regression of liver mass was neither associated with a loss of DNA nor an increase in apoptoses in the liver of C3H/He and C57Bl/6J mice; food restriction did not enforce the occurrence of apoptosis. Thus, the mouse strains did not differ with respect to the occurrence of apoptosis. In the long-term study, PB promoted liver tumor formation in all strains, exhibiting quantitative differences in growth kinetics of preneoplasia rather than a specific biological quality. Quantitative analysis of apoptosis in normal and (pre)neoplastic liver tissue of C3H/He and C57BL/6J mice revealed no clue to explain their different cancer susceptibility. Rather, cell proliferation seems to be the prevailing determinant of tumor promotion in the liver of both mouse strains.


*Keywords:* C3H/He; C57BL/6J; Heptocarcinogenesis; Tumor susceptibility; Apoptosis; Cell proliferation

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doi:10.1016/j.toxlet.2003.12.018

## 1. Introduction

Rodent species routinely used for life-time cancer studies on chemicals include mouse strains exhibiting a high (B6C3F1, C3H/He), but also a low (C57Bl/6J) susceptibility to spontaneous and chemically induced hepatocarcinogenesis (for review: Fausto, 1999, Gold et al., 2001). Elucidation the underlying causes is of great importance for risk assessment, but inspite of intense research efforts these still are a matter of debate (Maronpot and Boorman, 1996; Alden, 2000; van Ravenzwaay and Tennekes, 2002). At the cellular level, cell replication and apoptosis are processes integrating all the genetic and molecular features of the organism and, therefore may provide a clue to better understand specificities in cancer susceptibility. Apoptosis constitutes an innate tissue defense against carcinogens by preventing survival of genetically damaged cells; the balance between cell proliferation and cell death determines the net growth of preneoplastic and tumor cell populations (Bursch et al., 1984; Schulte-Hermann et al., 1990; Grasl-Kraupp et al., 1997; Luebeck et al., 1995, 2000; Pitot et al., 2000; Lee, 2000). Consequently, block of apoptosis has been elucidated as prevailing mechanism of liver tumor promotion in many rodent studies, mostly using rat liver models (Schulte-Hermann et al., 1990; Luebeck et al., 1995, 2000; Kolaja et al., 1996c; Schwarz et al., 2000; Pitot et al., 2000; Oliver and Roberts, 2002; Tharappel et al., 2002). Relatively little is known about the role of apoptosis in determining cancer susceptibility in different mouse strains. Rather, based upon different experimental models somewhat conflicting data on apoptosis in mouse liver tumor promotion have been reported. For instance, growth of preneoplastic lesions, at least in early stages, has been attributed to predominantly depend on the proliferative activity while other studies described apoptosis-inhibition by tumor promoting agents (phenobarbital, peroxisome proliferators) as important determinant for tumor development in mouse liver (Pereira, 1993;Kolaja et al., 1996b,c; James et al., 1998; James and Roberts, 1996; Stevenson et al., 1999; Sanders and Thorgeirsson, 2000; Goldsworthy and Fransson-Steen, 2002). Therefore, we initiated a series of comparative short-term in vivo studies on liver growth (cell proliferation) and involution (apoptosis) in C3H, C57Bl/6J and B6C3F1 mice using the “classical” tumor promoter Phenobarbital (PB) and the peroxisome proliferator nafenopin (NAF) as model compounds. The short-term experiments were complemented by a long-term carcinogenesis study with C3H/He, C57Bl/6J and B6C3F1 mice. Hepatocarcinogenesis was initiated by a single dose of *N*-nitrosodiethylamine (NDEA). Subsequently, we closely followed the growth of phenotypically distinct preneoplastic and neoplastic lesions as well as their DNA synthesis and apoptotic activitiy, without or with phenobarbital promotion. The experimental design was focussed to answer the question whether a high liver cancer susceptibility correlates with a failure of apoptosis. The present communication provides an excerpt of these studies to exemplarily highlight prominent species specificities of C3H/He and C57Bl/6J mice (parental strains of B6C3F1), C57Bl/6J both constituting the extremes in high and low tumor susceptibility.

## 2. Cell birth and cell death in normal mouse liver (short-term studies)

### 2.1. Liver growth

In male C57Bl/6J and C3H/He mice, PB treatment with doses of 500 ppm for 2 days followed by 750 ppm per day for 5 days via the diet caused an increase in liver weight by 40%–50% (Table 1). NAF treatment for 7 days, at the dose level chosen (500 ppm), caused a much more pronounced liver enlargement than PB in both strains of mice (85%–113% above controls, Table 1). Biochemical analysis revealed only a moderate increase in liver DNA (PB: about 8%–14%; NAF: 8%–28%, Table 1). Thus, mouse liver enlargement in response to PB and NAF is largely due to hypertrophy, the extent of which -at least for PB-tended to be somewhat more pronounced in the highly susceptible C3H/He-mice (49% above control) as compared to C57Bl/6J mice (36% above control). In the liver of PB-treated animals we also analysed the nuclear and cellular ploidy pattern by histological-morphometrical means. As summarized in Table 2, 66%–75% of the hepatocytes contained two nuclei and binuclearity did not change significantly upon PB-treatment. As to nuclear and cellular ploidy, C57Bl/6J mice exhibited a slight but not significant shift in favor of $2 \times 8n$, $1 \times 16n$ and $2 \times 16n$ hepatocytes, whereas in the liver of

Table 1
Relative liver weight and DNA content in male C57Bl/6J and C3H/He mice

| Strain | Treatment | Relative liver weight (g liver/100 g b.w.) | DNA content (mg DNA/100 g b.w.) |
|---|---|---|---|
| C57Bl/6J | Co (7 days) | 4.41 ± 0.14 | 4.53 ± 0.42 |
| | PB (7 days) | 6.01 ± 0.25 (+36%)** | 5.14 ± 0.46 (+14%)* |
| | NAF (7 days) | 9.39 ± 0.13 (+113%)** | 5.80 ± 0.43 (+28%)** |
| | Co (21 days) | 4.19 ± 0.17 | 4.66 ± 0.39 |
| | PB (7 days) → 0 (14 days) | 4.44 ± 0.19 (+6%) | 5.21 ± 0.50 (+12%) |
| | NAF (7 days) → 0 (14 days) | 4.47 ± 0.25 (+7%) | 5.47 ± 0.45 (+17%)** |
| C3H/He | Co (7 days) | 4.60 ± 0.30 | 4.82 ± 0.43 |
| | PB (7 days) | 6.84 ± 0.11 (+49%)** | 5.19 ± 0.41 (+8%) |
| | NAF (7 days) | 8.53 ± 0.48 (+85%)** | 5.18 ± 0.53 (+8%) |
| | Co (21 days) | 4.64 ± 0.35 | 4.57 ± 0.32 |
| | PB (7 days) → 0 (14 days) | 4.60 ± 0.15 (−1%) | 4.74 ± 0.47 (+4%) |
| | NAF (7 days) → 0 (14 days) | 4.34 ± 0.15 (−7%) | 4.94 ± 0.09 (+8%) |

Seven to eight week-old-male C57Bl/6J and C3H/He mice (obtained from Zentralinstitut für Versuchstierzucht, Hannover, Germany) were housed individually and adapted for 4–6 weeks to a reversed light–dark-rhythm (lights on 10 p.m. to 10 a.m., lights off from 10 a.m. to 10 p.m.) with food (standard maintenance diet Altromin 1321N, (Altromin, Lage, Germany) and tap water ad libitum. The substances were administered via the diet, PB: 2 days 500 ppm, followed by 5 days 750 ppm; NAF: day 1–7 500 ppm. PB or NAF (7d): animals killed after 7 days treatment, Co (d7): control for day 7 of the experiment. PB or NAF (7d) → 0 (14d): animals treated with PB or NAF for 7 days, followed by PB- or NAF-withdrawal for 14 days; Co (d21): controls at termination of the experiment ($7 + 14 = 21$ days). Animals were anaesthetized with isofluorane, decapitated and exsanguinated. The liver weight was taken upon removal of the gall bladder. For the biochemical determination of hepatic DNA content, liver specimens were processed as described in detail elsewhere (Schulte-Hermann et al., 1988). All animal experiments were performed according to the Austrian guidelines for animal care and treatment. Means (±S.D.) of 5–8 animals per group are given, $^{*}P < 0.05$, $^{**}P < 0.01$, PB/NAF vs. control, using, the two-tailed Mann–Whitney-test (non-parametric statistic test procedure); b.w.: body weight.

C3H/He mice a pronounced increase of $2 \times 8n$, $1 \times 16n$ and $2 \times 16n$ hepatocytes was observed (Table 2); the results of the image analysis were confirmed by flow-cytometry. This shift in ploidy pattern correlated with preceding increases in DNA synthesis (not shown). Taken together, the liver growth response to short-term administration of tumor promoters such as PB obviously parallels specificities in liver cancer susceptibility; hepatocelluar polyploidization (along with the preceding DNA synthesis) appears to constitute the major difference among the mouse strains under study. Likewise, previous studies revealed that the tumor promoting activity of PB, other drugs and industrial chemicals seem to be associated with their ability to induce liver growth, DNA synthesis, hepatocyte ploidy and specific drug metabolizing enzymes (Diwan et al., 1987; Nims et al., 1987; Schulte-Hermann et al., 1988; Carter et al., 1995, 2003; Kolaja et al., 1996d; Whysner et al., 1996; Hasmall and Roberts, 1997; Kamendulis et al., 2001). It might be argued that the effects as observed in the present study were only marginal and thus, are unlikely to explain the massive differences in susceptibility to hepatocarcinogenesis. However, previous studies of our own demonstrated that even small shifts in the balance of cell proliferation/cell death results in dramatic growth differences of preneoplastic lesions on a long-term basis (Schulte-Hermann et al., 1990; Luebeck et al., 1995, 2000; Grasl-Kraupp et al., 1997). In fact, the results of the present long-term study confirm this explanation (see below).

### 2.2. Liver regression

Discontinuation of PB or NAF treatment of C3H/He and C57BL/6J mice resulted in a decrease of liver weight back to control level within 14 days (Table 1). The enhanced liver DNA content, however, persisted at least for 14 days after cessation of PB or NAF treatment (Table 1). In both mouse strains, apoptoses were extremely rare in the liver of controls as well as at any time point after cessation of treatment (Table 3). The absence of increased numbers of apoptoses in involuting livers of mice does meet well with the persistent enhancement of liver DNA as determined biochemically. In an attempt to enforce liver regression and

Table 2
Binuclearity, nuclear and cellular ploidy in the liver of C57Bl/6J and C3H/He mice after PB treatment

| Strain | *n* | Treatment | Binuclearity (%) | Ploidy | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 2*n* | | 4*n* | | 8*n* | | 16*n* | | 32*n* | |
| | | | | 1 × 2*n* | 2 × 2*n* | 1 × 4*n* | 2 × 4*n* | 1 × 8*n* | 2 × 8*n* | 1 × 16*n* | 2 × 16*n* | 1 × 32*n* | 2 × 32*n* |
| C57Bl/6J | 3 | Co (7 days) | 66.8 ± 5.3 | 1.1 ± 0.5 | 20.5 ± 3.4 | 13.0 ± 4.8 | 26.6 ± 6.8 | 13.6 ± 3.9 | 16.6 ± 5.0 | 4.7 ± 3.2 | 2.5 ± 1.4 | 0.8 ± 0.8 | 0.6 ± 0.5 |
| | 4 | PB (7 days) | 67.4 ± 3.6 | 0.8 ± 0.7 | 19.1 ± 6.0 | 11.2 ± 4.3 | 25.3 ± 4.0 | 13.3 ± 2.7 | 19.1 ± 3.4 | 6.2 ± 2.6 | 3.7 ± 1.1 | 1.0 ± 1.3 | 0.2 ± 0.4 |
| C3H/He | 3 | Co (7 days) | 75.3 ± 1.3 | 1.6 ± 0.0 | 27.6 ± 2.7 | 13.1 ± 3.5 | 37.5 ± 3.8 | 7.4 ± 2.3 | 10.0 ± 6.7 | 2.2 ± 0.9 | 0.3 ± 0.5 | 0.3 ± 0.5 | 0.0 ± 0.0 |
| | 4 | PB (7 days) | 70.3 ± 3.6 | 1.4 ± 1.0 | 16.5 ± 5.3* | 13.2 ± 4.8 | 29.3 ± 6.4 | 10.3 ± 5.6 | 21.7 ± 5.1* | 4.1 ± 1.2 | 2.9 ± 2.6 | 0.6 ± 0.8 | 0.0 ± 0.0 |

Treatment: see legend to Table 1. Hepatocytes were isolated by in situ collagenase liver perfusion as described in detail elsewhere (Parzefall et al., 2002) and a smear of the cells was stained with H&E. Number of nuclei per cell and nuclear ploidy (nuclear diameter) of hepatocytes were determined by histological-morphometrical means by counting at least 1000 cells per animal. $n$ = number of livers analysed, means ± S.D. are given; $^{*}P < 0.05$, PB treatment vs. control using student's $t$-test.

Table 3
Hepatocellular apoptoses in C3H/He and C57Bl/6J mice

| Strain | Day | Control (%) | PB treatment (7 days) (%) | PB withdrawal (7 days → 0 for 2–14 days) (%) | NAF treatment (7 days) (%) | NAF withdrawal (7 days → 0 for 2–7 days) (%) |
|---|---|---|---|---|---|---|
| C57Bl/6J | 7 | 0 (0–0.17) | 0 (0–0.17) | | 0.2 (0.13–0.41) | |
| | 9 | | | 0 (0–0.17) | | 0 (0–0.17) |
| | 11 | | | 0.02 (0–0.17) | | |
| | 14 | 0 (0–0.17) | | 0 (0–0.17) | | |
| | 21 | 0 (0–0.17) | | 0.12 (0.06–0.29) | | 0 (0.06–0.29) |
| | 9–21 | | | FR 0.04 (0–0.14) | | |
| C3H/He | 7 | 0.02 (0–0.17) | 0.03 (0–0.17) | | 0.08 (0–0.17) | |
| | 9 | | | 0.02 (0–0.17) | | 0.08 (0–0.17) |
| | 11 | | | 0.02 (0–0.17) | | |
| | 14 | | | 0 (0–0.17) | | |
| | 21 | 0 (0–0.17) | | | | 0 (0–0.17) |
| | 9–21 | | | FR 0.05 (0–0.14) | | |

Treatment: see legend to Table 1. Liver specimens were cut into 4–5 mm thick slices and fixed with Carnoy's solution and processed for histological analysis as described in detail elsewhere (Grasl-Kraupp et al., 1994). Apoptoses were counted in hematoxylin and eosin (H&E) stained sections: hepatocytes with chromatin condensation typical of early stages of apoptosis as well as intra- and extracellular apoptotic bodies, with or without visible chromatin were recorded; their total number was expressed as percentage of the total number of hepatocytes scored in the respective cell population. The reliability and sensitivity of this procedure for quantitative determination of apoptoses has been verified (Chabicovsky et al., 2003). Data of two experiments are combined. means and 95% confidence limits (in brackets) are given. PB or NAF (7 days): animals sacrificed 1 day (day 7) after last treatment; PB or NAF (7 days) → 0: animals sacrificed two (day 9), four (day 11), seven (day 14) or fourteen (day 21) days after PB- or NAF-withdrawal; means of 5–6 animals are given. FR: food restriction along with PB-withdrawal. Data of day 9–14. i.e. 2,4,7 days after PB withdrawal and food restricton are summarized, in total data of 26 animals.

the occurrence of apoptoses, PB-pretreated mice were subjected to a 40% food restriction (FR) along with PB-withdrawal; FR has been previously shown to induce apoptoses in normal, hyperplastic as well as preneoplastic cell populations of rat liver (Grasl-Kraupp et al., 1994; Kolaja et al., 1996a; Hikita et al., 1998; Tomasi et al., 1999). Likewise, in C57Bl/6J × C3H F1 mice subjected to food restriction an increase in apoptotic activity was reported (James and Muskhelishvili, 1994; Kolaja et al., 1996a). In our study, body weight of FR-animals decreased steadily within the first week of food restriction, reaching the lowest levels (approx. 70% of the initial body weight) within 7–9 days. Liver weight decreased by approx. 55%–60% within 1 week of FR. Surprisingly, the hepatic DNA content was not affected by FR—even food restriction for 14 days did not result in a significant lower liver DNA content as compared to feeding animals ad libitum (not shown). Accordingly, histological analysis revealed no significant increase in apoptotic activity at all time points investigated (2, 4, 7, 14 days FR; Table 3). In summary, mouse liver hypertrophy induced by PB or NAF is completely reversible within a few days after cessation of treatment, similar to previous observations (for review—Schulte-Hermann, 1974; Bursch et al., 1992; Huber et al., 1996). In contrast to hypertrophy, the enhanced DNA content and ploidy persisted for at least 14 days after PB or NAF withdrawal and no increase of apoptoses occurred. Furthermore, food restriction did not facilitate the occurrence of apoptosis in mouse liver. Thus, the present findings of the short-term experiments strongly suggest that mouse hepatocytes do not enter apoptosis as readily as rat hepatocytes under these conditions. It should be emphasized that this conclusion applies to both compounds studied, namely PB and NAF, which exert their action on hepatocytes through different receptor-signal transduction pathways (Oliver and Roberts, 2002). Moreover, other in vivo as well as cell culture studies revealed that mouse hepatocytes are much less sensitive to the pro-apoptotic action of TGF-β1 as compared to rat hepatocytes (Parzefall et al., 2002; Chabicovsky et al., 2003). In conclusion, the present short-term studies reveal no difference with respect to the occurrence of apoptosis during liver regression among the mouse strains under study, but add support to observations

suggesting profound differences in apoptosis control in mouse as opposed to rat liver.

## 3. Cell birth and cell death in putative preneoplastic lesions, adenoma and carcinoma of mouse liver (long-term study)

### 3.1. Formation of liver tumors

Twenty weeks after a single dose of *N*-nitrosodiethylamine (NDEA), with or without subsequent PB-treatment, neither C57Bl/6J nor C3H/He developed macroscopically visible lesions (not shown). At 40 weeks of the experimental period, a low tumor incidence was found in "NDEA → PB (38w)" treated C57Bl/6J animals, followed by gradual increase until 74 weeks of PB treatment (Table 4). In contrast, at 40 weeks of the experiment "NDEA → PB (38w)" treated C3H/He mice exhibited already a high number of tumors (Table 4); C3H/He mice treated with either NDEA or PB alone developed liver tumors as well, but more slowly and to a lesser extent (Table 4). Furthermore, at 40 and 76 weeks of the experiment C3H/He mice in general exhibited a significantly higher liver tumor incidence than C57Bl/6J animals. In fact, because of the high liver tumor incidence "NDEA → PB" treated C3H/He mice could not be assigned for later time points of the study. These results confirmed the known interstrain differences between C3H/He and C57Bl/6J mice (for review: Gold et al., 2001; Fausto, 1999). Furthermore, according to histopathological criteria the macroscopically visible lesions were classified as either hepatocellular adenoma (HCA) or carcinoma (HCC)—(1) the majority revealed to be HCA (C57Bl/6J: 89%, C3H/He: 64%), (2) these data also indicate that progression from HCA to HCC occurs more readily in C3H/He (36% HCC) than in C57Bl/6J-mice (11% HCC). Likewise, the conversion rate of foci into tumors has been found to correlate with mouse strain susceptibility to hepatocarcinogenesis (Goldsworthy and Fransson-Steen, 2002).

### 3.2. Cell birth and cell death in PPF, HCA and HCC

Putative preneoplastic lesions (PPF) were diagnosed in HE-stained liver sections according to published criteria (Bannasch and Gössner, 1994; Hanigan et al.,

Table 4
Mouse liver tumor incidence in C57Bl/6J and C3H/He mice

| Strain/treatment | *n* | Number of tumors/animal |
|---|---|---|
| C57Bl/6J | | |
| 0 → 0 | | |
| (50w) | 6 | 0.2 ± 0.4 |
| (74w) | 4 | 0.3 ± 0.5 |
| NDEA → 0 | | |
| (38w) | 7 | 0.3 ± 0.8 |
| (50w) | 8 | 1.6 ± 1.2 |
| (74w) | 12 | 1.7 ± 1.7 |
| 0 → PB | | |
| (50w) | 6 | 0 |
| (74w) | 4 | 0 |
| NDEA → PB | | |
| (38w) | 5 | 0.1 ± 0.4 |
| (50w) | 8 | 2.5 ± 3.6 |
| (74w) | 12 | 5.2 ± 3.8[a] |
| C3H/He | | |
| 0 → 0 | | |
| (50w) | 6 | 0.7 ± 0.8 |
| (74w) | 6 | 0.6 ± 0.6 |
| NDEA → 0 | | |
| (38w) | 6 | 2.6 ± 1.9 |
| (50w) | 8 | 6.0 ± 2.0 |
| (74w) | 12 | 7.6 ± 2.8b[b] |
| 0 → PB | | |
| (50w) | 6 | 0.5 ± 1.2 |
| (74w) | 4 | 2.7 ± 1.9 |
| NDEA → PB | | |
| (38w) | 7 | 9.3 ± 2.5[c] |
| (50w) | 11 | 9.1 ± 2.2[d] |

Male C57Bl/6J and C3H/He (specific pathogen free; purchased from the Institut für Labortierkunde und –genetik (Himberg, Austria) were housed individually in Macrolon cages on standard softwood bedding (Altromin, Lage, Germany) under standardized environmental conditions and an inverted 12 h light–dark rhythm with light from 10 p.m. to 10 a.m. and dark from 10 a.m. to 10 p.m. Food (Altromin 1321 N) and tap water were provided ad libitum. Body weight and food consumption were recorded weekly. 5-weeks-old mice received a single intraperitoneal injection of *N*-nitrosodiethylamine (NDEA). 90 mg/kg of body weight (NDEA obtained from Sigma-Aldrich, Wien, Austria), freshly dissolved in sterile 0.9% saline (20 ml/kg of b.w.). After 2 weeks of recovery, mice were fed either standard diet ("NDEA → 0") or a diet containing phenobarbital (5-ethyl-5-phenyl-barbituric acid, (PB; Fluka Chemie, Buchs, Switzerland) for up to 90 weeks ("NDEA → PB"). Other groups of animals received PB alone ("0 → PB") or no treatment at all (control "0 → 0"). The PB concentration in the diet was adjusted in the range of 0.05%–0.07% to provide a constant daily intake of 90 mg PB/kg b.w. Mean number of macroscopically visible tumors per animal (±S.D.) are given. number of animals are given at the bottom. For statistical comparisons the two-tailed Mann–Whitney-test (non-parametric statistic test procedure) was used.

[a] $P < 0.05$ vs. control ("0_0").

[b] $P < 0.05$ vs. C3H/He control ("0 → 0") and "0 → PB" as well as C57Bl/6J. "NDEA → 0".

[c] $P < 0.05$ vs. C3H/He "NDEA → 0" and 57Bl/6J. "NDEA → PB".

[d] $P < 0.05$ vs. control ("0 → 0") and "0 → PB".

1993). In NDEA → PB-treated mice, eosinophilic and clear cell foci were found to constitute the vast majority of the preneoplastic cell population, similarly to previous observations (Lee, 1997, 2000; Wastl et al., 1998; Christensen et al., 1999; Goldsworthy and Fransson-Steen, 2002). In the present overview, the differences in the growth parameters of C3H/He and C57Bl/6J mice are described in principle for one interim time point of our long-term study, namely at 52 weeks of the experiment (Table 4). At this stage, in the liver of C57Bl/6J mice a few PPF (sum of all phenotypes, namely eosinophilic, clear cell, amphophilic, tigroid, basophilic, vacuolated cell foci) were detectable only in animals subjected to the initiating treatment with NDEA (Table 5). In contrast, in C3H/He mice PPF appeared more frequent and were found even in control animals ("0–0"; Table 5). This snapshot at 52 weeks of the experiment reflects a coherently different growth kinetics of PPF in C3H/He and C57Bl/6J mice over time with respect to foci number and size (not shown) and the development of HCA and HCC.

PPF, HCA and HCC were surveyed for cell birth (labeling index = LI) and cell death (apoptosis-index = AI), the results are summarized in Table 5 (note: for PPF, only data of eosinophilic and clear cell foci (=$PPF_{(EC)}$) are shown)—(1) in both strains, the cell birth rate was lowest in phenotypically normal liver (NL) and in general, gradually increased from NL to $PPF_{(EC)}$ to HCAs and finally, to HCCs, very much like our previous observations on hepatocarcinogenesis in rat and human liver (Grasl-Kraupp et al., 1997); (2) notably, C3H/He mice treated with PB exhibited a significantly higher rate of DNA synthesis in PPF, HCA and HCC cells as compared to the corresponding C57Bl/6J mice (2.2%–6.9% versus 0.23%–2.3%); (3) in NL of both mouse strains, apoptoses were undetectable or very few (C3H/He 0%–0.04%; C57Bl/6J 0%–0.03%); (4) $PPF_{(EC)}$, HCA and HCC exhibited a slightly increased apoptotic activity; the highest apoptotic activity tended to occur in HCC of C3H/He mice; (5) PB-treatment did not result in a consistent manifestation of an anti-apoptotic effect; (6) overall, histological analysis did not refer to a significant difference of apoptotic activity in the livers of C3H/He and C57Bl/6J mice. However, apoptotic activity in phenotypically normal as well as in (pre)neoplastic liver tissue was much lower as compared to previous observations on rat liver carcinogenesis (Schulte-Hermann et al., 1990; Grasl-Kraupp et al., 1997).

In a further approach to elucidate the role of cell proliferation versus apoptosis for growth of PPF we calculated the daily growth rates of the PPFs and HCAs from histologically determined labeling indices of the individual lesions. These values were compared with the daily growth rate estimated from the increase in cell number of lesions as described by Schulte-Hermann et al. (1990). Briefly, the growth rate was calculated using the following formula—$N = N_0 \bullet e^{k \bullet t}$, with $N_0$ = number of cells per lesion at start (i.e. at initiation with NDEA, $N_0$ was set 1; $t = 0$), $k$ = daily proliferation rate, and $N$ = number of cells per lesion after experimental period $t$ (in days). $N$ was obtained from the average size of PPFs, or HCAs, respectively, (not shown); the number of cells per cross section was transformed into number of cells per volume, assuming spherical shapes. As an estimate of $k$ it was assumed that the LI obtained reflected the number of cells replicating per day. Furthermore, an exponential growth of hepatocellular lesions was assumed. As shown in Table 6, the experimentally found and calculated daily growth rates were almost identical; it should be emphasized that this calculation does not account for apoptosis. Thus, these findings suggest that foci growth occurs without significant elimination of initiated cells. The molecular basis for the low rate of apoptosis in cancer prestages of mouse liver as observed by us and others is not yet clear. Interestingly, typical lesions resulting from the NDEA → PB-protocol, namely eosinophilic PPF and tumors, do not overexpress bcl-2 (Lee, 1997). Based upon results on chronic PB-treatment of B6C3F1 mice without initiation by a genotoxic agent Christensen et al. (1999) reported expression of $BclX_L$ in the majority of acidophilic foci and adenomas, but only a small fraction of these lesions to express bcl-2.

Consequently to the lack of apoptoses, the growth of PPF in mouse liver predominantly is affected by the rate of cell proliferation; cancer susceptible C3H/He mice exhibit a higher proliferative activitiy than C57Bl/6J-mice. Furthermore, the progression of cancer prestages to HCC may be associated with an increased sensitivity to pro-apoptotic signals, as indicated by the present data as well as by a previous independent analysis of HCCs of C3H/He mice

Table 5
Effect of NDEA and PB treatment on liver weight as well as DNA synthesis and apoptosis in stages of hepatocarcinogenesis in C57Bl/6J and C3H/He mice

| Strain/treatment | *n* | g liver/100g body weight | PPF (all phenotypes) number/cm$^2$ | DNA synthesis (%LI) | | | | Apoptoses (%AI) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | NL | $PPF_{(EC)}$ | HCA | HCC | NL | $PPF_{(EC)}$ | HCA | HCC |
| C57Bl/6J | | | | | | | | | | | |
| 0 → 0 (50w) | 6 | 4.09 ± 0.21 | 0 | 0.09(0.05–0.28) | – | – | – | 0(0–0.15) | – | – | – |
| NDEA → 0 (50w) | 8 | 4.19 ± 0.17 | n.e. | 0.03(0.02–0.13) | 0.93(0.73–1.26) | 1.18(0.94–1.55) | n.e. | 0.02(0.02–0.12) | 0.12(0.08–0.27) | 0.29(0.23–0.44) | 0(0–0.38) |
| 0 → PB (50w) | 6 | 5.47 ± 0.1 | 0 | 0.04(0.02–0.22) | – | – | – | 0(0–0.23) | – | – | – |
| NDEA → PB (50w) | 8 | 6.80 ± 3.29 | 0.6 ± 1.7 | 0.08(0.05–0.22) | 0.23(0.15–0.16) | 2.28(2.1–2.52) | 1.59(1.28–2.04) | 0.03(0.02–0.14) | 0.05(0.04–0.16) | 0.07(0.06–0.16) | 0(0–0.15) |
| C3H/He | | | | | | | | | | | |
| 0 → 0 (50w) | 8 | 4.12 ± 0.32 | 0.7 ± 1.7 | 0.09(0.06–0.3) | 0(0–4.86) | – | – | 0(0–0.14) | 0(0–2.72) | – | – |
| NDEA → 0 (50w) | 10 | 4.78 ± 0.60 | 7.7 ± 7.2 | 0.04(0.03–0.15) | 1.13(0.84–1.59) | 0.76(0.68–0.89) | 2.55(2.39–2.77) | 0.04(0.03–0.15) | 0.18(0.13–0.34) | 0.05(0.04–0.11) | 0.27(0.24–0.34) |
| 0 → PB (50w) | 8 | 5.82 ± 0.22 | 1.0 ± 2.0 | 0.11(0.06–0.31) | – | – | – | 0.02(0.01–0.18) | – | – | – |
| NDEA → PB (50w) | 10 | 18.91 ± 4.24 | 11.4 ± 7.1 | 0.03(0.02–0.11) | 2.23(2.04–2.49) | 2.75(2.42–3.19) | 6.92(6.48–7.43) | 0.03(0.02–0.11) | 0.03(0.02–0.1) | 0.04(0.03–0.13) | 0.27(0.21–0.41) |

Treatment: see legend to Table 4. Two weeks before sacrifice mice were subjected to a feeding rhythm with food offered 9 h per day (10 a.m. to 7 p.m.). This feeding rhythm serves to synchronize diurnal rhythms of liver cell proliferation and apoptosis; the hour of sacrifice (9 a.m.) was chosen to be at the daytime of maximal apoptotic activity in the liver which occurs several hours after the daily mitotic peak (Bursch and Schulte-Hermann, 1983; Grasl-Kraupp et al., 1994). Apoptoses were determined as described (Table 3). For quantitative determination of DNA synthesis. mice were pulse labeled with a single i.p. injection of a BrdU-solution (5-bromo-2′-deoxyuridine; Sigma Chemical, Germany; 20 mg BrdU per ml phosphate buffered saline); 100 mg BrdU/kg body weight was administered 14 h before sacrifice. BrdU-incorporation into DNA was visualized according to standard procedures, the number of BrdU-positive nuclei per total number of hepatocyte nuclei was calculated (%LI, labeling index; Grasl-Kraupp et al., 1997). Putative preneoplastic foci (PPF), hepatocellular adenoma (HCA) and carcinoma (HCC) were diagnosed in hematoxylin and eosin (H&E) stained sections according to published criteria (Bannasch and Gössner, 1994; Hanigan et al., 1993; Turusov and Turusov, 1979). Means (±S.D.) are given for relative liver weight (g liver/100 g b.w.) and number of PPF (all phenotypes of putative preneoplastic foci) per cm$^2$). The small size of many (pre)neoplastic lesions precluded a meaningful calculation of the labeling index (%LI, measure of DNA synthesis) and apoptoses (%AI) in individual PPF, HCA and HCC. Therefore, results obtained with individual lesions were combined for each group of animals. means and 95% confidence limits (given in brackets) were calculated. NL: phenotypically normal liver. $PPF_{(EC)}$: eosinophilic and clear cell phenotype; HCA: hepatocellular adenoma. HCC: hepatocellular carcinoma. $n$ = number of animals; –: no lesion detected; n.e.: lesion not evaluated.

Table 6
Calculated and experimentally found labeling indices (LI, %) of PPFs and HCAs

| Strain/treatment | *n* | Lesion | Calculated LI (%) | Found LI (%) |
|---|---|---|---|---|
| C3H/He | | | | |
| NDEA → 0 (50w) | 5 | PPF | 0.019 ± 0.003 | 0.014 ± 0.019 |
| NDEA → PB (50w) | 5 | PPF | 0.02 ± 0.003 | 0.015 ± 0.018 |
| NDEA → PB (50w) | 5 | HCA | 0.036 ± 0.003 | 0.026 ± 0.009 |

Treatment: see legend to Tables 4 and 5. $n$ = number of animals analysed. Mean ± S.D. are given.

(Frey et al., 2000). Likewise, Goldsworthy and Fransson-Steen (2002) reported an increase in apoptotic activity in late stages of hepatocarcinogenesis, possibly promoting focal cell selection. Finally, the present study also strongly suggests that the promoting action of phenobarbital in rats and mice is brought about by different mechanisms. In rat liver, as shown previously, PB stimulates DNA synthesis only transiently and tumor promotion is largely determined by inhibition of apoptosis (Bursch et al., 1984; Schulte-Hermann et al., 1990; Kolaja et al., 1996c).

In conclusion, the findings of the present study do not support a significant role of apoptotic cell suicide during tumor promotion in the mouse liver, at least in early stages. Thus, failure of apoptosis as a cancer preventive event does not appear to provide an explanation for the strain specificities in liver cancer susceptibility. Furthermore, our studies revealed profound species differences between mice and rats in regard to apoptosis control.

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