ACCELERATED PAPER

Xenomorphic hepatic precursors and neoplastic progression of tigroid cell foci induced in rats with low doses of N-nitrosomorpholine

Philipp Stroebel, Fritz Klimek, Heide Zerban, Annette Kopp-Schneider1 and Peter Bannasch2

Divisions of Cell Pathology and 1Biostatistics, Deutsches Krebsforschungszentrum, Abteilung Cytopathologie (C0100), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
2To whom correspondence should be addressed

Email: p.bannasch@dkfz-heidelberg.de

Tigroid cell foci (TCF) are a well-defined entity induced in rat liver by chemical carcinogens, their significance for hepatocarcinogenesis being controversial. Using cytomorphological, cytochemical and morphometric approaches, we studied the evolution and fate of TCF sequentially from 7 to 110 weeks in groups of 50 male Sprague–Dawley rats, which remained untreated or received N-nitrosomorpholine (NNM) orally at concentrations of 3 and 1 mg/kg body wt/day for 7 and up to 75 weeks, respectively. An increased incidence of hepatocellular neoplasms developed in exposed animals compared with controls, which was significant for adenomas at both dose levels, and for carcinomas (HCC) after the longer exposure to the lower dose level (P < 0.0001). TCF appeared frequently in addition to other types of proliferative foci of altered hepatocytes (FAH) including clear/acidophilic and mixed cell foci (MCF) in NNM-treated and rarely in untreated rats. Striking similarities in the cellular phenotypes of TCF and many hepatocellular neoplasms indicated the potential of TCF for progression to both adenomas and carcinomas. TCF emerged from xenomorphic cell foci (XCF), which consisted of hypertrophied hepatocytes typically presenting an enlarged nucleus, abundant glycogen, smooth and rough endoplasmic reticulum, altered activities of several enzymes of carbohydrate metabolism and an increased cell proliferation (P < 0.0001) compared with the extrafocal parenchyma. TCF shared many features with XCF, but their basophilia and proliferative activity was higher. The number of FAH appearing at the two dose levels of NNM was similar but the average size of TCF and MCF was frequently higher at late time points in the group developing a significantly higher incidence of HCC, which suggests a pronounced acceleration of neoeplastic conversion in established preneoplastic cell populations rather than the induction of additional FAH by sustained effects of low doses of carcinogens.

Abbreviations: ACF, acidophilic cell foci; ADC, adenylate cyclase; CCF, clear cell foci; DAB, diaminobenzidine; FAH, foci of altered hepatocytes; GLDH, glutamate dehydrogenase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; G6Pase, glucose-6-phosphatase; G6PDH, glucose-6-phosphate dehydrogenase; GSF, glycogen storage foci; GST-P, placental form of the glutathione S-transferases; γGT, γ-glutamyltransferase; H&E, haematoxylin and eosin; HCA, hepatocellular adenomas; HCC, hepatocellular carcinomas; HK, hexokinase; MCF, mixed cell foci; ME, malic enzyme; NNM, N-nitrosomorpholine; 8-OHG, 8-hydroxydeoxyguanosine; PCNA, proliferating cell nuclear antigen; PCNA-LI, PCNA-labelling-indices; PHO, glycogen phosphorylase; PK, pyruvate kinase; RER, rough endoplasmic reticulum; RT, room temperature; SER, smooth endoplasmic reticulum; TBS, Tris-buffered saline; TCF, tigroid cell foci; XCF, xenomorphic cell foci.

Introduction

Prenecrotic foci of altered hepatocytes (FAH) are consistent precursors of hepatocellular adenomas (HCA) and carcinomas (HCC) induced in different species including primates by a variety of chemical carcinogens, chronic hepaviral infection, transgenic manipulation and hormonal imbalance (1). There is increasing evidence for the early emergence of similar FAH in human hepatocarcinogenesis (2–5).

Based on cytomorphological and simple cytochemical criteria, at least eight different types of FAH may be distinguished in rodents, which apparently do not develop at random but are integrated within three cell lineages leading to hepatocellular neoplasms: (i) the glycogenotic-basophilic, (ii) the amphophilic, and (iii) the tigroid cell lineage (1,6). Whereas the sequential cellular changes characterizing the progression from the preneoplastic to the benign and malignant neoplastic phenotype have been largely elucidated for the glycogenotic-basophilic (1,7) and the amphophilic (8,9) lineage, the cellular origin and neoplastic potential of the tigroid cell population remains to be clarified. Since their first description as a pathomorphological entity in rats treated with a single oral dose of aflatoxin B1 (10) tigroid cell foci (TCF) were also found in rats after administration of other chemical carcinogens (11–16), particularly after low-dose treatment with N-nitrosomorpholine (14–16), in rats exposed to neutrons or α-particles of thorotrast (17), in streptozotocin-diabetic rats after intrahepatic transplantation of pancreatic islet cells (18) and in untreated control rats (10,19). The tigroid cell foci were considered preneoplastic lesions that potentially lead at least to hepatocellular adenomas (10,14–16,20), but this view has been challenged by some authors (21,22) who questioned the preneoplastic nature of TCF.

In close spatial relationship to TCF, markedly enlarged variants of hepatocytes are frequently found that are exceptionally rich in smooth endoplasmic reticulum (SER), randomly distributed aggregates of rough endoplasmic reticulum (RER) and glycogen, and often contain an enlarged nucleus with a prominent nucleolus. These xenomorphic cells (X-cells) were previously noticed in the extrafocal parenchyma (23–26) or in focal lesions (18) of pre-neoplastic rat liver, and were discussed as a possible precursor of tigroid cell populations (10), but this has not been established. Low-dose treatment of rats with NNM appeared to be a promising approach to further elucidate the role of xenomorphic and tigroid cell populations. In this experimental model we studied the early emergence, the progression and the proliferation kinetics of the altered hepatocellular populations sequentially, using cytomorphological, cytochemical and morphometric methods. Our results provide evidence for an origin of TCF from X-cells, and the potential of TCF to progress to both benign and malignant hepatocellular neoplasms.

Materials and methods

Animals and carcinogen treatment

Male Sprague–Dawley rats (Zentralinstitut für Versuchstierzucht, Hannover, Germany), weighing ~200 g at the start of the experiment, were randomly
distributed to cages (one per cage) and maintained under constant conditions (22°C room temperature, 12 h light–dark cycle), on a commercial laboratory chow (Altromin, Lage-Lippe, Germany) ad libitum. N-nitrosomorpholine (NNM) (a generous gift from Professor Preussmann, Abt. für Umweltcarcino- genesis, DKFZ, Heidelberg, Germany) was given in the drinking water. A total of 150 rats were divided into three groups of 50 animals each, including one control group. The second group received 3 mg NNM/kg body wt/day for 7 weeks, followed by tap water until being killed (medium-term stop experiment). The third group received 1 mg NNM/kg body wt/day for 7, 10, 17, 26, 30, 46, 50 and 75 weeks followed by tap water until being killed (long-term stop experiment). The daily NNM-dose given to the treated groups was recalculated according to the body weight at intervals of 4 weeks. Housing and treatment of the animals were in line with the guidelines of the Society for Laboratory Animal Service (GV-SOLAS) and the German animal protection law.

Five animals per group were killed at 7, 14, 21, 30, 40, 50, 60 and 77 weeks after the beginning of the experiment. The remaining animals were killed when they became moribund or were observed until they died spontaneously up to 110 weeks. Liver slices were fixed in Carnoy’s fluid and embedded in paraplast, or snap frozen in isopentane pre-cooled with liquid nitrogen at −180°C and stored at −80°C. In addition to the liver, tissue samples were taken and processed for histology from the kidneys, the lungs, the spleen, the heart, the pancreas and all other organs showing pathological changes at macroscopic inspection.

**Histology and classification of hepatocellular lesions**

Paraffin sections were stained with haematoxylin and eosin (H&E) or treated with the periodic acid-Schiff reaction (PAS), counterstained with orange G and iron haematoxylin. Hepatocellular lesions were classified as described previously (6). In addition to the well-known types of FAH, namely clear and acidophilic cell foci (CCF/ACF) excessively storing glycogen (GSF), mixed cell foci (MCF), and tigroid basophilic cell foci (TCF), focal lesions composed of X-cells (XCF) as described in detail earlier (23–25) were distinguished. HCA and HCC were distinguished according to published criteria (27), and HCA and HCC were classified as mixed and tigroid-basophilic HCA depending on the predominant cell type within the respective lesion. The incidence of HCA and HCC in the different groups was compared using the log-rank test.

**Electron microscopy of X-cell populations**

Liver samples (1 mm³) were fixed in 2.5% glutaraldehyde buffered with 0.05 M cacodylate and supplemented with ions (50 mM KCl and 2.5 mM MgCl₂), post-fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer and embedded in a mixture of Araldite and Epon resin after dehydration in graded series of ethanol and propylene oxide. Semithin sections were stained with toluidine blue. From these sections X-cell foci were selected, and re-embedded as described (28). Ultrathin sections were double-stained with a 1.5% ethanolic solution of uranyl acetate for 20 min and lead citrate for 10 min at room temperature (RT). The primary antibody was diluted 1:100 in 1% bovine serum albumin (BSA)/TBS and incubated for 2 h at RT. As secondary antibody, a peroxidase-conjugated goat-anti-mouse IgG (dilution 1:60 in 1% BSA/TBS) was applied for 1 h at 37°C. The chromogen used was 3,3’-diaminobenzidine (DAB).

**Enzyme and immunocytochemistry**

For enzyme cytochemistry, serial cryostat sections were mounted onto semi-permeable membranes (magnesium). The following enzymes (the activities of which have previously been shown to be frequently altered in FAH) were demonstrated histochemically: adenylate cyclase (ADC) (30), glycogen phosphorylase (PHO) (29), glucose-6-phosphatase (G6Pase) (29), hexokinasen (HK) (31), pyruvate kinase (PK) (32), glucose-6-phosphate dehydrogenase (G6PDH) (29), glycerol-3-phosphate dehydrogenase (G3PDH), glutamate dehydrogenase (GLDH), malic enzyme (ME) and γ-glutamyltranspeptidase (γ-GT) (33). The PAS-reaction and toluidine blue staining were applied to additional serial sections for the demonstration of glycogen and basophilic cellular components, respectively.

For most immunocytochemical studies 6–14 μm serial cryostat sections were air dried and fixed in acetone p.a. at −20°C for 10 min. The expression of two important liver glucose transporter proteins GLUT2 (typical of hepatocytes of adult livers) and GLUT1 (occurring in fetal liver parenchyma, perivenular hepatocytes of adult rats, and in late stages of hepatocarcinogenesis) was demonstrated immunohistochemically using the alkaline antibody and alkaline phosphatase technique (34). The antibodies were kindly provided by Dr Thorens (University of Lausanne, Switzerland). The placental form of the glutathione S-transferase (GST-P) was detected immunohistochemically according to Sato et al. (35). The proliferating cell nuclear antigen (PCNA) was demonstrated by the PC10 antibody (Dako, Hamburg, Germany) in 6 μm cryostat sections fixed with 10% formaldehyde/Tris buffered saline (TBS) for 10 min at room temperature (RT). The primary antibody was diluted 1:100 in 1% bovine serum albumin (BSA)/TBS and incubated for 2 h at RT. As secondary antibody, a peroxidase-conjugated goat-anti-mouse IgG (dilution 1:60 in 1% BSA/TBS) was applied for 1 h at 37°C. The chromogen used was diaminobenzidine (DAB).
Persistent and progressive histological liver lesions

At both dose levels of NNM, the number of FAH including CCF/ACF, MCF, XCF (Figure 1A and B) and TCF (Figure 1C and D) increased in treated rats compared with untreated controls. In both experiments, the first appearance of FAH preceded the manifestation of HCA and HCC (Figure 2) by ~30 weeks. The exact number, the size distribution, the proliferation kinetics and the time course of development of the different types of preneoplastic and neoplastic hepatocellular lesions will be given below. During the preneoplastic phase of both experiments FAH did not only persist but showed a progression to the more proliferative and larger TCF and MCF until HCA and HCC appeared.

In addition to parenchymal alterations, various mesenchymal neoplasms occurred at both dose levels of NNM (Table I), in particular a large number of spongiotic pericytomas, which have been described in detail elsewhere (39).

Fine structure of XCF

In contrast to all other types of FAH, the fine structure of XCF induced in rat liver by NNM has not been investigated previously in situ by target-directed tissue preparations as applied in this study. A typical example of a hepatocyte in this type of focus is given in Figure 3. The cells contain abundant SER, which is, in places, closely associated with β-particles of glycogen. Distinct and highly ordered aggregates of RER (Figure 3A and B) are interwoven into the SER. Mitochondria and peroxisomes exhibit a normal fine structure, and are predominantly localized in the vicinity of RER aggregates.

Enzyme and immunocytochemical patterns of FAH

The distribution of the activities of most enzymes investigated and of the expression of GLUT1 and GLUT2 within the liver lobules of the untreated controls was similar to that repeatedly described in male Sprague–Dawley rats (29–32,34), which were also used in this study. The activities of GLDH and ME showed a gradient from high activities in perivenular to lower activities in perportal zones of the parenchyma of control animals. GST-P was not expressed in the normal parenchyma of controls. Changes in enzyme and immunocytochemical patterns were related to certain phenotypes of FAH (Figure 4) in treated and untreated animals rather than to the dosing schedules applied, and were, hence, evaluated in specific types of FAH randomly selected from both experiments. As demonstrated in Figure 4, 60 XCF and TCF each were studied. In addition, 30 GSF and MCF each were selected for comparison of their cytochemical patterns with those of the same types of FAH, which were previously extensively investigated at other dose levels of NNM (1,6).

It is evident from Figure 4 that XCF show a number of enzyme and immunocytochemical changes that are largely similar to those of TCF, although the percentage of TCF with increased activities of the malic enzyme and two of the dehydrogenases (G3PDH, GLDH) studied is lower. XCF also exhibit similarities to the glycogenotic CCF/ACF and the MCF, but several striking differences to these two closely related types of FAH are obvious. This applies particularly to the much higher percentage of CCF/ACF and MCF characterized by a reduction in the content of GLUT2 and in the activities of enzymes involved in glycogen breakdown (ADC, PHO). The increase in the activities of the malic enzyme and two of the dehydrogenases (G6PDH, G3PDH) investigated is likewise especially pronounced in the glycogenotic CCF/ACF. Another striking difference between XCF and TCF on one hand, and CCF/ACF and MCF on the other, is that <30% of the former but >50 or 30% of the latter show increased expression of DST-P and activity of γ-GT, respectively.

Proliferation kinetics of FAH

Corresponding to the alterations in enzyme and immunocytochemical patterns of the focal hepatic lesions, changes in their proliferation kinetics were evaluated in specific types of FAH randomly selected from both experiments. As detailed in Figure 5, all types of FAH, including XCF, showed a highly significant (P = 0.0001) increase in the PCNA-LI compared with the extrafoveal parenchyma of both NNM-treated and untreated rats. The PCNA-LI increased significantly (P < 0.0001) from CCF/ACF to MCF, and was weakly significant (P < 0.1) from XCF to TCF. There was an additional significant increase of the PCNA-LI in HCCs (P < 0.05).

Sequential appearance of preneoplastic and neoplastic hepatocellular lesions

The number of animals in which the emergence of FAH was examined by a stereological approach, and the means ± SEM

**Table I.** Summary of basic data from animal experiments including daily and total doses of N-nitrosomorpholine (NNM), time schedules, number of effective animals and incidence of hepatic and extrahepatic neoplasms

<table>
<thead>
<tr>
<th>NNM (mg/kg body wt/day)</th>
<th>Duration of treatment (weeks)</th>
<th>Observation period (weeks)</th>
<th>Total NNM dose (mg/animal)</th>
<th>No. of effective animals</th>
<th>Hepatocellular neoplasms</th>
<th>Various other neoplasms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adenomas (mixed/tigroid)</td>
<td>Carcinomas</td>
</tr>
<tr>
<td>0</td>
<td>–</td>
<td>1–60</td>
<td>–</td>
<td>36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>61–110</td>
<td>–</td>
<td>37</td>
<td>1 (1/0)</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>61–110</td>
<td>50</td>
<td>13</td>
<td>2 (1/1)</td>
<td>1</td>
</tr>
</tbody>
</table>

4 One testicular Leydig cell tumor, one granulocytic leukemia.
5 One hemangiosarcoma, one histiocytic sarcoma, six spongiotic pericytomas.
6 One phaeochromocytoma, one esthesioneuroepithelioma, one renal hemangiosarcoma, one mesenchymal renal tumor, one transitional cell carcinoma of the urinary bladder, one lymphoma, one renal oncocytoma, one pancreatic acinar cell adenoma.
7 Five haemangiosarcomas, two spongiotic pericytomas.
8 One lymphoma, one nephroblastoma, one intra-abdominal sarcoma, one renal cell carcinoma.
Foci of altered hepatocytes induced in rats by exposure to low dose levels of $N$-nitrosomorpholine. (A) Xenomorphic cell focus (XCF) consisting of hypertrophied hepatocytes with slightly altered cytoplasmic fine structure and nuclear enlargement. (B) Portion of xenomorphic cell focus (XCF) showing scattered basophilic bodies on a weakly acidophilic background, and enlarged nuclei with prominent nucleoli in X-cells. (C) Appearance of small groups of tigroid cells in perivenular cell population also containing enlarged X-cells. (D) Small tigroid cell focus (TCF) characterized by increased cytoplasmic basophilia and enlarged nuclei with prominent nucleoli. CV, central vein. All figures H&E stained.

The numbers of hepatocellular neoplasms appearing at both dose levels of NNM are listed in Table I.
Fig. 2. Hepatocellular neoplasms induced in rats by exposure to low dose levels of N-nitrosomorpholine. (A) Peripheral portion of hepatocellular adenoma (HCA) composed of a mixture of X-cells, tigroid cells and cells exhibiting a homogeneous cytoplasmic basophilia. (B) Peripheral portion of hepatocellular carcinoma (HCC) containing tigroid cells (upper left) and cells with a more homogeneous cytoplasmic basophilia. (C) Portion of hepatocellular carcinoma (HCC) composed of considerably enlarged cells endowed with large nuclei and nucleoli, and scattered basophilic bodies in the cytoplasm, reminiscent of X-cells in preneoplastic focal lesions. (D) Portion of hepatocellular carcinoma (HCC) consisting of cells with large nuclei and nucleoli, and with a tigroid pattern of the cytoplasmic basophilia, reminiscent of tigroid cells in preneoplastic focal lesions. All figures stained with H&E.
Neither controls nor rats treated with NNM at both dose levels showed any FAH (Figure 6A–C) or hepatocellular neoplasms after 7 weeks. Exposure to the higher dose of NNM resulted in a small number of XCF and CCF/ACF at 14 and 21 weeks (Figure 6B), albeit carcinogen administration had been terminated 7 weeks before. At 21 weeks, a small number of XCF and CCF/ACF were also found in controls and in animals continuously exposed to the lower dose of NNM. In addition to these types of FAH, a few TCF were found in rats treated with NNM at both dose levels but not in controls.

During the following weeks, there was only a minor increase in the total number of FAH with age in the untreated controls (Figure 6A). In addition to XCF and CCF/ACF, a few TCF occurred after 40, and MCF after 60 weeks. The average size of CCF/ACF, MCF and TCF was always much smaller than that of XCF, which became slightly larger with age (Figure 7A).

Whereas the percentage of liver tissue (volume fraction) occupied by most types of FAH remained very small throughout the experiment, it increased from 1 to 6% between 21 and 77 weeks in the case of XCF (Figure 8A). After 79 weeks, one hepatocellular adenoma was observed in an untreated control animal.

In the rats exposed to 3 mg NNM/kg body wt/day for 7 weeks, the total number of FAH increased rapidly after 21 weeks up to a peak at 40 weeks (Figure 6B) when hepatocellular neoplasms started to develop (Table I). All types of FAH contributed to this increase in the total number, but the focal lesions predominantly accounting for the elevation in comparison to the untreated controls were CCF/ACF, TCF and MCF. CCF/ACF showed a particularly pronounced increase between 21 and 40 weeks. Whereas their number constantly increased after 40 weeks, MCF showed peaks at 40 and 60 weeks, and then decreased up to the end of the experiment. XCF were present in low numbers between 14 and 40 weeks, and showed a slight increase at later time points. Seven weeks after the first XCF were seen, TCF emerged and surpassed this lesion type in number up to 60 weeks. In addition to typical TCF, basophilic lesions with transitions from a tigroid to a more homogeneous basophilia were sometimes found. The average size of XCF did not differ significantly from that of the untreated controls up to 40 weeks but after this time point it was significantly ($P < 0.01$) smaller in the treated animals (Figure 7B). In contrast, compared with the controls, a significant ($P < 0.05$) increase in the average size was found for CCF/ACF at 30 and 50 weeks, and for MCF and TCF at 77 weeks. The volume fraction occupied by XCF was always higher than that of other types of FAH, and gradually increased throughout the experiment (Figure 8B). At a lower level, TCF and MCF also showed a gradual elevation of their volume fraction, in particular at the end of the experiment. Five hepatocellular adenomas appeared between 40 and 110 weeks (Table I). Three of these adenomas contained tigroid basophilic cell components, and two were of the mixed cell type. One hepatocellular carcinoma composed of homogeneously basophilic cells was found at 84 weeks. Compared with the untreated controls, the increase in the incidence of adenomas and carcinomas combined was statistically significant ($P < 0.05$).

An enhancement in the total number of FAH compared with the controls was also observed in rats exposed for >21 weeks to 1 mg NNM/kg body wt/day (Figure 6C). However, this increase was less pronounced than that produced by limited exposure to 3 mg NNM/kg body wt/day, although the animals received a total dose of NNM, which was higher by a factor of two to nearly six after 35 weeks. As to the individual types of FAH, the number of CCF/ACF increased up to 30 weeks, and subsequently steadily decreased up to the end of the experiment, whereas the number of MCF and, especially, TCF increased. There were small numbers of XCF since week 21. After week 40, TCF with transitions from typical tigroid to more homogeneous basophilia were also observed in small numbers. The average size of nearly all types of FAH increased up to 60 weeks. The average size of nearly all types of FAH was statistically significant ($P < 0.05$) increase in the average size was found for CCF/ACF at 30 and 50 weeks, and for MCF and TCF at 77 weeks. The volume fraction occupied by XCF was always higher than that of other types of FAH, and gradually increased throughout the experiment (Figure 8B). At a lower level, TCF and MCF also showed a gradual elevation of their volume fraction, in particular at the end of the experiment. Five hepatocellular adenomas appeared between 40 and 110 weeks (Table I). Three of these adenomas contained tigroid basophilic cell components, and two were of the mixed cell type. One hepatocellular carcinoma composed of homogeneously basophilic cells was found at 84 weeks. Compared with the untreated controls, the increase in the incidence of adenomas and carcinomas combined was statistically significant ($P < 0.05$).

An enhancement in the total number of FAH compared with the controls was also observed in rats exposed for >21 weeks to 1 mg NNM/kg body wt/day (Figure 6C). However, this increase was less pronounced than that produced by limited exposure to 3 mg NNM/kg body wt/day, although the animals received a total dose of NNM, which was higher by a factor of two to nearly six after 35 weeks. As to the individual types of FAH, the number of CCF/ACF increased up to 30 weeks, and subsequently steadily decreased up to the end of the experiment, whereas the number of MCF and, especially, TCF increased. There were small numbers of XCF since week 21. After week 40, TCF with transitions from typical tigroid to more homogeneous basophilia were also observed in small numbers. The average size of nearly all types of FAH increased up to 60 weeks. The average size of nearly all types of FAH was statistically significant ($P < 0.05$) increase in the average size was found for CCF/ACF at 30 and 50 weeks, and for MCF and TCF at 77 weeks. The volume fraction occupied by XCF was always higher than that of other types of FAH, and gradually increased throughout the experiment (Figure 8B). At a lower level, TCF and MCF also showed a gradual elevation of their volume fraction, in particular at the end of the experiment. Five hepatocellular adenomas appeared between 40 and 110 weeks (Table I). Three of these adenomas contained tigroid basophilic cell components, and two were of the mixed cell type. One hepatocellular carcinoma composed of homogeneously basophilic cells was found at 84 weeks. Compared with the untreated controls, the increase in the incidence of adenomas and carcinomas combined was statistically significant ($P < 0.05$).
untreated animals for CCF/ACF and MCF at 60 weeks, and for MCF and TCF at 77 weeks. When the average sizes of FAH at the two dose levels of NNM given were compared, several significant differences were observed in the last three groups. Compared with the rats exposed to the higher dose for 7 weeks (Figure 7B), the animals exposed to the lower dose for a much longer time period (Figure 7C) showed a significant increase in the average size of XCF ($P < 0.001$) and TCF ($P < 0.0001$) at 50 weeks, of MCF ($P < 0.0001$) and TCF ($P < 0.001$) at 60 weeks, and of MCF at 77 weeks. The volume fraction occupied by XCF was always higher than that of the other types of FAH, but slightly decreased at the end of the experiment (Figure 8C). After week 40, an increase in the volume fraction of MCF and TCF was observed. Thirteen hepatocellular adenomas and carcinomas each developed after 50 and 60 weeks, respectively (Table I). Fifty per cent of the adenomas were of the tigroid, and 50% of the mixed cell type. From the carcinomas that were often multicentric, four contained tigroid cell components (and sometimes even cells resembling X-cells), five mixed cell components and four were exclusively composed of homogeneously basophilic cells. Compared with the untreated controls, the increase in the incidence of hepatocellular neoplasms was highly significant for both adenomas ($P < 0.01$) and carcinomas ($P < 0.0001$). The increased incidence of hepatocellular carcinomas was also significantly different in the experimental groups receiving NNM at the two dose levels.

Intralobular localization of FAH
An unequivocal localization of the FAH within the liver lobule was not always possible. In the untreated controls 18–54%, and in the NNM-treated animals 21–39% of FAH were unclassifiable as to their intralobular localization. From the remaining FAH in untreated controls, the majority could be allocated to perivenular parts (Figure 9A). This applies particularly to XCF (73%), CCF/ACF (41%) and MCF (23%). However, periportal and/or intermediate positions were possible in all cases including TCF. After administration of 3 mg NNM/kg body wt per/for 7 weeks, a perivenular location of FAH likewise predominated (Figure 9B). Among the localizable FAH, 39–51% of XCF, CCF/ACF, MCF and TCF were found in the perivenular region, whereas 8–20% of all types of FAH were observed in periportal parts. When 1 mg NNM/kg body wt/day was given over longer time periods, the
Table II. Number (mean ± SEM) of FAH sections per cm² liver tissue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time period (weeks)</th>
<th>No. of animals</th>
<th>Xenomorphic cell foci</th>
<th>Tigroid basophilic cell foci</th>
<th>Clear/acidophilic cell foci</th>
<th>Mixed cell foci</th>
<th>All types of foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7</td>
<td>5</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>2</td>
<td>1.86 ± 1.86</td>
<td>0.00 ± 0.00</td>
<td>1.03 ± 0.41</td>
<td>0.00 ± 0.00</td>
<td>2.90 ± 1.45</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4</td>
<td>3.51 ± 2.11</td>
<td>0.00 ± 0.00</td>
<td>2.99 ± 0.67</td>
<td>0.44 ± 0.44</td>
<td>6.75 ± 2.86</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4</td>
<td>4.74 ± 0.90</td>
<td>1.56 ± 1.13</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>3.63 ± 1.71</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3</td>
<td>6.90 ± 2.54</td>
<td>1.31 ± 0.71</td>
<td>4.47 ± 2.87</td>
<td>0.00 ± 0.00</td>
<td>12.67 ± 4.73</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5</td>
<td>6.59 ± 1.04</td>
<td>1.48 ± 0.91</td>
<td>2.76 ± 1.44</td>
<td>1.25 ± 0.62</td>
<td>12.08 ± 2.08</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>4</td>
<td>10.86 ± 0.74</td>
<td>4.76 ± 1.14</td>
<td>0.00 ± 0.00</td>
<td>1.78 ± 0.82</td>
<td>17.32 ± 1.02</td>
</tr>
<tr>
<td>3 mg NNM/ 7 kg/day for 7 weeks</td>
<td>7</td>
<td>5</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>1 mg NNM/ kg/day up to 75 weeks</td>
<td>14</td>
<td>3</td>
<td>2.36 ± 2.36</td>
<td>0.00 ± 0.00</td>
<td>6.19 ± 4.95</td>
<td>0.00 ± 0.00</td>
<td>8.55 ± 3.93</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>4</td>
<td>4.45 ± 3.39</td>
<td>2.46 ± 1.24</td>
<td>2.49 ± 1.17</td>
<td>0.40 ± 0.26</td>
<td>9.20 ± 2.32</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4</td>
<td>3.88 ± 1.19</td>
<td>11.30 ± 1.43*</td>
<td>10.08 ± 1.78*</td>
<td>6.79 ± 2.48</td>
<td>12.50 ± 4.39*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4</td>
<td>6.03 ± 1.28</td>
<td>8.80 ± 2.33*</td>
<td>12.60 ± 3.56*</td>
<td>11.15 ± 1.41*</td>
<td>38.59 ± 2.98*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4</td>
<td>15.41 ± 3.04</td>
<td>11.90 ± 1.85*</td>
<td>3.29 ± 1.17</td>
<td>4.05 ± 1.70</td>
<td>34.65 ± 6.64</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>4</td>
<td>15.01 ± 0.67</td>
<td>15.09 ± 5.98*</td>
<td>1.67 ± 1.41</td>
<td>13.76 ± 3.52*</td>
<td>45.52 ± 12.10*</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>5</td>
<td>14.33 ± 3.49</td>
<td>12.98 ± 2.41*</td>
<td>0.56 ± 0.56</td>
<td>18.27 ± 7.50*</td>
<td>46.13 ± 12.35*</td>
</tr>
<tr>
<td>1 mg NNM/ 7 kg/day up to 75 weeks</td>
<td>14*</td>
<td>4</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4</td>
<td>0.96 ± 0.48</td>
<td>1.97 ± 0.25*</td>
<td>8.65 ± 3.83</td>
<td>0.46 ± 0.46</td>
<td>12.04 ± 2.49</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4</td>
<td>6.04 ± 2.60</td>
<td>9.11 ± 2.61*</td>
<td>7.59 ± 2.04*</td>
<td>6.14 ± 2.01*</td>
<td>28.87 ± 3.51*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>7.59 ± 2.58</td>
<td>10.26 ± 2.72*</td>
<td>1.06 ± 0.48</td>
<td>9.92 ± 4.15*</td>
<td>28.82 ± 4.82</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>3</td>
<td>8.73 ± 3.95</td>
<td>16.20 ± 6.70*</td>
<td>2.81 ± 1.68</td>
<td>11.16 ± 5.14</td>
<td>38.90 ± 6.92*</td>
</tr>
<tr>
<td>7 mg NNM/kg/day up to 75 weeks</td>
<td>7*</td>
<td>3</td>
<td>5.77 ± 3.38</td>
<td>8.62 ± 4.36</td>
<td>0.21 ± 0.21</td>
<td>4.35 ± 4.35</td>
<td>18.92 ± 9.60</td>
</tr>
</tbody>
</table>

*Significantly (P < 0.05) increased compared with untreated controls.
*Stop of NNM-treatment 4 weeks before being killed.
*Stop of NNM-treatment 2 weeks before being killed.

Discussion

The results reported provide evidence for the consistent early emergence of a variant hepatocellular population, designated as xenomorphic cell focus (XCF) when confined to distinct parenchymal areas during the evolution of FAH induced in rats by oral exposure to low doses of NNM or occurring spontaneously. XCF appear in addition to the well known glycogenotic CCF/ACF several weeks prior to other types of FAH, namely TCF and MCF, which in turn precede the manifestation of hepatocellular neoplasms frequently resembling in their cellular phenotypes the more advanced forms of FAH.

X-cells were first noted in the extrafocal parenchyma of the NNM-treated rat liver as considerably enlarged hepatocytes containing abundant but regularly distributed SER, RER and glycogen. As xenomorphic cell focus (XCF) when confined to distinct parenchymal areas. Nevertheless, among the localizable FAH, a large number of XCF (44%), TCF (40%), CCF/ACF (25%) and MCF (39%) occupied the perivenular parenchyma (Figure 9C). The remaining FAH emerged in periportal or intermediate parts of the liver lobules.

Fig. 5. Proliferation kinetics as determined by immunohistochemical demonstration of the proliferating cell nuclear antigen (PCNA) in untreated controls, and foci of altered hepatocytes, hepatocellular carcinomas, and extrafocal parenchyma of rats exposed to low dose levels of N-nitrosomorpholine. XCF, xenomorphic cell foci; TCF, tigroid cell foci; CCF/ACF, combined clear/acidophilic cell foci; MCF, mixed cell foci. The line inside the box represents the median and the boxes are delimited by the first and third quartile. Whiskers (dotted lines) are drawn to the nearest observed value not beyond ×1.5 (inter-quartile range). Points beyond are shown individually.
Preneoplastic hepatocellular foci

Fig. 6. Number of foci of altered hepatocytes (FAH) per cm$^3$ in rats exposed to low dose levels of N-nitrosomorpholine (NNM) and untreated controls. - - - xenomorphic cell foci; — tigroid cell foci; — clear/acidophilic cell foci; — mixed cell foci; — all types of foci.

to their ultrastructure, the X-cells share many features with the tigroid cells emerging during chemical or hormonal hepatocarcinogenesis (6,10,18). The most outstanding features of tigroid cells are abundant highly ordered stacks of the RER, whereas SER and glycogen are less prominent than in X-cells.

In addition to their fine structure, XCF deviate from the extrafocal parenchyma in their histochemical pattern. Enzyme and immunohistochemical approaches revealed a number of changes in XCF, showing some remarkable similarities to, but also interesting differences from, the CCF/ACF. Thus, the activity of enzymes involved in glycogen breakdown (ADC, PHO), the expression of GLUT2 (which facilitates glucose transport across the plasma membrane in both directions) and the activity of glucose-6-phosphatase (which produces free glucose from glucose-6-phosphate for release from the hepatocytes) are more frequently reduced in the glycogenotic CCF/ACF (29,30,34) than in XCF. This may explain the differences in the average amount of glycogen accumulated in these two types of FAH. The activities of the pyruvate kinase, the malic enzyme and some dehydrogenases, including the rate limiting enzyme of the pentose phosphate pathway (G6PDH), were often elevated in both XCF and CCF/ACF. However, with the exception of the G3PDH these enzymatic changes affected more CCF/ACF, in which several of these enzymes were found to be similarly altered earlier (29,40,41). The most striking enzymatic difference between XCF and CCF/ACF observed in this study concerned the well known ‘marker’ enzymes of FAH, GST-P (35) and γ-GT (42), the expression of which was often increased in CCF/ACF but only rarely in XCF. In this respect XCF behave like TCF (10,20). This applies also to the expression of GLUT2 and changes in the activities of several enzymes such as ADC and HK. For two of the dehydrogenases (G6PDH, GLDH) studied, and for malic enzyme there is a similar trend in changes of activities in XCF and TCF, although somewhat less TCF than XCF are involved.

The cytomorphological and cytochemical findings discussed indicate that XCF represent a cell population closely related to CCF/ACF on the one hand, and TCF on the other. This notion is strongly supported by the observation that all of these foci show a significantly increased cell proliferation (as demonstrated
Fig. 8. Volume fraction (%) of foci of altered hepatocytes in rats exposed to low dose levels of N-nitrosomorpholine (NNM) and untreated controls. -- xenomorphic cell foci; — tigroid cell foci; — clear/acidophilic cell foci; — mixed cell foci; — all types of foci.

by PCNA-labelling) compared with the extrafocal parenchyma of treated and untreated animals. XCF show even a higher PCNA-LI than CCF/ACF, the DNA-synthesis of which has previously been shown by [3H]thymidine-labelling to be significantly elevated over the level of the normal parenchyma (43). Recently, a significantly increased cell proliferation compared with the extrafocal parenchyma was also found in XCF, which emerged early during hepatocarcinogenesis in streptozotocin-diabetic rats after intrahepatic transplantation of pancreatic islet cells (18). In MCF induced in rat liver by low doses of NNM, the PCNA-LI was significantly higher than in XCF and CCF/ACF. For MCF induced in rats by a higher dose of NNM (12 mg/kg body wt/day) we have earlier shown that their cell proliferation is significantly higher than that of CCF/ACF (43). In line with results obtained in a number of stereological studies in NNM-treated rats (14–16,44,45), the recent mathematical modelling of some of these data as a sequence of epigenetic events (46) and findings in hormonal hepatocarcinogenesis (18) indicate that MCF represent a more advanced type of FAH than CCF/ACF. The same seems to apply to TCF compared with XCF.

At both dose levels of NNM investigated in this study, all types of FAH, in particular XCF and TCF, appeared predominantly in perivenular parts of the liver lobules. This finding is in contrast to the preferential occurrence of FAH in peripheral and intermediate lobular regions (corresponding to acinar zones 1 and 2) in rats treated with various hepatocarcinogens, including NNM (7,47). However, similar observations were previously made after continuous oral exposure of rats to 6 mg NNM/kg body wt/day (48) and to low doses of thioacetamide that at higher dose levels also preferentially produced FAH in acinar zones 1 and 2 (49). This dose-dependence of the location of FAH may mainly be due to the pronounced cytotoxic changes regularly found in perivenular parenchyma after administration of high doses of hepatocarcinogens, which apparently preclude the development of FAH in this lobular region under many experimental conditions. The predominant perivenular localization of both XCF and TCF induced by low doses of NNM indicates that these two types of proliferative cell populations, which in addition also share a number of other cytomorphological and cytochemical features, are jointly involved in the process of hepatocarcinogenesis.

Several stereological studies on the appearance of the different types of FAH in rats administered NNM at various dose levels between 6 and 320 mg/kg body wt/day revealed a predominant hepatocellular lineage leading from the early glycogenotic CCF/ACF via MCF and glycogen-poor, basophilic cell populations
to HCA and HCC (14–16,44,45). This lineage develops also at the lower doses of NNM investigated, but is regularly accompanied by the tigroid cell lineage. The frequent occurrence of TCF in rats after continuous administration of 1 mg NNM/kg body wt/day for up to 75 weeks, and after exposure to 3 mg NNM/kg body wt/day for 7 weeks supports the concept that this lesion phenotype results from gentle effects of carcinogenic agents on the hepatocytes, which, nevertheless, provide the potential for neoplastic cell conversion (20). This notion is endorsed by the observation that FAH resembling TCF often appear in untreated aged rats of different strains, which may also develop ‘spontaneous’ hepatocellular neoplasms (50–54), possibly resulting from contamination of the diet with low doses of hepatocarcinogens (10) or from hormonal imbalance as demonstrated recently (18).

Similar considerations apply to XCF, which emerged in older untreated controls. Although the number of XCF was relatively low in all groups, their average size and volume fraction was always much higher than that of all other types of FAH. However, the average size of XCF significantly decreased in NNM-treated compared with untreated rats at later time points when the number and size of more advanced types of preneoplastic foci and neoplastic lesions increased, suggesting a multicentric evolution of FAH and subsequently hepatocellular neoplasms from XCF. This holds particularly true for TCF, which share their preferential intralobular localization, and many features of their fine structure and enzymic pattern with XCF. Since there are also a number of similarities in all of these parameters with CCF/ACF, this type of FAH might also originate from XCF, and vice versa, under certain conditions, as discussed by Dombrowski et al. (18), for the induction of FAH by hypersecretion of insulin. Insulinomimetic effects of chemical and viral hepatocarcinogens have been considered to play a decisive role in the induction of FAH, especially the glycogenotic CCF/ACF, in recent years (1,31,40,56). This hypothesis received strong support by the discovery of an early NNM-induced overexpression of the insulin receptor substrate-1 eliciting the preneoplastic hepatic glycogenesis in rat liver (56). Thus, it is conceivable that changes in the hormonal balance during ageing might be an important causal factor for the frequent development of FAH, including XCF and CCF/ACF, in untreated control animals, and, perhaps, also for synergistic effects of such endogenous events with the exogenous action of carcinogenic agents. Although XCF and CCF/ACF are usually well separated from each other, the borderline may be ill-defined. It is, therefore, impossible to exclude that fluctuations between these two cell populations may occur in both directions.

The time-course and frequency of appearance of TCF were similar to those of MCF until HCA started to develop at both dose levels of NNM administered, indicating that both types of FAH participated in the progression from preneoplastic to neoplastic lesions. In line with the well-established property of MCF to give rise to HCA and HCC (6,7), about half of the HCA and HCC occurring showed a mixed cell type or at least mixed cell components. However, all remaining HCA and even four of the HCC contained tigroid cell components, confirming earlier observations in rats developing HCA after treatment with a single dose of aflatoxin B1 (10) or higher doses of NNM (14–16), and demonstrating in addition the potential of TCF to progress to HCC.

An intriguing result of our stereological investigations is that neither the total number of FAH nor the number of any subtype of the focal lesions showed significant differences at the two dose levels of NNM, although the total dose administered, and the incidence of HCC observed differed markedly. Non-linearity of the dose–response in the induction of FAH at low doses of hepatocarcinogens has been reported by several groups recently (57–60). Interestingly, after administration of NNM at dose levels of 6–60 mg/l (corresponding to 0.006–6 mg/kg body wt for the rats at the beginning of the experiment) the dose–effect curves for the number of enzyme-altered FAH became non-linear at a dose level below 4 mg/kg body wt (60), which was also used in this study. However, when we compared the average size of MCF and TCF induced at the two dose levels of NNM we found much larger lesions at several late time points after the longer exposure to the lower dose level, which also resulted in a significantly higher incidence of HCC. As to the mechanism of hepatocarcinogenesis, these findings suggest that the much higher incidence of HCC after long-term administration of 1 mg NNM/kg body wt/day compared with the 7 weeks’ exposure to 3 mg NNM/kg body wt/day results from a pronounced acceleration of neoplastic cell conversion by sustained effects of the carcinogen on established preneoplastic cell populations rather than the induction of additional FAH.

Acknowledgements

The authors gratefully acknowledge the excellent technical assistance of Gabrielle Schmidt, Rita Büttner, Gabriele Becker and Ditmar Greulich, the expert photographic assistance of Joachim Hollatz and Dirk Nehrbass, and the secretarial help of Brigitte Peilinóll.

References


