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ORIGINAL ARTICLE

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Expression of pyruvate kinase M_2 in preneoplastic hepatic foci of *N*-nitrosomorpholine-treated rats

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Abstract The expression of the pyruvate kinase (PK) isoenzymes L and M_2 was analysed in the livers of rats treated with the hepatocarcinogenic agent *N*-nitrosomorpholine (NNM) in the drinking water. In control animals L-PK expression was restricted to liver parenchymal cells, whereas M_2-PK was detected in bile duct epithelial, blood vessel wall, endothelial and Kupffer cells. In rats treated with NNM proliferating oval cells were consistently L-PK negative and M_2-PK positive, while the ductal cells of cholangiofibroses were clearly L-PK positive and coexpressed M_2-PK. However, no morphological differentiation of ductal cells into hepatocyte-like cells was observed. In the clear and acidophilic cell foci storing glycogen in excess strong staining for L-PK was observed. In glycogen-poor foci induced by NNM a shift from L-PK to M_2-PK expression takes place.

Key words Pyruvate kinase isoenzymes · *N*-Nitrosomorpholine · Rat

Introduction

The sequential cellular changes occurring during the development of hepatocellular and cholangiocellular carcinomas induced in the rodent liver by a variety of chemical carcinogens have been analysed extensively. The predominant cell lineage observed during the development of hepatocellular carcinomas in the rat ranges from clear and acidophilic cell foci, which store glycogen in excess and may be acidophilic due to the proliferation of smooth endoplasmic reticulum, through mixed and basophilic (ribosome-rich) cell foci to hepatocellular adenomas and carcinomas [3]. The development of cholangiocellular neoplasms proceeds in four stages: proliferation of oval cells in response to the toxic effects of the chemicals on the liver parenchymal cells; appearance of cholangiofibroses, in which the bile duct epithelial cells store and secrete neutral and acidic mucopolysaccharides; appearance of cholangiofibromas, which grow expansively and produce collagen fibres and also mucous substances; appearance of cholangiocarcinomas, which grow invasively and show a gradual decrease of mucus production [4, 5, 19, 30]. Oval cells are liver epithelial cells that proliferate during the early stages of hepatocarcinogenesis and severe liver injury induced by many chemicals [1]. It has been suggested that oval cells can not only give rise to cholangiocellular carcinomas, as mentioned above, but can also differentiate into basophilic parenchymal cells under particular experimental conditions and lead to the formation of hepatocellular carcinomas [11, 12, 14, 23, 24, 27, 28]. These authors postulate that oval cells should be viewed as bipotent tumour precursor cells that can give rise to hepatocellular as well as cholangiocellular carcinomas.

Pyruvate kinase (PK) is one of the key enzymes of carbohydrate metabolism, controlling the glucose flow throughout the later part of glycolysis [9]. There are several PK isoenzymes: the L-type is present in liver parenchymal cells and proximal kidney tubules, while the M_2-type is detected in liver mesenchymal and bile duct epithelial cells and also in distal kidney tubules.

Findings published on the expression of L- and M_2-PK during hepatocarcinogenesis are controversial. Early biochemical studies showed that the expression of L-PK was down-regulated and that of M_2-PK up-regulated within transplantable and primary hepatocellular tumours [8, 13, 26, 29, 32, 37–39]. Whereas a number of

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later biochemical and immunohistochemical studies confirmed the decrease in the expression of L-PK during the development of hepatocellular tumours [15–18, 21, 22, 25], this shift from L- to M_2-PK could not be reproduced when the phenomenon was investigated by immunohistochemistry [25]. Furthermore, Klimek and Bannasch [21] reported that PK activity was actually increased in the clear and acidophilic cell foci that appeared early in the process and that it decreased both in mixed and basophilic cell foci and in basophilic hepatic tumours, (during the later stages of hepatocarcinogenesis). Very recently Hacker et al. [20] have been able to show for the first time, in serial liver sections from rats fed a choline-deficient/DL-ethionine CDE-supplemented diet, that a shift from L- to M_2-PK expression takes place in mixed cell foci and hepatocellular adenomas.

In cholangiocarcinogenesis, it has been shown that M_2-PK is present in proliferating oval cells as well as in the ductal cells of cholangiofibroses and cholangiofibromas of CDE-fed rats [31, 33–36]. Interestingly, in this experimental model the proliferating oval cells and the ductal cells of cholangiofibroses and cholangiofibromas also express L-PK [31, 33–36]. The interpretation of this observation has varied. According to Yeoh et al. [35, 36], the presence of L-PK, a specific parenchymal cell marker in untreated rat liver, in oval cells strongly suggests that oval cells are able to differentiate into liver parenchymal cells. However, Steinberg et al. [31] have clearly shown that, according to morphology, the ductal cells expressing L-PK within cholangiofibroses and cholangiofibromas are part of the bile duct epithelial cell lineage.

To test whether the shift from L-PK to M_2-PK expression in hepatocellular lesions reported by Hacker et al. [20] was exclusively due to CDE feeding or whether it represented a biochemical phenomenon observed in other models of chemically induced hepatocarcinogenesis, we analysed the expression of L-PK and M_2-PK in the cholangiocellular and hepatocellular lesions of rats that had been exposed to *N*-nitrosomorpholine (NNM) for up to3 weeks. NNM was chosen because it has been used in the past to induce hepatocellular neoplasms in rats [4, 5, 7, 10]. In the present study we show that: M_2-PK and L-PK are coexpressed in ductal cells within cholangiofibroses of NNM-treated rats and that in glycogen-poor basophilic cell foci appearing in the livers of NNM-treated animals a shift from L-PK to M_2-PK expression takes place.

Materials and methods

Forty-eight male outbred 6-week-old Wistar rats from Charles River (Sulzfeld, Germany) were housed in plastic cages on a fixed day and night cycle and exposed to 500 mg NNM/l drinking water for 5, 10, 14 or 21 days. Animals were either sacrificed at these time-points or kept for another 4 weeks without being further exposed to NNM. Forty-eight rats not given NNM were used as controls and were sacrificed in groups of six at the given time-points. Livers were quickly removed under light ether anaesthesia, and liver slices ~2 mm thick were prepared. Slices of liver were fixed in 10% formalin in phosphate-buffered saline, and sections of formalin-fixed liver samples were stained with haematoxylin and eosin for the light microscopic examination of liver structure. Other slices of liver were fixed in acetone for 4 days at 4°C (acetone being changed three times during the 4-day period), and then laid in isopropanol for 4–8 h and in xylol for another 2 h. Thereafter, paraffin blocks were prepared and sections of these liver samples were incubated with monoclonal antibodies against L-PK (clone AD12) and M_2-PK (clone DF4) (ScheBoTech, Wettenberg, Germany). The site of antibody binding was revealed by using the peroxidase–antiperoxidase method as previously described [15], and the liver slices were counterstained with Mayer's haematoxylin.

Results

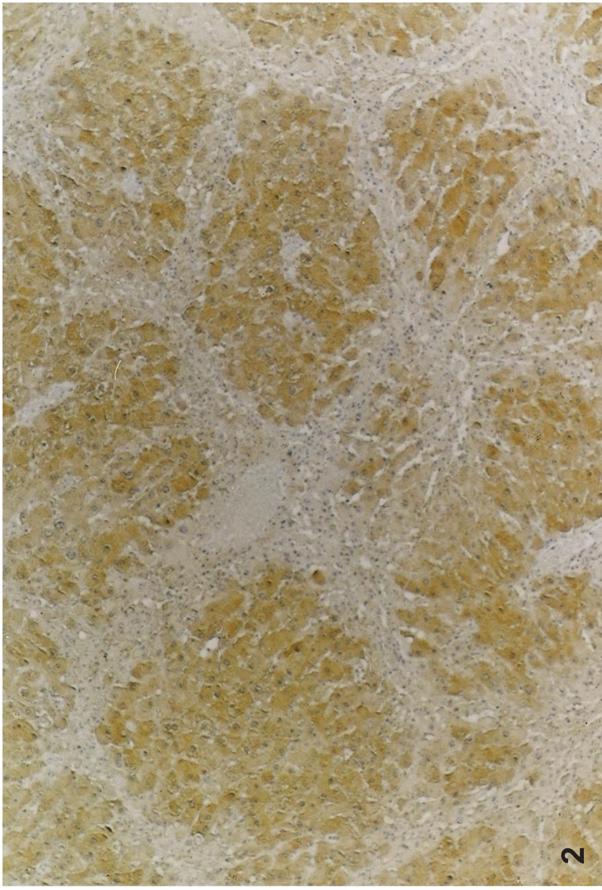
The histopathological features of the livers from rats exposed to NNM were described in detail by Bannasch's group a number of years ago [4, 5]. Since our own light microscopic observations in no way differed from those recorded by Bannasch's group [4, 5], only a brief description of the hepatic lesions observed in the course of this study is presented here. Five days after the beginning of the NNM treatment, necrosis of a significant number of liver parenchymal cells situated centrilobularly was observed and the area was largely replaced by red blood cells. Up to day 21 liver parenchymal cell necrosis became increasingly pronounced, and within the necrotic areas liver parenchymal cells with an eosinophilic cytoplasma and polymorphic nuclei were observed. Mild oval cell proliferation was observed in the periportal areas of the lobules as soon as 5 days after the start of the NNM treatment. By day 21 a marked oval cell proliferation had developed, and areas of cholangiofibrosis containing ducts formed (in part) by tall columnar cells were observed.

In rats that had received NNM for 5 or 10 days and were sacrificed 4 weeks later, no necrotic areas were visible and most of the oval cells had disappeared. Furthermore, up to three clear and acidophilic cell foci/liver lobe were observed. In animals that had been treated with NNM for 14 or 21 days and had then been kept without NNM for another 4 weeks, the normal architecture of the liver was completely distorted and pseudolobules had formed. The pseudolobules were sorrounded by fibrotic septa including many oval cells and contained mainly clear and acidophilic cell populations: these cells were confined to focal lesions (up to 10 foci/liver lobe) or occupied large areas of the pseudolobules. In addition, in those animals that had received NNM for 21 days and

Fig. 1 **a** Expression of L-PK in the liver of an untreated rat: only parenchymal cells are stained. ×100. **b** Expression of M_2-PK in the liver of an untreated rat: only bile duct epithelial, blood vessel wall and sinusoidal lining cells are stained. ×400 ▶

Fig. 2 L-PK distribution in the liver of a rat treated 10 days with *N*-nitrosomorpholine (NNM): L-PK is not detected in the centrilobular necrotic areas, while its expression is strongly reduced in the remaining hepatocytes. ×100

Fig. 3 M_2-PK expression is restricted to oval and ductal cells in the liver of a rat treated for 14 days with NNM. ×400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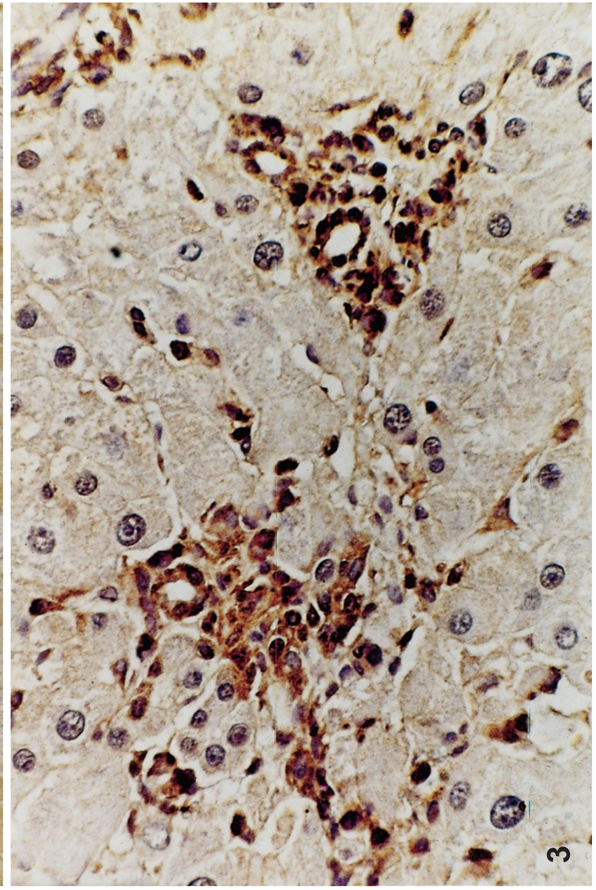
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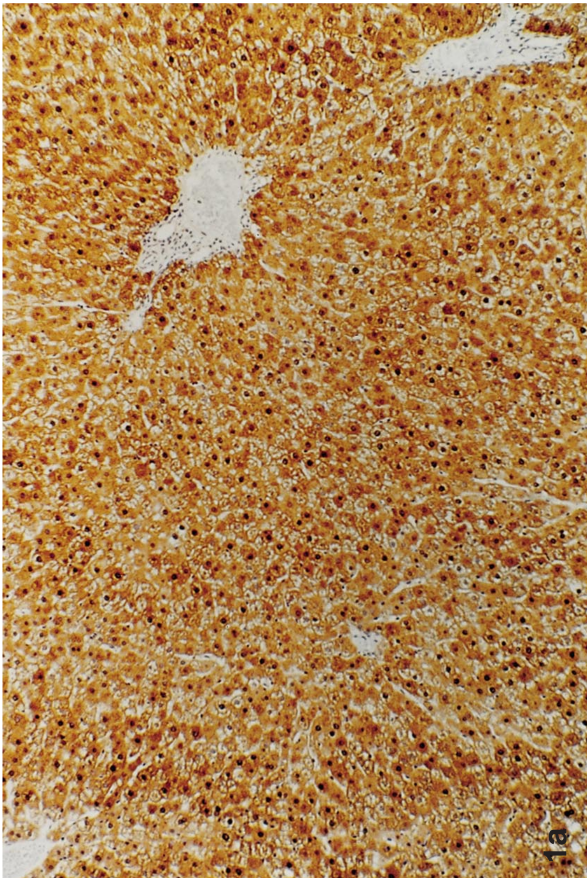
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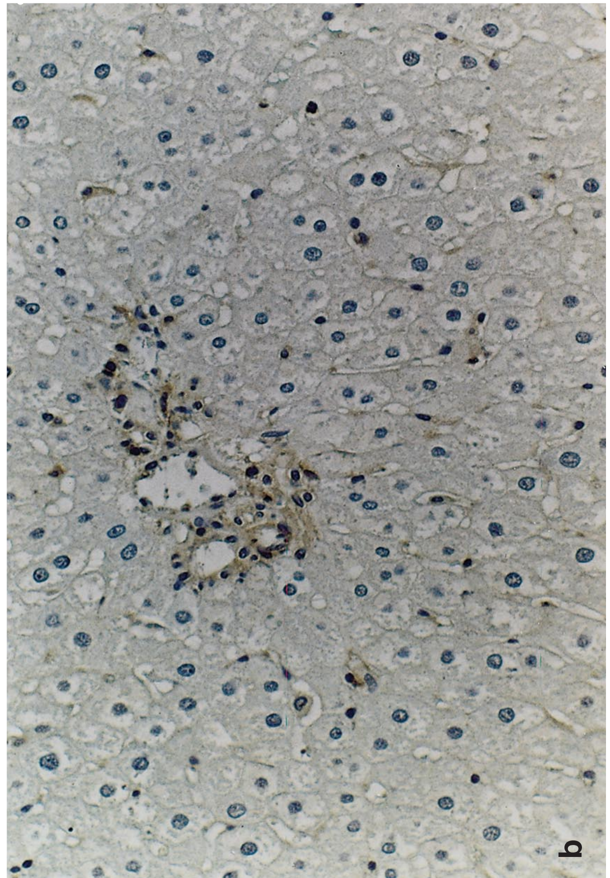
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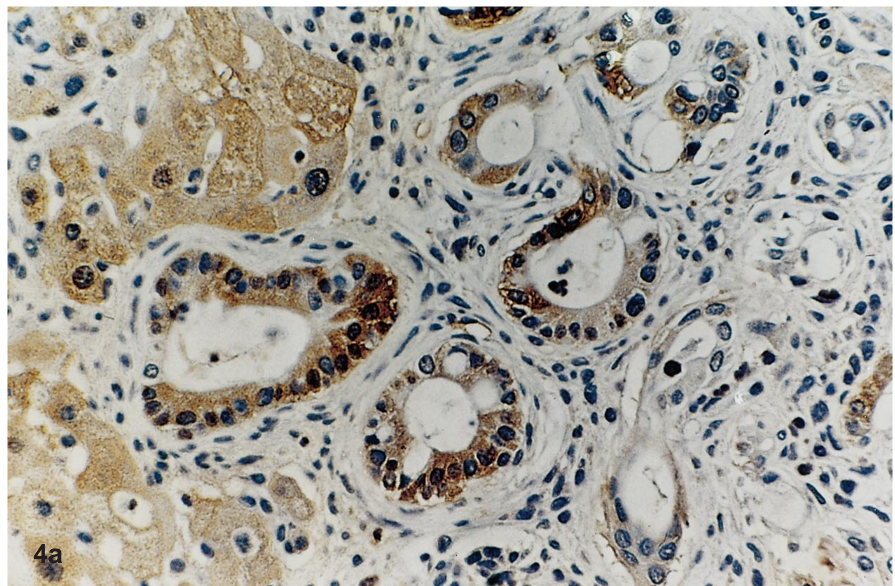
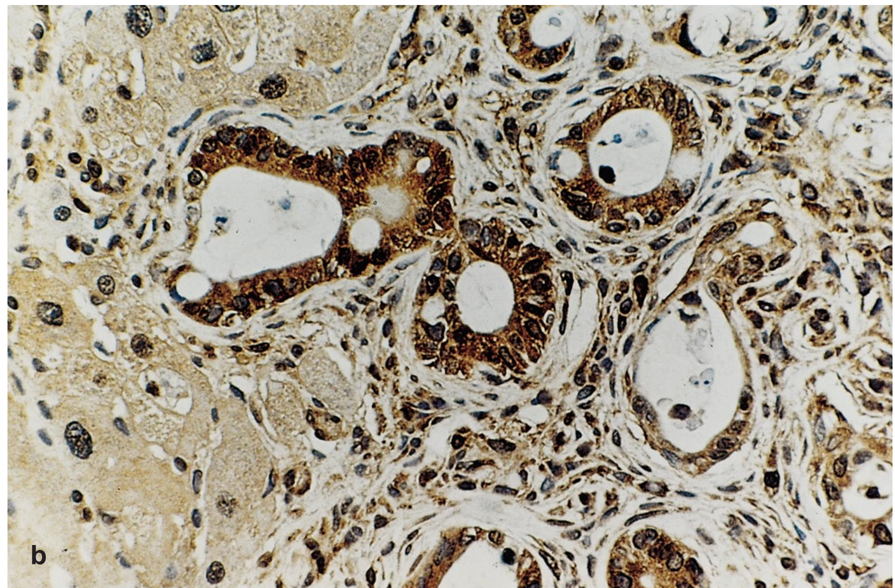
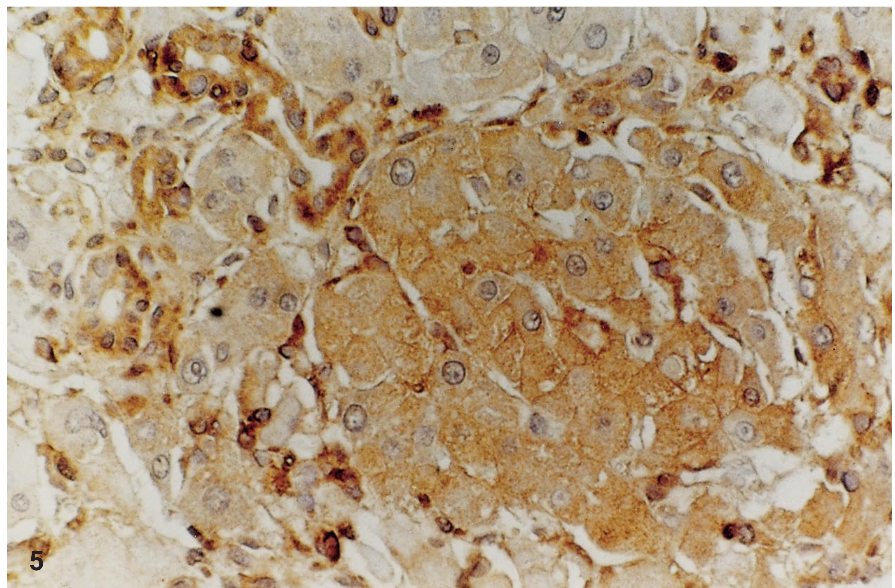
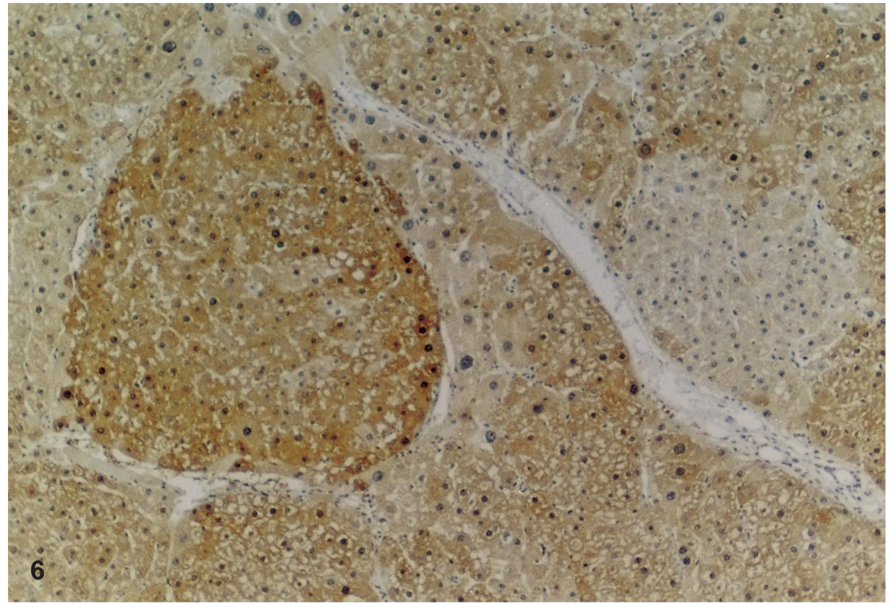
Fig. 4 **a** L-PK and **b** M_2-PK are present in the ductal cells of a cholangiofibrosis that developed within the liver of a rat treated for 21 days with NNM. Serial sections, ×400

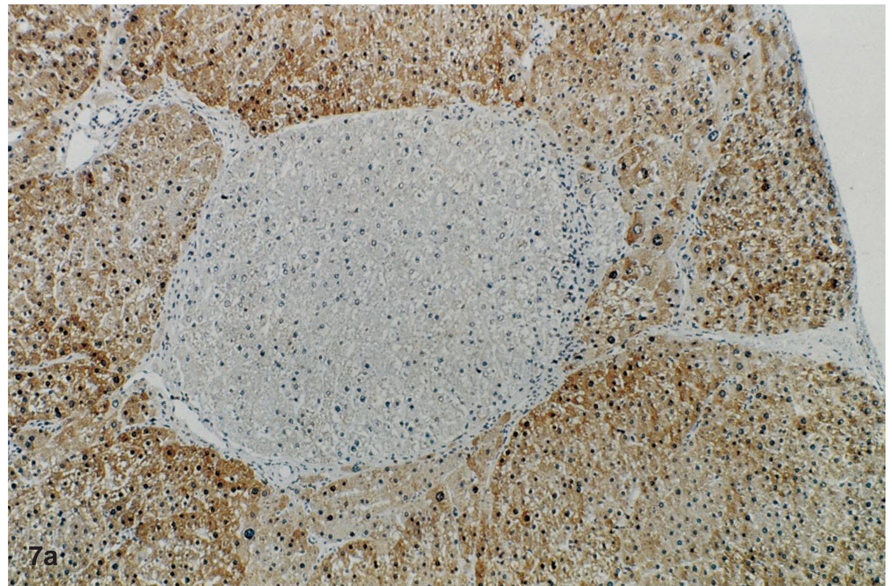
Fig. 5 M_2-PK-positive focus adjacent to M_2-PK-positive oval and ductal cells in a rat treated for 21 days with NNM. ×400











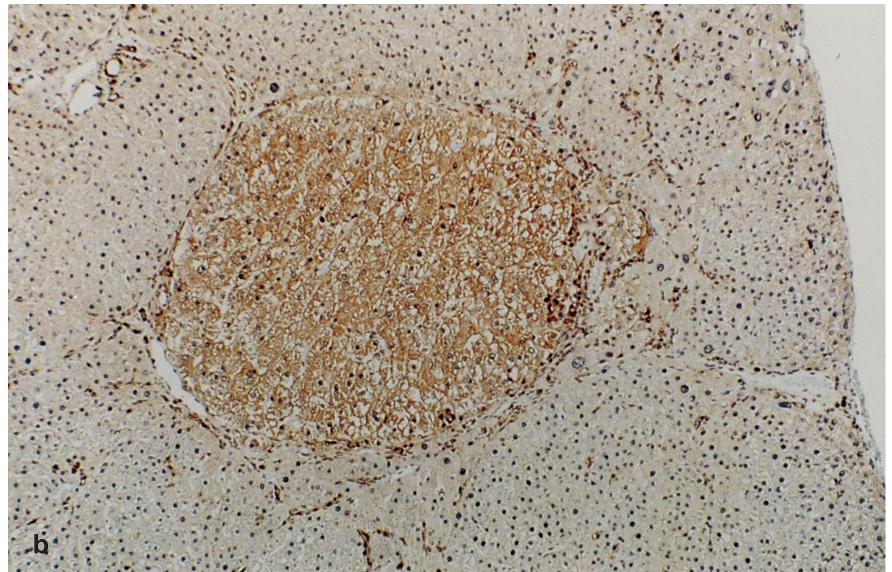


Fig. 6 L-PK-positive and -negative foci in the liver of a rat treated for 21 days with NNM and sacrificed 4 weeks later. ×100

Fig. 7 **a** L-PK and **b** M_2-PK expression in a liver focus of a rat treated for 21 days with NNM and sacrificed 4 weeks later. ×100

were sacrificed 4 weeks later up to three mixed and basophilic cell foci/liver lobe as well as cholangiofibroses were observed.

The specificity of the monoclonal antibodies against L-PK and M_2-PK was tested on liver slices from untreated rats. L-PK expression was restricted to liver parenchymal cells (Fig. 1a), whereas M_2-PK was detected in bile duct epithelial, blood vessel wall, endothelial and Kupffer cells (Fig. 1b). In rats sacrificed immediately after NNM treatment no L-PK was present within the centrilobular necrotic areas and its expression was markedly reduced in the remaining hepatocytes (Fig. 2) compared with the control tissue (Fig. 1a). While proliferating oval cells were consistently L-PK negative and M_2-PK positive (Fig. 3), staining of serial sections revealed that the ductal cells of the cholangiofibroses were clearly L-PK positive (Fig. 4a) and coexpressed M_2-PK (Fig. 4b). In addition, in animals receiving NNM for 21 days isolated M_2-PK-positive foci surrounded by M_2-PK-positive oval cells were observed (Fig. 5).

In rats that had received NNM for 5, 10, 14 or 21 days and were kept without NNM for another 4 weeks, L-PK expression in the liver parenchyma was similar to that in control animals, except for the hepatocytes arranged in foci, as described below. The number of L-PK- or M_2-PK-positive foci appearing in these animals was highly dependent on the duration of the NNM treatment. In rats receiving NNM for 5 or 10 days and sacrificed 4 weeks later 3–10 glycogen-rich, L-PK-positive foci per liver lobe were observed. In animals that had been treated with NNM for 14 or 21 days and were then were maintained without NNM for 4 weeks, 10–20 L-PK-positive foci per liver lobe were seen (Fig. 6). However, up to 3, 10 and 20 glycogen-poor, M_2-PK-positive foci per liver lobe were observed in rats that had received NNM for 10, 14 or 21 days, respectively, and were sacrificed 4 weeks later. The staining of serial liver sections from these animals showed consistently that L-PK was no longer present in the M_2-PK-positive foci (Fig. 7a, b). In the four groups of animals M_2-PK again proved to be an excellent marker for all cholangiocellular lesions and L-PK was again detected in the ductal cells forming part of the cholangiofibroses but not in the proliferating oval cells (data not shown).

Discussion

In an attempt to improve our understanding of the role of different cell lineages (liver parenchymal and oval cells) in the development of experimentally induced cholangiocellular and hepatocellular carcinomas, we chose to analyse the expression of L- and M_2-PK in the livers of rats treated with a high dose of NNM for up to 3 weeks. First, the PK isoenzymes were known to be expressed in a cell type-specific way in the untreated liver (L-PK is present in liver parenchymal cells, whereas M_2-PK is restricted to bile duct epithelial, sinusoidal lining and blood vessel wall cells). Second, it had been reported that L-PK was detectable in oval and ductal cells within the livers of rats fed CDE, while M_2-PK was regarded as a bile duct epithelial cell lineage-specific marker in the same experimental model [31, 33–35]. Third, in the past Bannasch's group had provided extensive documentation confirming that the appearance of oval cells, cholangiofibroses, clear and acidophilic cell foci, and mixed and basophilic cell foci could be induced very efficiently in rats by adding NNM to the drinking water [4, 5, 7, 10]. The decision to include animals treated with NNM for a defined time period and then maintained without NNM for 4 weeks in this study is based on the fact that in a number of experimental models foci of phenotypically altered hepatocytes were shown to disappear ("revert") after the discontinuation of carcinogen administration [2]. Therefore, Bannasch et al. [6] proposed that stop experiments should be conducted whenever foci with a disputed significance develop after the administration of a compound.

The number of oval cells remaining in the livers of rats fed NNM for 5 or 10 days and then left NNM-free for 4 weeks was far lower than the number in the livers of rats sacrificed immediately after the 5- or 10-day NNM treatment. In contrast, in animals that were treated for 14 or 21 days with NNM and then left NNM free for 4 weeks oval cell proliferation did not regress. Hacker et al. [19] showed earlier that in rats fed CDE for 4 or 6 weeks and then maintained on a standard lab chow for up to 62 weeks only a low number of oval cells were visible, whereas in rats sacrificed immediately after the 4- or 6-week CDE feeding period oval cell proliferation had been shown to be massive [30]. If the rats were fed the carcinogenic diet for 10, 14 or 22 weeks and then left on a standard lab chow for 1 year a pronounced oval cell proliferation was still observed [17]. The data obtained in the two experimental models [19, 30] (this study) suggest that many of the oval cells proliferating in the early stages of the carcinogenic process undergo necrosis and disappear with time, whereas those oval cells that have been in contact with a carcinogen for a longer period might have developed a sort of survival mechanism and remain as they were in the liver tissue or give rise to further lesions such as cholangiofibroses, cystic cholangiomas and cholangiofibromas [19, 30] (this study). In contrast to the early proliferating oval cells, the cholangiofibroses developing in the livers of NNM-treated animals in no way reverted, and these are therefore viewed as persistent cholangiocellular lesions.

As in the oval cells proliferating within the livers of CDE-fed rats [31, 33–36], M_2-PK proved to be an excellent marker for the oval cells and the ductal cells in NNM-treated animals. Interestingly, the duct cells not only expressed M_2-PK, but also stained positive for L-PK. This observation, which also applies to the ductal cells in the CDE model [31, 33–36], has been interpreted by Yeoh's group [35, 36] as strong support for the hypothesis that oval cells are able to differentiate into hepatocytes. However, in our investigations the ductal cells that stained positive for L-PK in the CDE [31] and the

NNM model [this study] showed no morphological signs of differentiation towards hepatocyte-like cells. Thus, the expression of the hepatocyte marker L-PK in a cell of the bile duct epithelial cell lineage does not allow the conclusion that such a differentiation has taken place.

The most striking result in this study is the unequivocal demonstration that during the NNM-driven hepatocarcinogenic process in rats a shift from L-PK to M_2-PK expression occurred within glycogen-poor foci. This is in accordance with a very recent paper published by Hacker et al. [20], who reported the same shift in a low number of mixed and basophilic cell foci as well as in hepatocellular adenomas developing in CDE-fed rats. In contrast to the report by Hacker et al. [20] and this study, Reinacher et al. [25] were unable to show that a decrease in L-PK in preneoplastic foci induced in rats by a single dose of NNM followed by the administration of phenobarbital or α-hexachlorocyclohexane in the diet was accompanied by the expression of M_2-PK in these foci. This discrepancy is most probably due to the fixation procedure used by Reinacher et al. [25]. As shown in this study and in that by Hacker et al. [20], acetone alone is not sufficient to fix M_2-PK within the preneoplastic lesions. In both cases a further step involving isopropanol (this study) or ethanol [20] as fixative was needed to obtain positive staining for M_2-PK in the liver slices.

Because of the expression of the oval cell marker M_2-PK in preneoplastic hepatocytes, it is tempting to suggest that the precursor cells of these M_2-PK-positive hepatocytes are the oval cells. However, it should be taken into account that most of the M_2-PK-positive foci were not surrounded by oval cells and that no oval cells were seen within these foci. Therefore, in this case we conclude that the shift of L-PK to M_2-PK occurs within the same cell type (the hepatocyte). The situation is different in the case of the few M_2-PK-positive foci that were surrounded by M_2-PK-positive oval cells (such as that depicted in Fig. 5); in this particular case it is not possible to discern whether the cell of origin of the M_2-PK-positive foci was a pre-existing hepatocyte or an oval cell.

By using a laser micro-dissection device and performing a microbiochemical analysis, Klimek and Bannasch [21] had previously found that in NNM-treated animals PK activity was enhanced in clear and acidophilic cell foci, and on the basis of kinetic data they concluded that the increase in PK activity was due to L-PK. Furthermore, they reported that in the same model mixed and basophilic cell foci L-PK activity decreased. The results presented in this immunohistochemical study clearly confirm the earlier work by Klimek and Bannasch [21]; the foci storing glycogen in excess overexpressed L-PK, whereas those that were poor in glycogen were L-PK negative and M_2-PK positive.

In conclusion, during the NNM-induced hepatocarcinogenic process a shift in the expression of L-PK to M_2-PK occurs in glycogen-poor foci and the ductal cells within the cholangiocellular lesions coexpress the two PK isoenzymes. In neither case is there evidence for morphological differentiation of oval cells into hepatocytes.

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