**Pathogenesis of Liver Cell Carcinoma**

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Hepatocellular carcinoma (HCC) is one of the most frequent malignant neoplasms worldwide. Chronic infections with the hepatitis viruses B (HBV) and C (HCV), contamination of foodstuffs with chemicals, particularly the mycotoxin aflatoxin B1, and chronic abuse of alcoholic beverages are considered major risk factors for the development of HCC. The results of a number of epidemiologic studies suggest that hepatocarcinogenesis in high risk areas is due to synergistic effects of chronic viral hepatitis and chronic dietary exposure to low doses of aflatoxins.

We were able to provide conclusive evidence for this notion in an experimental model. Woodchucks inoculated as newborns with woodchuck hepatitis virus (WHV), which is closely related to other hepadnaviridae including HBV, and exposed to low doses of dietary aflatoxin B1, developed liver cell cancer more rapidly and at a higher incidence compared to animals chronically infected with WHV alone [2]. In this animal model we studied the relationship between hepadnaviral infection and the development of hepatocellular neoplasms in early stages of this process [3, 4], and compared the results with those obtained in experimental chemical hepatocarcinogenesis [1, 5-13]. The experimental results were adopted for human pathology by studying more than 200 tissue specimens from resected livers which we received from the University Hospitals in Heidelberg and Hannover [14-16]. In addition, the mechanism of hepatocarcinogenesis was further studied in an *in vitro* model of hepatocellular preneoplasia [17, 18] and in different models of hormonal hepatocarcinogenesis [19-27], most of which were established in close cooperation with the Institute of Pathology of the University of Bonn [26, 27].

**Hepatocellular glycogenosis and hepatocarcinogenesis**

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In the liver of different species exposed to various oncogenic agents, the prevailing phenotype of parenchymal preneoplastic lesions is characterized by an excessive storage of glycogen (glycogenosis) in foci of altered hepatocytes [1, 2]. The results of previous enzyme histochemical and microbiochemical studies in rat liver suggested that the *preneoplastic hepatic glycogenosis* is elicited by an insulin-like (insulinomimetic) effect of the oncogenic agents, and this hypothesis has been strongly supported by the induction of glycogenotic foci frequently progressing to hepatocellular neoplasms by low-number pancreatic islet transplants in the liver of diabetic rats. To further clarify...
the molecular mechanisms underlying these changes we investigated several members of the **insulin signaling cascade** during chemical hepatocarcinogenesis induced in rats by limited exposure (stop model) to N-nitroso-morpholine [1, 11, 12]. Using immunohistochemistry and Western blot analysis, we studied the expression patterns of the insulin receptor, the insulin-like growth factor-I receptor, the insulin receptor substrate-1, the insulin receptor substrate-2, the mitogen-activated extracellular-regulated kinase (MEK-1), as well as phosphotyrosine in different types of preneoplastic hepatic focus and in hepatocellular neoplasms. All of these proteins were strongly overexpressed in the early preneoplastic glycogenotic foci and in glycogen-rich mixed cell foci. However, in later stages of hepatocarcinogenesis, particularly in glycogen-poor hepatocellular neoplasms, the expression of the proteins involved in insulin signaling was gradually downregulated, while the glycogen was lost and the number of ribosomes (basophilia) and cell proliferation increased. These results indicate that the preneoplastic hepatic glycogenosis is indeed initiated by a transient insulinimimetic effect. During phenotypic conversion from the preneoplastic glycogenotic to the glycogen-poor malignant neoplastic phenotype the early insulinimimetic events may be replaced by the effects of growth factors such as insulin-like growth factor-II, stimulating cell proliferation without leading to an accumulation of glycogen. In contrast to previous investigations, our studies revealed that the inhibitor-1 of the protein phosphatase 1 (PP1), which plays a key role in the regulation of metabolic processes including glycogen metabolism by both insulin and glucagon signaling, is expressed in normal rat liver at the mRNA-level, albeit in low copy number [17]. The intact open reading frame was sequenced and found to be identical to that of the muscle type of inhibitor-1 described by others.

The question whether the **preneoplastic hepatic glycogenosis** is due to genetic defects or to epigenetic events was studied by molecular biologic methods in rat liver and in the **in vitro** system established in our laboratory. In early glycogenotic and late glycogen-poor passages of a non-tumorgenic (preneoplastic) epithelial liver cell line and in malignant hepatocellular neoplasms propagated in **in vitro** or induced in **in vivo**, we analysed the expression and the structure of the coding regions of a number of genes involved in glycogen metabolism. In the glycogenotic liver cell line, genes coding for the following proteins were overexpressed at the mRNA-level: the catalytic and the glycogen binding subunit of PP-1, the γ-subunit of phosphorylase kinase, and the K-ras oncprotein. Whereas the abundance of mRNA for protein kinase A was not significantly changed, the mRNA expression of glycogen phosphorylase was strongly reduced. This finding indicates that the previously observed decrease in the amount and activity of the glucagon-regulated glycogen phosphorylase (which seems to be mainly responsible for the accumulation of glycogen) are predominantly due to alterations in gene expression. In addition, an inhibition of the enzyme activity by dephosphorylation resulting from an overexpression of both subunits of PP-1 has to be taken into account. The expression of inhibitor-1 was slightly reduced or normal in the preneoplastic glycogenotic cells but overexpressed in the glycogen-poor malignant cell populations. The activation of PP-1 in the glycogenotic cells is compatible with an insulinomimetic effect, but the expression of the insulin receptor substrate-1 and the glycogen synthase kinase-1 were not increased in these cells. In hepatocellular carcinomas, the expression of the genes studied usually increased with increasing dedifferentiation. The changes in the expression of three important genes (coding for glycogen phosphorylase, the regulatory subunit of PP-1, and K-ras) were also studied at the cDNA-level in the glycogenotic liver cell line (corresponding to preneoplastic focal liver lesions observed in situ) and in neoplastic cell populations. No mutations in the coding region of these genes were detected in both the preneoplastic and the neoplastic state. Although the molecular basis of the metabolic aberrations during neoplastic conversion of epithelial liver cells has not been completely clarified and some discrepancies between the observations in **in vitro** and in **in situ** are evident, we conclude from our findings that these fundamental changes are most probably due to epigenetic rather than genetic changes. This conclusion is in line with theoretical considerations in a new mathematical model for hepatocarcinogenesis [7].

**Hepadnaviral hepatocarcinogenesis**

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In the **Woodchuck model of chronic hepatitis** (with and without dietary exposure to aflatoxin B1) preneoplastic hepatocellular lineages emerging in hepadnaviral hepatocarcinogenesis were studied in situ by electron microscopic, enzyme histochemical, immunohistochemical, and molecular biological approaches [3,4]. The earliest phenotypic changes of the liver parenchyma detected were proliferative areas which showed minimal structural deviation from the normal state and were composed of three types of altered hepatocytes: glycogen-rich cells, amphiphilic cells (poor in glycogen but rich in mitochondria), and ground glass cells well known from chronic viral hepatitis in humans. In almost all of these altered hepatocytes replication of viral DNA and expression of viral core (WHcAg) and surface antigens (WHsAg) were demonstrable. The minimal deviation areas give rise to two main preneoplastic hepatocellular lineages: the glycogenotic-basophilic and the amphiphilic lineage. In both lineages, particularly in the glycogenotic-basophilic lineage, viral replication and expression of viral antigens usually decrease during progression from preneoplastic to neoplastic lesions. The preneoplastic focal glycogenosis is associated with a pattern of enzyme expression indicating an initial activation of the insulin-stimulated signal transduction
cascade in this cell lineage of the evolution of HCC. This observation is in accordance with previous findings during chemical hepatocarcinogenesis in the rat [1, 5, 9, 11, 12]. In contrast to this insulinoimetic effect of the chronic WHV-infection, which also occurs after exposure to aflatoxin B1, the enzymatic pattern of the amphophilic cell lineage of hepatocarcinogenesis suggests a thyromimetic effect of the WHV-infection. This effect may also be due to early changes in cellular signal transduction pathways as discussed for similar phenotypic changes induced in other species by nongenotoxic chemicals of the peroxisome proliferator type or hormones [1, 9, 12, 19-22]. The striking similarities in altered cellular phenotypes of preneoplastic lineages appearing in both viral and chemical hepatocarcinogenesis argue for closely related underlying molecular mechanisms that may be mainly responsible for synergistic effects of these oncogenic agents [1-5].

An early functional inactivation of the tumor suppressor gene p53 by the formation of complexes with the X antigen of HBV or WHV has been regarded as an important early event in the mechanism of hepatocarcinogenesis by some authors [2]. Using several carefully tested antibodies to HBx obtained from different laboratories [14] and commercially available antibodies to p53, we have examined this hypothesis by immunohistochemistry and immunoprecipitation in specimens from 149 resected human livers, including 39 cases of chronic HBV-infection and 35 HCC [16]. In 51% of the HCC, p53 was immunohistochemically demonstrable, and was predominantly found in poorly differentiated, rapidly growing tumor components. The accumulation of p53 was verified in most of the positive cases by immunoprecipitation. In a total of 354 preneoplastic hepatic foci, p53-positive cells were never detected. We conclude from these results, that at least in areas with low exposure to aflatoxins mutations of p53 are not an early event, but represent secondary changes occurring during tumor progression. The intracellular distribution of p53 and HBx was different: while the immunohistochemical reaction for p53 was invariably found in the cell nucleus, the expression of HBx was limited to the cytoplasm. HBx and p53 did not co-immunoprecipitate. These results indicate that a functional inactivation of p53 by binding of HBx is of at best a very rare event, which cannot be considered a general mechanism of HBV-associated hepatocarcinogenesis. Occasionally, we observed an accumulation of p53, which was also independent of the expression of HBx, in cells of the liver parenchyma and biliary tree in cases of fulminant hepatitis. This alteration is most probably due to stress reactions associated with tissue damage and regeneration.

**Hormonal hepatocarcinogenesis**


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**Dehydroepiandrosterone (DHEA)** is an adrenal steroid hormone circulating in high concentrations in the human blood [19, 25]. In rats, high oral doses of DHEA, which also acts as a peroxisome proliferator in this species, produce a high incidence of hepatocellular adenomas and carcinomas, particularly in females [19-21]. However, the peroxisome proliferation is apparently not essential for the hepatocarcinogenic effect of DHEA. Our cytomorphological and cytochemical studies on rats chronically exposed to DHEA revealed that the hepatocellular neoplasms induced by this hormone originate from amphophilic focal parenchymal lesions which are mainly characterized by a proliferation of mitochondria closely associated with cisternae of the rough endoplasmic reticulum [21] and by an increase in the amount and/or activity of several mitochondrial enzymes [9, 22]. Additional biochemical effects of DHEA are described in the report by the research group Hormones and Signal Transduction (B0810). During progression from preneoplastic amphophilic cell foci to hepatocellular neoplasms the number of cytoplasmic ribosomes and, hence, basophilia frequently increases. Biochemical studies *in situ* have shown that the amphophilic cell foci express a pattern of enzymes resembling the effect of thyroid hormones [5, 9, 22]. In both their morphological and their biochemical appearance the preneoplastic and neoplastic parenchymal alterations induced by DHEA correspond to preneoplastic and neoplastic liver lesions elicited by other peroxisome proliferators. Thus, the studies on rat liver treated with DHEA are of more general importance, having clarified the intriguing amphophilic preneoplastic hepatocellular lineage which differs in many respects from the glycogenotic-basophilic lineage of hepatocarcinogenesis [5, 9, 12, 19-22]. From the toxicological point of view the distinction of the amphophilic cell lineage is of great significance since the peroxisome proliferators represent an important class of chemicals, including pharmacological substances and industrial chemicals, which have been shown to produce a high incidence of liver neoplasms in rodents. In addition, the amphophilic cell lineage has also been observed in experimental hepatadnaviral hepatocarcinogenesis [1-5]. Similar hepatocellular changes were also found in human chronic liver diseases [15] but their possible relationship to the development of human HCC remains to be demonstrated [2, 15].

The notion of a thyromimetic effect of chemical hepatocarcinogens of the peroxisome proliferator type and of oncogenic viruses has been supported by recent studies in rats [26]. In an experimental animal model established by Frank Dombrowski, we were able to demonstrate that *intraportal transplntation of thyroid tissue* into thyroidectomized rats results in amphophilic proliferative paren-
chymal changes downstream of the transplants, which resemble morphologically and biochemically those induced in rodents by DHEA, other peroxisome proliferators or hepadnaviridae. It has been verified by immunohistochemistry, autoradiography, and measurement in the blood serum that the intrahepatic thyroid transplants actually synthesize and secrete thyroxine and 3,5,3′-triiodo-L-thyronine (T₃) under these experimental conditions.

In a similar experimental model, which has also been established by Frank Dombrowski, focal proliferative liver lesions were produced in ovariectomized rats by intraperitoneal transplantation of ovarian tissue [27]. The parenchymal alterations produced by this procedure largely resembled the amphophilic cell foci, but showed some differences in their histochemical pattern compared to this well characterized type of focus. It is most likely, however, that the variant of the amphophilic cell focus is a preneoplastic cell population since hepatocellular adenomas and carcinomas developed in this animal model in a long-term pilot study. The new model provides for the first time an opportunity to study long-term effects of natural estrogens on hepatocytes and to contribute to the clarification of the well known hepatocarcinogenic effects of synthetic estrogens in humans.

Pathogenesis of Renal Cell Carcinoma

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In comprehensive experimental studies which were mainly conducted in the rat kidney exposed to N-nitrosomorpholine we had previously shown that at least four types of renal cell tumor may be distinguished by cytornorphological and cytochemical methods, each of which originates from a defined segment of the renal tubular system [28]. While many of these experimental observations were applied to human pathology and stimulated the development of new classifications for experimental and human renal cell tumors, the histogenesis and cyto genesis of the clear (glycogenotic) and granular renal cell carcinoma in rat and man has remained controversial. In humans the proximal nephron is considered the site of origin of this type of renal cell carcinoma since the 50ies of the last century. In the rat, however, we have demonstrated that at least the majority of these tumors derives from the collecting duct system, and this finding has been confirmed by a Japanese group recently. Since the clear/granular renal cell carcinoma is by far the most frequent malignant human renal cell neoplasm we have reinvestigated its histogenesis and cyto genesis by immunohistochemistry. Using an approach previously applied to studies on the expression of epithelial mucins (MUC1, MUC2) as well as the Thomson-Friedenreich-antigen and related antigens in preneoplastic and neoplastic liver lesions [28], we systematically investigated the expression of these antigens in tissue specimens from 58 resected human renal cell tumors and the surrounding renal tubular system [30]. Our studies revealed that the antigen expression in human clear and granular renal cell carcinomas corresponds in many respects to that of the collecting duct system. In addition, clear cells excessively storing glycogen were frequently found in the neighborhood of these tumors within renal tubules, the epithelia of which were morphologically and cytochemically classified with the collecting duct system. We conclude from these results that at least a large part of the human clear and granular cell tumors originate from the collecting duct system as the corresponding tumor type in the rat.

Publications (* = external coauthor)


