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FIELD OF VISION

Glycogenotic hepatocellular carcinoma with glycogen-ground-glass hepatocytes: A heuristically highly relevant phenotype

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Abstract

Glycogenotic hepatocellular carcinoma (HCC) with glycogen-ground-glass hepatocytes has recently been described as an allegedly "novel variant" of HCC, but neither the historical background nor the heuristic relevance of this observation were put in perspective. In the present contribution, the most important findings in animal models and human beings related to the emergence and further evolution of excessively glycogen storing (glycogenotic) hepatocytes with and without ground glass features during neoplastic development have been summarized. Glycogenotic HCCs with glycogen-ground-glass hepatocytes represent highly differentiated neoplasms which contain subpopulations of cells phenotypically resembling those of certain types of preneoplastic hepatic foci and benign hepatocellular neoplasms. It is questionable whether the occurrence of glycogen-ground-glass hepatocytes in a glycogenotic HCC justifies its classification as a specific entity. The typical appearance of ground-glass hepatocytes is due to a hypertrophy of the smooth endoplasmic reticulum, which is usually associated with an excessive storage of glycogen and frequently also with an expression of the hepatitis B surface antigen. Sequential studies in animal models and observations in humans indicate that glycogen-ground-glass hepatocytes are a facultative, integral part of a characteristic cellular sequence commencing with focal hepatic glycogenosis potentially progressing to benign and malignant neoplasms. During this process highly differentiated glycogenotic cells including ground-glass hepatocytes are gradually transformed *via* various intermediate stages into poorly differentiated glycogen-poor, basophilic (ribosome-rich) cancer cells. Histochemical, microbiochemical, and molecular biochemical studies on focal hepatic glycogenosis and advanced preneoplastic and neoplastic lesions in tissue sections and laser-dissected specimens in rat and mouse models have provided compelling evidence for an early insulinomimetic effect of oncogenic agents, which is followed by a fundamental metabolic switch from gluconeogenesis towards the pentose-phosphate pathway and the Warburg type of glycolysis during progression from preneoplastic hepatic glycogenosis to the highly proliferative malignant phenotype.

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Key words: Acquired focal hepatic glycogenosis; Inborn hepatic glycogenosis; Hepatic preneoplasia; Hepatic neoplasia; Early metabolic aberrations; Progression-linked metabolic switch

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INVITED COMMENTARY ON HOT ARTICLES

The glycogenotic hepatocellular carcinoma (glycogenotic

HCC) with glycogen-ground-glass hepatocytes has recently been described as a "novel variant" of HCC by Callea *et al*[1]. Relating the excessive storage of glycogen (glycogenosis) to a complete absence of glucose-6-phosphatase activity as measured by a microbiochemical approach, the authors referred to the long known finding of a focal hepatic glycogenosis (FHG) as an early event in experimental hepatocarcinogenesis[2], continuing "however, ground-glass cells have not been reported in FHG". In contrast to this statement, the characteristic hepatocellular phenotype addressed was not only explicitly described for the first time in preneoplastic and neoplastic lesions in rodents[3,4] and in humans[5,6] (Figure 1) decades ago but has also been proven to be heuristically highly relevant in numerous publications since then. Glycogenotic ground-glass hepatocytes (GGH) predominate in subpopulations of many focal precancerous hepatocellular lesions, particularly in preneoplastic FHG and benign hepatocellular neoplasms such as hepatocellular adenomas and focal nodular hyperplasia, but may also occur in more or less extended subpopulations of HCC as observed in various species, including non-human primates and human beings[7,8]. However, glycogen-GGH hardly ever account for whole neoplasms. This also applies to the glycogenotic HCC depicted by Callea *et al*[1] in which the GGH are mixed with "clear" (glycogenotic) cells without ground-glass features. It is, hence, questionable whether glycogenotic HCC with glycogen-GGH should be considered a specific entity as proposed by Callea *et al*[1]. Extensive investigations in models of chemical, viral, and hormonal hepatocarcinogenesis and some observations in humans suggest that FHG with and without GGH indicates a critical early metabolic aberration in the pathogenesis of benign and malignant hepatocellular neoplasms[7,8].

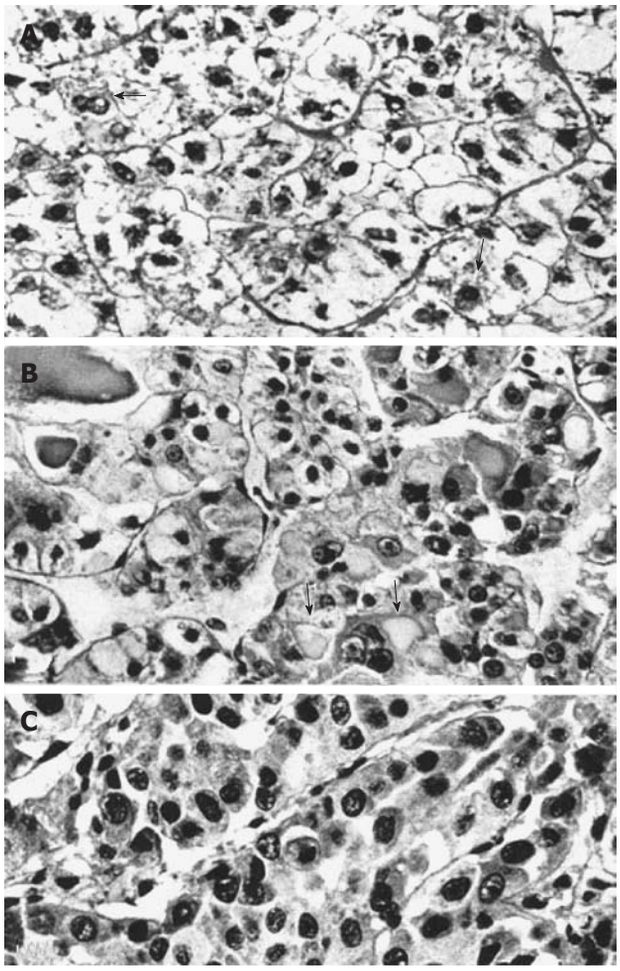

Figure 1 Light micrographs of portions from human hepatocellular neoplasms with and without glycogenosis. A: Clear-cell hepatocellular adenoma consisting predominantly of glycogenotic cells. In some cells (arrows) there is a reduction of glycogen and focal increase in cytoplasmic basophilia; B: Highly differentiated hepatocellular carcinoma (HCC) composed of a mixed population of clear (glycogenotic) cells, acidophilic cells (ground-glass hepatocytes, arrows), and some glycogen-poor, basophilic cells; C: Poorly differentiated, glycogen-free, basophilic HCC. All: Hematoxylin and eosin stain, x 460, from Bannasch *et al*[6].

Animal models of hepatocarcinogenesis

The observations in humans were preceded by several seminal findings in animal models of hepatocarcinogenesis as repeatedly reviewed[2,9]. In their pioneering electron microscopic investigations in rats continuously exposed to 3-methyl-dimethylaminoazobenzene, Porter *et al*[10] detected a hypertrophy of the smooth endoplasmic reticulum in many hepatocytes, and addressed its light microscopic counterpart as "hyaline degeneration"[11]. The authors related this characteristic subcellular change to a decreased rather than an increased storage of glycogen and felt "that only cells which, through mutation, loose the normal tendency to differentiate for glycogenesis will survive and so will be selected out for continued growth and differentiation"[10]. However, investigations in other models of rat hepatocarcinogenesis, employing both continuous or limited (stop model) exposure to N-nitrosomorpholine at various dose levels and time schedules[3,4], or ethionine for up to approximately 20 wk[12] revealed that hypertrophy of the smooth endoplasmic reticulum is often associated with an excessive storage of glycogen, the smooth membranes forming either a typical network or peculiar lamellar complexes which often show a close spatial relationship with glycogen particles (Figure 2) but may also be free of glycogen forming "fingerprints"[4]. Steiner *et al*[12] designated the complexes of the smooth endoplasmic reticulum associated with glycogen as "glycogen-bodies", and speculated that these formations indicate a "resistance" to the carcinogen, reflecting a reactivation of the glycogen-storing ability after an early loss of glycogen in response to toxicity. In contrast to both of these considerations, many studies on experimental hepatocarcinogenesis in different species revealed that FHG composed of glycogenotic clear and/or acidophilic cells, the latter showing a pronounced hypertrophy of the smooth endoplasmic reticulum (corresponding to glycogenotic GGH), regularly occur in a multi-centric fashion in early stages of neoplastic development induced in small rodents by a variety of chemicals[3,4,9,13]. More recently, typical glycogenotic GGH were also found after chronic infection of woodchucks with hepadnaviridae[14-16], in hepatitis B virus (HBV)-transgenic mice[17], and during hormonal hepatocarcinogenesis in

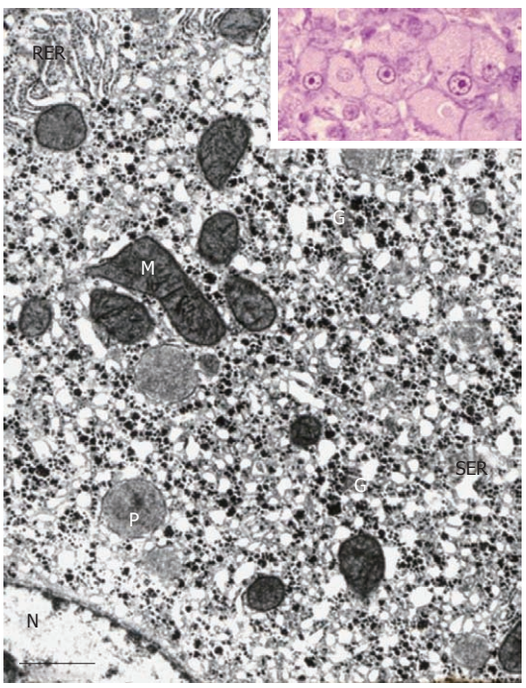

Figure 2 Portion of a glycogenotic acidophilic hepatocyte (corresponding to glycogen-ground-glass hepatocyte) induced in rat liver by N-nitrosomorpholine. Note abundant α- and β-glycogen particles (G) in close spatial relationship with large network complexes of proliferated smooth endoplasmic reticulum (SER). Rough endoplasmic reticulum (RER), mitochondria (M), peroxisomes (P), and nucleus (N). Inset: Group of acidophilic hepatocytes as seen under the light microscope. Transmission electron microscopy, lead citrate. Bar: 1 μm.

diabetic animals after intrahepatic transplantation of pancreatic islets in rats and mice[18,19].

As demonstrated particularly in stop models of hepatocarcinogenesis in rats exposed to N-nitrosomorpholine or thioacetamide, glycogenotic hepatocytes with and without hypertrophy of the smooth endoplasmic reticulum may persist for weeks and months after withdrawal of the carcinogen, and may persist as almost entire populations of hepatocellular adenomas ("hyperplastic nodules", "neoplastic nodules") and in subpopulations of highly differentiated HCCs[4,20,21]. These observations are neither compatible with the idea of a preferential cellular survival by loss of differentiation for glycogenesis[10] nor with the notion of a "resistance" of these cells to carcinogen[12,22,23]. The functional significance of the persistent hypertrophy of the smooth endoplasmic reticulum has remained obscure, but many findings suggest that the organelle represents a metabolic compartment whose increase is a facultative consequence of the disturbed carbohydrate metabolism characterizing the acquired hepatocellular glycogenosis[4,24].

Independent of the observation of an acquired FHG produced in rat liver by N-nitrosomorpholine[5], Gössner *et al*[25] described a focal reduction in the activity of glucose-6-phosphatase in rats exposed to N-nitrosodiethylamine. A causal relationship between this enzyme deficiency and the accumulation of glycogen in preneoplastic FHG and "hyperplastic liver nodules" has been suggested by a number of authors[3,4,26,27]. This conclusion appears to be supported by the well known high risk of children suffering from an inborn hepatic glycogenosis type I , especially type I a (von Gierke), due to a genetically fixed deficiency of the glucose-6-phosphatase, to develop hepatocellular adenomas and carcinomas when passing through adolescence[2,9,28]. This interpretation is in line with the recent finding that the targeted deletion of liver glucose-6-phosphatase in a knock-out mouse model results in hepatic glycogenosis and steatosis, and eventually also in multiple hepatocellular adenomas in all animals beyond 18 mo of age[29].

It is important to realize, however, that correlative cytochemical studies in rodent models of hepatocarcinogenesis have shown that the focal decrease of glucose-6-phosphatase activity in FHG is regularly combined with decrease or increase in the activity of many other enzymes[7,8,30], especially enzymes of the carbohydrate metabolism[2,24,31]. In addition, over-expression of the key enzyme of *de novo* fatty acid synthase has been described in FHG in the N-nitrosomorpholine stop-model[32]. In rats exposed to N-2-fluorenylacetamide, Williams *et al*[33] demonstrated that the preneoplastic glycogenotic clear cell foci are resistant to the storage of iron.

For a long time the cause of these complex metabolic alterations in FHG remained elusive. More recently, however, histochemical, microbiochemical and molecular biochemical studies on FHG and advanced preneoplastic and neoplastic liver lesions in tissue sections and laser-dissected specimens obtained from small rodents exposed to chemical carcinogens or oncogenic viruses provided evidence for an early insulin-like (insulinomimetic) effect of these agents[7,24,34-36]. This notion has been corroborated by a number of studies on hormonal hepatocarcinogenesis induced in diabetic rats and mice by local hyperinsulinemia[18,19,32,37-39].

The phenotype of preneoplastic FHG is not stable, but undergoes dramatic changes during progression to the benign and/or malignant neoplastic phenotype[3,4]. Collectively, all types of specific focal hepatocellular lesions appearing during the preneoplastic phase in rodents have been termed foci of altered hepatocytes, and have been widely used as early indicators of neoplastic development in toxicologic pathology[40-42]. The characteristic sequence of cellular changes starting with FHG follows an ordered pattern, passing through intermediate or mixed cell foci composed of glycogenotic, intermediate, and glycogen-poor basophilic (ribosome-rich) cell types, the latter corresponding to the typical cell type in poorly differentiated HCCs[2,4,24]. Detailed morphological analysis of intermediate cell types at the light- and electron microscopic level has suggested that the hypertrophied smooth endoplasmic reticulum is usually transformed into rough endoplasmic reticulum by the addition of ribosomes during this phenotypic conversion[4,9,20]. Frequently, the intermediate cells show an accumulation of neutral fat, often leading to a combination of glycogenosis and steatosis[2,20]. Evidence of this sequence of cellular changes was originally provided by light- and electron-microscopic studies in rats

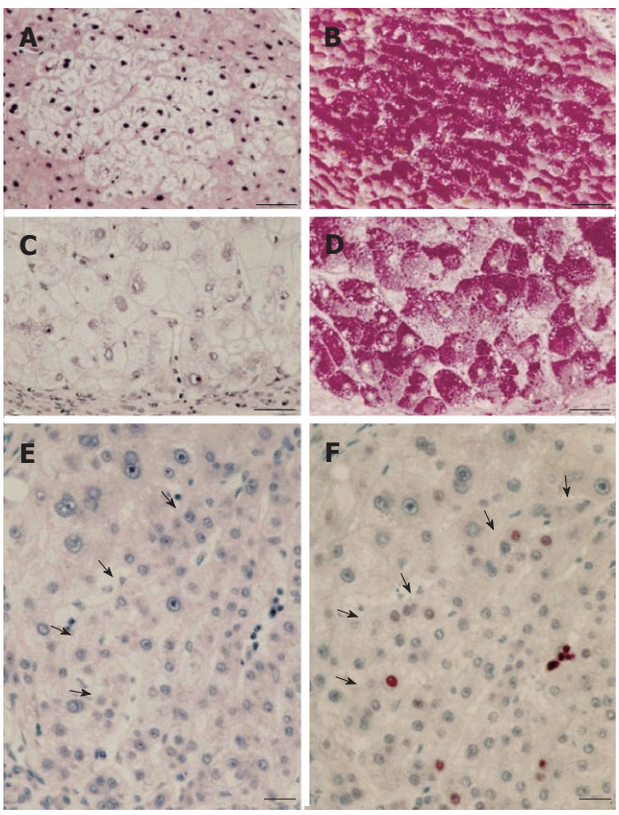

Figure 3 Human focal (A, B) and nodular (C, D) hepatic glycogenosis, and more advanced mixed cell populations (E, F) with intrafocal small cell change. A, B: Hepatic vein. Perivenular glycogenolytic (clear cell) focus in an hepatocellular carcinoma (HCC)-bearing liver with hepatitis B virus (HBV)-associated cirrhosis, demonstrated in serial sections with hematoxylin and eosin (A) and periodic acid schiff (PAS) (B)-reaction counterstained with orange G and iron hematoxilin (Tri-PAS). Bar: 100 μm; C, D: Glycogenotic (clear cell) nodule in an HCC-free liver with HBV-associated cirrhosis demonstrated in serial sections with hematoxylin-eosin (C) and Tri-PAS (D). Bar: 100 μm; E, F: Hematoxylin and eosin (E), proliferating cell nuclear antigen (F) immunostaining. Increased cell proliferation (arrows) in a mixed cell focus with less pronounced glycogen storage (not shown) and with intrafocal small cell change in liver with cryptogenic cirrhosis, demonstrated in serial sections. Bar: 50 μm, from Su *et al*[68].

exposed for the lifetime or for limited time periods to N-nitrosomorpholine[3,4]. And it has since been substantiated by a series of morphometric studies[43-45] and by similar observations in other rodent models of hepatocarcinogenesis elicited by several "genotoxic" and "non-genotoxic" chemicals[7,41], by local hyperinsulinemia[18], by hepadnaviridae[14,15], and by oncogenic transgenes[17,46]. Most recently, multiple FHG, more advanced types of foci of altered hepatocytes, hepatocellular adenomas and HCC indicative of such a sequence, including an intermediate steatosis, were also observed in a knock-out mouse model with a reduced expression of the mitochondrial protein frataxin, which is responsible for the inherited neurodegenerative disease Friedreich´s ataxia in humans[47].

The conversion of the highly differentiated glycogenotic clear or acidophilic to the de-differentiated glycogen-poor, basophilic (ribosome-rich) phenotype is associated with a fundamental metabolic switch characterized by a reduction in gluconeogenesis, an activation of the pentose phosphate pathway and the Warburg type of glycolysis as detailed elsewhere[7,8,24], and by an ever increasing cell proliferation which is inversely related to the gradual reduction of the glycogen initially stored in excess[48]. Based on these observations, Kopp-Schneider *et al*[49] developed the so-called color-shift model of hepatocarcinogenesis considering epigenetic changes in parenchymal colonies rather than multiple successive genomic mutations in single cells as the main cause of neoplastic cell conversion induced in the liver by exogenous oncogenic agents. The importance of epigenetic events in chemical hepatocarcinogenesis has been discussed by several authors previously[7,50], and has been emphasized in recent years by Pogribny *et al*[51,52].

Human hepatocarcinogenesis

In human pathology, the predominance of clear (glycogenotic) cells (Figure 1A and B) in a minor proportion of HCCs[6,53], comprising about 8% in 150 cases studied by Buchanan *et al*[54], and in many hepatocellular adenomas[6,53-56] is well known. A favorable prognosis of the clear-cell variant of HCC has been reported[57]. Sasaki *et al*[58] described two cases of clear-cell HCC associated with hypoglycemia and hypercholesterolemia, and postulated a disturbed glucose metabolism of the tumor tissue, directed to lipogenesis and/or glucogenesis. Acquired FHG has been considered a preneoplastic condition in humans[6]. This idea was supported by the fortuitous observation of FHG (clear-cell foci) in HCC-bearing livers of children suffering from different disorders[59,60], in women after long-term use of oral contraceptives[61], in about 12% of 95 males studied in a consecutive autopsy series in Finland[62], in patients with Crohn's disease treated over years with azathioprine which is apparently also responsible for associated HCC development[63-67], and in a variety of other chronic liver diseases prone to develop HCC[8,24,53,68,69]. Special cases are patients with genetic hemochromatosis endowed with a high risk of developing HCC, which frequently show FHG excluding iron similar to the iron-resistant FHG observed in experimental hepatocarcinogenesis in rodents[8,53,69-73].

Particularly relevant are systematic histochemical and histological investigations on the phenotype and proliferation kinetics of foci and nodules of altered hepatocytes in more than 150 explanted and resected human livers with and without HCC[68,74,75]. The results suggest that foci of altered hepatocytes are proliferative preneoplastic lesions, mixed cell foci (frequently with "small cell change") being more advanced than FHG (Figure 3), potentially transforming into nodules of altered hepatocytes, highly differentiated HCC containing glycogenotic clear and ground-glass hepatocytes (Figure 4), and eventually also glycogen-poor, basophilic HCC (Figure 1C)[68]. In keeping with these findings, clear-cell change, steatosis and small cell change have been considered histological features predicting malignant transformation in non-malignant hepatocellular nodules[76]. Analysis of clonality and chromosomal aberrations in nodules of altered hepatocytes microdissected from cirrhotic livers revealed

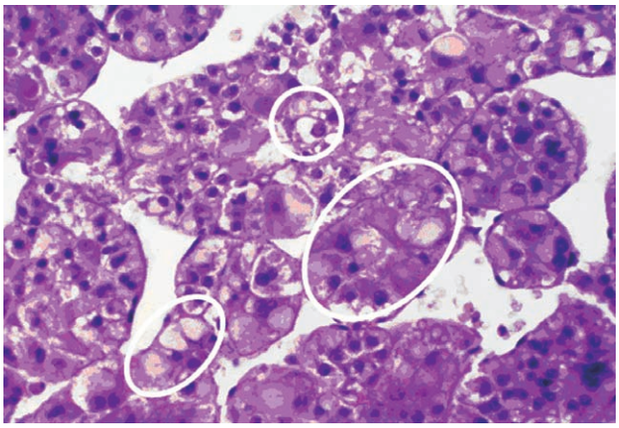

Figure 4 Portion of a human trabecular hepatocellular carcinoma with mixed populations, including clear (glycogenotic) cells (small upper white circle), acidophilic (glycogenotic) ground-glass cells (somewhat larger white circle at the bottom, left), transitions from ground-glass hepatocytes into basophilic cell populations (largest white circle in the middle, right), and basophilic cell populations in the middle and at the bottom right (not marked by circles). Hematoxylin and eosin.

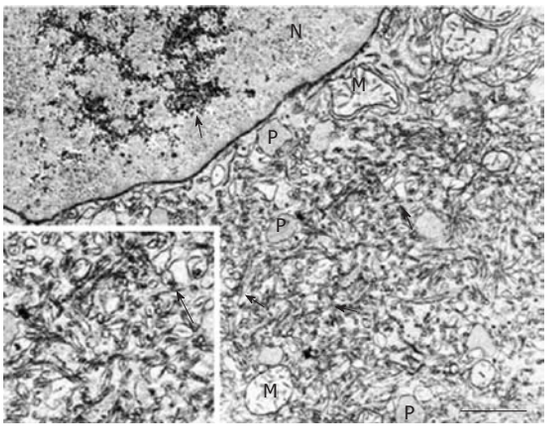

Figure 5 Portion of a ground-glass hepatocyte from a human liver with hepatitis B virus-associated cirrhosis, showing an accumulation of glycogen granules (long arrow) in the nucleus (N) and abundant smooth endoplasmic reticulum containing filamentous hepatitis B surface antigen (short arrows). M: Mitochondria: P: Peroxisome. Transmission electron microscopy, lead citrate. Bar: 1 μm.

a loss of chromosomal inactivation mosaicism in three large "regenerative" nodules and in all (12) nodules of altered hepatocytes with small cell change, indicating their neoplastic nature[77]. Even among 60 nodules of altered hepatocytes without small cell change, almost 50% (29) were shown to be monoclonal, whereas FHG and 14 "regenerative" nodules were found to be polyclonal. Interestingly, Cai *et al*[78] using a similar approach to analyze focal nodular hyperplasia, the pathogenesis and neoplastic nature of which has been debated for decades[53], found that this lesion, as a whole, is polyclonal, but represents a cluster of nodules of altered hepatocytes, some of which are monoclonal harboring chromosomal aberrations as in hepatocellular adenomas. The lack of genomic alterations in fatty and clear-cell changes in HCC and precursor nodular lesions in cirrhotic livers emphasized by some authors[79] is in line with the polyclonal nature of many of these lesions[77], but in view of the increasing evidence for a decisive role of epigenetic events in the development and progression of human HCC[80] the findings by Laurent *et al*[79] do not argue against a preneoplastic nature for these cellular changes.

A pronounced hypertrophy of the smooth endoplasmic reticulum was discovered in biopsies from cirrhotic livers and liver cell carcinomas at the light and electron microscopic level (Figure 1B) almost half a century ago, and related to aberrations of glycogen metabolism including glycogenosis from the very beginning[5,6]. The altered hepatocytes (Figures 1B and 4) were designated as "acidophilic" (or "eosinophilic"). Later on, Popper *et al*[81,82] found frequent association of this phenomenon with the expression of the hepatitis B surface antigen (HBsAg) localized in the lumen of hypertrophied smooth endoplasmic reticulum (Figure 5), and coined the term "ground glass hepatocyte" which has become an important diagnostic entity in chronic liver diseases elicited by HBV and has dominated the literature since then[83]. Wills[84] described HBsAg-free "ground glasslike hepatocytes" exhibiting "glycogen bodies" in liver biopsies from an immunosuppressed (azathioprine, prednisone, clonidine, frusemide) renal transplant patient, similar to those observed in experimental chemical hepatocarcinogenesis[4,12].

Irrespective of the expression of HBsAg, glycogenotic hepatocytes showing hypertrophy of the smooth endoplasmic reticulum (corresponding to GGH) have often been observed in human FHG, hepatocellular adenomas, and HCC and considered preneoplastic or highly differentiated neoplastic phenotypes[6,53,68]. In 30 specimens of HBV-associated cirrhosis, GGH were identified in 17 of 25 showing HBsAg expression[85] (Figure 5). Without mentioning any particular relationship to glycogen, others described GGH containing pre-S mutants in chronic HBV infection, postulating that they represent preneoplastic lesions[83]. Wisell *et al*[86] depicted partially persisting "glycogen pseudoground glass hepatocytes" in 12 patients immunosuppressed for numerous indications, but detected neither viral particles nor hypertrophy of the smooth endoplasmic reticulum under the electron microscope. Similar observations were reported by Bejarano *et al*[87] but no efforts were made in either of these studies to directly compare GGH in serial sections of defined focal lesions at the light and electron microscopic level. Different types of altered hepatocytes, which superficially resemble glycogenotic GGH appearing during hepatocarcinogensis, have also been observed in several other diseases but will not be further discussed in this context[83,86,87].

An intriguing form of acquired hepatic glycogenosis was discovered by Mauriac *et al*[88] in a child with poorly controlled insulin-dependent diabetes type 1. The excessive storage of glycogen resulted in hepatomegaly and was associated with growth retardation, delayed puberty, and a cuchingoid face (named after Harvey Williams Cushing). Many additional cases resembling Mauriac's

syndrome, especially with respect to hepatic glycogenosis, have subsequently been described[89-91]. Torbenson *et al*[91] emphasized that this "glycogenic hepatopathy" is an underrecognized complication of diabetes mellitus. In addition to children with insulin-dependent diabetes, hepatomegaly due to glycogen storage has also been recognized in adults afflicted by non-insulin-dependent diabetes type 2 with poor glycemic control[91-93]. To the best of my knowledge neither GGH nor a relationship of glycogenic hepatopathy to the evolution of HCC in patients suffering from diabetes mellitus has hitherto been described, but it might be timely to take a closer look into this possibility.

The high risk of diffuse glycogenosis characterizing inborn hepatic glycogen storage diseases, particularly glycogen storage disease type I due to glucose-6-phosphatase deficiency, developing into hepatocellular adenomas potentially progressing to HCC has been well established[2,9,28,94] since the first description of a case by Mason *et al*[95]. In the meantime, hepatocellular neoplasms are now known to also be occasionally found in other types of glycogen storage disease, namely glycogenoses type Ⅲ (amylo-1,6-glucosidase deficiency), type Ⅳ (α-1,4-glucan:α-1,4-glucan-6-glycosyl transferase deficiency) and type Ⅵ (phosphorylase deficiency, Hers disease)[94,96-99]. I am not aware of any explicit report of GGH in inborn glycogenoses, but from the findings outlined it is obvious that a more detailed comparison of the molecular, metabolic, and morphological aspects of hepatocarcinogenesis in inborn and acquired (focal) hepatic glycogenosis should help to further elucidate the pathogenesis of hepatocellular neoplasms, and facilitate development of appropriate measures for the prevention and therapy of this frequently fatal disease. From a diagnostic point of view it appears to be of great advantage to use the characteristic changes in hepatocellular glycogen content during hepatocarcinogenesis as simple "superficial" histochemical markers of complex basic aberrations at the molecular and metabolic level.

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