Specific alterations of carbohydrate metabolism are associated with hepatocarcinogenesis in mitochondrially impaired mice

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Received September 13, 2011; Revised and Accepted October 26, 2011

Friedreich's ataxia is an inherited neurodegenerative disease caused by the reduced expression of the mitochondrial active protein frataxin. We have previously shown that mice with a hepatocyte-specific frataxin knockout (AlbFxn⁻²⁻) develop multiple hepatic tumors in later life. In the present study, hepatic carbohydrate metabolism in AlbFxn⁻²⁻ mice at an early and late life stage was analyzed. In young (5-week-old) AlbFxn⁻²⁻ mice hepatic ATP, glucose-6-phosphate and glycogen levels were found to be reduced by ∼74, 80 and 88%, respectively, when compared with control animals. This pronounced ATP, G6P and glycogen depletion in the livers of young mice reverted in older animals: while half of the mice die before 30 weeks of age, the other half reaches 17 months of age and exhibits glycogen, G6P and ATP levels similar to those in age-matched controls. A key event in this respect seems to be the up-regulation of GLUT1, the predominant glucose transporter in fetal liver parenchyma, which became evident in AlbFxn⁻²⁻ mice being 5–12 weeks of age.

The most significant histological findings in animals being 17 or 22 months of age were the appearance of multiple clear cell, mixed cell and basophilic foci throughout the liver parenchyma as well as the development of hepatocellular adenomas and carcinomas. The hepatocarcinogenic process in AlbFxn⁻²⁻ mice shows remarkable differences regarding carbohydrate metabolism alterations when compared with all other chemically and virally driven liver cancer models described up to now.

INTRODUCTION

One of the most consistent features observed during chemically induced liver cancer development in laboratory animals is an aberrant carbohydrate metabolism (1,2). By making use of the model compound N-nitrosomorpholine, Bannasch and colleagues (3–8) were able to show that the predominant sequence of cellular changes during hepatocarcinogenesis leads from clear and acidophilic cell foci storing glycogen in excess through mixed cell foci and nodules, which consist of glycogen-poor as well as glycogen-rich cells, to basophilic cell populations prevailing in hepatocellular carcinomas characterized by glycogen depletion, an enhanced amount of rough endoplasmic reticulum and an increased number of ribosomes. Furthermore, in glycogen-storing cells, decreased glycogen phosphorylase a (GPa) and glucose-6-phosphatase activities and an enhanced glucose-6-phosphate dehydrogenase (G6PDH) activity, a key enzyme of the pentose phosphate pathway, were observed (2,4). Concomitantly, an increase in glucose-6-phosphate (G6P) levels at an early stage of the hepatocarcinogenic process elicited by N-nitrosomorpholine was reported (8). At later stages of liver cancer development,
when glycogen stores are strongly reduced, the activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase is enhanced and that of G6PDH remains high (2,4). These results are consistent with the hypothesis that the initial glycogenosis leads to a series of adaptive changes in certain enzyme activities redirecting carbohydrate metabolism towards other metabolic pathways, such as glycolysis and the pentose phosphate pathway (9,10), thereby providing energy and precursors for nucleic acid synthesis and cell proliferation (11). All of these metabolic pathways have in common that they depend on the supply of glucose. In this context Bannasch and colleagues (12) reported that GLUT2, the typical glucose transporter in hepatocytes of adult liver, is markedly reduced whereas GLUT1, the typical glucose transporter in hepatocytes of fetal liver, is markedly increased. Interestingly, patients suffering from inborn hepatic glycogen storage disease type 1 (i.e. with an inborn glucose-6-phosphatase defect) in many cases develop hepatocellular neoplasms (10,17).

Friedreich’s ataxia is an inherited neurodegenerative disease (18) caused by the reduced expression of the mitochondrial protein frataxin (19). Cancer is not considered to be a typical feature of the disorder; however, Friedreich’s ataxia patients exhibit various types of cancer atypical for their young age (20,21). Thierbach et al. (22) reported that livers of mice with a hepatocyte-specific frataxin knockout (AlbFxn−/− mice) showed increased oxidative stress, impaired respiration, reduced ATP levels, reduced activity of iron-sulfur cluster-containing proteins as well as enhanced apoptosis and cell proliferation. Furthermore, these animals exhibited a significantly decreased life expectancy: about half of the animals died before they reached an age of 30 weeks. Unexpectedly, the mice living >30 weeks developed multiple hepatic tumors at late life stages. In the present study, the hepatic carbohydrate metabolism in AlbFxn−/− mice at an early and late life stage was analyzed and the possible relevance of the alterations described for the hepatocarcinogenic process in AlbFxn−/− mice is discussed.

RESULTS

Hepatocyte-specific disruption of frataxin expression

Frataxin expression was totally abolished in young (i.e. 5-week-old) mice and remained disrupted to a great extent in old (i.e. 17-month-old) animals (Fig. 1, upper blot), the faint remnant signals most probably being due to non-parenchymal cells present in the liver samples analyzed.

Mice with a hepatocyte-specific frataxin knockout are known to gain weight extremely slowly and to exhibit a significantly decreased life expectancy, leading to a reduction in the number of knockout animals of ~50% at 30 weeks of age. Therefore, the histopathological analysis was limited to AlbFxn−/− mice that were either young (≤3 months of age) or old (≥17 months of age), only sacrificing two animals at 10 months of age, and the biochemical analyses were only performed in 5-week- and 17-month-old AlbFxn−/− mice.

Histopathological alterations in the livers of AlbFxn−/− mice

Supplementary Material, Table S1 summarizes the histopathological findings in the livers of AlbFxn−/− mice at different life stages. The preponderant feature in the liver of AlbFxn−/− mice being up to 3 months old was a massive glycogen depletion (Fig. 2A and Supplementary Material, Fig. S1A). Furthermore, liver parenchymal cells undergoing apoptosis and liver parenchymal cells undergoing (in numerous cases a disturbed) mitosis (Supplementary Material, Fig. S1B), a polypliodization as well as an enlargement of nucleoli in liver parenchymal cells (Supplementary Material, Fig. S1C) were observed in young as well as old AlbFxn−/− mice.

In AlbFxn−/− mice being 8–12 weeks old, liver lobule architecture was strongly disturbed by proliferating inflammatory, connective tissue and bile duct epithelial cells, while in AlbFxn−/− mice being 10, 17 or 22 months of age, the liver architecture was almost normal and only a slight proliferation of inflammatory and bile duct epithelial cells was observed. Moreover, in animals being >10 months of age, glycogen stores were replenished (Fig. 2B). The most significant histological findings in animals being 17 or 22 months of age were the appearance of multiple clear cell, mixed cell and basophilic foci throughout the liver parenchyma (Supplementary Material, Fig. S2) as well as the development of hepatocellular adenomas and carcinomas (Fig. 2C and D and Supplementary Material, Figs S3 and S4).
G6P, ATP and glycogen levels as well as carbohydrate metabolizing enzyme activities in the livers of AlbFxn\(^{2/2}\) mice

In 5-week-old AlbFxn\(^{2/2}\) mice, hepatic G6P, ATP and glycogen levels were reduced by \(\sim 80, 74\text{ and }88\%\), respectively, when compared with control animals (Fig. 3A–C). These dramatic decreases were accompanied by a reduction in hepatic GPa and PK-L activities of \(\sim 63\) and \(25\%\), respectively (Fig. 3D and E), whereas no statistical differences in hepatic pyruvate kinase type M 2 (PK-M 2), G6PDH and GK activities between 5-week-old control and AlbFxn\(^{2/2}\) mice were observed (Fig. 3F–H). In 17-month-old AlbFxn\(^{2/2}\) mice, G6P, ATP and glycogen levels recovered and were similar to those in the corresponding control animals (Fig. 3A–C). Furthermore, in 17-month-old AlbFxn\(^{2/2}\) mice, hepatic PK-L, PK-M 2 and G6PDH activities increased by 45, 220 and 256%, respectively, when compared with control animals (Fig. 3E–G), while hepatic GPa activity was similar in 17-month-old control and AlbFxn\(^{2/2}\) mice (Fig. 3D).

DISCUSSION

Mice with a hepatocyte-specific disruption of frataxin expression show an impaired mitochondrial function leading to a decreased oxidative phosphorylation, a reduced respiration rate and a dramatic decay of ATP levels at very early life stages (22). As shown in the present study, the reduced ATP levels are accompanied by a strong decrease in G6P levels and an extremely pronounced depletion of glycogen stores in the 5-week-old AlbFxn\(^{2/2}\) mice livers. The very low G6P levels in the hepatocytes could be ascribed in a very first moment to a reduced GK activity or to the fact that glucose taken up by hepatocytes cannot be adequately phosphorylated via GK because of ATP scarcity. Since GK activities in young AlbFxn\(^{2/2}\) and control mice were similar, the decrease in the hepatocellular ATP concentration most probably is the key

Expression of phosphorylated glycogen synthase (GS), phosphorylated glycogen synthase kinase 3\(\alpha/\beta\) (GSK3\(\alpha/\beta\)), GLUT1 and GLUT2 in the livers of AlbFxn\(^{2/2}\) mice

The expression of phosphorylated (i.e. the inactive form of) GS was decreased in 5-week-old AlbFxn\(^{2/2}\) mice when compared with the corresponding control animals (Fig. 1, middle blot). In contrast, in 17-month control and AlbFxn\(^{2/2}\) mice, phosphorylated GS levels were similar. Moreover, the phosphorylated (i.e. inactive) forms of GSK3\(\alpha\) and GSK3\(\beta\) were enhanced, GSK3\(\alpha\) more strongly than GSK3\(\beta\), in 5-week- as well as in 17-month-old AlbFxn\(^{2/2}\) mice when compared with the corresponding control animals (Fig. 1, lower blot).

An immunohistochemical analysis revealed that in 5–12-week-old AlbFxn\(^{2/2}\) mice, GLUT1 expression was clearly enhanced when compared with the corresponding control groups, while in animals being \(\geq 10\) months old, GLUT1 levels returned to those observed in control animals (Fig. 4). In contrast, GLUT2 expression in control and AlbFxn\(^{2/2}\) mice was largely similar throughout the whole experimental period, the expression levels reaching a maximum in 5-week-old animals and returning back to the levels observed in the 3-week-old mice in elder animals (Fig. 4).
Figure 3. Biochemical alterations in young and old AlbFxn\(^{-/-}\) mice. The results are shown as median and quartile values of the various parameters measured. G6P, ATP and glycogen levels were determined in four mice of each experimental group, GPa, PK-L, PK-M2, G6PDH and GK in 5–6 5-month-old control and AlbFxn\(^{-/-}\) mice and in 4–8 17-month-old control and AlbFxn\(^{-/-}\) mice. The asterisk indicates significantly different from the corresponding control group (Mann–Whitney U-test; \(* P < 0.05; **P < 0.01\)).

Figure 4. GLUT1 and GLUT2 expression in AlbFxn\(^{-/-}\) mice and age-matched controls. The GLUT1 and GLUT2 expression was evaluated semi-quantitatively by making use of score points, the product of occurrence and intensity of the staining. Results are shown in form of box plots, each dot representing one animal. The median value is depicted by the thick line in the box plot.
factor responsible for the strong reduction in G6P levels in the hepatocytes of young AlbFxn−/− mice.

GS, GSK3α/β and GPa are regulated in such a way in the livers of 5-week-old AlbFxn−/− mice that glycogen synthesis should occur. First, the expression of the phosphorylated (i.e. the inactive form of) GS was reduced. Secondly, the expression of phosphorylated (i.e. inactive) GSK3α/β, which in the non-phosphorylated form catalyze the conversion of the active GS a form into the inactive GS b form, were enhanced. Thirdly, the activity of GPa, which catalyzes the degradation of glycogen, was significantly reduced. However, as mentioned above, livers of 5-week-old AlbFxn−/− mice were almost devoid of glycogen. This is most probably due to the very low G6P levels within the hepatocytes of the young AlbFxn−/− mice. On the other hand, the G6P units are needed to build up glycogen molecules through the cascade glucose-6-phosphate → glucose-1-phosphate → UDP-glucose → glycogen. On the other hand, G6P produced by GK effectively promotes the activation of GS (23–25).

One could argue that glycogen depletion and the extremely low G6P levels within the hepatocytes are the consequence of redirecting carbohydrate metabolism towards the pentose phosphate shunt pathway and glycolysis. However, the activity of G6PDH, the rate-limiting enzyme in the pentose phosphate shunt pathway, is not enhanced in the livers of 5-week-old AlbFxn−/− mice when compared with the age-matched controls. Furthermore, glycolysis is not activated as demonstrated by PK activity, which catalyzes the conversion of phosphoenolpyruvate to pyruvate at the end of the glycolytic cascade and is not increased in young knockout animals when compared with the corresponding controls. Under conditions in which oxidative phosphorylation is impaired and ATP levels dramatically fall, one would expect that glycolysis is up-regulated. Nevertheless, this is not the case and may be explained by the fact that hexokinase and phosphofructokinase-1, two enzymes involved in the glycolytic pathway, need ATP in order to phosphorylate glucose and fructose-6-phosphate, respectively.

The most outstanding feature of the AlbFxn−/− mice model regarding alterations of the carbohydrate metabolism is that the drastic ATP, G6P and glycogen depletion observed in the livers of young mice is reverted in old animals: while one half of the mice die before they reach an age of 30 weeks, the other half reaching 17 months of age show glycogen, G6P as well as ATP levels similar to those in age-matched controls. A key event in this respect seems to be the up-regulation of GLUT1, the typical glucose transporter in fetal liver parenchyma (12), that became evident in AlbFxn−/− mice being 5–12 weeks of age. Moreover, GLUT2 was concomitantly up-regulated in mice being 5 weeks of age, but the enhanced GLUT2 expression was also observed in age-matched control animals. In mice being >10 months old, i.e. those animals in which glycogen stores had been replenished, GLUT1 as well as GLUT2 expression returned to the basal levels observed in 3-week-old animals. The alterations of GLUT1 and GLUT2 expression in mice with a hepatocyte-specific fraatrixin knockout are in sharp contrast to the changes observed during chemically induced hepatocarcinogenesis. Grobholz et al. (12) showed that in rats having been treated with N-nitrosomorpholine GLUT1 up-regulation occurred at late stages of hepatocarcinogenesis, whereas GLUT2 expression was already reduced in early preneoplastic hepatic foci and remained low in hepatocellular adenomas and carcinomas. The fact that one half of the animals still dies before they reach an age of 30 weeks is most probably explained by the massive ATP, G6P and glycogen depletion that cannot be reverted in time. In the present study, a reduced expression of active GSK3α/β as well as energy depletion was accompanied by an enhanced GLUT1 expression. These observations are in accordance with previous reports showing that the inhibition of GSK3 by lithium chloride or SB216763 leads to an increase in GLUT1 expression (26) and that energy depletion activates AMP-activated protein kinase, thereby enhancing GLUT1 transport activity (27,28). However, AMP-activated protein kinase expression was not increased in AlbFxn−/− mice throughout the entire experimental period (R. Thierbach, unpublished data), which in turn indicates that in this animal model GLUT1 expression is up-regulated via an AMP-activated protein kinase-independent and up to now not identified mechanism.

In 17-month-old AlbFxn−/− mice, hepatic G6PDH activity was strongly increased when compared with that of age-matched controls. G6PDH mediates the conversion of G6P to 6-phosphogluconate and the concomitant reduction in NADP+ to NADPH in the pentose phosphate pathway. Since it is the main NADPH-generating enzyme in cells, it was earlier postulated that its primary function is to control cellular redox state instead of the synthesis of ribose-5-phosphate, which is essential for nucleic acid synthesis (29). However, very recently it has been shown in colon cancer cells that the oxidative branch of the pentose phosphate pathway, controlled by G6PDH, is specifically up-regulated during the late G1 and S phases of the cell cycle, thereby underlining the eminent role of G6PDH in supporting nucleic acid synthesis and cell proliferation (30). The strongly enhanced G6PDH activity observed in the AlbFxn−/− mice has also been reported in chemically (2,4,10) and virally induced liver tumors (13–15), the increased G6PDH activity being in line with the high cell proliferation rate in the preneoplastic and neoplastic hepatic lesions described in the different liver cancer models.

In chemically induced liver cancer models, a shift from PK-L to PK-M2 expression was reported (31,32). Since PK-M2 has a higher affinity for its substrate phosphoenolpyruvate than PK-L and is insensitive to modulation by ATP and fructose-1,6-biphosphate, the above-mentioned shift most probably leads to an enhanced glycolysis by ensuring an efficient substrate utilization. In the livers of 17-month-old AlbFxn−/− mice, PK-L as well as PK-M2 activities were significantly increased when compared with the corresponding control animals. Hence, regarding PK-L and PK-M2 expression, the hepatocarcinogenesis model described in this study differs from all other experimental liver cancer models, in which PK-L and PK-M2 expression was analysed. However, the molecular basis for the concomitant increase in PK-L and PK-M2 activities remains unexplained at the present time.

In AlbFxn−/− mice being 17 or 22 months of age, multiple clear cell, mixed cell and basophilic foci throughout the liver parenchyma as well as hepatocellular tumors (adenomas and
carcinomas) were observed. Taking into account that mice with a hepatocyte-specific frataxin knockout exhibit a significantly decreased life expectancy (22) and in order to have enough old animals (i.e. being ≥17 months of age) for the histopathological and biochemical analyses, no animals being more than 10 months and less than 17 months old were sacrificed. Thus, although it is most probable that the sequence of cellular changes regarding carbohydrate metabolism observed during chemically (3–8) and virally driven hepatocarcinogenesis by Bannasch and colleagues (13–16) also occurs in mice with a liver-specific disruption of frataxin, this assumption cannot be definitely deduced from the results obtained so far.

Taken together, the results obtained in the present study show that the hepatocarcinogenic process in mice with a hepatocyte-specific frataxin knockout is different regarding carbohydrate metabolism alterations when compared with all other chemically and virally driven liver cancer models described up to now and can be divided in three phases. In an early phase, a massive depletion of ATP, G6P and glycogen as a consequence of frataxin disruption occurs. Thereafter, in an intermediate phase, the hepatocytes seem to ‘recover’ regarding carbohydrate metabolism, a key element in this phase being the up-regulation of GLUT1 expression. During the late phase of the process, preneoplastic and neoplastic lesions develop, most probably as a consequence of the biochemical alterations having occurred in the second phase. The histopathological changes are accompanied by an activation of glycolysis (enhanced PK activity) and the pentose phosphate pathway (enhanced G6PDH activity), thereby ensuring energy supply and supporting nucleic acid synthesis.

Data by Su and colleagues (15,16) suggest that foci of altered hepatocytes including glycogen-storing and mixed cell foci as well as the resulting hyperproliferative nodular lesions are indeed associated with the development of hepatocellular carcinomas in humans. While Friedreich’s ataxia is a disease predisposing to occasional tumors at young age (20,21), cancer is not considered to be a mandatory complication of the disease. This may be due to the fact that Friedreich’s ataxia patients typically exhibit a decreased life expectancy of 38 years on average, the period of time needed for liver tumors to develop being most probably insufficient.

Regarding the molecular mechanisms leading to malignant cell transformation in mice with a liver-specific frataxin, knockout oxidative stress (22) and defective DNA repair (33) have been discussed. Thierbach et al. (22) showed that disruption of frataxin causes increased formation of reactive oxygen species, as indicated by enhanced levels of thiobarbituric acid reactive substances and oxidized glutathione in liver samples from knockout mice. Nevertheless, the cellular buffering capacity against reactive oxygen species remained unaffected, as indicated by the unaltered levels of reduced glutathione in the above-mentioned liver samples (22), thus suggesting that oxidative stress most probably plays a limited role in the development of the Friedreich’s ataxia phenotype (34). Consistent with the fact that frataxin is essential for the mitochondrial biosynthesis of iron-sulfur clusters and that certain DNA repair enzymes contain iron-sulfur clusters, it has recently been shown by Thierbach et al. (33) that an altered expression of frataxin modulates DNA repair activity, correlates with an enhanced mutagenicity and culminates in tumor formation in mice. These results suggest that one of the mechanisms by which a frataxin gene disruption may lead to tumor initiation is the alteration of the biosynthesis of iron-sulfur cluster-containing DNA repair enzymes, thereby resulting in a reduced ‘caretaker function’ of these DNA protecting enzymes and an enhanced gene mutation rate.

MATERIALS AND METHODS

Generation of liver-specific frataxin knockout mice

AlbFxn<sup>−/−</sup> mice were generated (35), bred (36) and maintained (36) as previously described with the exception that beta-cell-specific Ins2-cre mice were replaced by hepatocyte-specific Alb-cre animals (37), which were 67% C57Bl6 and 33% FVB of the origin, whereas frataxin loxP animals were at least 90% of the C57Bl6 origin. Genotyping and detection of knockout animals at the genomic, transcriptional and translational level were previously described (22). Mice were kept in accordance with the regulations of the Federation of European Laboratory Animal Science Associations and all experiments were approved by the corresponding institutional review board.

Tissue preparation

Mice were sacrificed by cervical dislocation and the left, right and middle liver lobes were immediately excised. Liver tissue was fixed in 4% neutral buffered formalin for 24 h and washed for 24 h under tap water. Then specimens were dehydrated and embedded in paraffin wax. Serial tissue sections (2 μm) were prepared for histology [hematoxylin and eosin stain for the morphological examination of liver structure, periodic acid-Schiff stain for glycogen localization (38)] and immunohistochemistry.

Immunohistochemistry

Rabbit anti-GLUT1 as well as anti-GLUT2 antibodies (39) were a gift from A. Schürmann (German Institute for Human Nutrition, Nuthetal, Germany) and the staining of liver samples was performed as described in Supplementary Material and Methods.

Measurement of ATP, G6P and glycogen levels

ATP content was measured as previously described (40). Hepatic G6P and glycogen levels were determined according to the modified versions of the methods of Lang and Michal (41) and Roe and Dailey (42), respectively, as described in Supplementary Material and Methods.

Enzyme activity assays

Glucokinase (GK) and GPa activities were determined according to the modified versions of the methods of Walker and Rao (43) and Sutherland and Wosilait (44) coupled to the method
of Fiske and Subbarow (45) for phosphorus determination, respectively, as described in Supplementary Material and Methods. G6PDH as well as pyruvate kinase type L (PK-L) and type M2 (PK-M2) activities were measured according to the previously described protocols (46,47). Protein concentration was determined according to the method of Bradford (48).

Western blotting

Fratixin was analyzed according to Shoichet et al. (49) and that of glycogen synthase kinase 3α/β (GSK3α/β) as described in Supplementary Material and Methods.

Statistical analyses

The significance of the differences between the experimental groups was analyzed by using the Mann–Whitney U-test. All statistical analyses were performed with SPSS 11.0 (Chicago, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank Mrs Elisabeth Meyer (German Institute of Human Nutrition, Nuthetal, Germany) and Mrs Gabriele Schmitt (Division of Cellular and Molecular Pathology, German Cancer Research Center, Heidelberg) for her excellent technical assistance. Support by the DKFZ Light Microscopy Facility is gratefully acknowledged.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by a grant from the German Research Association (Deutsche Forschungsgemeinschaft) (TH 1392/1-1).

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