Hepatocellular Neoplasms Induced by Low-Number Pancreatic Islet Transplants in Autoimmune Diabetic BB/Pfd Rats

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Abstract

It has been shown that combined high local hyperinsulinism and hyperglycemia after low-number islet transplantation into the livers of streptozotocin-diabetic rats lead to the development of hepatocellular neoplasms but in a substantial cocarcinogenic effect of genotoxic streptozotocin could not be ruled out completely. Thus, we herein investigated this model in BB/Pfd rats (n = 805; nine experimental groups), which develop spontaneous autoimmune diabetes similar to human type 1 diabetes. After low-number islet transplantation (n = 450), the liver acini downstream of the islets show insulin-induced alterations: massive glycogen and/or fat accumulation, translocation of the insulin receptor, decrease in glucose-6-phosphatase activity, increase in expression of insulin-like growth factor (IGF)-I, IGF-II/mannose-6-phosphate receptor, insulin receptor substrate-1, Raf-1, and Mek-1, corresponding to clear cell preneoplastic foci of altered hepatocytes known from chemical hepatocarcinogenesis and identical to that in streptozotocin-diabetic Lewis rats. After 6 months, many altered liver acini progressed to other types of preneoplasias often accompanied by an overexpression of the glutathione-S transferase (placental form), IGF-I receptor, and transforming growth factor (TGF)-α. After 12 to 15 and 15 to 18 months, 52% and 100% of the animals showed one or two hepatocellular adenomas or hepatocellular carcinomas (HCCs), respectively. Conclusively, this study identifies combined hyperinsulinism and hyperglycemia as a carcinogenic mechanism for the development of HCCs in diabetic rats. Hepatocarcinogenesis is independent from additional genotoxic compounds (i.e., streptozotocin), but is primarily triggered by increased intracellular insulin signaling via pathways associated with cell growth and proliferation, such as the Ras-Raf-mitogen-activated protein kinase pathway and the IGF system, and secondarily involves other growth factors, such as TGF-α. (Cancer Res 2006; 66(3): 1833-43)

Introduction

Recent clinical trials introducing new immunosuppressive regimens and improved islet preparation techniques have shown that transplantation of islets of Langerhans into the liver of type I diabetic patients could represent an alternative to exogenous insulin treatment and allows for the normalization of metabolic control, which cannot be achieved by administration of exogenous insulin alone (1–5). Although a few cases have been published in the English literature, detailed histopathologic studies of the livers of long-term recipients have not yet been conducted (5–8). Thus, little is known about the paracrine effects of insulin on the adjacent hepatocytes, which may turn out to be of major clinical importance in the long term.

On the other hand, diabetes mellitus has been identified as a risk factor for hepatocellular carcinoma (HCC) in humans in Western Europe and the United States (9–15). However, the mechanisms by which diabetes may contribute to the formation of HCCs in humans are poorly understood. This led to a controversy about whether diabetes mellitus itself or rather associated diseases, such as obesity or hepatitis C, are mainly responsible for tumor induction (11, 16, 17), and the need for experimental studies revealing the underlying mechanisms is emphasized (18).

A possible explanation for the relationship of diabetes mellitus and liver cancer is provided by our previous studies in an animal model of hormonally induced hepatocarcinogenesis in which intrahepatic low number (i.e., 350–450 islets) pancreatic islet transplantation in streptozotocin-diabetic Lewis rats seemed to be the primary trigger for carcinogenesis (19–26). Sole high number (i.e., 1,000–2,000 islets) transplantation in streptozotocin-diabetic Lewis rats, in which the β-cells of the grafts are not maximally stimulated to secrete insulin and the resulting local hyperinsulinemia is relatively slight, does not suffice to induce the carcinogenic process (19, 21). Carcinogenesis starts with hepatocellular alterations, which, on the one hand, correspond to known insulin effects and, on the other hand, resemble the so-called clear cell focus (CCF) of preneoplastic hepatocytes, known from many other models of hepatocarcinogenesis (27). These alterations are reflected in an increase in glycogen and lipid storage, in an increase in cell-turnover (i.e., high proliferative activity and apoptotic elimination of preneoplastic hepatocytes), as well as in characteristic alterations in the activities of key enzymes, in particular of the carbohydrate and fatty acid metabolism (19–21, 23, 25). These include an up-regulation of enzymes of glycogenolysis (hexokinase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase), de novo lipid synthesis (fatty acid synthase), and the pentose phosphate pathway (glucose-6-phosphate dehydrogenase), whereas key enzymes of gluconeogenesis (glucose-6-phosphatase), glycogenolysis (glycogen phosphorylase), and adenylate cyclase activity were down-regulated (20, 23). Insulin effects in the CCF also manifested in an overexpression of apolipoprotein A-IV (25) and in an altered expression of proteins of the insulin-like growth factor (IGF) pathway in the CCF, including IGF-I and its binding proteins, such as IGF binding protein (IGFBP)-1 and IGFBP-4 (22). Moreover, we have recently shown strongly increased insulin signaling in CCF after islet transplantation, reflected in a translocation of the insulin receptor and in an overexpression of several insulin signal transduction proteins of the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway, such as insulin receptor substrate-1 (IRS-1), Raf-1, and Mek-1 (24). In the beginning, the CCF were always strictly confined to the liver acini located downstream of the islets but gradually expanded into the neighborhood when...
undergoing neoplastic transformation (19, 21). After 15 to 22 months, 86% and 19% of the animals developed at least one hepatocellular adenoma (HCA) or HCC, respectively (21).

There are additional data obtained in other animal models that showed that the phenotypes of preneoplasias can mimic responses to insulin action (28), and Nehrbass et al. (29, 30) have shown that IRS-1 overexpression is an early event in chemical hepatocarcinogenesis of rats, given p.o. N-nitrosomorpholine. From these studies, it can be concluded that increased insulin action or insulinomimetic effects may constitute an interesting carcinogenic mechanism in the development of HCC in rats and possibly also in humans.

However, a serious drawback in the interpretation of the results from the islet transplantation model was the administration of streptozotocin to achieve diabetes. Streptozotocin is genotoxic and carcinogenic in rats (31, 32). Okawa and Doi (33) have reported the development of hepatocellular and cholangiocellular tumors in streptozotocin-treated Sprague-Dawley rats, although a complete hepatocarcinogenic potential in rats is not sufficiently proved by this study in which only 16 animals were investigated. Nevertheless, at least a significant cocarcinogenic contribution of streptozotocin has to be taken into account. To finally exclude an influence of streptozotocin acting as an incomplete hepatocarcinogen in this experimental setting and for reasons of better comparability with the situation in human diabetes mellitus, we thus investigated in this long-term study the local influence and the carcinogenic potential of insulin on the adjacent hepatocytes in a model of autoimmune transplantation.

### Table 1. Experimental design, preneoplastic foci, and hepatocellular neoplasms

<table>
<thead>
<tr>
<th>Streptozotocin administration</th>
<th>Inbred BB/Pfd rats (n = 805)</th>
<th>Spontaneous autoimmune diabetes (n = 338)</th>
</tr>
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<tbody>
<tr>
<td>Ilet transplantation</td>
<td>Low-number (n = 450)</td>
<td>High-number (n = 1,200)</td>
</tr>
<tr>
<td>Spontaneous reestablishment of self-tolerance</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Experimental group</td>
<td>MG (n = 148)</td>
<td>CG 1 (n = 42)</td>
</tr>
<tr>
<td>Blood glucose (mean ± SE; mmol/L)</td>
<td>11.6 ± 0.7</td>
<td>19.9 ± 0.9</td>
</tr>
<tr>
<td>Body weight at sacrifice (mean ± SE; g)</td>
<td>288 ± 5</td>
<td>262 ± 9</td>
</tr>
<tr>
<td>Relative number/main type of foci</td>
<td>0-3 mo p.t.</td>
<td>+++/CCF 0 (15)</td>
</tr>
<tr>
<td></td>
<td>3-6 mo p.t.</td>
<td>+++/CCF 0 (18)</td>
</tr>
<tr>
<td>Relative no. animals bearing a HCA/HCC (%)</td>
<td>6-9 mo p.t.</td>
<td>+++/CCF 0 (33)</td>
</tr>
<tr>
<td></td>
<td>9-12 mo p.t.</td>
<td>+++/MCF 14/0 (51)</td>
</tr>
<tr>
<td>No. investigated animals (in parentheses)</td>
<td>12-15 mo p.t.</td>
<td>+++/MCF 52/0 (25)</td>
</tr>
<tr>
<td></td>
<td>15-18 mo p.t.</td>
<td>+++/MCF 83/50/0 (6)</td>
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</table>

NOTE: The number of foci was estimated semiquantitatively using three grades: +, single (sporadic) foci in single animals; ++, single number of foci in every animal; +++, numerous foci in every animal. Yellow box, significantly more tumor-bearing animals than in CG 2–5 and CG 7–8. Orange box, significantly more tumor-bearing animals than CG 5, 7, and 8 (others not tested).

Abbreviations: MG, main group; CG, control group; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma; CCF, clear-cell focus; MCF, mixed cell focus, p.t., post-transplantation.

*Significantly more HCA-bearing animals than in CG 2–5 and CG 7–8. All data refer to the right liver part and were tested using Fisher’s exact test and accepted if P < 0.05.

cSignificantly more HCC-bearing animals than in CG 2.
diabetes, using autoimmune-diabetic BioBreeding (BB) rats. BB rats became diabetic by an autimmunologic disorder similar to human type 1 diabetes mellitus (34), which is not completely understood but involves mutations of the $lyp$ locus on chromosome 4, lymphopenia, and dysregulation of inflammatory cells, including eosinophils, mast cells, and lymphocytes, which, in turn, lead to insulinitis and $\beta$-cell destruction (35–37). The Pfd substrain of BB rats is characterized by the reestablishment of self-tolerance to the $\beta$-cells after previous destruction of the $\beta$-cells in the pancreas (38, 39). Therefore, these animals tolerate the isologous islet grafts that were transplanted in the course of the experiment without the use of immunosuppression and its possible side effects on the hepatocytes. We also included streptozotocin-diabetic control groups of the BB strain to compare carcinogenesis between these different types of diabetes induction and with the Lewis rat model.

### Materials and Methods

**Animals.** Eight hundred five animals were investigated in this study and housed as described previously (21). Only nondiabetic littermates were paired, which also served as islet donors. Spontaneous diabetes was defined by a nonfasting blood glucose level higher than 22.2 mmol/L (400 mg/dL). To avoid greater weight loss and to have normoglycemia by the time of transplantation, the diabetic rats were treated with s.c. injections of insulin (Insulin Protaphan HM 40, NOVO, Mainz, Germany) as described previously (21), a procedure that was immediately stopped after islet transplantation.

The spontaneous diabetic animals were finally subdivided into five experimental groups according to whether the rats reestablished self-tolerance (MG and CG 2) or not (CG 1 and CG 3) and according to the modus of transplantation [low number (MG and CG 1), high-number (CG 2 and CG 3), or no (transplantation CG 4)], resulting in one main group (MG) and four control groups (CG 1-4; Table 1). CG 1 and CG 3 were composed of animals that never developed self-tolerance or lost it again and rejected the islet grafts during the experimental course.

Rats not having developed autoimmune diabetes were left completely untreated as normoglycemic controls (CG 5) or received a single i.v. injection of streptozotocin (65 mg/kg). Two weeks later, they were again subdivided (CG 6-8) according to the respective transplantation procedure (Table 1). All animals were inspected daily. Blood glucose and body weight were measured monthly and also 1 and 3 days after transplantation, as well as immediately before and 2 days after streptozotocin treatment. Animals showing a weight loss of more than one third of their maximal body weight posttransplantation were given a s.c. insulin implant (Linplant, Linshin.

| Table 1. Experimental design, preneoplastic foci, and hepatocellular neoplasms (Cont’d) |
|---------------------------------|---------------------------------------------------------------|
| **Streptozotocin administration** | **No spontaneous diabetes (n = 467)** |
| Islet transplantation | No | Diabetes induction by streptozotocin (65 mg/kg body weight) |
| Spontaneous reestablishment of self-tolerance | Tolerance never lost | Tolerance never lost |
| Experimental group | CG 5 (n = 165) | CG 6 (n = 144) | CG 7 (n = 118) | CG 8 (n = 40) |
| Blood glucose (mean ± SE; mmol/L) | 5.0 ± 0.1 | 10.8 ± 0.6 | 4.2 ± 0.1 | 17.8 ± 0.6 |
| Body weight at sacrifice (mean ± SE; g) | 328 ± 7 | 296 ± 6 | 367 ± 7 | 289 ± 8 |

Relative number/main type of foci

| 0-3 mo p.t. | 0 | 0 (15) | +++/CCF | 0 | 0 (15) | 0 | 0 (0) |
| 3-6 mo p.t. | 0 | 0 (10) | +++/CCF | 0 | 0 (15) | 0 | 0 (5) |
| 6-9 mo p.t. | 0 | 0 (33) | +++/CCF | 0 | 0 (23) | 0 | 0 (7) |
| 9-12 mo p.t. | 0 | 0 (50) | +++/MCF | 15/0 (39) | 0 | 0 (33) | 0 | 0 (12) |
| 12-15 mo p.t. | 0 | 0/0 (32) | +++/MCF | 54/0 (28) | +/CCF | 0/0 (20) | 0 | 0/0 (10) |
| 15-18 mo p.t. | +/CCF | 4/0 (25) | +++/MCF | 75/25 (12) | +/CCF | 8/0 (12) | 0 | 0/0 (6) |
Canada, Scarborough, ON, Canada) to prevent death by diabetes. Animal treatment was in line with the guidelines of the Society for Laboratory Animal Service and the strict German Animal Protection Law.

**Transplantation procedure.** Animals were anesthetized (100 mg/kg ketamin and 4 mg/kg xylazin) and islets of Langerhans were isolated from nondiabetic littersmates and transplanted into the liver via the portal vein as described in detail previously (19). During infusion, the branch supplying the left part of the liver (i.e., left lobe and left part of the middle lobe) was clamped, thus making sure that the transplants were emobilized only into the right part of the livers (i.e., right lobe, right part of the middle lobe, caudate lobe, and anterior and posterior papillary processus) and the left part served as an intraindividual control. Islets for the left part lasted for 1 minute. Rats of the MG, CG 1, and CG 6 received a low number (n = 450) of islet grafts; CG 2, CG 3, and CG 7 animals received a high number (n = 1,200) of islet grafts; and CG 4, CG 5, and CG 8 were not transplanted.

**Animal sacrifices and 5-bromo-2-deoxyuridine application.** Animals were killed because of severe complications or were matched in time groups (Table 1). Rats were anesthetized, the aorta was cannulated, the inferior caval vein was cut, and the vessels were then rinsed for 2 minutes with Ringer’s solution, mixed with 0.5% procain and 4% dextran, followed by perfusion fixing using a cocktail of aqua dest containing 4% dextran, 0.5% glutaraldehyde, and 3% paraformaldehyde. All animals received a single dose of BrdUrd i.p. (50 mg/kg) 1 hour before sacrifice as described previously (21).

**Tissue sampling and processing.** After fixation, the livers were removed, cut into slices (0.5 mm), and examined with a stereomicroscope. All macroscopic liver lesions and at least 10 additional slices, as well as specimens from the heart, lung, kidneys, adrenal glands, small intestine and colon, pancreas, spleen, thyroid gland, muscle, and pituitary gland were embedded in paraffin. Serial sections (2-3 μm) of the liver specimens were stained with H&E and with the periodic acid-Schiff (PAS) reaction. Additional sections were made for immunohistochemistry. The other organs were stained with H&E.

The morphologic classification of lesions was done as described previously (21). Briefly, preneoplastic foci composed exclusively of glycochen- and fat-storing cells were classified as CCF; a glycogen-depleted, exclusively basophilic cell population as basophilic cell foci; and those with both cell types as mixed cell foci. HCA extended beyond the original liver acini, were sharply limited, and compressed the surrounding liver parenchyma. Tumors being larger than 5 mm in diameter, exhibiting trabeculae thicker than three cell layers in at least two separate areas, and showing a higher number of mitotic figures, vascular invasion, or metastases were classified as HCC.

Insulinasomas were diagnosed if they fulfilled the following criteria: intrahepatic islet graft larger than 2 mm; immunohistochemical positivity for insulin, mitotic activity of insulin-positive cells, and severe hypoglycemia (blood glucose lower than 1.11 mmol/L; < 20 mg/dL).

For electron microscopy, appropriate tissue specimens of 2 × 2 mm size were postfixed in OsO₄ and embedded in Epon. Semithin sections were stained according to Richardson et al. (40); ultrathin sections were stained with uranyl acetate and lead citrate. The examination was done using a Philips (Eindhoven, the Netherlands) CM10 electron microscope.

For enzyme histochemistry, the middle lobes of selected livers were removed before fixation, cut into slices, and immediately frozen in −80°C cold isopentane. Cryostat sections of 6 μm thickness were cut, stained according to Benner et al. (41) for glucose-6-phosphatase activity, and semiquantitatively evaluated by comparison with the adjacent unaltered liver parenchyma. Additional sections were also stained with the PAS reaction and fat stain (Sudan red).

**Immunohistochemistry.** Paraffin sections were incubated with the following primary polyclonal rabbit antibodies: anti-insulin receptor (A1314, 0.5 mg/mL, kindly provided by Dr. J.W. Unger, Department of Anatomy, University of Munich, Munich, Germany), anti-IRS-1 (dilution: 1:50; Upstate Biotechnology, Inc., New York, NY), anti-Raf-1 (dilution: 1:50; Santa Cruz Biotechnology, Heidelberg, Germany), anti-Mek-1 (dilution: 1:100; Santa Cruz Biotechnology), anti-IGF-I (dilution: 1:250; DLS, Webster, TX), anti-IGF-I receptor (Santa Cruz Biotechnology), anti-IGF-II/mannose-6-phosphate-receptor [purified from rat liver (42) and kindly provided by Dr. J.G. Scharf, Division of Gastroenterology and Endocrinology, Department of Medicine, University of Göttingen, Göttingen, Germany], anti-gluthathione S-transferase placental form (dilution: 1:100, no pretreatment; Biogenex, San Ramon, CA), anti-TGF-α (final concentration 10 μg/mL; Oncogen Sciences, Cambridge, MA), as well as anti-insulin, antiglucagon, and antisomatostatin (dilution: 1:200; all from DAKO, Hamburg, Germany). The anti-BrdUrd antibody (dilution: 1:100; DAKO) was monoclonal. Negative controls without usage of the primary antibody were done in each run. Further details of the staining procedures, including antigen retrieval methods, secondary antibodies, blocking of endogenous peroxidase, counterstaining, and mounting of the tissue sections, were described previously (21, 22, 24).

**Results**

**Blood glucose and body weight.** Spontaneous diabetes manifested in ~10% of male and in 2% of female 3- to 6-month-old rats. All animals of CG 4 (and also animals of CG 1 and CG 3) developed hyperglycemia (27-33 mmol/L), which was so severe that insulin depots had to be implanted to prevent death. Thus, blood glucose of CG 4 was lowered to a mean of 20.4 mmol/L, but was still higher than in untreated streptozotocin diabetic rats (CG 8: 17.8 mmol/L). Animals after low-number transplantation persisted in a mild diabetic state as was intended (MG, mean blood glucose 11.6 mmol/L). This was the result of previous autoimmunologically induced self-destruction of the β-cells in the pancreas and the subsequently transplanted and, owing to the reestablished self-tolerance now tolerated, low number of intrahepatic islet grafts. However, 22% of these animals failed to reestablish long-lasting self-tolerance and resumed β-cell destruction during the experimental course, this time in the intrahepatic islet grafts, as revealed by postmortem liver examination. The mean blood glucose level in these animals was 19.9 mmol/L. They had to be excluded from the MG and formed CG 1. As expected, high-number transplantation initially established normoglycemia in all animals but only 80% stayed normoglycemic owing to the reestablishment of self-tolerance in these animals (CG 2, mean blood glucose: 4.7 mmol/L). Twenty percent resumed β-cell destruction in the islet grafts, proved by postmortem examination, and became diabetic again 1 to 6 months posttransplantation. They had to be excluded from CG 2 and formed CG 3 (mean blood glucose: 16.2 mmol/L).

On average, the blood glucose level in the streptozotocin-treated animals was 7% to 13% lower when compared with the corresponding autoimmune diabetic groups (Table 1) and they did not need insulin implants. They also showed mild hyperglycemia after low-number transplantation (CG 6, mean blood glucose: 10.8 mmol/L) and normoglycemia after high-number transplantation (CG 7, mean blood glucose: 4.2 mmol/L). Completely untreated rats without spontaneous diabetes stayed normoglycemic throughout the entire experiment (CG 5, mean blood glucose: 5.0 mmol/L). Single animals of the MG and CG 6 reached normoglycemia after several months owing to hyperplasia of the islet grafts. The three animals that developed graft insulinasomas (see below) were hypoglycemic; the lowest blood glucose measured was 0.4 mmol/L.

Body weight was inversely correlated with blood glucose; that is, it was highest in normoglycemic animals of the CG 2, CG 5, and CG 8, and lowest in the completely untreated diabetic animals of CG 4 (Table 1).

**Intercurrent diseases and causes of spontaneous deaths.** Some rats were affected by other severe diseases that were not located in the liver and had no effect on hepatocarcinogenesis. When there were no complications of the diabetes, they were affected by other severe diseases that were not located in the liver and had no effect on hepatocarcinogenesis. They included malignant lymphomas, pituitary gland adenomas, and, most frequently, a transmural eosinophilic inflammation of the
colon and sometimes also the small intestine, leading to fibrosis, stenosis, and consecutive megacolon and massive obstipation as reported by Meehan et al. (43). These animals were usually killed in time to prevent further suffering and spontaneous deaths. Unfortunately, owing to this high morbidity, only a small number of animals stayed alive for 15 to 18 months after transplantation and prolongation of the experiment exceeding 18 months was not possible. As reported in previous studies (35, 44), some animals also showed signs of an autoimmune enteropathy (i.e., decreased villous and enlarged crypt length) as well as an increase in the number of intraepithelial lymphocytes; however, this was not the focus of our study. We also observed several renal cell carcinomas showing a strong predilection for the diabetic groups, indicating diabetes mellitus to be a risk factor also for renal cell carcinomas. The relationship between diabetes mellitus and renal carcinogenesis will be investigated in detail in a future study.

**Macroscopy and stereomicroscopy of the livers.** With the exception of very few sporadic CCF in the control livers of late-stage animals, all focal liver alterations were observed in the right part of the livers of the MG, CG 6, and, to a lesser extent, of CG 1. This was the result of the transplantation procedure, as the branches of the portal vein that supply the left liver part were clamped during infusion of the islets. Focal white lesions were observed on the liver surface and on the cut surface of liver slices, identified under a stereomicroscope as yellow-white liver acini draining the blood from the transplanted islet grafts (Fig. 1A). In the first 3 months, they were always confined to the anatomic borders of the respective liver acini and limited by the draining hepatic venules at the border to the adjacent acini (Fig. 1B). With increasing time, they expanded around the hepatic venules (Fig. 1C), extended into the neighboring liver parenchyma (Fig. 1D), and became inhomogenous in color and irregular in shape (Fig. 1E). Tumors of several millimeters in size began to develop after 9 months (Fig. 1F-I).

**Light and electron microscopy of the liver parenchyma.** Light microscopy confirmed that in the first 3 months, the hepatocellular alterations were strictly limited to the liver acini, draining the blood from the transplanted islets. The hepatocytes at this stage were of uniformly clear cell morphology in the H&E stain and showed strong purple staining in the PAS reaction, resulting from a high amount of glycogen (Fig. 2A). Electron microscopy revealed that glycogen was deposited in α-particles in the cytoplasm (Fig. 2E). In addition, these hepatocytes displayed an increase in fat storage in the form of single or multiple lipid droplets.
vacuoles within the cytoplasm (Fig. 2B and E). These CCF were also characterized by an increase in mitotic activity and in apoptotic elimination. In addition, they showed a mean 39-fold increase in BrdUrd-positive nuclei when compared with the unaltered liver parenchyma (Fig. 2C; Table 2). Approximately 6 months posttransplantation, a subpopulation of altered hepatocytes lost their glycogen and fat, now displaying a basophilic cytoplasm (Fig. 3C). In addition to the reduced glycogen content, these cells were ultrastructurally characterized by abundant ribosomes and rough endoplasmic reticulum. In the beginning, these basophilic cells were intermingled with hepatocytes that retained the clear cell morphology and such lesions were classified as mixed cell foci. However, some of the lesions gradually progressed to pure basophilic foci. Mixed cell foci and basophilic cell foci showed an increase in mitotic activity and BrdUrd-labeled hepatocyte nuclei when compared with the adjacent unaltered liver tissue, albeit not as strong as in CCF (Table 2). It is noteworthy that part of the preneoplastic foci in the MG and CG 6 persisted, although the respective animals became normoglycemic and the insulin secretion of the respective islet graft probably strongly decreased. Moreover, we also observed persistence of some preneoplasias in CG 1, although the islet grafts were rejected, became fibrotic, and the β-cells completely vanished (Fig. 1I, 3G, and 4I).

The first hepatocellular neoplasms developed after 9 months. Most HCA and HCC in this study were composed of a mixed population of clear and basophilic cells possibly originating from mixed cell foci. Some small HCA were purely clear cell. Pure basophilic or clear cell carcinomas were rare (Fig. 3D-F). They were found to contain remnants of islet grafts in most cases. HCA were characterized by expansive growth into the neighborhood without cytologic atypia. HCCs were highly differentiated, showed a trabecular growth pattern with trabecules thicker than three cell

Figure 2. Histochemical and immunohistochemical alterations of liver acini downstream of transplanted islets and HCCs in the main group. A, strongly positive PAS reaction in the liver acini downstream of the transplanted islet (arrow) owing to increased glycogen storage. Arrowheads, hepatic venules at the border to the neighboring unaltered liver acini. Other altered liver acini show predominant storage of fat droplets (B). Glycogen particles and fat droplets are also visible using electron microscopy (E). Glycogenosis in altered liver acini is due to a strong decrease in the activity of glucose-6 phosphate (D, islet in the center). Increase in proliferative activity of the altered hepatocytes (and islet cells) is shown by BrdUrd immunostaining in (C): left, islet; middle, altered acinus; right third of the panel, unaltered acinus. The insulin receptor (IR) is translocated from the cell membrane into the cytoplasm of the altered glycogen-storing hepatocytes (hyperinsulinemic acinus in the left part of F) and is increased in HCC (I). CCF are defined by their clear appearance in H&E stain (G). As a consequence of insulin action, IGF-I is up-regulated (H). Signal transduction proteins IRS-1, Raf-1, and Mek-1 are up-regulated in the altered acini (I, PAS; J, M, and N) and are increased in glycogen-storing HCCs (L). Whereas all these phenomena are present a few days after transplantation, TGF-α is detectable not earlier than 3 months after transplantation, beginning in acinar zone 3, and is stronger in mixed cell foci (O). In addition to TGF-α, the placental form of glutathione S-transferase (GST-P) is also strongly increased in glycogen-poor neoplasms, such as this HCA (P and Q). Time after transplantation and length of the lower edge of the panel: A, 3 weeks, 2.2 mm; B, 3 weeks, 360 μm; C, 3 months, 720 μm; D, 2 months, 2.2 mm; E, 1 month, 10.6 μm; F, 1 month, 140 μm; G and H, 3 months, 3.6 mm; I, 18 months, 560 μm; J and K, 4 months, 2.9 mm; L (same tumor as Fig. 1H), 16 months, 1.1 mm; M and N, 1 month, 1.5 mm; O, 3 months, 900 μm; P and Q, 14 months, 1.5 mm; A, B, and D, frozen sections; E, electron micrograph of an ultrathin section; all others are paraffin sections.
biochemically characterized by alterations reflecting typical streptozotocin diabetic Lewis rats (20–22, 24). Briefly, CCF were
The results were identical in autoimmune and streptozotocin diabetic and that received a low number of islets (i.e., MG, CG 1, and CG 6). Tumorigenesis was only weak in animals of CG 1, which developed at least one hepatocellular neoplasm. Unfortunately, the metastases were not observed. Between 15 and 18 months, 100% increase in basophilic HCCs (Table 2). Vascular invasion or metastases were not observed. Between 15 and 18 months, 100% of the MG animals (6 of 6) and 83% of CG 6 animals (10 of 12) developed at least one hepatocellular neoplasm. Unfortunately, the diabetic BB/Pfd rats did not survive longer than 18 months posttransplantation as most HCCs had developed between 18 to 24 months in the Lewis rats in previous studies. Some proliferative cholangiocellular lesions and cholangiomas, but no cholangiocellular carcinomas, also developed in the draining area of the islets in the MG and were more pronounced in the CG 6.

Enzyme histochemistry and immunohistochemistry. Enzyme and immunohistochemical results are given in detail in Table 2. The results were identical in autoimmune and streptozotocin diabetic BB/Pfd rats and were in line with previous results in streptozotocin diabetic Lewis rats (20–22, 24). Briefly, CCF were biochemically characterized by alterations reflecting typical insulin effects (i.e., overexpression of the fatty acid synthase and down-regulation of the glucose-6-phophatase; Fig. 2D). The insulin receptor was translocated from the membrane into the cytoplasm (Fig. 2F), triggering increased intracellular signaling via the Ras-Raf-MAPK pathway as reflected in the overexpression of IRS-1, Raf-1, and Mek-1 (Fig. 2J, K, M, and N). Altered expressions were also found for proteins of the IGF axis (Fig. 2G and H). Clear-cell HCAs and HCCs retained many of these alterations but additionally showed a moderate expression of the IGF-1 receptor and TGF-α. Corresponding to the proportion of basophilic cells, mixed cell foci, basophilic cell foci, and basophilic tumors displayed not only a gradual normalization of the increased insulin receptor signaling but also a strong overexpression of TGF-α (Fig. 2O and P).

Islet graft morphology. Islet grafts can already be identified using a stereomicroscope (Fig. 1A and B). Histologically, they were found within terminal portal venules (Fig. 2A and 3A-D) and consisted of different types of endocrine cells, which were immunohistochemically discriminated using antibodies directed against insulin, glucagon, and somatostatin (Fig. 4E and F). They were richly vascularized and sometimes showed small ductular proliferations at the border to the neighboring hepatocytes (Fig. 4A). Some islets displayed mild lymphocytic infiltration (Fig. 3A). Distinct differences were found regarding the ultrastructure of α- and β-cells in the hyperglycemic animals but not in the normoglycemic rats. The stimulated β-cells were enlarged, showed massive hyperplasia of the rough endoplasmic reticulum and the Golgi complex, and were nearly completely degranulated (Fig. 4C). By contrast, the α-cells (and also the δ-cells) were atrophic, were reduced in number, and stored many electron-dense secretion granules (Fig. 4C). At later stages, islets were often observed in the center of small hepatocellular tumors that had originated from a downstream preneoplastic focus. In larger HCAs and in HCCs, the

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<th>Table 2. Expression of insulin-related proteins and BrdUrd index in preneoplasias and hepatocellular tumors</th>
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<td>Insulin receptor</td>
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<td>Intracytoplasmic</td>
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<td>IGF-II/M6P receptor</td>
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<td>TGF-α</td>
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<td>Glucose-6-phosphatase</td>
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<td>Fatty acid synthase*</td>
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<td>Increase of BrdUrd labeling index</td>
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NOTE: The intensity of the immunohistochemical variables in focal lesions was estimated semiquantitatively compared with unaltered liver tissue of the same sections using the following grades: [ ], strong decrease; [ ], decrease; = equal; ↑, increase; ↑↑, strong increase. If staining was not uniform in one lesion, the secondary pattern is given in parentheses.

Abbreviations: BCF, basophilic cell focus; cHCA/cHCC, clear cell HCA/clear cell HCC; bHCA/bHCC, basophilic HCA/basophilic HCC; GST-P, glutathione S-transferase (placental form); N.D., not determined.

*Data obtained from a previous study (see ref. 23).

1.X-fold increase when compared with the adjacent unaltered liver tissue.
integrity of the islets became disrupted and small clusters or single islet cells were scattered within the tumor.

In the few animals of the MG, CG 1, and CG 6, which became normoglycemic after ≥10 months posttransplantation, the overall number of islets was reduced but the remaining islets were considerably enlarged and consisted predominantly of large β-cells (Fig. 4I). In addition, we observed two and one insulinomas that had originated 15 to 18 months after transplantation from grafts in the MG and CG 6, respectively (Fig. 4K and L). No insulinomas in the pancreas have emerged. The liver tissue surrounding the insulinomas showed small areas of glycogen storage; however, owing to the systemic hypoglycemia in these animals, this was not as pronounced and is much more variable than in the CCF of hyperglycemic animals. Nevertheless, these animals had classic preneoplastic foci in other parts of the liver that were obviously progressed to a state in which they became independent from the previously important hyperglycemia. We did not observe a topographic relation between insulinomas and hepatocellular tumors.

Discussion

In this experiment, we confirmed previous findings made in streptozotocin diabetic Lewis rats (19–25), showing that low-number transplantation of pancreatic islets into the liver exerts a strong carcinogenic influence on the downstream hepatocytes in streptozotocin diabetic BB/Pfd rats (CG 6-8). This study is the first to show that the same carcinogenic process can be induced in spontaneously autoimmune diabetic BB/Pfd rats (MG and CG1), providing conclusive evidence that hepatocarcinogenesis in this model is a result of islet-derived factors and is independent from streptozotocin.

Figure 3. Histomorphology of altered liver acini, hepatocellular neoplasms, and control livers. A, in CG 2, animals stayed normoglycemic owing to the high number of transplanted islets; thus, no hepatocellular alterations emerged downstream of the islets. At the transplantation site, a few lymphocytes were often visible (top right part of the islet in A), even when the transplants were not rejected. B to F, stepwise development to a HCC in the MG, beginning with a slightly progressed CCF (B), early mixed cell focus with areas of glycogen depletion (C), early clear cell HCA (D); large clear cell HCA (E), and basophilic (glycogen-poor) HCC (F). Arrows (B-D), islet grafts belonging to the respective liver lesion. In CG 1 (G and H), the transplanted islets were rejected after low-number transplantation, but the respective hepatocellular lesions persisted; arrows, mononuclear cell infiltrate in the area of the rejected islet. On serial sections of these animals, no vital islet epithelial cells were present. G, mixed cell focus; H, a very early HCA with nodular expansive growth and mitotic figures (arrowheads). A to H, PAS reaction. Time after transplantation and length of the lower edge of the panel: A, 12 months, 1.1 mm; B, 7 months, 1.8 mm; C, 7 months, 1.5 mm; D, 14 months, 2.2 mm; E, 14 months, 720 μm; F, 18 months, 900 μm; G, 8 months 720 μm; H, 13 months, 720 μm.
The sequence of hepatocellular alterations in the BB/Pfd rats, beginning with the early formation of CCF even a few days after islet transplantation and progressing partly via mixed cell foci and basophilic cell foci to HCAs and HCCs, was qualitatively and quantitatively not different from our previous results (19–25). In the beginning, CCF were always confined to the anatomic borders of the liver acini that drain the hyperinsulinemic blood from the islet grafts. Moreover, all metabolic and morphologic alterations, without exception, are typical insulin effects, and we showed increased intracellular insulin signaling in these cells via IRS-1 and the Ras-Raf-MAPK pathway. Thus, CCF must be interpreted as adaptive alterations resulting from increased insulin action, although minor additional effects of other islet hormones cannot be ruled out. This is also corroborated by the fact that these alterations in preneoplasias and the subsequent development into hepatocellular tumors only took place in the right part of the liver in which the islet grafts intermingled with the liver parenchyma (B). Activated \( \beta \)-cells showed degranulation and hyperplasia of smooth endoplasmic reticulum and Golgi fields in electron microscopy (C). Note the atrophy of the \( \alpha \)-cell, packed with electron-dense secret granules (top right part of C). D to F, the same lesion showing an islet graft surrounded by a CCF of altered hepatocytes (E and F, insulin immunostain). In CG 1 (G and I) and CG 3 (H), mononuclear inflammatory infiltrates and rejection of the islets were visible. \( l \), an insulin immunostain showing a few \( \beta \)-cells during rejection. \( l \), a fibrotic scar marks the former place of a rejected islet in the center of a glycogenotic CCF (note strong PAS reactivity in the surrounding hepatocytes); thus, this CCF persists without depending on continuing insulin action. Insulinomas occurred only in MG and CG 6. The \( \beta \)-cells of the transplanted islets in these groups proliferated until the animal became normoglycemic at 10 months or later. Then, single large islets were visible (J). In single animals, these large islets continued to proliferate and secrete insulin, resulting in the formation of insulinomas and severe hypoglycemia (K and L show the same insulinoma). A, B, and G, semithin sections stained according to Richardson; \( C \), electron micrograph of an ultrathin section; \( D \) and \( K \), unstained liver slices; \( E \), \( F \), \( H \), and \( L \), insulin immunostain; I and J, PAS stain. Time after transplantation and length of the lower edge of the panel: A, 13 months, 360 \( \mu \)m; B, 9 months, 360 \( \mu \)m; C, 5 months, 9.6 \( \mu \)m; D-F (same lesion), 11 months, \( D \), 3.6 mm; \( E \), 2.9 mm; \( F \), 570 \( \mu \)m; G, 8 months, 360 \( \mu \)m; H, 1 month, 290 \( \mu \)m; I, 13 months, 360 \( \mu \)m; J, 13 months, 1.1 mm; K and L (same lesion), 14 months, K, 14.5 mm, L, 1.1 mm.

Figure 4. Morphology of transplanted islets (A-F), islet grafts during rejection (G-I), and development of insulinomas (J-L). After high-number islet transplantation and resulting normoglycemia, the transplants remained more compact (CG2 in A; CG7 not shown), whereas after low-number transplantation (MG in B-F) the islet cells intermingled with the liver parenchyma (B). Activated \( \beta \)-cells showed degranulation and hyperplasia of smooth endoplasmic reticulum and Golgi fields in electron microscopy (C). Note the atrophy of the \( \alpha \)-cell, packed with electron-dense secret granules (top right part of C). D to F, the same lesion showing an islet graft surrounded by a CCF of altered hepatocytes (E and F, insulin immunostain). In CG 1 (G and I) and CG 3 (H), mononuclear inflammatory infiltrates and rejection of the islets were visible. \( l \), an insulin immunostain showing a few \( \beta \)-cells during rejection. \( l \), a fibrotic scar marks the former place of a rejected islet in the center of a glycogenotic CCF (note strong PAS reactivity in the surrounding hepatocytes); thus, this CCF persists without depending on continuing insulin action. Insulinomas occurred only in MG and CG 6. The \( \beta \)-cells of the transplanted islets in these groups proliferated until the animal became normoglycemic at 10 months or later. Then, single large islets were visible (J). In single animals, these large islets continued to proliferate and secrete insulin, resulting in the formation of insulinomas and severe hypoglycemia (K and L show the same insulinoma). A, B, and G, semithin sections stained according to Richardson; \( C \), electron micrograph of an ultrathin section; \( D \) and \( K \), unstained liver slices; \( E \), \( F \), \( H \), and \( L \), insulin immunostain; I and J, PAS stain. Time after transplantation and length of the lower edge of the panel: A, 13 months, 360 \( \mu \)m; B, 9 months, 360 \( \mu \)m; C, 5 months, 9.6 \( \mu \)m; D-F (same lesion), 11 months, \( D \), 3.6 mm; \( E \), 2.9 mm; \( F \), 570 \( \mu \)m; G, 8 months, 360 \( \mu \)m; H, 1 month, 290 \( \mu \)m; I, 13 months, 360 \( \mu \)m; J, 13 months, 1.1 mm; K and L (same lesion), 14 months, K, 14.5 mm, L, 1.1 mm.
grafs were transplanted. Similar to streptozotocin diabetic Lewis rats, preneoplastic CCF virtually did not develop in animals after high-number islet transplantation, which fully compensates the diabetic state and establishes normoglycemia, illustrating that hyperglycemia is also of relevance for tumor development. However, even high-number islet transplantation in conjunction with normoglycemia has at least cocarcinogenic potential, as it strongly promotes hepatocarcinogenesis in Lewis rats, initiated by administration of the hepatocarcinogen N-nitrosomorpholine (26), indicating a dose-dependent effect of insulin and glucose levels. Preneoplastic foci did not regress in late-stage animals of the MG or CG 6 that became normoglycemic or even hypoglycemic owing to excessive insulin production by hyperplastic transplants or graft insulinomas, which corroborates similar observations formerly made in Lewis rats (45). However, a new and interesting finding was that CCF also did not regress in animals of CG 1, which showed a rejection of islet grafts after several months of tolerance and local hyperinsulinism (Fig. 11 and 41). These observations clearly indicate that at this time point, the primary adaptive nature of these CCF has already changed and that neoplastic transformation no longer depends on insulin action. In this context, the overexpression of other tumorigenic growth factors or their receptors, such as TGF-α, which has been shown to promote hepatocarcinogenesis in transgenic mice (46, 47), in the late stage lesions and neoplasms of the present model is interesting. The point of transformation of the purely adaptive alterations of the liver acini into genetically or, probably initially more likely, epigenetically fixed preneoplasias that did no longer spontaneously regress must lie between 3 and 12 months after transplantation. The clarification of this important biological alteration and the underlying mechanisms is one of the most interesting aims for future studies.

To the best of our knowledge, no detailed histopathologic studies of human recipient livers in clinical islet transplantation have been done nor has the occurrence of hepatocellular neoplasms been reported. However, Hirshberg et al. (48) conducted a histopathologic study of livers in a nonhuman primate model and showed glycogenic CCF, which only developed in one single animal that was insufficiently treated by a too low number of functioning islet grafts and that stayed hyperglycemic. Histologic reports in humans generally deal with the islet graft morphology and do not describe the liver morphology in detail (8). However, there are a few recent single case reports or small series of cases that describe focal, mostly steatotic, or glyco-genic alterations in the livers of islet transplant recipients that are strikingly similar to our observations (5–7, 49). The macroscopic descriptions and the histopathologic depictions of these alterations, as well as the clinical data (recurrence of hyperglycemia, high fasting glucose levels) in these patients, are virtually identical to the alterations seen in our rats, illustrating obvious similarities in the metabolic situation and its influence on the hepatocytes in our model and in a group of clinically transplanted patients.

The increased incidence of HCC in human diabetic patients reported in epidemiologic and case-control studies is not well understood. We suggest, although not always being clearly stated, that these patients are suffering from type 2 diabetes mellitus, which is usually characterized by hyperglycemia and hyper-insulinemia. On the one hand, the metabolic situation in these patients is similar to that in the altered liver acini of our model and some researchers have indeed proposed that insulin and glucose may directly be involved in the carcinogenic process in humans (11, 14). On the other hand, the occurrence of hepatocellular CCF in human livers that resemble preneoplastic CCF known from a variety of animal models has been shown (50), and even indications for their involvement in human hepatocarcinogenesis have been found (51). Therefore, our results may help to understand how the combination of insulin action with the diabetic state can alter the expression of growth factors and their receptors, intracellular signaling, enzyme activities, morphology, and proliferative activity of hepatocytes, thus inducing and/or promoting hepatocarcinogenesis. In addition, they may also help to explain the increase in HCC incidence in human type 2 diabetic patients and warrant a careful observation of liver alterations in patients having undergone clinical islet transplantation.

**Acknowledgments**

Received 8/5/2005; revised 10/14/2005; accepted 11/16/2005.

**Grant support:** Deutsche Forschungsgemeinschaft (German Research Foundation) grant Do622/1-5.

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We thank Gabriele Becker, Jörg Bedorf, Danuta Chmihok, Mariana Dombrowski, Mathilde Hau-Liersch, Regine Landeck, and Claudia Miethke for technical assistance; Yvonne Fischer and Kurt Büdel for animal care; and Bernd Wüsthoff for editing the manuscript.

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