American Journal of Pathology, Vol. 150, No. 3, March 1997

Hepatocellular Neoplasms Induced by Low-Number Pancreatic Islet Transplants in Streptozotocin Diabetic Rats

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We have previously demonstrated in short-term experiments that altered hepatocytes in liver acini draining the blood from intraportally transplanted pancreatic islets in streptozotocin-induced diabetic rats with mild persisting diabetes resemble those in preneoplastic foci of altered hepatocytes. We now present the results of long-term studies (up to 22 months) in this animal model. Glycogen-storing foci (which were the first parenchymal alteration observed some days after transplantation) persisted at least for 6 months, when the first mixed-cell foci and the first hepatocellular adenoma emerged. After 15 to 22 months, 86% of the animals exhibited at least one hepatocellular adenoma and four animals (19%) showed a hepatocellular carcinoma. The transplants were found in a close spatial relationship with the preneoplastic foci and the hepatocellular neoplasms. The mitotic indices, the 5-bromo-2'-desoxyuridine labeling indices and the apoptotic indices showed significant differences between the unaltered liver parenchyma, different types of preneoplastic foci, and hepatocellular neoplasms. The immunohistochemical expression of transforming growth factor-α increased during the stepwise development from glycogen-storing liver acini to hepatocellular carcinomas. Hepatocarcinogenesis in this new animal model is probably due to the hormonal and growth-stimulating effects of insulin secreted by the intraportally transplanted islets of Langerhans in diabetic rats. (Am J Pathol 1997, 150:1071–1087)

Transplantation of islets of Langerhans *via* the portal veins into the livers of diabetic individuals has been studied in many trials during the last 23 years.[1–8] The clinical aim of such a treatment is to restore a regulated insulin delivery. In a recently described model of low-number islet transplantation into the livers of streptozotocin-induced diabetic rats, the animals persisted in a mild diabetic state and the transplanted islets showed morphological signs of increased insulin synthesis and secretion due to the persisting hyperglycemia.[7] In this model, alterations of the liver acini draining the blood from the islet grafts were induced, which resembled early preneoplastic parenchymal liver lesions in 1) their focal character, 2) their altered enzymic pattern, 3) their alterations in glycogen content, and 4) their hyperproliferative state and increased apoptosis.[7,9] The observation period in the low-number islet transplantation model had been only 21 days. In long-term experiments, we have now investigated whether or not the altered liver acini do actually represent preneoplastic lesions and might proceed to hepatocellular neoplasms.

Materials and Methods

Animals

Highly inbred male Lewis rats (from Harlan and Winkelmann, Borchen, Germany) weighing 250 to 300 g at the beginning of the experiment were made diabetic by a single intravenous dose of 80 mg of streptozotocin per kg body weight. Diabetes was defined by a nonfasting blood glucose level greater

Dedicated to Hans-Werner Altmann on the occasion of his 80th birthday.

Supported by the BONFOR-program (grant 158/01 BONFOR).

Accepted for publication November 18, 1996.

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Table 1. *Animal Data*

Number	Month	Diabetic or Normoglycemic	Types of foci	HCA	HCC	RCC	Others
Main group							
1	1	d	GSF	–	–	–	–
2	1	d	GSF	–	–	–	–
3	1	d	GSF	–	–	–	–
4	1	d	GSF	–	–	–	–
5	1	d	GSF	–	–	–	–
6	1	d	GSF	–	–	–	–
7	2	d	GSF	–	–	–	–
8	3	d	GSF	–	–	–	–
9	3	d	GSF	–	–	–	–
10	3	d	GSF	–	–	–	–
11	3	d	GSF	–	–	–	–
12	4	d	GSF	–	–	–	–
13	4	d	GSF	–	–	–	–
14	4	d	GSF	–	–	–	–
15	6	d	MCF, GSF	1	–	–	Lymphoma
16	7	d	MCF, GSF	1	–	–	Megacolon
17	8	d	MCF, GSF, BCF	2	–	X	–
18	10	d	GSF, MCF	–	–	X	Dental deviation
19	11	d	MCF	–	–	X	Megacolon
20	11	d	MCF, GSF	1	–	–	Lymphoma
21	11	d	MCF	–	–	–	Megacolon
22	13	d	MCF, GSF	2	–	X	–
23	13	n (10 m)	MCF, GSF, BCF	–	–	X	–
24	13	d	MCF, GSF, BCF	2	–	X	–
25	14	d	MCF	–	–	X	–
26	15	d	MCF, GSF	–	–	X	–
27	15	d	MCF, GSF	2	–	X	–
28	16	d	GSF, MCF, BCF	1	–	–	Sarcoma of the urinary bladder
29	16	d	MCF	–	–	X	–
30	17	d	MCF, GSF, BCF	–	–	X	–
31	17	d	MCF, BCF, TCF	1	–	X	Sarcoma of the cecum
32	17	n (16 m)	MCF, BCF, TCF	1	–	X	–
33	17	d	MCF, BCF, TCF	1	–	X	–
34	18	d	MCF, BCF	3	–	X	–
35	19	n (15 m)	MCF	2	–	X	–
36	19	n (12 m)	BCF, MCF, GSF	1	1	X	–
37	19	n (18 m)	MCF, BCF, GSF	1	–	X	Sarcoma of the neck
38	19	d	MCF, BCF	2	1	X	–
39	19	d	MCF, BCF	2	1	X	–
40	20	n (17 m)	MCF, BCF, GSF	1	–	X	–
41	20	d	MCF, GSF, BCF	1	–	X	–
42	21	d	MCF, BCF	1	–	X	–
43	21	n (16 m)	MCF	1	–	X	–
44	22	n (13 m)	MCF	1	–	X	–
45	22	d	MCF, BCF	3	–	X	–
46	22	d	MCF	1	1	X	–
Control group I							
1	1	n	–	–	–	–	–
2	3	n	–	–	–	–	–
3	4	n	–	–	–	–	–
4	8	n	–	–	–	–	–
5	11	n	–	–	–	–	–
6	13	n	–	–	–	X	–
7	13	n	–	–	–	X	–
8	13	n	–	–	–	X	–
9	15	n	–	–	–	X	–
10	16	n	–	–	–	X	–
11	19	n	GSF	–	–	X	–
12	20	n	GSF	–	–	X	–
13	20	n	–	–	–	X	–
14	22	n	–	–	–	X	–
15	22	n	–	–	–	X	–
16	22	n	GSF	–	–	X	–
17	22	n	–	–	–	X	–

Table 1. *Continued*

Number	Month	Diabetic or Normoglycemic	Types of foci	HCA	HCC	RCC	Others
Control group II							
1	1	d	–	–	–	–	–
2	3	d	–	–	–	–	Megacolon
3	4	d	–	–	–	–	Megacolon
4	8	d	–	–	–	–	Megacolon
5	8	d	–	–	–	–	Megacolon
6	11	d	–	–	–	X	Megacolon, dental deviation
7	13	d	–	–	–	X	Megacolon
8	14	d	–	–	–	X	General weakness
9	15	d	–	–	–	X	Dental deviation
10	15	d	–	–	–	X	General weakness
Control group III							
1	3	n	–	–	–	–	–
2	7	n	–	–	–	–	–
3	11	n	–	–	–	–	–
4	13	n	–	–	–	–	–
5	16	n	–	–	–	–	–
6	19	n	GSF	–	–	–	–
7	19	n	–	–	–	–	–
8	20	n	–	–	–	–	–
9	22	n	–	–	–	–	Emphysema of the lung
10	22	n	GSF	–	–	–	–

Month indicates the month after islet transplantation when the animal died or was killed. It is indicated whether the animal was normoglycemic (n, blood glucose < 150 mg/dl) or diabetic (d, blood glucose > 150 mg/dl) after islet transplantation. Some animals from the main group became normoglycemic during the experiment. They are indicated with the number of the month (m) when they became normoglycemic. GSF, glycogen-storing foci (clear-cell foci/acini and X-cell acini/foci together); MCF, mixed-cell foci; TCF, tigroid-cell foci; BCF, homogeneously basophilic-cell foci. The relative frequency of types of foci/acini is indicated; the most frequent type is listed first. The numbers of HCAs and HCCs of the respective livers are shown. The presence of a renal cell carcinoma (RCC) is indicated by X.

than 400 mg/dl, which was reached 1 to 3 days after streptozotocin injection. The body weight and capillary blood glucose (tip of the tail) were measured (Haemo-Glucotest 1–44 R and Reflolux II, Boehringer-Mannheim, Mannheim, Germany) daily (between 0800 and 1000 hours) for 1 week after transplantation, then weekly for the first 3 months, and thereafter once a month and immediately before sacrifice. Housing and treatment of the animals were in line with the guidelines of the Society for Laboratory Animal Service (GV-SOLAS) and strict German animal protection law.

Main Group

Isologous islet transplantation of 250 to 450 islets *via* the portal vein was carried out with 47 animals as described earlier.[7] We chose a smaller mass of islet tissue compared with the previous experiments of low-number islet transplantation,[7,9] because in preliminary experiments the animals became normoglycemic some weeks after islet transplantation when the number of islets was 500 or higher.

Thirty-five animals were killed at different time intervals after islet transplantation (Table 1). They were perfused with 0.2% glutaraldehyde and 3% paraformaldehyde as described earlier.[7] Twelve animals died spontaneously due to different causes (see Results). The tissue of these animals was fixed for 24 hours by immersion in buffered 3% formaldehyde, freshly prepared from paraformaldehyde.

The islets of the main group and the control group I (see below) were transplanted only into the right part of the liver as follows. Before infusion of the islets into the portal vein, the branch of the portal vein that supplies the left lobe and the left part of the middle lobe was occluded by a vessel clamp. After infusion of the islets into the portal vein, the clamp was removed (maximal time of ischemia, 1 minute). With this procedure, it was possible to infuse the islet transplants only into the right part of the liver, ie, the right lobe, the caudal lobes, and the right part of the middle lobe (the border of the right part and the left part of the middle lobe is marked by the falciform ligament). Thus, the left part of the liver, ie, the left part of the middle lobe and the left lobe, could be taken as an internal control in the experiments of the main group and the control group I.

Control Groups

Seventeen diabetic animals received 1000 to 2000 islets of Langerhans (control group I), which was expected to result in persisting normoglycemia. In our earlier experiments, no altered liver acini emerged under these conditions.[7]

Ten diabetic animals had a sham operation (control group II; infusion of 1 ml of Hanks' solution instead of islets).

Ten nondiabetic animals remained completely untreated (control group III).

5-Bromo-2'-Desoxyuridine (BrdU) Application According to Eldridge et al[10]

Six days before killing, sixteen animals of the main group were anesthetized as described above, and osmotic minipumps (Alzet model 2ML1, Alza Corp., Palo Alto, CA, filled with 40 mg of BrdU, Sigma, Heidelberg, Germany) were surgically implanted subcutaneously over the dorsal thoracolumbal area. Seventeen animals of the main group received a single dose of 50 mg/kg body weight BrdU intraperitoneally 1 hour before sacrifice.

Tissue Preparation

Sections (0.5 to 1.0 mm thick) of the fixed livers were examined under water with a stereomicroscope. Appropriate specimens from unaltered liver tissue, from focally altered liver tissue, from conspicuous foci, and from all liver tumors (see Results) were processed for electron microscopy as described earlier.[7] The remaining liver sections and additional tissue samples from the heart, lung, both kidneys, adrenals, small intestine, colon, pancreas, spleen, muscle, and skin were embedded in paraffin. From the liver specimen, serial sections of 2 to 3 μm thickness were stained with hematoxylin and eosin (H&E) and with the periodic acid-Schiff (PAS) reaction. Additional sections were made for immunohistochemistry. Specimens from the other organs were stained with H&E.

Immunohistochemistry

After examination of the H&E and the PAS stains, appropriate sections were selected and the corresponding sections were processed for immunohistochemistry. Endogenous peroxidase was inhibited with 1% hydrogen peroxide. Polyclonal anti-insulin, anti-glucagon, and anti-somatostatin antibodies (DAKO, Hamburg, Germany) were diluted 1:200 and incubated for 20 hours at 4°C. Antigen retrieval for the detection of transforming growth factor (TGF)-α was performed by cooking the deparaffinized slides in a citrate buffer (pH 6.0) for 30 minutes at 750 W. Monoclonal anti-TGF-α antibody (Oncogene Sciences, Cambridge, MA) was used in a final concentration of 10 μg/ml. Nonspecific binding was eliminated and antibody detection was done with the LSAB kit and the aminoethylcarbazole substrate (DAKO). Slides were rinsed with Tris buffer (0.05 mol/L Tris/aminomethane in 0.9% NaCl, pH 7.6) between all incubations. Tissue sections were counterstained with hematoxylin and coverslipped with Aquatex (Merck, Darmstadt, Germany).

BrdU immunohistochemistry (monoclonal antibody from DAKO) was performed as described earlier.[7]

Classification of Acini/Foci of Altered Hepatocytes and Hepatocellular Neoplasms

Foci of altered hepatocytes (which in early stages were confined to liver acini) were classified according to Bannasch and Zerban[11] into glycogen-storing foci, mixed-cell foci, acidophilic-cell foci, tigroid-cell foci, and homogeneous basophilic-cell foci. Glycogen-storing foci were subdivided into clear-cell foci and X-cell foci.[12,13] Hepatocellular adenomas (HCAs) were diagnosed when the lesions were sharply limited and compressed the surrounding liver parenchyma. Hepatocellular carcinomas (HCCs) were diagnosed when the lesion exhibited trabeculae thicker than three cell layers in at least two separate areas, showed high numbers of mitotic figures, and had a diameter larger than 5 mm (according to Metzger et al[14]). Unequivocal evidence of malignancy such as metastases or vascular invasion has not been observed up to the present.

Determination of Mitotic, Apoptotic, and BrdU Labeling Indices and Statistical Analysis

The mitotic indices (MI) and the apoptotic indices (AI) as well as the 6-day and the 1-hour BrdU labeling indices (BrdU-LI 6d and BrdU-LI 1h) of the hepatocytes in altered liver acini and altered foci of hepatocytes as well as in hepatocellular neoplasms and the extrafocal liver tissue of the animals of the main group were determined as described in our short-term study.[7] The MI, AI, BrdU-LI 6d, and BrdU-LI 1h of the different types of foci, neoplasms, and the extrafocal tissue were compared with the Wilcoxon-Mann-Whitney test. Significance was accepted when $P < 0.01$.

Results

Blood Glucose and Weight Gain

In line with our expectations, all animals from the main group showed a mild diabetic state after islet transplantation. The mean level of blood glucose was approximately 270 mg/dl. At the end of the experiment, some animals from the main group slowly became normoglycemic, which was defined as a blood glucose level below 150 mg/dl (Figure 1a). The diabetic or normoglycemic status at death is indicated in Table 1. The animals from control group I became and stayed normoglycemic, whereas the animals from control group II stayed hyperglycemic and the animals from the untreated control group III stayed normoglycemic. Weight gain (Figure 1b) from the main group rats was meager as a consequence of persistent mild diabetes. Between 4 and 19 months after islet transplantation, the mean body weight of the animals from the main group was nearly constant. With the exception of the first few days after transplantation, body weight was lower in the main group than in control groups I and III and higher than in control group II. Signs of the diabetic state (ie, polydypsia, polyphagia, polyuria, and diarrhea) were improved in the main group compared with control group II. Control group I animals and the untreated rats (control group III) did not show any signs of diabetes during the long-term observation period.

Intercurrent Diseases and Causes of Spontaneous Deaths

Some of the diabetic animals (main group and control group II) showed complications of diabetes, ie, abscesses, obstipation, and megacolon (Table 1). In control group II, no animal survived more than 15 months due to severe diabetic complications, most often megacolon (Table 1). The most frequent cause of spontaneous death and sacrifice in the main group and in control group I was renal cell carcinoma. These carcinomas were up to 4 cm in size and exhibited a basophilic papillary and sometimes cystic histological pattern. When these carcinomas were complicated with macrohematuria, the animals were killed and perfused. Intraperitoneal bleeding of kidney tumors was the most frequent cause of spontaneous death. Metastases were not seen, even in the large carcinomas. In three animals of the main group, sarcomas were observed (neck, urinary bladder, and cecum). It should be mentioned that three animals that received a high number of pancreatic islets (and were consecutively normoglycemic) also exhibited sarcomas of the skin. These neoplasms were resected in early tumor stages and the animals are still alive and are not shown in this study. Two animals from the main group exhibited non-Hodgkin lymphomas. One animal from the main group suffered from a 3-cm chronical fungal abscess in the left liver lobe 9 months after islet transplantation. The multiple hepatocellular neoplasms in the right part of the liver of this animal were excluded from the study, because there might have been co-carcinogenic effects of fungal products or the chronic inflammation *per se* and, therefore, this animal is not shown in Table 1. One animal from the main group and two animals from control group II showed dental deviation of the rodents and therefore had to be killed. In the untreated control group, only one animal showed severe illness (emphysema and chronic pneumonia) at the age of 2 years.

Alterations of the bile duct epithelia were often seen after long lag periods after low-number islet transplantation in the right part of the liver. These

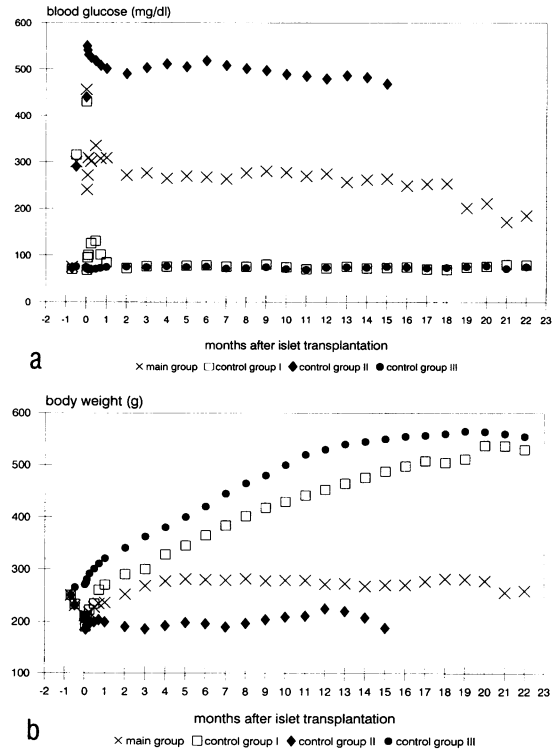


Figure 1. *Mean values of blood glucose* (a) *and body weight* (b). *Forty-six animals that received a small mass of islets (main group) returned to a mild diabetic state. Seventeen animals that received a high mass of islet grafts (control group I) became normoglycemic after islet transplantation, as did ten completely untreated animals (control group III). Ten animals that had a sham operation (control group II) stayed diabetic. Animals from the main group and control group II had a meager weight gain.*

Table 2. *Mitotic Index, Apoptotic Index, and BrdU Labeling Indices (1-Hour Pulse Labeling and 6-Day Continuous Administration by Osmotic Pumps) of the Animals from the Main Group (Low-Number Islet Transplantation*

	MI (n)	AI (n)	BrdU-LI 1h (n)	BrdU-LI 6d (n)
CCF	3.5 ± 0.9 (10)	4.6 ± 1.1 (10)	9.7 ± 1.6 (10)	278.0 ± 64.4 (6)
XCF	0.4 ± 0.2 (19)	1.4 ± 1.0 (19)	1.5 ± 0.4 (5)	60.4 ± 12.7 (9)
MCF	1.0 ± 0.4 (30)	0.5 ± 0.3 (30)	5.8 ± 1.7 (9)	221.5 ± 35.9 (8)
BCF	2.2 ± 0.6 (16)	0.2 ± 0.1 (16)	6.8 ± 1.3 (4)	269.3 ± 36.3 (5)
HCA	4.1 ± 0.6 (22)	1.0 ± 0.3 (22)	13.5 ± 4.5 (8)	344.1 ± 23.2 (4)
HCC	6.1 ± 1.2 (4)	1.0 ± 0.4 (4)	16.4 (1)	
Extrafocal tissue	0.1 ± 0.04 (46)	0.1 ± 0.08 (46)	0.2 ± 0.1 (17)	2.7 ± 1.9 (16)

Mean values ± SEM are shown. The number of animals is shown in parentheses. Indices are the number of events per 100 hepatocytic nuclei. CCF, clear-cell foci; XCF, X-cell foci; MCF, mixed-cell foci; BCF, homogeneous basophilic-cell foci.

(cystic) alterations could be clearly distinguished from the hepatocytic alterations. They will be described in detail in another paper.

Control Livers

At early stages, the livers of control animals and the left part of the livers of the animals from the main group did not show any preneoplastic or neoplastic lesions. At 19 to 24 months, small glycogen-storing foci were rarely observed. Hepatocellular neoplasms were never found.

Macroscopy and Stereomicroscopy of the Unstained Liver Sections

Except for these very few foci in controls, altered liver acini and foci of altered hepatocytes were detected in the main group only in the right side of the middle lobe and the right lobe of the recipient livers. Up to 6 months after islet transplantation, yellow-white liver acini draining the blood from the transplanted islets could be identified under the stereomicroscope. These altered liver acini were sharply limited at the hepatic venules during the first 3 months after islet transplantation (Figure 2a). Thereafter, the acinar architecture of the focally altered parenchyma was disturbed as follows. In some foci, the altered hepatocytes increased in size (Figure 2b) and expanded around the hepatic venules (Figure 2c), and more and more lesions exhibited irregular shape (Figure 2, d–e) and inhomogeneous color. After 6 months, expanding nodules were observed (Figure 2, f–h). Four tumors of 6 to 10 mm diameter (Figure 2i) emerged in four animals 19 months and 22 months after low-number islet transplantation (main group).

Light and Electron Microscopy of the Focal Liver Alterations (Main Group)

With the H&E stain and the PAS reaction, it was easy to identify altered liver acini draining the blood from the transplanted islets of Langerhans at every stage of the experiment in the main group. During the first 2 months of the experiment, the altered liver acini did not differ from the alterations described earlier.[7] By electron microscopic examination, it was evident that the enlarged hepatocytes within the PAS-positive altered liver acini stored abundant glycogen α-particles (Figure 3a). MI, AI and BrdU-LIs were significantly higher in the glycogen-storing foci than in the extrafocal liver tissue (Table 2).

At the end of the 3rd month after islet transplantation, the hepatocytes of many altered liver acini showed less intense glycogen storage, partly associated with smooth endoplasmic reticulum and larger fields of rough endoplasmic reticulum, and a smaller size of the hepatocytes than in the typical glycogen-storing foci (Figure 3b). These altered liver acini were classified as X-cell foci. Within these foci, some hepatocytic nuclei exhibited cytoplasmic inclusions (Figure 3b, inset), which had not been observed in the short-term studies[7,9] or in the unaltered hepatocytes of the same livers. The MI, AI, and BrdU-LI in the X-cell foci were significantly lower than in the more intense glycogen-storing foci but were, nevertheless, significantly higher than in the extrafocal liver tissue (Table 2).

By 6 months after islet transplantation, more and more basophilic, glycogen-poor hepatocytes began to emerge within the glycogen-storing and X-cell foci. By electron microscopic examination of mixed-cell foci, glycogen-rich and glycogen-poor hepatocytes were often seen with a close spatial relationship (Figure 3c). The MI, AI, and BrdU-LI were significantly higher than in the extrafocal tissue. Although the MI and the BrdU-LI were significantly increased in the mixed-cell foci compared with the X-cell foci, the AI was significantly lower in the mixed-cell foci than in the X-cell foci (Table 2). Rarely, acidophilic cell foci were seen in the neighborhood of the transplanted islets of the animals from the main group during the 2nd year after islet transplantation. The acidophilic hepatocytes showed

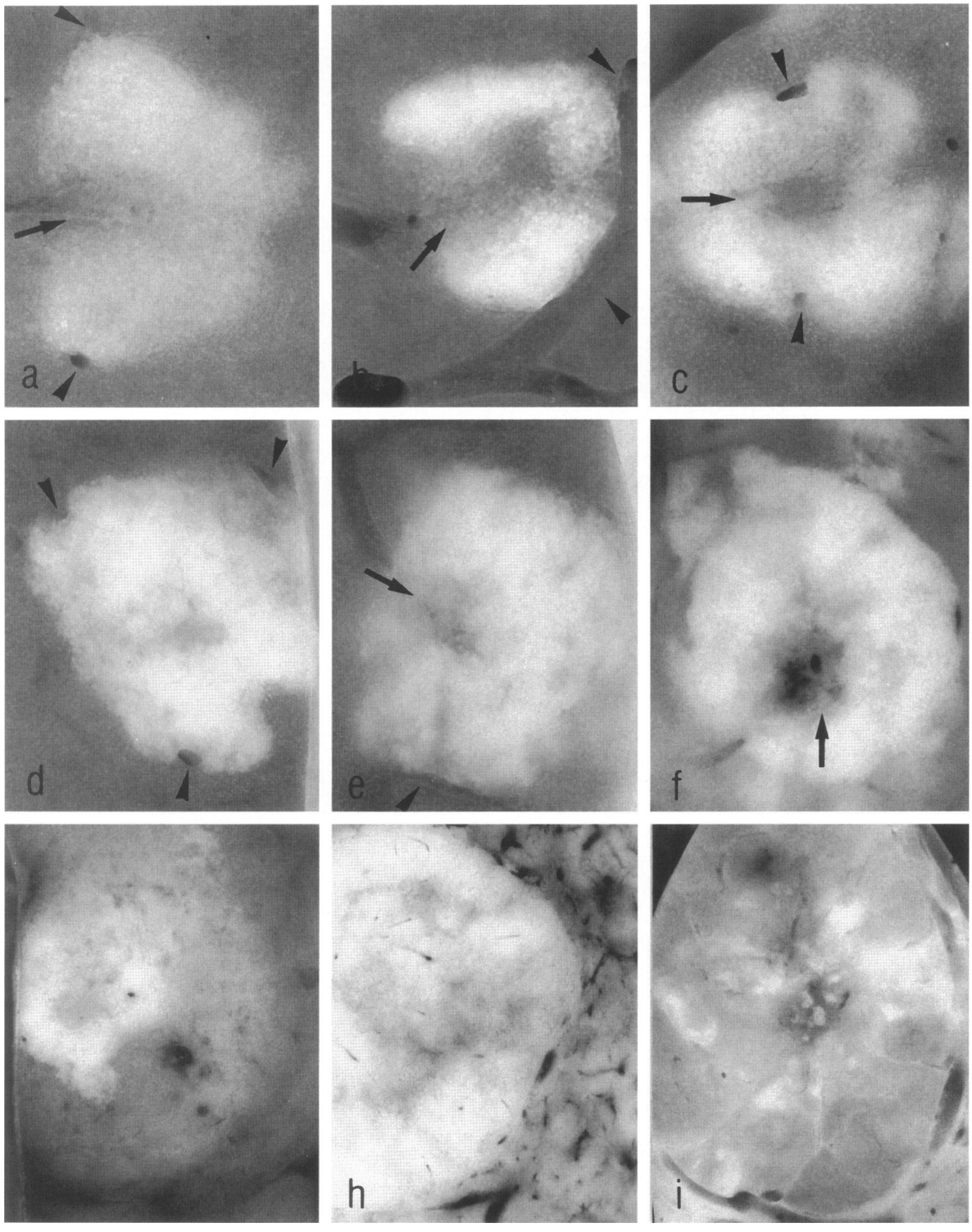
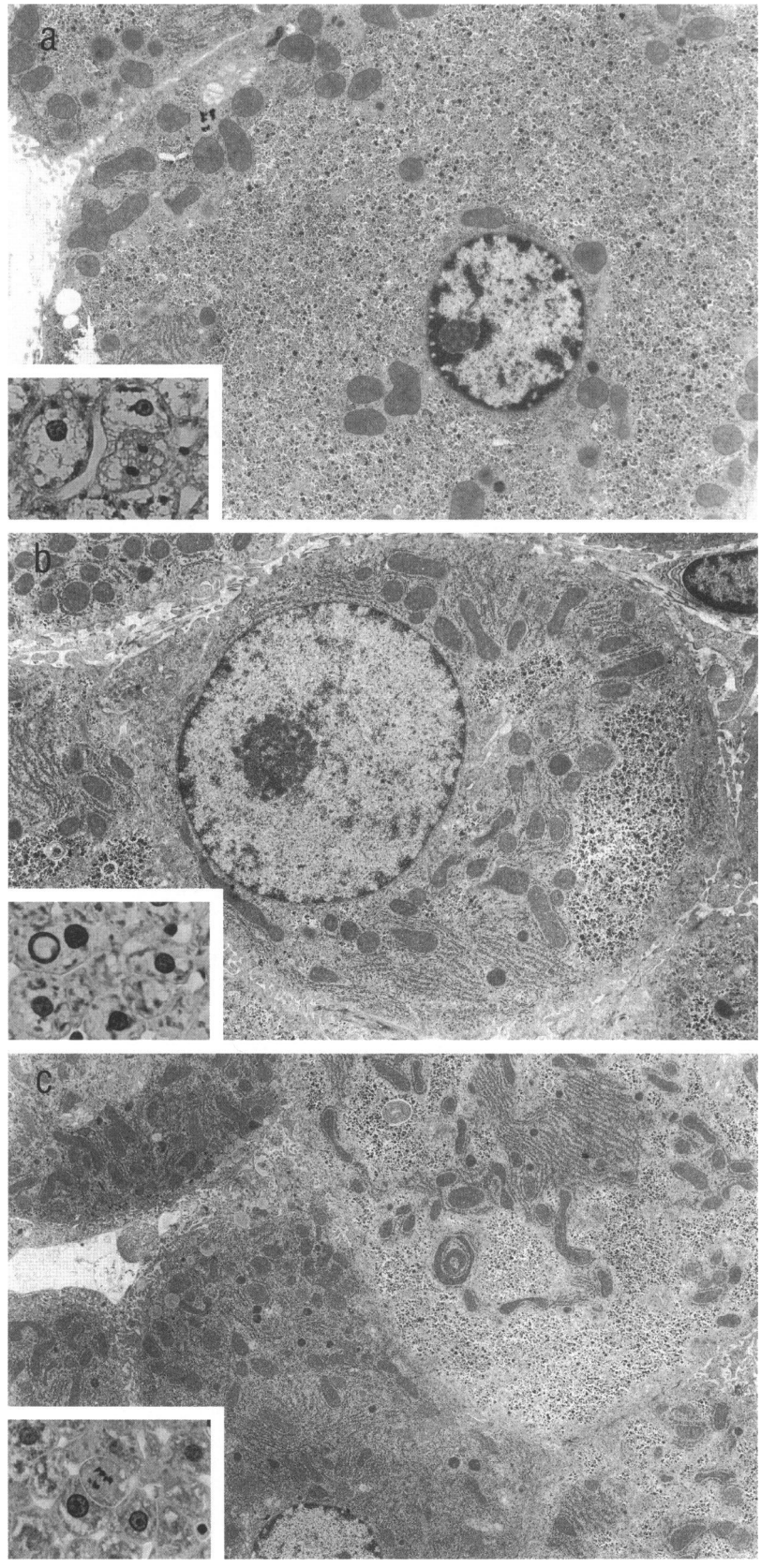


Figure 2. *Stereomicroscopic aspects of unstained liver slices of the main group show the stepwise development from altered liver acini* (a) *to hepatocellular carcinoma* (i). *Transplanted islets are indicated by arrows in* a *to* c, e, *and* f. *Hepatic venules (central venules) are indicated by* arrowheads *in* a *to* e. *During the first 3 months after islet transplantation, the focal liver alterations had exclusively the shape of liver acini* (a *and* b). *After 3 months, the acinar borders were exceeded by the growing foci* (c). *After 6 months, the shape of the foci became irregular and the color inhomogeneous, representing mixed cell foci* (d *and* e) *as demonstrated by comparative histological investigations. Nodules appeared for the first time after 6 months but were mainly observed during the second year* (f *to* i), *being much larger than the acini and foci and showing an expansive growth.* f *to* h: *Hepatocellular adenomas.* i: *Hepatocellular carcinoma of animal 36 (Table 1). The increasing size of the lesions is shown by the decrease in the magnifications used:* ×*50* (a), ×*44* (b), ×*34* (c), ×*30* (d), ×*28* (e), ×*18* (f), ×*14* (g), ×*12* (h), *and* ×*7.5* (i).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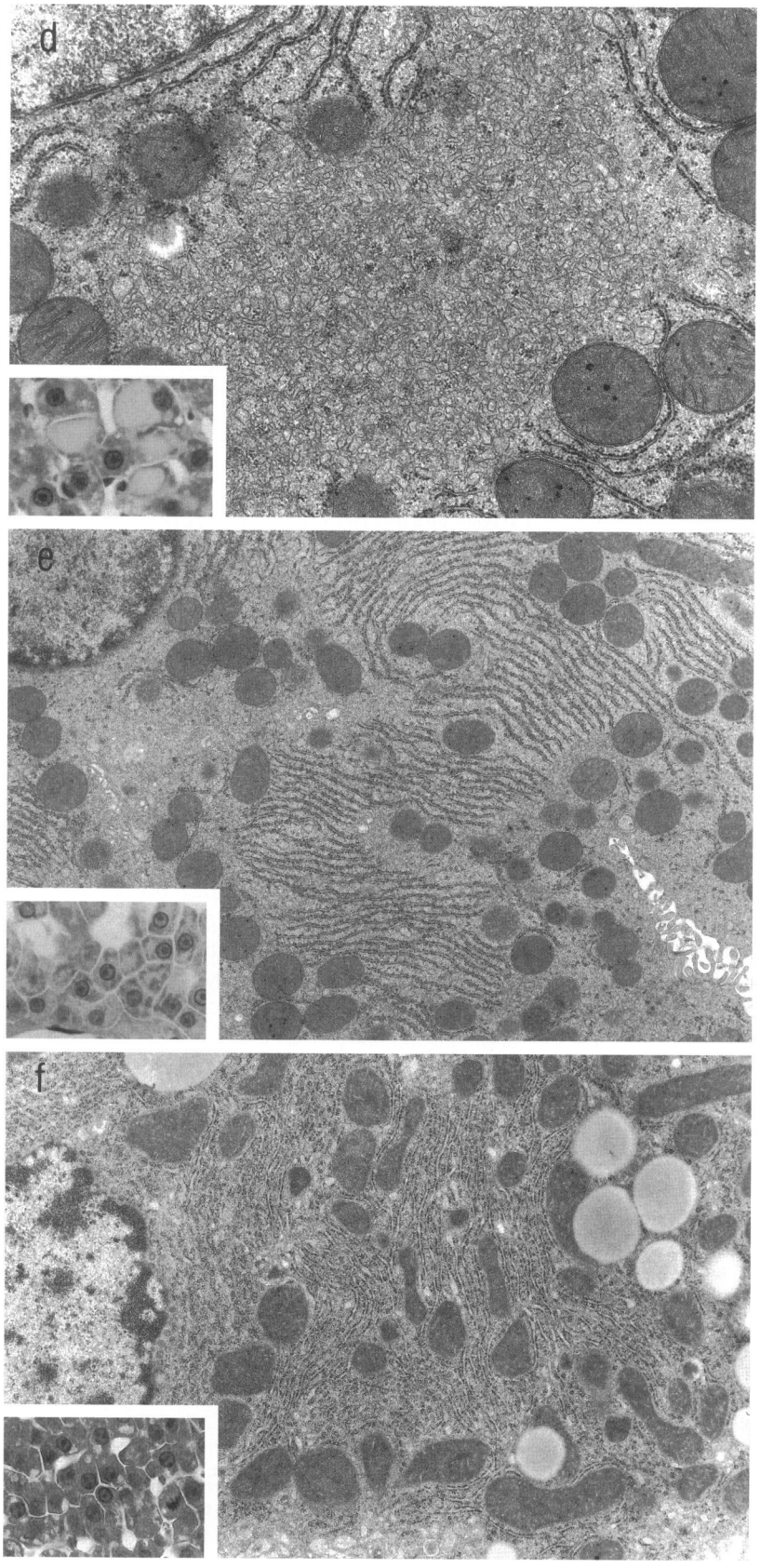

Figure 3. *Light microscopic* (insets) *and electron microscopic aspects of cells characterizing the different types of foci of altered hepatocytes during hepatocarcinogenesis after low-number islet transplantation (main group).* a: *In early glycogen-storing foci/acini, the enlarged hepatocytes contain abundant glycogen α-particles.* b: *When the glycogen storage of the hepatocytes was less intense than shown in* a, *the glycogen-storing foci were classified as X-cell foci, which appeared 3 months after islet transplantation. The hepatocytic nuclei of the X-cell foci often showed cytoplasmic inclusions* (inset). c: *Six months after islet transplantation, more and more basophilic, glycogen-poor hepatocytes* (left) *replaced the glycogen-storing hepatocytes* (right) *and built mixed-cell foci.* d: *Acidophilic cells, which show large areas of smooth endoplasmic reticulum, were seldom seen.* e: *Some foci of altered hepatocytes during the 2nd year after islet transplantation showed a striped (tigroid) basophilia* (inset), *which had its ultrastructural equivalent in highly organized rough endoplasmic reticulum.* f: *Diffusely basophilic foci were composed of small glycogen-poor hepatocytes, which were rich in rough endoplasmic reticulum. Mitotic figures are shown in the* insets *of* a, c, *and* f. *H&E; magnification, ×6,600* (a *and* b), *×3,700* (c), *×25,800* (d), *×11,600* (e), *×14,800* (f), *and ×462* (insets).

large areas of smooth endoplasmic reticulum (Figure 3d). In some animals from the main group, hepatocytes with tigroid basophilic cytoplasm emerged within the altered liver acini. The striped basophilia of the hepatocytes within altered liver acini had its ultrastructural equivalent in highly organized rough endoplasmic reticulum (Figure 3e). Because amphophilic and tigroid cell foci emerged in only a few animals, the LIs of these types of foci were not determined. Foci of diffusely basophilic hepatocytes emerged for the first time 8 months after islet transplantation and increasingly replaced the mixed-cell foci. The basophilia of these hepatocytes had its ultrastructural equivalent in abundant rough endoplasmic reticulum and strongly reduced glycogen (Figure 3f). The MI and the BrdU-LI were significantly increased in the diffusely basophilic foci compared with the extrafocal tissue. The AI of the basophilic foci was significantly lower than in the mixed-cell foci (Table 2).

Unequivocal neoplastic lesions occurred for the first time 6 months after islet transplantation (Table 1). We found adenomas (Figures 2, f–h, 5d, and 7a) and highly and moderately differentiated trabecular HCCs of >5 mm in diameter (Figures 2i, 4, 5, e–h, and 7b) that were of the mixed-cell or of the basophilic-cell phenotype. One HCC showed in parts a pseudoglandular pattern as a result of a dilatation of the bile canaliculi (Figure 4*b*). Not more than three neoplasms per liver emerged. The HCAs could easily be distinguished from altered liver acini because they were always larger (Figure 2, f–h) and had an abnormal histological architecture (Figure 5d). The HCAs and the HCCs showed strongly increased proliferative activities. The MI and the BrdU-LI were significantly higher in HCA and HCC than in the extrafocal tissue, the X-cell foci, the mixed-cell foci, and the homogeneous basophilic-cell foci (Table 2). There was no significant difference between the MI of the HCA and the early glycogen-storing foci, but the AI was significantly decreased in the HCA. The mitotic activities of the HCCs were inhomogeneously distributed within the tumors. All of the neoplasms were well delineated from the liver parenchyma (Figures 2, f–i, 4a, and 5, d–f). Vascular invasion and metastases were not detected. The incidence of HCA and HCC during the time from 15 to 22 months after islet transplantation was 86% (18 of 21 animals) and 19% (4 of 21 animals), respectively.

TGF-α Immunohistochemistry

The hepatocytes of extrafocal liver tissue of the animals from the main group and the livers of the control animals were negative for TGF-α. The different foci of altered hepatocytes and the liver neoplasms of the main group showed positive intracytoplasmic immunohistochemical reactions for TGF-α. The number of positive hepatocytes and the intensity of the reactions increased as follows. Glycogen-storing foci showed a weakly positive reaction only in the hepatocytes of the acinar zone 3 (Figure 5a). The reaction was stronger in the mixed-cell foci, where the smaller and more basophilic, glycogen-poor hepatocytes showed intense staining (Figure 5b). In homogeneous basophilic-cell foci (Figure 5c) and in HCAs (Figure 5d), every cell was positive for TGF-α. The HCCs were strongly positive for TGF-α, especially within the areas with glycogen-poor (PAS-negative) tumor cells (Figure 5, e–h).

Observations on the Islet Grafts

The islet grafts could sometimes be seen even in the unstained liver sections under the stereomicroscope (Figure 2, a–c). In the light microscope, they were identified in the H&E- or PAS-stained paraffin sections as well as in semi-thin sections stained according to Richardson. The different types of islet cells could be discriminated by insulin, glucagon, and somatostatin immunohistochemistry and by electron microscopy. When the hepatocytic lesion was cut in the periphery of the respective acinus or focus or neoplasm, serial sections were necessary to identify the respective islet graft. As shown previously in the short-term experiments,[7] the β-cells of the transplanted islets within the hyperglycemic animals from the main group exhibited nearly complete degranulation of the specific granula and hyperplasia of rough endoplasmic reticulum and Golgi complex (Figure 6) at any time of the study. In the animals that were in a bad condition during the last hours of their life or that died spontaneously, the β-cells were usually not degranulated (Figure 7, a and b). The β-cells of the normoglycemic animals from control group I and of the main group (see above) stored large amounts of granules and did not show hyperplasia of rough endoplasmic reticulum or Golgi fields. The α-cells and the δ-cells were reduced in their number and were often atrophic.

In the centers of the hepatocellular adenomas the islet epithelial cells were intermingled with the tumor cells, which could be demonstrated by insulin (Figure 7a, inset) and glucagon immunohistochemistry as well as by electron microscopy (Figure 7a). Within the HCCs, the islet epithelial cells were scattered about at large distances of 1 to 2 mm and could be identified by insulin immunohistochemistry and elec-

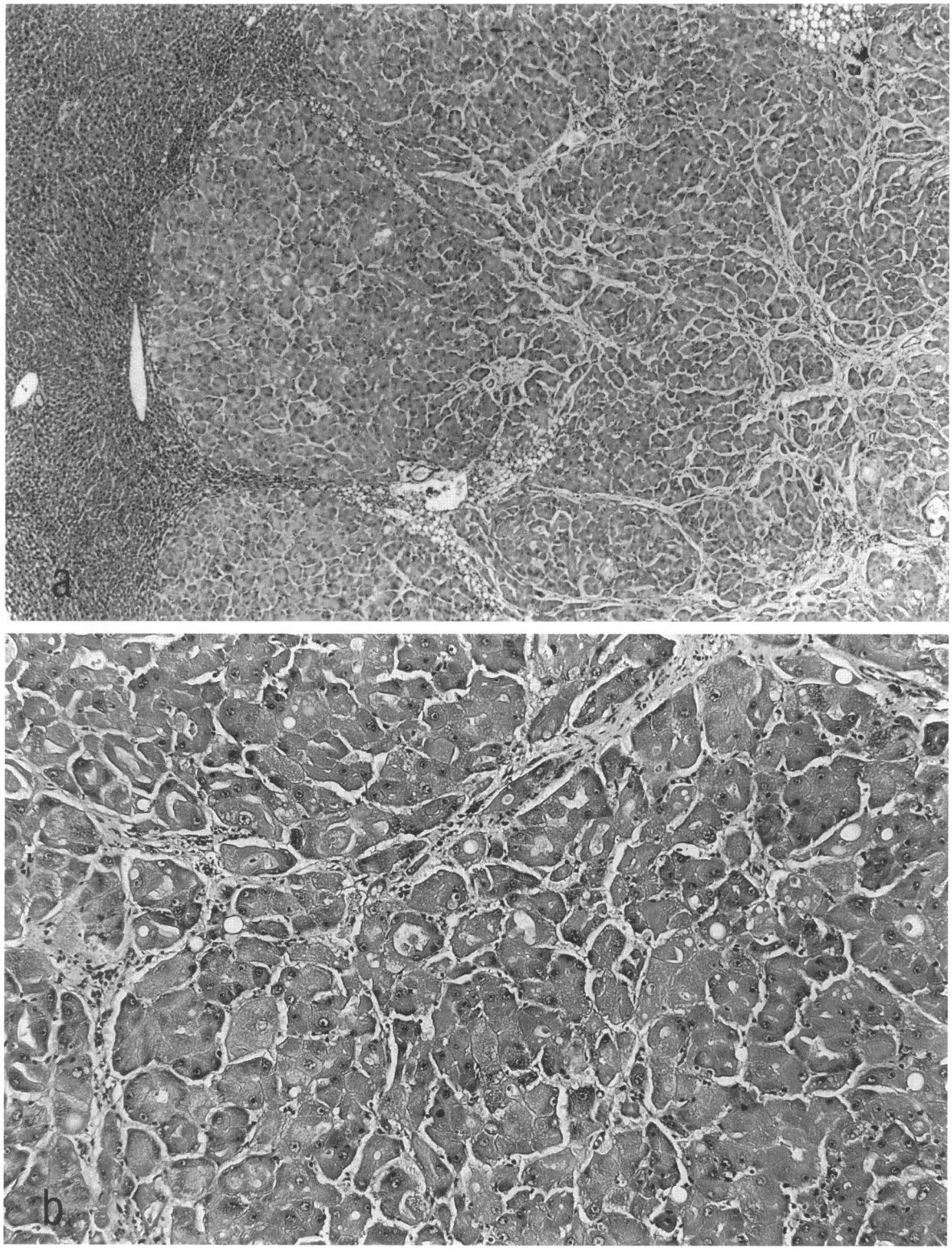

Figure 4. a: *This hepatocellular carcinoma (12 mm in diameter) emerged in the right liver lobe 22 months after islet transplantation into the right part of the liver (animal 46 in Table 1).* a: *Border to the adjacent liver parenchyma (right margin of the figure).* b: *Trabecular and, in parts, pseudoglandular growth pattern of this carcinoma. H&E; magnification, ×77.5 (a) and ×138 (b).*

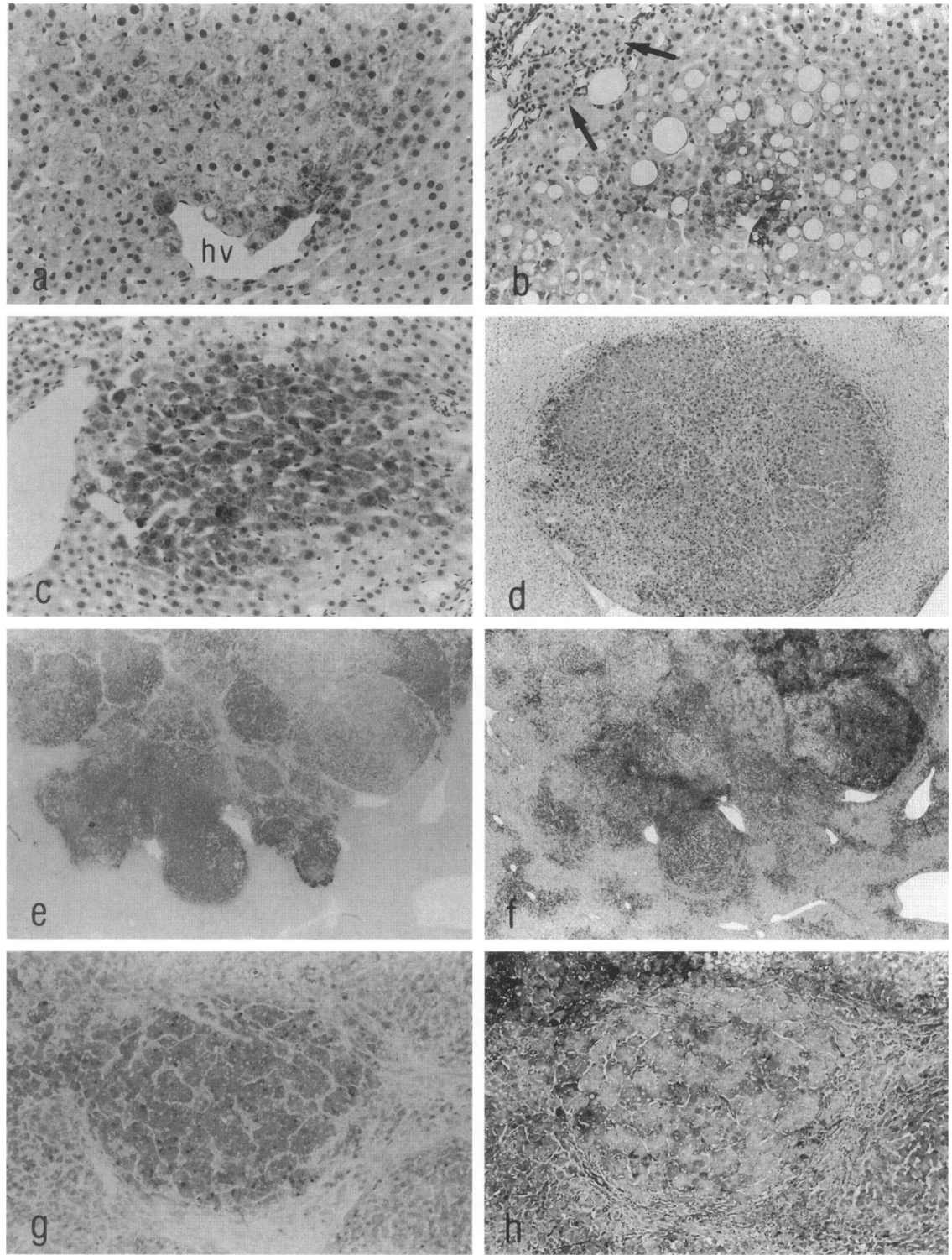

Figure 5. *The increase in the expression of TGF-α during the stepwise development from altered liver acini to HCC is shown by immunohistochemistry.* a: *Glycogen-storing altered liver acinus (upper part of the figure) shows a weak expression of TGF-α only in the acinar zone 3 (hv, hepatic venule) 3 weeks after islet transplantation. In mixed-cell foci* (b), *the small, basophilic hepatocytes show a moderate to strong expression of the increased expression of TGF-α. The transplanted islet that induced the focus in* b *is shown in the left upper corner* (arrows). *Homogeneous basophilic cell foci* (c; *17 months after islet transplantation*), *hepatocellular adenomas* (d; *19 months after islet transplantation*), *and HCC* (e *to* h; *19 months after islet transplantation*) *showed strong expression of TGF-α. The border of the HCC in* e *is well defined by the expression of TGF-α. The surrounding liver tissue is completely negative for TGF-α. The PAS reaction of serial sections* (f *and* h) *show that the glycogen-poor (PAS-negative) tumor cells exhibit a particularly increased TGF-α expression.* e *to* h: *Same HCC as Figure 7b (animal 38 in Table 1).* a *to* e *and* g: *TGF-α immunohistochemistry.* f *and* h: *PAS. Magnification, ×140* (a), *×108* (b *and* c), *×27* (d), *×13.5* (e *and* f), *×54* (g *and* h).

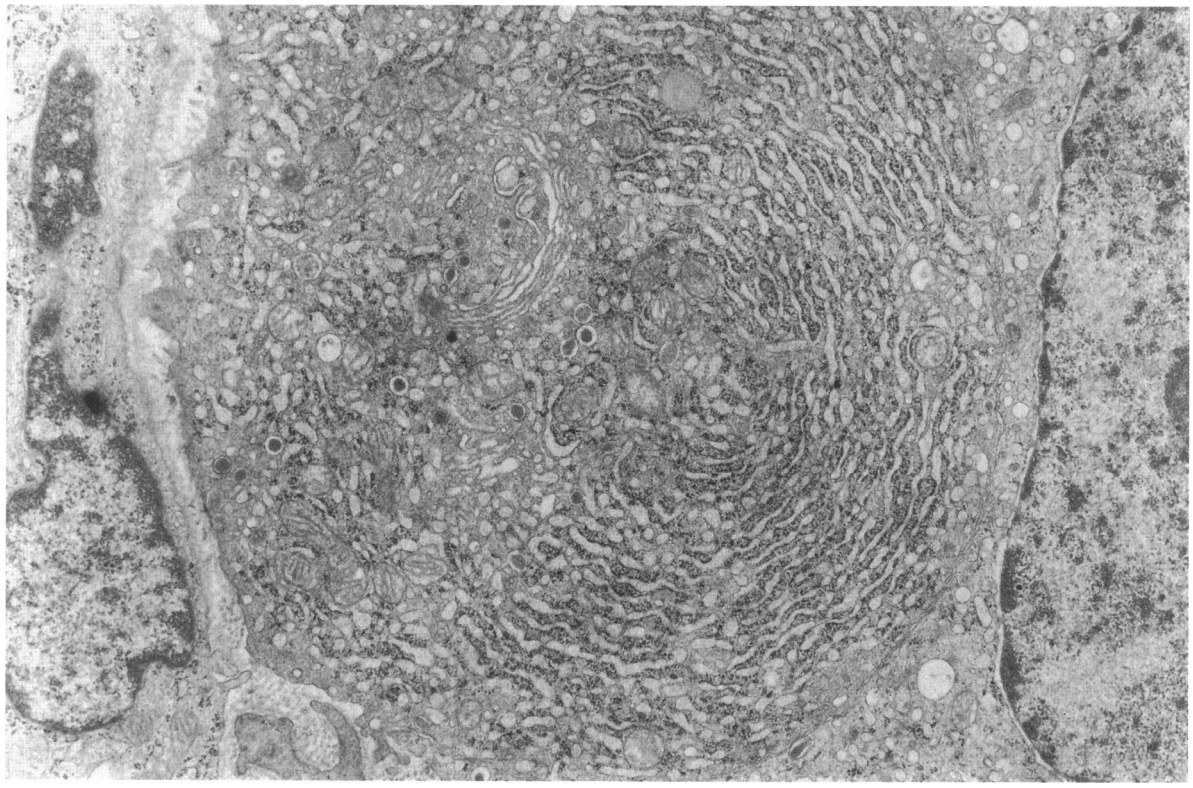

Figure 6. *Electron micrograph of two β-cells* (middle *and* right) *and a mesenchymal cell* (left) *of an islet of Langerhans 13 months after its transplantation into the liver. The strong reduction in the size and the number of electron-dense granules and the hyperplasia of rough endoplasmic reticulum were the typical changes of β-cells of animals that stayed hyperglycemic after low-number islet transplantation (main group). These changes indicate an increased synthesis and excretion of insulin. Magnification, ×11,700.*

tron microscopy in many serial sections (Figure 7b). No hepatocellular neoplasm was observed without an islet graft in its center.

The islet grafts within the control group I animals were compact, and they were not intermingled with hepatocytes.

Discussion

This study shows for the first time that low-number islet transplantation into the livers of streptozotocin-induced diabetic rats causes hepatocellular neoplasms. Advanced preneoplastic lesions and the HCAs and HCCs observed in this study emerged only in the right part of the liver, into which the islets were transplanted. Furthermore, the lesions exhibited a close spatial relationship to the transplanted islets of Langerhans. In contrast, the left part of the liver, the internal control, which was free of islets, was also free of both altered liver acini and liver neoplasms. Causative factors resulting in the glycogen- and fat-storing liver acini other than products of islet secretion, most probably insulin, have been shown to be unlikely in our short-term experiments (up to 21 days after islet transplantation).[7] Usually there was a sequence of clear glycogen- and fat-storing foci over X-cell foci, mixed-cell populations, and sometimes also homogeneous basophilic-cell foci to the HCAs and HCCs. In addition, tigroid-cell foci and acidophilic-cell foci were observed as variants of preneoplastic hepatic lesions. A similar stepwise development is well known to occur in different species and in various models of hepatocarcinogenesis, which include chemical, viral, transgenic, and radiation carcinogenesis.[11,15–18] It has been shown that the morphological development is accompanied by changes in the mitotic and apoptotic activities of the different types of foci and neoplasms.[19] Our results largely confirm these data. However, there is an obvious difference between the clear-cell foci in chemical hepatocarcinogenesis induced in rats by exposure to *N*-nitrosomorpholine (stop model), which show only a very low proliferative activity, and the clear-cell foci/acini after islet transplantation, which show a high MI and BrdU-LI (Table 2). Apparently, the effect of the secretion products of the islet grafts on the proliferation of the hepatocytes is very strong. Despite the high proliferative activity, the

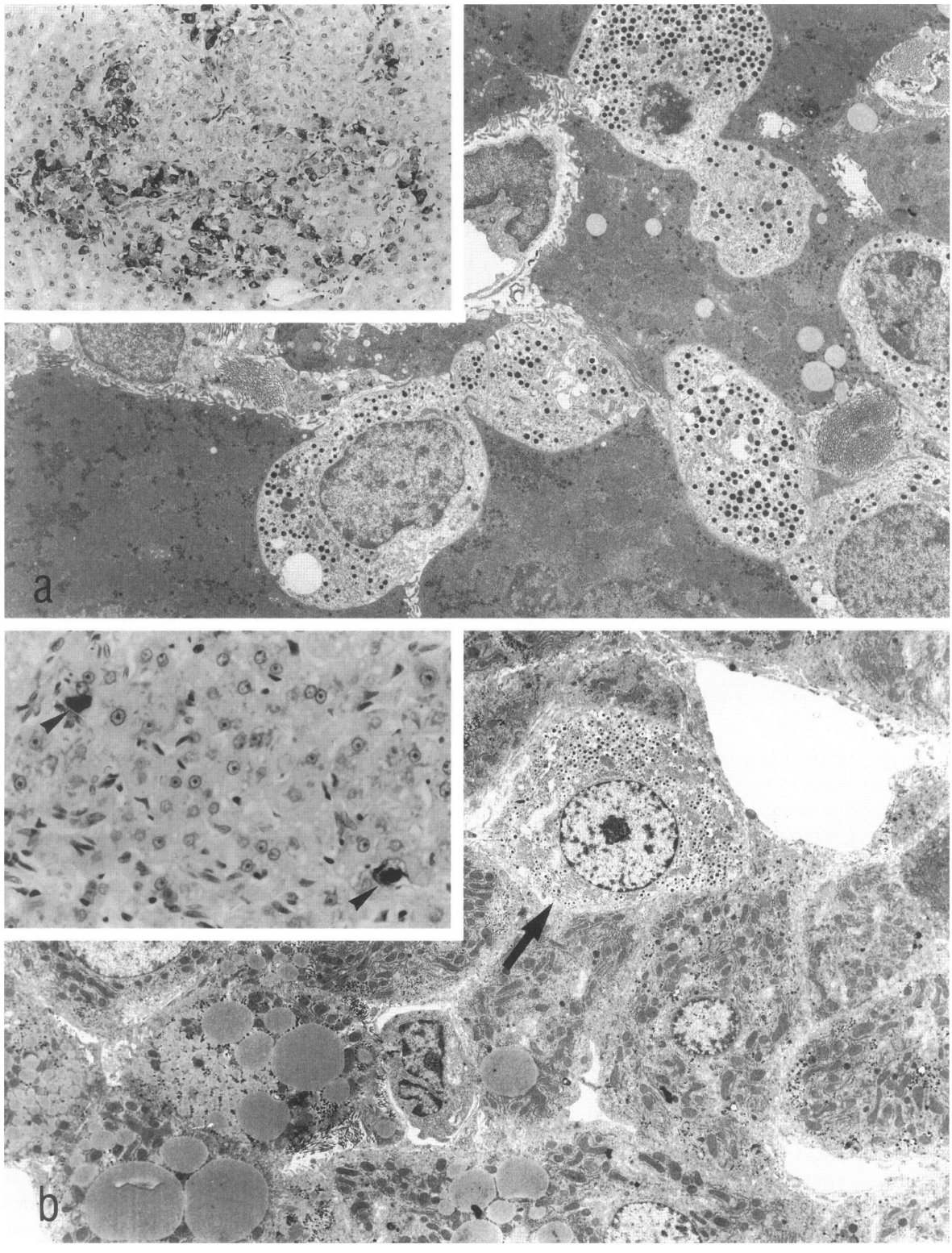

Figure 7. *Light and electron microscopic aspects of islet cells within HCAs and HCCs.* a: *In the center of the HCA, the islet epithelial cells were intermingled with the tumor cells.* b: *In the HCC, the islet epithelial cells were scattered over large distances. One single β-cell is shown* (arrow) *in the electron micrograph, and two insulin-positive cells are indicated* (arrowheads) *in the* inset. b: *Same HCC as Figure 5, a–b* (*animal 38 in Table 1*). *The β-cells that are shown in this figure are not degranulated* (*compare with Figure 6*). *These well granulated β-cells were particularly seen within the animals that were in bad condition during the last hours of their life. Magnification, ×3700* (a), *×160* (inset), *×2700* (b), *and ×277* (inset). Insets *show paraffin sections stained by insulin immunohistochemistry. Glucagon and somatostatin immunohistochemistry of parallel sections* (*not shown*) *indicated very few α- and δ-cells.*

growth of the clear-cell foci/acini was limited because of the simultaneously high AI. The differences between the clear-cell foci and the X-cell foci might be an indicator for less intense effects of the islet hormones on the hepatocytes in the case of the X cells. During the development from mixed-cell foci to HCA and HCC, the MI and the BrdU-LI increased, whereas the AI was low. These indices correlate with the growth of the foci and the stepwise progression to neoplasms. In this study, there was also a strong correlation between the evolution of the types of foci to hepatocellular neoplasms and the expression of TGF-α, which has been found to be a marker of progression in hepatocarcinogenesis.[20–23]

Streptozotocin is known to cause renal-cell carcinomas and endocrine pancreas tumors in rats after a single dose.[24] In our experiment, a high incidence of renal-cell carcinoma was observed (95% at 15 to 22 months after islet transplantation in the main group and 100% in control group I). No neoplasms of the pancreas were observed in this experiment. An increase in the incidence of hepatocellular tumors after streptozotocin administration was not observed in rats,[24] and in our control groups I and II and in the left parts of the livers of the animals from the main group, neither advanced preneoplastic liver lesions nor hepatocellular neoplasms emerged. Strong evidence for a local hyperinsulinism as a causative agent in this experimental model is provided by the finding that, after high-number islet transplantation (control group I), neither advanced preneoplastic hepatocellular lesions nor hepatocellular neoplasms have been observed. Only a few foci of glycogen-storing hepatocytes have been seen after more than 18 months in the part of the liver not carrying islet grafts in the main group and in the livers of the animals from control groups I and III. The spontaneous appearance of such lesions in aged animals is well known in different rat strains.[11] A role for the carcinogen streptozotocin in the development of the present hepatocellular preneoplastic and neoplastic lesions is not completely excluded, as this genotoxic compound might initiate hepatocytes, the neoplastic transformation of which is promoted by the sustained secretion of insulin (and possibly other factors) from the implanted islets. However, even under these conditions, hormonal influences would play a decisive role in the realization of hepatic neoplasia. Additional studies have been started to avoid any involvement of an exogenic genotoxic agent in this model of carcinogenesis.

The β-cells of the islet grafts of the hyperglycemic animals from the low-number islet transplantation group showed morphological signs of maximal protein (probably insulin) synthesis and secretion. Degranulation of the β-cells after islet transplantation in combination with glycogen storage of surrounding hepatocytes has been reported from different groups,[5–8] but these phenomena were only temporary when normoglycemia was restored after high-number islet transplantation.[5,6,8] In the present study (main group), the focal alterations of the liver parenchyma persisted even when the animals became normoglycemic after many months of hyperglycemia (Table 1). In these animals, hepatocellular neoplasms were also observed.

In different studies, it was found that pancreatic islets play a role in experimental pancreatic carcinogenesis.[25,26]

Insulin is well known to have anticatabolic, growth-promoting effects.[27–34] A systemic hyperinsulinemia in a normal individual causes hypoglycemia, and therefore, a long-term study of carcinogenic actions of hyperinsulinemia seemed difficult. In our model, a long-term hypersecretion of insulin is possible because the hyperinsulinemia is not systemic. The animals are hyperglycemic and, therefore, probably systemically hypoinsulinemic. The persisting hyperglycemia keeps the regulated but maximal insulin secretion going.

Recently, it has been discussed that the phenotype of early preneoplastic liver lesions induced by various oncogenic agents, namely, glycogen-storing foci and in some points also mixed-cell foci, resemble insulin effects.[18,35] *Vice versa*, it has been shown in our previous studies that alterations of liver acini induced by insulin resemble preneoplastic liver lesions in their focal character, their morphology, and their enzymic pattern.[7,9] In the present study it is shown that these alterations do actually represent preneoplastic lesions and may proceed to neoplasia.

Carcinogenic effects of a long-term hyperproduction of growth factors, eg, TGF-α and growth hormone, have been studied in transgenic mice.[20,21,36,37] The time of onset of hepatocellular tumors was 8 to 12 months in these mouse models. In comparison with these data, the time interval between islet transplantation and the hepatocellular neoplasia does not seem to be very long.

In conclusion, we have established a new model of hepatocarcinogenesis that is most probably due to the hormonal and growth-stimulating effects of insulin secreted by the intraportally transplanted islets of Langerhans in streptozotocin-induced diabetic rats.

Acknowledgments

We thank Jörg Bedorf, Mathilde Hau-Liersch, and Inge Heim for skillful technical assistance, Doris Giessen for immunohistochemistry, Kurt Rüdel for animal care, and Gerrit Klemm and Claudia Müller for photographic artwork.

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