Insulinomas Derived from Hyperplastic Intra-Hepatic Islet Transplants

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Insulinomas were induced in a new animal model by transplanting a low number of isologous pancreatic islets via the portal vein into the livers of 66 streptozotocin-induced diabetic rats. In contrast to high-number islet transplantation, which restored normoglycemia in 25 control animals, the low-number islet transplantation was followed by persisting hyperglycemia for at least 13 months. Hyperplasia of islet cells developed in the transplanted islets as a consequence of hyperglycemia, which for the beta cells is not only a secretory but also a proliferative stimulus. Six of thirty-three animals between the 18th and the 24th month after islet transplantation changed from hyperglycemia to severe hypoglycemia, due to insulinomas that had developed in the liver from the transplanted islets. In contrast to other animal models, insulinoma development in this new model does not result from DNA damage by chemicals or radiation or from the expression of transgenes, but starts from apparently normal islets prepared from untreated isologous donors, which are exposed to an imbalance in glucose metabolism. The persistent proliferative stimulus and the metabolic alterations caused by the longstanding hyperglycemia seem to be the most relevant oncogenic factors in this model. (Am J Pathol 1998, 152:1025-1038)

Pancreatic islets of Langerhans can be transplanted via the portal vein into the liver.1 The clinical implication of this procedure is to treat diabetes.2 When only a low number of islets are transplanted into diabetic rats, the diabetic state is ameliorated but not completely compensated.3 Under these conditions the beta cells of the transplanted islets show signs of an increased activity of insulin synthesis and secretion.

As has been described recently, the hepatocytes in the liver acini downstream of these islets are altered in their morphology, in the activity of a number of their enzymes, and in their proliferative state.3,4 These hepatocytic alterations are in line with the effects of an increased insulin concentration, ie, a local hyperinsulinemia caused by the stimulated beta cells. Long-term studies of this model demonstrated that the altered liver acini represent neoplastic lesions, which after months may proceed to hepatocellular adenomas and carcinomas.5 It is known that an increased glucose concentration is a stimulus not only for synthesis and secretion of insulin but also for beta cell proliferation in vitro and in vivo.6–10 In short-term studies, 5-bromo-2′-desoxyuridine (BrdU)-labeled nuclei of islet epithelial cells have been demonstrated days and weeks after islet transplantation.11,12 As an unexpected long-term effect of the persisting hyperglycemia on the transplanted islets, we have now observed in a long-term study the development of insulinomas from the transplanted islets.

Materials and Methods

Transplantation Procedure and Experimental Groups

Adult male Lewis rats (inbred strain) weighing 250 to 300 g were made diabetic by streptozotocin (80 mg per kg body weight). Diabetes was defined by a nonfasting blood glucose level greater than 400 mg/dl, which was reached 1 to 3 days after streptozotocin injection. In the main group, a small number of islets of Langerhans (n = 250 to 450) freshly isolated from isologous healthy male rats was transplanted via the portal vein into the right part of the livers of 66 animals.

The number of islets chosen was so low that mild hyperglycemia persisted for many months after islet transplantation. In 25 control group animals, a high number of islets (n = 1000 to 2000) were transplanted.

The donor animals were anesthetized with a mixture of ketamine (100 mg/kg) and xylidine (4 mg/kg). Between 60 and 80 ml of a freshly prepared solution of neutral red (60 mg of neutral red dissolved in 500 ml of 0.9% sodium chloride) was retrogradely injected into the abdominal aorta with persistent pressure. The pancreas was dissected, finely chopped with scissors, and rinsed in cold Hanks’ solution. The fragments were digested at 37°C for 20 minutes in 10 ml of Hanks’ solution containing colla-
BrdU Application According to Eldridge et al\textsuperscript{13}

Six days before they were killed, 23 animals from the main group and 11 animals from the control group were anesthetized, 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 thoracolumbar area. These pumps continuously delivered BrdU until the animals were sacrificed. Thirty animals of the main group and twelve animals of the control group received a single dose of 50 mg of BrdU per kg body weight intraperitoneally 1 hour before sacrifice. BrdU administration was not possible in 13 animals of the main group that died spontaneously.

Preparation of Tissues

The animals were perfused with 0.2% glutaraldehyde and 3% paraformaldehyde as described earlier.\textsuperscript{5} Immediately after perfusion, 50 to 100 slices per animal were cut from the fixed liver lobes at a thickness of 0.5 to 1.0 mm. These slices were placed into PBS and were systematically examined with a stereomicroscope. With some experience, it was possible to identify transplanted islets in these unstained liver slices as well as after embedding (see Results). The transplanted islets could be detected by this procedure even after low-number islet transplantation, and corresponding slices of the same islets were embedded in Epon and in paraffin. The pancreatic tissue was spread on blotting paper before fixation. After fixation, it was cut into three parallel slices, which, after examination under the stereomicroscope, were embedded in paraffin blocks. From the paraffin-embedded liver and pancreas specimens, serial sections of 2 to 3 μm thickness were stained with hematoxylin and eosin (H&E) and with the periodic acid Schiff reaction (PAS). Additional sections were used for immunohistochemistry. In the six cases of insulomas that were found in the livers (see Results), the pancreatic paraffin blocks were completely cut in many section levels. In addition, 10 small cubes (1 mm\textsuperscript{3} in size) were cut from each specimen and were embedded in Epon. Semi-thin sections of the Epon-embedded specimens were stained according to Richardson.\textsuperscript{14} Thin sections for electron microscopy were stained with uranyl acetate and lead citrate and were examined with a Phillips CM10 electron microscope (Eindhoven, The Netherlands).
Immunohistochemistry

After examination of the H&E and the PAS stains, appropriate sections were selected and the corresponding sections were processed for immunohistochemistry. Single immunostains for BrdU, insulin, glucagon, and somatostatin (antibodies from DAKO, Hamburg, Germany) were performed as described earlier. Double stains for insulin and PAS were started with the insulin immunohistochemistry, followed by the PAS reaction. Double immunostains were started with the stain for the respective hormone using the LSAB*-Kit (DAKO) and the DAB*-Kit (DAKO). For BrdU immunohistochemistry, antigen retrieval steps were performed as described earlier and the BrdU primary monoclonal antibody (from DAKO; dilution 1:100) was incubated overnight. The primary antibody was detected using the alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) method (secondary antibody and complex from DAKO). Alkaline phosphatase was detected by trizolium blue (Merck, Darmstadt, Germany). Single immunostains were counterstained with hematoxylin, and double immunostains were counterstained with Nuclear fast red (Merck), dehydrated, and coverslipped with Pertex (Medite, Burgdorf, Germany).

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

Epoxy resin sections stained according to Richardson were viewed systematically for apoptotic and mitotic cells at a magnification of ×1000. Apoptotic and mitotic indices (AI and MI) were calculated for all epithelial cells of the transplanted islets as the number of apoptotic bodies and of mitotic figures per 1000 epithelial cell nuclei. At least 1500 nuclei were counted per animal. Paraffin sections, double immunostained for BrdU and insulin were examined at a magnification of ×400. BrdU labeling indices (BrdU-LI) were calculated for insulin-positive cells as the number of BrdU-labeled nuclei per 1000 beta cell nuclei. At least 1200 cells were counted per animal.

As glucagon- or somatostatin-positive cells were negative for BrdU in the islet grafts, BrdU-LI of these cell types could not be calculated. The AI, MI, and BrdU-LI at 1 hour, and the BrdU-LI at 6 days, and the blood glucose and serum insulin (see below) of the different animal groups and the different times after islet transplantation (Table 1) were compared with the Wilcoxon-Mann-Whitney test. Significance was accepted when \( P < 0.05 \).

Results

Blood Glucose and Serum Insulin Levels

As expected, all of the animals from the high-number islet transplantation group (control group) became normoglycemic after islet transplantation. Their blood glucose levels ranged from 60 to 80 mg/dl during the whole experiment. In the course of the first 10 months after islet transplantation the animals from the main group exhibited mild hyperglycemia (blood glucose, 200 to 350 mg/dl). Although a very low number of transplanted islets was chosen, 49% of the animals from the main group that lived long enough became normoglycemic (blood glucose < 150 mg/dl) 10 to 18 months after islet transplantation (Figure 1). The mean blood glucose of the animals from the main group decreased significantly from months 1 to 3 to months 4 to 24 (Table 1). Unexpectedly, six animals from the main group trembled and showed se-
The animals from hypoglycemia to (50 mg/dl) and six (Figure 2, a, b, c, and d) stayed normoglycemic for a relatively long time until they became hypoglycemic. The animals d and f showed a relatively rapid change from hyperglycemia to hypoglycemia. Typically, the animals had a better weight gain during the change from hyperglycemia to normoglycemia. The insulinoma of the animal in a is shown in Figure 8. b–d, and 9. The insulinoma of the animal in c is shown in Figures 3d and 8a.

Figure 2. Blood glucose and body weight data from the six animals of the main group that developed an intrahepatic insulinoma. The range of normoglycemia (50 to 150 mg/dl) is marked by the dotted area. The animals in a, b, c, and d stayed normoglycemic for a relatively long time until they became hypoglycemic. The animals d and f showed a relatively rapid change from hyperglycemia to hypoglycemia. Typically, the animals had a better weight gain during the change from hyperglycemia to normoglycemia. The insulinoma of the animal in a is shown in Figure 8. b–d, and 9. The insulinoma of the animal in c is shown in Figures 3d and 8a.

Severe hypoglycemia (blood glucose, 12 to 36 mg/dl) between the 18th and the 24th month after islet transplantation (Figure 2). The serum insulin of two of these insulinoma-bearing animals (Figure 2, a and e) was found to be significantly increased when it was compared with the serum insulin of six untreated normoglycemic animals and six hyperglycemic animals from the main group (Table 1).

Light and Electron Microscopy of the Transplanted Islets and the Insulinomas

The stereomicroscopic examination of the unstained liver slices was a great help for finding the islets in the livers, especially after low-number islet transplantation (Figure 3). Although the islets of the control group persisted as small organs in the portal tracts during the whole exper-
iment (Figures 3a and 4a), islets with an irregular shape and mitotic figures of endocrine epithelial cells that were positive for insulin in the immunohistochemistry were found in the animals of the main group (Figure 4, b–d). The MI, the AI, and the BrdU-LI of the islets were significantly increased in the main group when compared with the control group (Table 1). Electron microscopic examination of the islets of the hyperglycemic animals (main group) revealed degranulated beta cells with hyperplastic Golgi fields and rough endoplasmic reticulum, whereas the beta cells of the control group did not show hyperplasia of these organelles but storage of many electron-dense secretory granules.

In the animals from the main group that became normoglycemic more than 10 months after islet transplantation, only very few large hyperplastic islets could be detected (Figure 5, a–d), but the other transplanted islets seemed to have vanished or were very small, sometimes consisting only of some non-beta cells (Figure 5d, inset). The hyperplastic islets consisted predominantly of beta cells (Figure 5, a and c) but always contained small groups (Figure 5b) or disseminated (Figure 5d) alpha cells and delta cells (not shown).

In most animals from the main group, cells with a PAS-positive cytoplasm were seen in the transplanted islets at short (Figure 6b) and long (Figure 6, c and d) time intervals after islet transplantation. Many of these PAS-positive cells were positive for insulin (Figure 6d) but negative for glucagon and somatostatin. The electron microscopic examination of these cells (Figure 7) showed abundant glycogen particles, beta-cell-typical electron-dense granules, and mitochondria that were typical for
Figure 4. Examples of islet grafts after high-number (a) and after low-number (b to d) transplantation are shown at different time intervals (a, 15 months; b, 1 month; c and d, 13 months) after islet transplantation. Whereas in the high-number example the islet graft stays largely unaltered within the portal triad as a compact organ without signs of cell proliferation, the grafts after low-number transplantation show mitotic figures in their beta cells (arrows in b and d), and they became hyperplastic (c and d) and grew infiltratively in the surrounding liver parenchyma (c and d). This latter phenomenon is seen in conventional Epon sections with their weakly stained beta cells (c) as well as in paraffin sections immunostained for insulin (d). Magnification, ×310 (a and c) and ×400 (b and d).
Figure 5. Insulin (a and c) and glucagon (b and d, inset) immunohistochemistry of two hyperplastic islets, which typically contain many insulin-positive beta cells and only few glucagon-positive cells. The latter are either located in small groups at the border of the islet (upper margin in b) or are disseminated in the islet (d). When the animals of the main group became normoglycemic more than 10 months after islet transplantation, then often only one or two hyperplastic islets could be detected and the other islets were atrophic, sometimes consisting of only few glucagon-positive cells surrounded by hepatocytes (inset in d). This phenomenon is interpreted as an adaptive atrophy of the beta cells, which is the consequence of an inadequate high insulin secretion of the hyperplastic islets. After low-number islet transplantation, a proliferation of bile ducts (upper part in a and b) has frequently been observed in the portal triads of the transplantation site. Magnification, ×100 (a and b), ×125 (c and d), and ×400 (inset).
Figure 6. Large, pale, and vacuolated, weakly insulin-positive (a and d) beta cells that were PAS positive (b to d) were seen only after low-number islet transplantation, either at an early time when the animal was hyperglycemic (a and b, 3 weeks) or at a later time when the animal had become normoglycemic (c and d), 17 months after islet transplantation. Insulin immunohistochemistry (a), PAS reaction (b and c); double stain for insulin (brown) and PAS (red) (d; double-stained cells are indicated by arrowheads); magnification, ×310 (a to c) and ×620 (d).
Figure 7. Electron micrographs of beta cells of islets of Langerhans 17 months (a) and 3 months (b) after islet transplantation into the liver. The storage of glycogen was seen only within hyperplastic beta cells of the main group (low-number islet transplantation). The islet in (a) is the same as in Figures 3, b and c, and 6d. N, nucleus; G, glycogen field; m, mitochondria of the beta cell; M, mitochondrion of a neighboring hepatocyte in b. Arrows show beta cell granules. Magnification, \( \times 8100 \) (a) and \( \times 19,000 \) (b).
the islet epithelial cells and differed from the surrounding hepatocytes (m and M in Figure 7b).

All of the animals that showed severe hypoglycemia (Figure 1) exhibited one intrahepatic islet cell tumor. The size of these tumors varied between 3 mm (Figures 3d and 8a) and 11 mm (Figure 8b). The tumor cells were immunohistochemically positive for insulin (Figure 8, a–d) and contained typical electron-dense granules in the electron microscope (Figure 9). Single glucagon-positive cells occurred in the tumors. The insulinomas showed an invasive growth pattern and a high mitotic activity (Table 1; Figure 8, c and d). The MI and the BrdU-LI of the insulinomas were significantly higher when compared with the beta cells in the transplanted islets of the animals from the main group and the control group (Table 1). One insulinoma showed marked nuclear atypia: increased nuclei, increased nuclear/cytoplasmic ratio, and prominent and enlarged nucleoli (Figure 8d). In two of the insulinoma cases, no other transplanted islet was present. Representative slides from the lungs, heart, both kidneys, adrenals, stomach, intestine, abdominal lymph nodes, brain, and hypophysis were examined, but metastases of the insulinomas were not observed.

Neither hyperplastic islets nor insulinomas occurred in the livers when the number of transplanted islets was high and the animals became normoglycemic immediately after transplantation (control group).

Light and Electron Microscopy of the Intra-Pancreatic Islets

The intra-pancreatic islets of the recipients of both experimental groups, as to be expected as the consequence of streptozotocin damage, consisted predominantly of alpha cells and contained also single delta cells but only a very small number of beta cells. The beta cells of the hyperglycemic animals from the main group were enlarged and showed similar signs of high synthetic and secretory activity as the transplanted intra-hepatic beta cells, such as degranulation and hyperplasia of endoplasmic reticulum and Golgi fields under the electron microscope. Mitotic figures or apoptotic bodies of the intra-pancreatic islets were not observed. As some preliminary BrdU immunostains of the pancreata did not show labeled intra-pancreatic endocrine epithelial cell nuclei, double immunostains were not performed. Nevertheless, there must have been some proliferation of the beta cells, as in the main group, animals with persisting hyperglycemia showed more intra-pancreatic beta cells during the second year after islet transplantation than control animals. On the other hand, intra-pancreatic beta cells were reduced in number and atrophic in the animals that had developed large hyperplastic transplanted islets or an insulinoma. Only one animal from the main group that became normoglycemic 24 months after islet transplantation showed a single intra-pancreatic hyperplastic islet (0.8 mm in size), consisting of beta cells, but no hyperplasia of intra-hepatic islets. No other hyperplastic islet and no endocrine tumor occurred in the pancreata of the other animals from both experimental groups. This was true also in the six animals that had developed an insulinoma in the liver, where the complete pancreatic tissue was examined in many section levels.

Relationship between Hepatocellular Neoplasms and Insulinomas

Four of the six animals that developed an insulinoma simultaneously exhibited at least one hepatocellular adenoma, and one of these four animals showed in addition a hepatocellular carcinoma. But the insulinomas were not in topographical proximity to the hepatocellular tumors. In the hepatocellular tumors, small islets or dispersed islet cells could be detected by insulin immunohistochemistry.6 Two insulinomas were surrounded by multiple pre-neoplastic glycogen-storing liver acini.

Discussion

In previously published studies as well as in this study it has been shown that after low-number islet transplantation into the liver the beta cells of the transplants exhibited signs of high activity in synthesis and secretion of insulin.3–5,11 Obviously, due to the hyperglycemia, which is a known proliferative stimulus for beta cells,6–10 the intrahepatic islets in animals of the main group showed a significantly higher proliferative activity than in normoglycemic animals after high-number islet transplantation (control group). Interestingly, the apoptotic elimination of the islet epithelial cells was increased, too, after low-number islet transplantation. This indicates that an increased cell turnover takes place during the compensatory growth of the beta cells.

The glycogen storage as observed in the stimulated islets of the main group (Figures 6 and 7) is an unusual finding for beta cells of the rat but has been observed in different other species that have been treated with high doses of glucose15 or that developed a spontaneous diabetes mellitus.15–18 The high blood glucose might be a causative factor resulting in an altered metabolic activity of the beta cells leading to an accumulation of glycogen. Interestingly, the alpha cells of the transplanted islets did not contain significant amounts of glycogen in their cytoplasm. The glycogen-storing phenotype of some beta cells persisted, even if the animal became normoglycemic more than 10 months after islet transplantation. In analogy to the well known glycogenotic foci of the liver parenchyma,19–21 we interpret this as a preneoplastic beta cell type.

The phenomenon that all of the animals that became normoglycemic after more than 10 months exhibited only few large hyperplastic intra-hepatic islets (the only exception was the single animal with the intra-pancreatic hyperplastic islet; see above), whereas the other intrahepatic islets were atrophic, can be interpreted as a sign of a beginning autonomy of beta cells in the hyperplastic islets and a consecutive atrophy of the beta cells in nonautonomous islets. In two of the insulinoma cases, no other islet transplants could be detected, indicating a
Figure 8. Positive insulin immunohistochemistry of the smallest, 3 mm in diameter (a), and the largest, 11 mm in diameter (b to d), insulinoma, which both induced severe hypoglycemia 19 months (a) and 24 months (b to d) after low-number islet transplantation. The double immunostain for insulin and BrdU (blue-stained nuclei in c), which was given 1 hour before sacrifice, shows proliferative activity of insulin-positive tumor cells that, at higher magnification (d), showed nuclear atypia, ie, large nucleoli and an increased nuclear/cytoplasmic ratio, and mitotic figures (arrowheads). Insulin immunohistochemistry (a, b, and c); double stain for insulin (brown) and BrdU (blue) (c); hematoxylin (a, b, and d); nuclear fast red (c); magnification, ×40 (a), ×20 (b), ×200 (c), and ×400 (d).
complete adaptative atrophy of the nontumorous islets. Neither in this study nor in our earlier studies3-5,11 were any signs of rejection of the isologous islet transplants observed.

The fact that only one hyperplastic islet but no insulinoma had developed in the pancreas of the recipients is noteworthy, because streptozotocin has been shown to induce insulinomas in the pancreas22-24 when given at lower doses than in our study. Probably more beta cells survived the toxic damage in these studies. It should be noted that in the present experiment the islet transplants did not have any contact either with streptozotocin, which has a half-life of 15 minutes at 37°C,25 and was intravenously injected to the recipients 7 to 20 days before islet transplantation, or with any other known carcinogen. Neutral red, which was used for isolation of the islets, is not known to be carcinogenic. In contrast to other animal models, insulinoma development in this new model does not result from DNA damage by chemicals22-24 or radiation26 or from the expression of transgenes27-32 Rather, it starts from apparently normal islets prepared from untreated isologous donors, which are exposed to an imbalance in glucose metabolism. The persistent proliferative stimulus and the metabolic alterations caused by the longstanding hyperglycemia seem to be the most relevant oncogenic factors in this model.

As the method of islet transplantation is becoming more and more effective also in human diabetes mellitus,5 post-transplantation control of the diabetes has to be considered extremely important to prevent the possible development not only of hepatomas5 but also of insulinomas. Our results suggest that the long-term persisting hyperglycemia and consecutive hyperproliferation per se are adequate and sufficient causative factors for the induction of insulinomas via beta cell hyperplasia. To a certain degree, this could be seen in analogy to the development of tumors in animals transgenic for growth factors33-35 if hyperglycemia is in the widest sense inter-
Interpreted as a growth factor for beta cells. Additional examples of tumorigenesis of endocrine cells via long-acting functional and proliferative stimulation are 1) the induction of a tertiary (autonomous) hyperparathyroidism and adenomas of the parathyroid gland in humans by a long-persisting secondary (functional) hyperparathyroidism and 2) the sequence of achlorhydria of the stomach: hyperplasia of gastrin-producing cells and enterochromaffin-like cells of the stomach resulting in carcinoid. There are few reports on insulinomas emerging in human cases of non-insulin-dependent diabetes mellitus, the most common type of diabetes in which the beta cells might become hyperplastic as a consequence of hyperglycemia due to peripheral insulin resistance. Our findings may help to understand these cases. The remaining question, however, of why insulinomas are so rare in this frequently seen type of diabetes is difficult to answer. One could speculate that the not yet understood increase in glucose tolerance, as characteristic for this type of diabetes, is not only due to an alteration of the beta cells but may also be due to an alteration of the glucose sensitivity of the beta cells resulting in an inadequate insulin release and an inadequate proliferative response of these cells (concept of glucose toxicity). The concept of a long-acting functional activation or proliferative stimulation of primarily nonmutated cells at the very beginning of a tumorigenic process might be an interesting alternative or addition to the most favored initiation promotion concept of carcinogenesis in which primary genetic damage is followed by positive or negative selection of the so-called initiated cells.

Conclusion

This new model of tumorigenesis does not start with a genotoxic (chemical or radiation) or a transgenic manipulation but works via long-term activation and proliferative stimulation of transplanted pancreatic islets. In the present experiments, the only evident hit for the cells that transform to tumor cells (i.e., beta cells) is their overstimulation by the major physiological stimulating agent, i.e., glucose. Such a model may open ways to analyze the steps between a long-term proliferative stimulus and the uncontrolled up-regulation of oncogenes.

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References


