Insulin Receptor, Insulin Receptor Substrate-1, Raf-1, and Mek-1 during Hormonal Hepatocarcinogenesis by Intrahepatic Pancreatic Islet Transplantation in Diabetic Rats

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ABSTRACT

Low-number transplantation of pancreatic islets into the livers of diabetic rats leads to transformation of the downstream liver acini into clear-cell foci of altered hepatocytes (FAHs). These FAHs correspond to the glycogen-storing (clear-cell) phenotype of hepatocellular preneoplasias and develop into hepatocellular adenomas (HHCAs) and hepatocellular carcinomas (HCCs) within 6 to 24 months. In addition, they show metabolic alterations that resemble well-known insulin effects, most likely constituting the result of the local hyperinsulinemia. Thus, we investigated FAHs, HCCAs, and HCCs for altered expression of insulin receptor, insulin receptor substrate-1 (IRS-1), Raf-1 and Mek-1. Light and electron microscopic immunohistochemistry revealed a translocation of insulin receptor from the plasma membrane (normal tissue) into the cytoplasm in clear-cell FAHs and an increase in insulin receptor expression in HCCAs and FAHs. IRS-1 also showed an increase in IRS-1 gene expression, investigated by in situ hybridization and quantitative reverse transcription-PCR. IRS-1, Raf-1, and Mek-1 proteins were strongly overexpressed in FAHs and tumors, as compared with the unaltered liver tissue. These overexpressions were closely linked to the clear-cell phenotype of preneoplastic and neoplastic hepatocytes, because basophilic FAHs (later stages) and basophilic tumors showed no overexpressions. In this endocrine model of hepatocarcinogenesis, severe alterations of insulin signaling were induced by the pathological local action of islet hormones in the livers and may substantially contribute to the carcinogenic process.

INTRODUCTION

Insulin is a pleiotropic hormone exerting different metabolic and mitogenic effects on hepatocytes (1–6). Although insulin signaling is transduced intracellularly via an extensive signaling network with multiple alternative pathways, insulin receptor substrate-1 (IRS-1) is believed to be the first basic cytosolic mediator (7–10). IRS-1 is activated by phosphorylation of tyrosine residues, mediated by the β-subunit of the insulin receptor, which acts as a tyrosine kinase after extracellular binding of insulin to the α-subunit. Phosphorylated IRS-1 acts as a docking protein and can associate with proteins containing Src homology 2 (SH2) domains, such as GRB-2. GRB-2 links to IRS-1, and, thus, insulin signaling to the Ras-complex, which is the most important downstream signaling cascade besides the phosphoinositol-3-kinase pathway. Ras has been demonstrated to initiate a stepwise activation of cytosolic kinases, i.e., Raf-1 and, hence, Mek-1/MAPKK (mitogen-activated kinase (MAPK) and pp90 S6 kinase, thereby inducing increased cellular proliferation and anabolic alterations in the carbohydrate and fatty acid metabolism. This pathway is believed to be essentially involved in the hepatotropic and hepatocellular mitogenic effects of insulin (7, 10–11).

Several studies have shown that insulin signal transduction proteins, in particular insulin receptor and IRS-1, may also contribute to the development of hepatocellular carcinomas (HCCs) and other tumors (12–24). However, it has not yet been demonstrated that this process can be induced at the very beginning, i.e., via insulin itself.

In a model of hormonal hepatocarcinogenesis, long-term local effects of insulin appear to induce the carcinogenic process (25–27). After 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 morphologic signs of increased insulin synthesis and secretion because of the continuous hyperglycemia. As a response to the local hyperinsulinemia, the hepatocytes downstream of the transplanted islets show an increase in glycogen and lipid storage, accompanied by corresponding alterations in their carbohydrate metabolism, as well as a strong increase in proliferative and apoptotic activity (25, 27). As a result, these foci of altered hepatocytes (FAHs) bear morphologic similarities to preneoplastic hepatocellular foci, known from animal models of chemical hepatocarcinogenesis (26, 28), and may develop into hepatocellular adenomas (HHCAs) and HCCs within 6 to 24 months (27). We previously investigated the insulin-like growth factor (IGF) axis in this model and found that even a few days after transplantation, IGF-I, IGF-II receptor, and IGF-binding-protein (IGFBP)-4 were overexpressed, whereas IGFBP-1 was down-regulated and IGF-II was not detectable within the FAHs (29–30). IGF-I receptor expression, however, remained on the low level of the normal parenchyma. Thus, at this early stage, not only insulin but possibly also IGF-I-signaling was more likely transduced via the insulin receptor.

The aim of the present study was to determine whether the hepatocytes of the preneoplastic foci and tumors in this model show alterations in their intracellular signaling that can be attributed to the local hyperinsulinemia and that may contribute to the carcinogenic process. Thus, we examined insulin receptor and IRS-1 for their function as the basic mediator molecules of insulin action, as well as the Raf-1/Mek-1/MAPKK pathway as one of the principal downstream cascades involved in insulin action.

MATERIALS AND METHODS

Islet Transplantation Experiment. Diabetes was induced in adult inbred male Lewis rats (n = 75; 250 to 300 g) by treatment with a single subcutaneous dose of streptozotocin (80 mg per kg body weight) and was defined by a nonfasting blood glucose level higher than 400 mg/dL, manifesting between 1 and 3 days after the administration of streptozotocin. The transplantation procedure was performed as described previously (25, 27). We chose a low number of islets (250–450 islet grafts per animal) so that mild hyperglycemia...
(250–300 mg/dL) persisted for at least 10 months after islet transplantation. The animals were killed under anesthesia between 2 days and 24 months after transplantation. Fourteen nondiabetic rats did not undergo transplantation and served as a control group regarding the spontaneous development of preneoplastic hepatocellular foci, which is known to occur in the experimental group in animals that are ≥2 years old. They were killed simultaneously with the animals of the experimental group.

**Fasting-Refeeding Experiment.** In addition, we investigated 10 nondiabetic control rats that had fasted for 24 hours. Six animals were directly killed, and four animals received food 2 hours before death. This was done to evaluate physiologic insulin receptor distribution at low (fasting) and high (postprandial) intrahepatic insulin concentrations, simulating the conditions in the transplantation experiment. Insulin concentration in the portal vein was measured with Linco’s rat insulin radioimmunoassay kit (Linco, St. Charles, MO). Housing and treatment of all animals conformed to the guidelines of the Society for Laboratory Animals Service (GV-Solas) and the German animal protection law.

**Processing of Tissue.** Tissue was processed as described previously (27). Approximately 2- to 3-mm-thick sections were dehydrated and embedded in paraffin. Serial sections were cut and stained with hematoxylin and eosin or cresyl-violet or with periodic acid Schiff reaction (PAS). Additional sections were analyzed immunohistochemically. FAHs were histologically classified into clear-cell foci (CCF), mixed-cell foci (MCF), and basophilic-cell foci (BCF) as described previously (31). CCF showed enlarged cell bodies with extensive glycogen storage (PAS-positive). The basophilic cytoplasm of BCFs was PAS-negative because of loss of glycogen, and the nuclear-to-cytoplasmic ratio was increased. The basophilia is a result of an increase in the number of ribosomes. FAHs that were composed of glycogen-rich and glycogen-poor hepatocytes in a close spatial relationship were designated as MCF. Lesions were classified as HCCs when they were sharply demarcated, compressed the surrounding liver parenchyma, and showed loss of acinar architecture. HCCs were diagnosed when the lesion exhibited trabeculae thicker than three cell layers in at least two separate areas or showed unequivocal signs of malignancy, such as vascular invasion.

Tissue for Western blotting, quantitative reverse transcription-PCR and in situ hybridization was obtained from the middle lobe of the liver, removed before perfusion with the fixation cocktail; the tissues were then cut in slices, were immediately frozen in isopentane (at −120°C), and were stored at −80°C. RNA, DNA, and proteins were isolated with the TRIZOL method (Life Technologies, Inc., Gaithersburg, MD).

**Immunohistochemistry.** Seventy-eight CCF, 20 MCF, 3 BCF, 14 HCCs, and 7 HCCs were morphologically classified and subsequently investigated immunohistochemically. The β chain of insulin receptor was detected with two different primary polyclonal rabbit antibodies, A1314 (kindly provided by Dr. J. W. Unger, Department of Anatomy, University of Munich, Munich, Germany) and SC-711 (Santa Cruz Biotechnology, Heidelberg, Germany), each at a concentration of 0.5 μg/mL (secondary antibody was goat-antirabbit-IgG, biotinylated, 1.0 g/mL; Dianova, Hamburg, Germany), detected with the CSA-Kit (Dako, Hamburg, Germany) according to the manufacturer’s instructions. Both antibodies resulted in a similar granular staining of the plasma membrane. A1314 was used for semiquantitative analysis.

Polyclonal rabbit antibodies against IRS-1 (Upstate Biotechnology Inc., Lake Placid, New York; dilution: 1:50), Raf-1 (Santa Cruz Biotechnology; dilution: 1:50), and Mek-1 (Santa Cruz Biotechnology; dilution 1:100) were detected with the LSAB+/–Kit (Dako).

Microwave treatment (10 min) was used for antigen retrieval. Rabbit IgG without preliminary immunization served as a negative control in all cases (Oncogen Science/Dianova, Hamburg, Germany). The results were evaluated semiquantitatively by comparing the immunohistochemical reaction within the lesions and the adjacent unaltered liver tissue. The specificity of all primary antibodies was tested by Western blotting, except for A1314, which has already been characterized successfully in a former study (32). Finally, polyclonal anti-insulin, antiglucagon and antisomatostatin antibodies (Dako; dilution 1:200; 4°C incubation for 20 hours) were used as described previously to prove the existence of the transplanted islets or smaller remnants within the foci or tumors (27).

**Immunoelectron Microscopy of the Insulin Receptor.** For electron microscopic evaluation of the ultrastructural localization of insulin receptor, the tissue was embedded in Lowicryl K4M according to Roth et al. (33), with few modifications. Briefly, perfusion-fixed liver tissue was cut into pieces of 0.5 mm in length and fixed for 2 hours in a solution containing 3% paraformaldehyde and 0.2% glutaraldehyde in 0.1 mol/L PBS. The tissue was then rinsed twice for 2 minutes with 0.1 mol/L PBS and put into 30 mmol/L ammonium chloride solution for 2 × 30 minutes. After rinsing in 0.1 mol/L PBS (2 × 5 minutes), the tissue was dehydrated in a graded series of EtOH, beginning with 30% EtOH at 4°C. The last two steps (90% and 100% EtOH) were performed at 35°C; followed by infiltration with Lowicryl in four steps: Lowicryl/H100% EtOH (1:1) at −35°C overnight. Lowicryl/100% EtOH (2:1) at −35°C for 4 to 6 hours, and finally twice with pure Lowicryl at −35°C overnight. UV polymerization of the embedded tissue was performed in a nitrogen atmosphere at −35°C for 12 to 16 hours, followed by a 48-hour period at room temperature.

IR was immunostained with the primary antibody A1314, which was also used for light microscopy. Staining was performed with a two-step method with colloidal gold, bound to the secondary antibody (12 nm-gold-donkey-antirabbit-IgG, dilution 1:50; Dianova, Hamburg, Germany), or by interposition of an amplifying step with a streptavidin-biotin system (34–37). Goat-antirabbit-IgG (1 μg/mL) and 12 nm-gold-donkey-antigoat-IgG (dilution 1:30, Dianova) served as secondary and tertiary antibodies, respectively. Sections were subsequently stained with uranylacetate (10 seconds) and examined on a Phillips CM 10 electron microscope (Eindhoven, the Netherlands).

**In situ Hybridization of IRS-1.** Forty-six CCF and 6 hepatocellular tumors were investigated. A 550-bp cDNA fragment [3092–3642 bp of rat insulin receptor substrate-1 homologue (hIRS-1) DNA; GenBank accession no. S85963] was used for generating RNA probes. The probe was a kind gift of Dr. L. Mohr (Molecular Hepatology Laboratory, Massachusetts General Hospital Cancer Center, Charlestown, MA). After subcloning and plasmid amplification, 35S-labeled UTP labeling of the RNA probes was performed with SP6 or T7 polymerase. Probes were hydrolyzed to a length of ~200 bp. We performed prehybridization, hybridization, and washing procedures, including the removal of nonspecifically bound probe by RNase A digestion for both antisense and sense strand 35S-labeled RNA probes, exposition for 2 to 6 weeks and autoradiographic detection of activity as described previously (38).

**Quantitative Reverse Transcription-PCR of IRS-1.** Pooled tissue of several early clear-cell FAHs of two animals and six hepatocellular tumors was investigated. Kryostat sections were laser-microdissected for harvesting mRNA of the FAHS and the unaltered control tissue of the same animal as described previously (39). After RNA preparation (RNAeasy kit, Qiagen, Hilden, Germany) and reverse transcription (Reverse transcription system, Promega, Madison, WI), cloning and PCR amplification were done as described elsewhere (39). Briefly, IRS-1 and β-actin (internal control) were amplified with the LightCycler-FastStart DNA Master Hybridization Probes kit (Roche, Mannheim, Germany). Sequences of primers, hybridization probes, length of PCR-products, and annealing temperatures are given in Tables 1 and 2. Relative expression levels of different samples of FAHs were calculated by quantification of IRS-1 levels, normalized to the endogenously expressed house gene β-actin according to the formula 2(Rt−Et−Etn), where $R_t$ and $E_t$ are the threshold cycle numbers for the reference gene β-actin and the IRS-1 gene in FAHS, respectively, and $R_0$ and $E_0$ fulfill the same purpose for the unaltered tissue.

**RESULTS**

**Insulin Receptor**

**Zonal Distribution and Localization in Normal Tissue.** Immunohistochemical detection of insulin receptor resulted in a predomi-
nant reaction of hepatocytes, whereas nonparenchymal cells showed only weak staining (Fig. 1A). Signal intensity was higher in diabetic than in healthy control rats. In most animals, there was a zonal distribution with a predominance of insulin receptor expression in acinar zone 3 (Fig. 1B). Cytoplasmic staining was weak, whereas membranous staining was strong, particularly in the sinusoidal part.

**Fasting-Refeeding Experiment.** Feeding resulted in an approximately 15-fold increase in portal vein blood insulin concentration (0.1 ng/mL in fasted versus 1.54 ng/mL in re-fed animals). As expected, this increase in sinusoidal insulin concentration in the re-fed group was accompanied by translocation of the insulin receptor from the cell membrane into the cytoplasm, resulting in diffuse or sometimes granular staining of hepatocytes (Fig. 1C and D). In contrast to fasted animals, in which the hepatocytes were glycogen-depleted, re-fed animals showed increased glycogen storing as expected. Thus, high insulin concentrations in sinusoidal blood lead to translocation of a large part of the membrane-bound insulin receptor into cytoplasm, resembling the initial step of insulin signaling in hepatocytes.

**Expression and Localization in FAHs and Hepatocellular Tumors.** The morphology of the FAHs was not different from the former experiments. FAHs developed within the first days after transplantation and had a clear-cell morphology (CCF) in the beginning because of increased glycogen and storing of fat. The clear-cell phenotype was also overall predominant (78 of 101 FAHs).

### Table 2. Sequences of primers, hybridization probes, and lengths of PCR product

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence</th>
<th>Length</th>
<th>Annealing Temperature</th>
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<tbody>
<tr>
<td>β-Actin-F</td>
<td>5'-CAC ggC ATT gTA ACC AAC Tgg gAC</td>
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<tr>
<td>β-Actin-R</td>
<td>5'-CAg Tgg TAC gAC CAg Agg CAT ACA gg</td>
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<tr>
<td>hyb β-Actin-X</td>
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<tr>
<td>hyb β-Actin-LC</td>
<td>5'-LC-CGC AAg TCA TAg AAA gTg Tgg TgC CA-p</td>
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Note. For rat β-actin, GenBank accession no. was NM 031144; length was 232 bp; and annealing temperature was 71°C.

Abbreviations: -F and -R, forward and reverse primer; hyb, hybridization probe; X, fluorescein; LC, light cycler red 640; p, phosphate.

Fig. 1. Immunohistochemical expression of the insulin receptor in nonneoplastic tissue (A–D), clear-cell FAH (E–F), and hepatocellular neoplasms (G–H). Insulin receptor in normal hepatocytes is nearly exclusively located on the sinusoidal membrane (A). A zonal distribution with predominant expression in the acinar zone 3 (Fig. 1B). Cytoplasmic staining was weak, whereas membranous staining was strong, particularly in the sinusoidal part.

- In animals that fasted for 24 hours, this membranous pattern of insulin receptor staining was stable (c).
- Feeding the fasted animals resulted in high sinusoidal insulin concentrations and provoked a translocation of the receptor into the cytoplasm (D), but membranous staining is still visible. In E, a transplanted islet is located in the lower left corner. The hepatocytes of the surrounding preneoplastic focus, corresponding to the liver acinus, which drains the hyperinsulinemic blood from the islet graft, show a translocation of the insulin receptor into the cytoplasm, whereas the hepatocytes from a neighboring acinus (right edge and upper right corner) maintain their membranous staining pattern. At higher magnification (F), the clear-cell morphology and the lack of membranous insulin receptor staining of the pre-neoplastic hepatocytes (left part) as well as the sharp border to hepatocytes of the neighboring non-altered acinus (right part) can be seen. (G) and (H) show a HCA (G, upper left part) and a HCC (H, left part) at the border to the adjacent unaltered parenchyma, respectively. Both tumors were of clear-cell morphology, because of the maintenance of increased glycogen and lipid storage, and show a pronounced intracytoplasmic localization of the insulin receptor, when compared not only to the adjacent unaltered liver tissue but also to preneoplastic foci. ×452 (A); ×577 (B); ×220 (C); ×141 (D, E, G); ×550 (F); ×110 (H), antibody A1314.
types of foci, i.e., MCF (n = 20) or BCF (n = 3), developed after several weeks.

Because of the hyperinsulinemic environment, FAHs, indeed, showed an altered signaling pattern in insulin receptor immunohistochemistry when compared with the adjacent hypoinsulinemic liver tissue. CCF showed a considerable increase of granular cytoplasmic signals with simultaneously decreased or sometimes unaltered membrane staining, resembling the same translocation of the receptor from membrane into cytoplasm, as it was induced in the re-fed group of the fasting experiment (Fig. 1E and F; Table 3).

Ultrastructurally, the immunogold-labeled insulin receptor-antibody detected most of the receptor molecules on the microvilli in the space of Disse and at the border between the sinusoidal and the lateral cell surface in normal tissue (Fig. 2A). Adjacent collagen fibers were free of gold particles. They were also rare in endothelial cells. The hepatocyte nuclei, mitochondria, lipid droplets, and peroxisomes were virtually free of gold staining; few granules were sometimes noted within the membrane system of rough endoplasmic reticulum. Most intracellular, i.e., non-membranous gold particles in the hepatocytes, appeared in groups within vesicles, particularly near the sinusoidal membrane. By contrast, in the preneoplastic hepatocytes within the FAHs, most of the gold particles were located within these cytosolic vesicles, and the membranous portion was decreased (Fig. 2B), supporting the results obtained by light microscopy.

Pure BCFs with a homogenous basophilic-cell population showed no differences in membrane-bound staining, and cytoplasmic staining was equal or even slightly decreased when compared with the adjacent parenchyma (Table 3). The individual glycogen-storing and basophilic hepatocytes within the MCF had the same signaling pattern as in CCF and BCF, respectively, i.e., the clear cells showed insulin receptor translocation, whereas the basophilic cells did not. Interestingly, all of the very few spontaneous FAHs, observed in 2-year-old untreated animals of the control group belonged to the clear-cell type and showed the same alterations.

The 14 HCAs and 7 HCCs of this experiment can be subdivided into tumors composed predominantly of glycogen-storing (11 adenomas and 4 carcinomas) or basophilic cells (3 adenomas and 3 carcinomas). The glycogen-storing phenotype still predominated in the adenomas, but was less frequent in the carcinomas. Cytoplasmic insulin receptor expression in the clear-cell tumors was higher when compared not only with the unaltered adjacent parenchyma but also with the FAHs, and it was still strongly associated with increased glycogen storage (Fig. 1G and H), while basophilic neoplasms, analogous to the preneoplasias, showed no translocation. Membrane-staining was difficult to assess, because the tumors do not possess the sinusoidal architecture that can be preserved by perfusion fixation. In cases in which evaluation was possible, there was no difference from the unaltered liver tissue. In addition, no difference was found to exist between adenomas and carcinomas. Thus, the majority of tumors showed insulin receptor overexpression. As in the fasting experiment, one might conclude that a high insulin concentration in the sinusoidal blood within early FAHs leads to permanent internalization of receptor-bound insulin into the hepatocytes. It is of particular interest that even in later stages, i.e., after several months, this pattern was maintained, and no adaptive receptor down-regulation, which might have been expected, was observed.

Insulin Receptor Substrate-1, Raf-1, Mek-1

In control animals and in the unaltered liver tissue of diabetic rats, IRS-1, Raf-1 and Mek-1 proteins were detected immunohistochemically in hepatocytes and in nonparenchymal cells, presenting as weak granular staining of the cytoplasm. In the hepatocytes of all of the CCF examined, there was a striking increase in protein...
immunoreactivity (Fig. 3A–H; Table 3). The majority of the MCF also showed an increase in signaling when compared with the unaltered adjacent tissue, although the amount of protein was smaller than in CCF and was closely correlated with the portion of glycogen-storing cells within the MCFs (Fig. 3I–N). In the few pure BCF, the overall extent of IRS-1 was as low as in the adjacent unaltered parenchyma or was slightly decreased (Fig. 4A and B).

The amount of the different proteins in HCAs and HCCs was similarly correlated with the ratio of glycogen-storing-cells to basophilic-cells as in FAHs, and the overall increase in signal reactivity for these proteins in the neoplasms was about the same as for insulin receptor (Fig. 4C–F). Analogous to insulin receptor, the few spontaneous clear-cell preneoplastic hepatocellular foci in the 2-year-old animals of the untreated control group showed the

![Fig. 3. Immunohistochemical expression of IRS-1, Raf-1, and Mek-1 in clear-cell preneoplastic foci (A–H) and a MCF (I–N). Liver tissue 6 months after islet transplantation (A–D) containing three FAHs. In the PAS reaction (A), the FAHs were highlighted because of their increased glycogen and lipid content. Serial sections were stained with antibodies directed for IRS-1 (B), Raf-1 (C), and Mek-1 (D) and show an increase in expression of these proteins within the FAHs, even at low magnification. In E, a clear-cell FAH in more detail is depicted in PAS reaction. The lesion is restricted within the liver acinus (hepatic venule in the lower right part), draining the blood from the islet graft, which is located within a portal tract (arrow). F–H, serial sections to E, with antibodies against IRS-1 (F), Raf-1 (G), and Mek-1 (H). Normal protein expression is visible in the unaltered liver tissue of the adjacent parenchyma. Serial sections of a MCF are shown in I–N. In the PAS reaction (I), the majority of the hepatocytes within the MCF is pale, only a few single cells dispersed and a group of cells (arrow) have maintained the glycogen-storing phenotype. Vice versa, these latter cells were not stained with the basophilic reagent cresyl-violet (J), whereas the basophilic cell population stains deeply blue-violet. Interestingly, the glycogen-storing cells in this focus do not show the translocation of the insulin receptor from the membrane to the cytoplasm (K), which is characteristic for the early foci of pure clear-cell phenotype and as it is depicted in Fig. 1. The IRS-1 (L), Raf-1 (M), and Mek-1 (N) overexpression is strictly connected with the clear-cell (glycogen-storing) phenotype of the preneoplastic hepatocytes. The content of the respective proteins within the basophilic cell population is slightly decreased in this focus, when compared with the unaltered hepatocytes of the adjacent parenchyma (lower left parts of the panels). A–D, ×7; E, ×71; F–H, ×47; I–N, ×62.

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same staining pattern as did their insulin-induced counterparts (Fig. 4G–I).

In situ hybridization for IRS-1 mRNA revealed specific positive signals in the cytoplasm of hepatocytes in normal, preneoplastic, and neoplastic liver tissue. Sinusoids and nuclei, however, were virtually devoid of any signals. When compared with unaltered adjacent liver parenchyma, the FAHs showed a slight increase in signal density (Fig. 5); in a minority of the cases, no difference was noted. As with in situ hybridization, quantitative reverse transcription-PCR of laser-microdissected liver tissue also showed an overexpression of pooled IRS-1 mRNA in the FAHs when compared with the unaltered liver tissue in both animals, 6.0 ± 1.9-fold and 6.3 ± 1.9-fold, respectively.

DISCUSSION

It is generally accepted that insulin signaling is primarily accomplished by binding to the membranous insulin-receptor, although there is also evidence that insulin can enter the cytoplasm of hepatocytes via fluid-phase-endocytosis (8). A recent investigation of the normal rat liver has shown that the insulin receptor protein is predominantly localized in hepatocytes of the perivenous zone, mainly at the plasma...
membrane (40), which is corroborated by our observation in control tissue of normoglycemic and diabetic, *i.e.*, systemically hyperglycemic and hypoinsulinemic, animals. In addition, we confirmed the results obtained in earlier studies according to which, hepatocytes of streptozotocin-diabetic rats contain more insulin receptor than do normoglycemic animals (41–42).

The main alteration regarding the insulin receptor in early preneoplastic CCF, which drain the blood from the islet grafts, was a translocation from the plasma membrane into the cytoplasm, demonstrated by light and electron microscopy. At the beginning, this appears to be the consequence of constant extracellular insulin receptor binding caused by the local hyperinsulinemia and subsequent receptor internalization. This is supported by the finding that we were able to induce a similar translocation of the insulin receptor by refeeding fasting rats, which showed a high postprandial insulin concentration in their portal-venous blood. However, despite the translocation, overall protein expression of the insulin receptor was not altered in early CCF, which stands in contrast to the observation of insulin receptor down-regulation in (hyperinsulinemic and hyperglycemic) type 2 diabetes in humans, and to a former study, in which insulin-treated streptozotocin-diabetic rats and rat hepatoma cells have shown a decrease in insulin receptor mRNA or protein expression, measured indirectly by the binding of 125I-labeled insulin (43–44). This absence of receptor down-regulation is most likely the consequence of the systemic hyperglucagonemia caused by insulin deficiency (45), which has been demonstrated in streptozotocin-diabetic rats, and can be restored by exogenous insulin treatment or high-dose islet transplantation (46–47). The absence of insulin receptor down-regulation might be important for the development and maintenance of carcinogenesis in this model.

It also intriguing that glycogen-storing HCAs and HCCs maintain the cytosolic pattern of insulin receptor expression and even show increased expression. This pattern can be easily explained for the early FAHs, which are confined to the anatomic borders of the liver acinus and drain the hyperinsulinemic blood from the islet grafts. With the increasing size of the lesions, particularly after neoplastic transformation, however, the often scattered islet graft remnants cannot be made responsible for this overexpression, although they can be regularly demonstrated within the tumors (27). Although the intraslesional insulin concentration cannot be measured, it can be assumed that the former hyperinsulinemia, induced by the islet grafts, has diminished or is completely absent within these tumors. Thus, the insulin receptor overexpression seen in the tumors cannot exclusively be explained as a consequence of increased insulin internalization. Additional factors, such as impaired or altered degradation, lack of retranslocation to the plasmamembrane or genetic and epigenetic mechanisms have to be taken into account.

Overexpression of insulin receptor and IRS-1 has been shown in human and rodent HCC and in other neoplasms (12–14, 19). Moreover, the transforming potential of IRS-1 in hepatic and nonhepatic cell lines has been well documented (16, 48). IRS-1 overexpression also protects HCC cells against transforming growth factor-β-induced apoptosis (21). *Vice versa*, the expression of an inhibitory COOH-terminal-truncated IRS-1 molecule resulted in reversal of the malignant phenotype of human HCC cells (20). Conversely, transgenic mice overexpressing IRS-1, do not develop hepatocellular tumors, although they show increased liver growth in the first 3 months of life (49). Particularly interesting is the overexpression of IRS-1 as an early event in a rat model of *N*-nitrosomorpholine-induced hepatocarcinogenesis, because this model is similar to ours in some aspects, particularly regarding the insulinomimetic phenotype of the preneoplastic hepatocytes (17). In that study, the authors also observed a strong correlation between the IRS-1 overexpression and the extent of glycogen-storage in the preneoplastic and neoplastic cells, suggesting that the phenotype of preneoplastic hepatic glycososis is elicited by an overexpression of IRS-1. This was also demonstrated by the same authors in another investigation, in which amphophilic (non-glycogen-storing) FAHs did not show overexpression of IRS-1 (18). However, it is unclear which mechanisms lead to the overexpression of IRS-1 in this chemically induced model of hepatocarcinogenesis. In our model, the alterations in the insulin signaling transduction cascade have also been observed from the beginning of the formation of preneoplastic foci, even 2 days after islet transplantation; therefore, they should be regarded as a direct consequence of the local hyperinsulinemia. Although Ras itself has not yet been investigated, the overexpression of several upstream and downstream proteins of the Ras-Raf-1/Mek-1 pathway as shown in this study indicates that this pathway is essentially involved in mediating the insulin signal in this model, thus contributing to the alterations in cell metabolism, proliferation kinetics, and the development and maintenance of the preneoplastic phenotype. Although the phosphorylation status of the signaling proteins is difficult to establish by immunohistochemistry alone, and the FAHs in this model are too small for Western blotting after laser microdissection, it is very likely that the overexpression of the proteins is accompanied by phosphorylation as has been already shown. Toyoda et al. (24), with another model of hepatocarcinogenesis in rats, have recently demonstrated that the expression and phosphorylation statuses of IRS-1, ERK-1 and ERK-2 are altered in the same manner so that overexpression is indeed accompanied by increased activity.

At later stages of the carcinogenic process, the FAHs become more diverse, showing a decrease in glycogen storage in part of (MCF) or in all preneoplastic hepatocytes (BCF), although the majority maintain their glycomicogenic appearance even after neoplastic transformation. It is unclear, however, whether the maintenance of the glycogen-storing preneoplastic character of the altered hepatocytes at this time point still depends on continuous insulin signaling in principle, or on other mechanisms that have meanwhile been activated, fixing the carcinogenic process and making it “irreversible.” In this context it is noteworthy that transforming growth factor-α is overexpressed in late-stage foci and particularly in the glycogen-poor HCCs, and that single HCCs in this model display strong IGF-II overexpression (27, 29).

In conclusion, we showed that in a model of low-number islet transplantation into streptozotocin-diabetic rats, insulin induces a morphologic and metabolic transformation of hepatocytes by activation of its intracellular signaling cascade, involving the Ras-Raf-MAPKK pathway. This transformation results in the clear-cell “insulinomimetic” phenotype of preneoplastic FAH, which is known to be induced by chemical carcinogens, such as *N*-nitrosomorpholine. Future studies will show whether other intracellular transduction cascades of the insulin signaling network, in particular the phosphoinositide-3-kinase/AKT-pathway and related transcription factors, such as nuclear factor κB or the forkhead transcription factor FKHR, are activated, and whether this activation also contributes to the carcinogenic process in this model.

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