Hyperproliferative Hepatocellular Alterations after Intraportal Transplantation of Thyroid Follicles

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The thyroid hormone 3,5,3'-triiodo-L-thyronine (T3) is a strong direct hepatocyte mitogen *in vivo*. The effects of T3 resemble those of peroxisome proliferators, which are known to induce hepatocellular tumors in rats. With the aim of studying long-term local effects of thyroid hormones on liver parenchyma, small pieces of thyroid tissue were transplanted via the portal veins into the livers of thyroidectomized male Lewis rats. At 1 week, 3 weeks, 3 months, and 18 months after transplantation, the transplants were found to proliferate, to synthesize thyroglobulin, and to release thyroxine and T3. At 3 and 18 months after transplantation, the hepatocytes of the liver acini downstream of the transplanted follicles showed an increase in cytoplasmic basophilia, a loss of glycogen, an enlargement and hyperchromasia of their nuclei, and a strong increase in cell turnover compared with unaltered liver acini. The altered hepatocytes exhibited an increase in the activities of glucose-6-phosphate dehydrogenase, glucose-6-phosphatase, malic enzyme, mitochondrial glycerol-3-phosphate dehydrogenase, cytochrome-c-oxidase, and acid phosphatase; the activities of glycogen synthase and glycogen phosphorylase were strongly decreased. The hepatocytic alterations downstream of the transplanted follicles could be explained by effects of T3. On the other hand, they resembled alterations characteristic of amphophilic preneoplastic liver foci observed in different models of hepatocarcinogenesis. (Am J Pathol 2000, 156:99–113)

Although T3 has been shown to be a strong mitogen on hepatocytes *in vivo*,^{1,2} little is known about long-term effects of thyroid hormones on liver parenchyma.³ The idea to transplant thyroid follicles into the liver of thyroidectomized rats was born from a similar transplantation model, in which a long-term increase in locally secreted pancreatic islet hormones (especially insulin) induced

alterations of liver acini downstream of intrahepatically transplanted islets of Langerhans in diabetic rats.⁴ The hepatocellular alterations were in many respects in line with insulin effects and simultaneously similar to those observed in glycogen-storing foci, which are well known from different models of hepatocarcinogenesis.⁵ The glycogen-storing liver acini downstream of the transplanted islets proceeded to hepatocellular neoplasms as shown in a long-term study.⁶

One type of preneoplastic liver focus, namely the amphophilic cell focus, which is well known to occur in different models of hepatocarcinogenesis in different species^{7,8} and which is one of the most often observed types of focal lesion in human liver cirrhosis,⁹ was not induced by the islet transplants.⁶ This type of focus of altered hepatocytes is characterized by amphophilic (basophilic and eosinophilic) cytoplasm due to a proliferation of mitochondria, which are closely associated with rough endoplasmic reticulum, and a strong decrease in glycogen.⁷ Alterations of enzyme activities in this type of preneoplastic liver focus show an energy-wasting metabolism and have been suggested to reflect thyromimetic effects of the responsible oncogenic agents.^{10,11} We thus decided to investigate whether intrahepatic thyroid follicle transplants are able to induce amphophilic cell foci in thyroidectomized rats.

Materials and Methods

Experimental Design and Transplantation Procedure

Male Lewis rats (inbred strain) weighing about 200 g were used. In the main group (MG), the animals were thyroidectomized; 2 weeks later thyroid tissue pieces were transplanted via the portal vein into the right part of the liver. The animals of control group 1 (CG1) were not thyroidectomized; thyroid tissue pieces were transplanted via the portal vein into the right part of the liver. Control group 2 animals (CG2) were thyroidectomized but did not receive a transplant. The thyroidectomy was done under an anesthesia with diethylether. Housing and

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Table 1. Effect of Thyroid Follicle Transplantation on Body Weight

Animal group*	% Transplantation body weight at time of sacrifice			
	1 week	3 weeks	3 months	18 months
MG	101 ± 2 (6)	101 ± 3 (6) [†]	137 ± 13 (6)	221 ± 35 (3)
CG1	99 ± 4 (6)	123 ± 6 (6)	145 ± 11 (6)	—
CG2	104 ± 2 (6)	107 ± 5 (6)	111 ± 3 (6) [†]	—

Body weight at the day of transplantation was set as 100% for MG and CG1. CG2 animals were matched without a transplantation. Mean values ± SEM are shown. The number of animals (*n*) is shown in parentheses.

*MG, Main group (thyroidectomy and intrahepatic thyroid follicle transplantation); CG1, control group 1 (intrahepatic thyroid follicle transplantation and no thyroidectomy); CG2, control group 2 (thyroidectomy and no transplantation).

[†]Mean was significantly different from CG1.

treatment of the animals were in line with the guidelines of the Society for Laboratory Animals Service (GV-Solas) and the strict German animal protection law.

In preliminary experiments, it was found that thyroid follicles or small pieces of thyroid tissue dissected by razor blades were too fragile for an effective transplantation. The rigidity of intrafollicular thyroglobulin was suspected to be the main problem. Therefore a pretreatment of donor animals was necessary to get follicles almost depleted of thyroglobulin. Donor animals received an iodine-poor diet (<0.04 µg iodine/g) with 0.05% propylthiouracil and tap water for 10 days, followed by an iodine-poor diet and H₂O containing 1% KClO₄ for 2 days; and finally an iodine-poor diet and H₂O for 3 days. The following day the donor animals were killed, and the thyroid gland was prepared. The pretreatment resulted in an about fourfold enlargement of the gland with almost empty follicular lumina. The glands were washed with Hanks' solution (pH 7.2) and dissected with razor blades into small cubes of about 0.2 mm³. One gland was used for two recipient animals. The small cubes were dispersed in ice-cold Hanks' solution (pH 7.2) with 1% Dextran (*M_r* 35,000–50,000; Merck, Darmstadt, Germany) and were aspirated with 500 µl of this mixture into a syringe with a needle of 23G × 1". The addition of dextran reduced the attachment of the tissue cubes to each other and to the wall of the syringe. The recipient animals were

anesthetized with diethyl ether, and the thyroid tissue pieces were transplanted only into the right part of the liver as follows. 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 thyroid transplants into the portal vein, the clamp was removed (maximal time of ischemia, 1 minute). With this procedure it was possible to infuse the thyroid tissue pieces 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 between the right part and the left part of the middle lobe is marked by the falciform ligament). Thus, the left part of the middle lobe and the left lobe could be taken as an internal control in the experiments of the MG and the CG1.

At 1 week, 3 weeks, and 3 months after transplantation, six animals of each experimental group were killed between 10:00 a.m. and 11:30 a.m. An additional three animals of the MG were killed 18 months after transplantation. Additional MG animals were killed at days 3 (three animals) and 4 (three animals) without 5-bromo-2'-desoxyuridine (BrdU) application and without serum sampling (see below). Two hours before killing, another MG animal at 2 months after transplantation received 200 µCi ¹²⁵I intravenously, and the intrahepatic thyroid transplants were prepared for autoradiography.

Table 2. Effect of Thyroid Follicle Transplantation on Serum

Animal group* and serum factor	Serum ft3 and ft4 (pg/ml) and TSH (ng/ml) at time of sacrifice [†]			
	1 week	3 weeks	3 months	18 months
MG				
ft3	1.2 ± 0.2 (3) [‡]	1.1 ± 0.2 (5) [‡]	1.2 ± 0.2 (5) [‡]	3.4 ± 0.3 (3) ^{§¶}
ft4	3.9 ± 1.1 (3) [‡]	4.1 ± 2.0 (5) [‡]	5.9 ± 2.2 (5) [‡]	20.7 ± 0.6 (3) ^{§¶}
TSH	58.7 ± 4.3 (3) [‡]	61.3 ± 1.2 (5) [‡]	62.1 ± 1.7 (5) [‡]	9.3 ± 0.3 (3) ^{§¶}
CG1				
ft3	3.2 ± 0.5 (3)	3.2 ± 0.3 (5)	3.4 ± 0.2 (5)	—
ft4	23.5 ± 2.7 (3)	26.5 ± 2.6 (6)	21.6 ± 4.1 (5)	—
TSH	11.8 ± 1.4 (3)	12.9 ± 1.1 (6)	12.4 ± 1.6 (5)	—
CG2				
ft3	0.8 ± 0.1 (3)	0.5 ± 0.3 (6)	0.7 ± 0.0 (4)	—
ft4	0.8 ± 0.1 (3)	2.4 ± 1.3 (5)	2.8 ± 1.1 (4)	—
TSH	63.1 ± 0.9 (3)	62.0 ± 1.3 (6)	61.1 ± 0.3 (4)	—

*MG, Main group (thyroidectomy and intrahepatic thyroid follicle transplantation); CG1, control group 1 (intrahepatic thyroid follicle transplantation and no thyroidectomy); CG2, control group 2 (thyroidectomy and no transplantation).

[†]Mean values ± SEM are shown. The number of animals (*n*) is shown in parentheses.

[‡]Significantly different from CG1 and not different from CG2.

[§]Significantly different from all of the preceding times.

[¶]Not different from data of CG1 at 3 months.

^{||}Significantly different from CG2.

Figure 1. The method of perfusion fixation and stereomicroscopic preparation of unstained liver slices allowed finding of small transplants **a** and **b**: The same tissue piece 1 week after transplantation (MG). In the MG, the transplants were completely vascularized during the first 3 weeks. (**c**, 3 days after transplantation; **d**, 3 weeks after transplantation. The transplants of CG1 underwent fibrosis, obviously because the serum levels of TSH were not increased (**e**, 3 weeks after transplantation; **f**, 3 months after transplantation). **a**: Unstained liver slice; **b-d**: Epon-sections stained by the method of Richardson; **e** and **f**: paraffin sections stained with H&E. Original magnifications, $\times 27$ (**a**), $\times 40$ (**b**), $\times 240$ (**c** and **d**), $\times 254$ (**e**), and $\times 200$ (**f**).

Figure 2. Three weeks after transplantation the thyroid follicle cells of the transplants of the MG showed regular organelles and microvilli. At this time the process of transplant vascularization was finished. The endothelial cell of the capillary, which is situated close to the thyroid follicle, shows regular fenestrations (**arrowheads**). The lumen of the follicle is at the top of the figure. Electron micrograph; original magnification, $\times 9500$.

BrdU Application by the Method of Eldridge et al¹²

Seven days before killing, half of the animals to be killed at 1 week, 3 weeks, and 3 months and all three animals to be killed at 18 months were anesthetized, and osmotic pumps (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. These pumps continuously delivered BrdU until the animals were sacrificed. The remaining animals to be killed at 1 week, 3 weeks, and 3 months received a single dose of 50 mg BrdU/kg body weight intraperitoneally 1 hour before sacrifice.

Preparation of Tissues

Slices from the right part and from the left part of the middle lobe of the liver were snap-frozen and were used for enzyme histochemistry (see below). After removing the middle lobe of the liver, the animals were perfused with a mixture of 0.5% glutaraldehyde and 3% formaldehyde as described earlier.⁴ Immediately after perfusion, about 40 slices per animal were cut from the fixed liver lobes. These slices were transferred into phosphate-buffered saline (PBS) and were systematically examined with a stereomicroscope. With some experience it was possi-

Figure 3. Three weeks after transplantation the follicles of this transplant of the MG show positive staining for TG (**A**), T4 (**B**), and T3 (**C**). The transplant is typically situated within a small branch of the portal vein. Serial paraffin sections; immunofluorescence; original magnification, $\times 150$.

ble to identify transplanted thyroid tissue pieces in these unstained liver slices as well as after embedding (see Results). Corresponding slices of the same transplants were embedded in Epon and in paraffin. Additionally, pituitary gland, kidney, adrenal glands, lung, heart,

Figure 4. This autoradiogram shows the regular uptake of ^{125}I in the intrahepatic thyroid follicles of an animal of the MG 2 months after transplantation. Original magnification, $\times 250$.

spleen, and pancreas were embedded in paraffin. Four horizontal sections of the upper trachea and larynx together with surrounding tissue were embedded in paraffin to check the area of thyroidectomy (completeness of thyroidectomy) of the MG and the CG2 or the thyroid gland of CG1.

From the paraffin-embedded specimen, serial sections of 2 to 3 μm in thickness were stained with hematoxylin and eosin (H&E) and with the periodic acid-Schiff reaction (PAS). Additional sections were used for immunohistochemistry. In addition, 10 small cubes (1 mm^3) were cut from each liver and were embedded in Epon. Semithin sections of the Epon-embedded specimens were stained by the method of Richardson et al.¹³ 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 PAS stains, appropriate sections were selected and the corresponding sections were processed for immunohistochemistry. Immunostains of the liver for BrdU (monoclonal primary antibody from DAKO, Hamburg, Germany; dilution 1:100) and transforming growth factor- α (monoclonal primary antibody from Oncogene Science, Cambridge, MA; final antibody concentration 10 $\mu\text{g}/\text{ml}$) with antigen retrieval steps were performed as described earlier.^{4,6} Glutathion S-transferase placental form (GST-P; polyclonal primary antibody from Biogenex, San Ramon, CA; dilution 1:100) was analyzed without antigen retrieval steps, using the LSAB⁺-Kit (DAKO) and the DAB⁺-Kit (DAKO). Immunostains of the hypophysis for thyroid-stimulating hormone (TSH) were done with a monoclonal antibody from DAKO

Figure 5. The strong proliferative activity of the follicular epithelial and mesenchymal cells 3 weeks after transplantation is shown by the immunohistochemical localization of BrdU-labeled nuclei in **a** and **b** (left side in **b**). The first alteration of the hepatocytes within the acini downstream of the transplanted thyroid follicles was an increase in proliferative activity (**b** and **c**) 3 weeks after transplantation (compare with Table 3). **c**: The sharply defined border between a hyperproliferative acinus in the left part of the figure, which is under the influence of a transplant (not shown) and the nonaltered liver tissue in the right part of the figure. Immunostains for BrdU (administered via subcutaneously implanted osmotic pumps for 7 days); original magnifications, $\times 142$ (**a** and **b**) and $\times 111$ (**c**).

(dilution 1:50) using the LSAB⁺ and the DAB⁺ Kits. Sections were counterstained with hematoxylin, dehydrated, and coverslipped with Pertex (Mediate, Burgdorf, Germany).

Anti-T3 and anti-T4 (polyclonal primary antibodies from ICN Biomedicals, Eschwege, Germany) were applied at a dilution of 1:250, anti-TG (polyclonal antibody, Institute of Cell Biology, University of Bonn, Bonn, Germany) and anti-calcitonin (monoclonal antibody from DAKO) were used at a

Figure 6. PAS-stained section shows glycogen loss in the altered liver acini downstream of a thyroid transplant, which is situated in a portal triad, 3 months after transplantation (thyroid follicles are strongly PAS-positive in the center of the figure). The topography of the central veins (arrowheads) shows that the altered areas represent acini. Only animals of the MG exhibited these altered liver acini. Original magnification, $\times 140$.

dilution of 1:50. Secondary fluorescein isothiocyanate-conjugated goat anti-rabbit or goat anti-mouse antibodies (Sigma Chemical Co., Heidelberg, Germany) were used at a dilution of 1:50. In controls primary antibodies were omitted.

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

H&E-stained paraffin sections were viewed systematically for apoptotic and mitotic cells at a magnification of $\times 400$. Mitotic and apoptotic indices (MI and AI) were calculated as the number of mitotic figures and of apoptotic bodies, respectively, per 1000 hepatocytic nuclei. Paraffin sections immunostained for BrdU were examined at a magnification of $\times 400$. BrdU labeling indices (BrdU-LI) were calculated as the number of BrdU-labeled nuclei per 1000 hepatocytic nuclei. 5000–15,000 hepatocytic nuclei were counted per animal.

For the MG and the CG1, all indices were calculated separately for the liver acini downstream of the transplanted thyroid follicles in the right part of the liver, and in the left part of the liver which was free of transplants

(intraindividual control). When no alterations were identifiable downstream of the transplants (MG at 1 week; CG1 at all times), the indices for the right part of the liver were calculated with the hepatocytes at a distance of about 1 mm from the transplants. For CG2 the indices were calculated only for the right lobe.

The body weight, AI, MI, BrdU-LI at 1 hour, BrdU-LI at 7 days, serum T3, serum thyroxine (T4), and serum TSH of the different animal groups and the different times after thyroid follicle transplantation (Table 1) were compared with the Wilcoxon-Mann-Whitney test. Significance was accepted when $P < 0.05$.

^{125}I Application and Autoradiography

Two hours before sacrifice, 200 μCi of ^{125}I were injected into the tail vein of an animal from the MG at 2 months after transplantation. The liver tissue was fixed in 4% formaldehyde and embedded into paraffin. Autoradiography was done with 3- μm -thick paraffin sections and

Figure 7. 18 months after thyroid follicle transplantation, single very large transplants were found, which are surrounded by altered liver acini. **a:** The stereomicroscopic aspect of an unstained liver slice. **b–g:** Paraffin sections of the same liver slice. The thyroid follicles are PAS-positive (**c, e, h**) but the altered liver acini are PAS-negative and show an amphophilic cytoplasm (**b, d, g**). **d–f:** An area of **a–c** at higher magnification. **f:** Immunohistochemical localization of BrdU-labeled nuclei exhibits an increase in proliferative activity in the altered liver acini associated with the transplanted follicles. **g:** Close spatial relationship and the synchronous appearance of mitotic figures (**arrows**) and apoptotic bodies (**arrowheads**) of hepatocytes within the altered acini. **h:** The phenomenon that some hepatocytes within the altered acini exhibited areas of glycogen accumulation. **i:** At 18 months the nuclei of the altered hepatocytes were enlarged and showed multiple condensations of their chromatin and several nucleoli (Epon-section stained by the method of Richardson et al¹³). Original magnifications, $\times 24$ (**a**), $\times 30$ (**b** and **c**), $\times 155$ (**d–f**), $\times 310$ (**g**), $\times 242$ (**h**), and $\times 385$ (**i**).

Table 3. Effect of Thyroid Follicle Transplantation on Hepatocytic Proliferation and Apoptotic Elimination

Animal group,* index, and liver part	Index count at time of sacrifice†			
	1 week	3 weeks	3 months	18 months
MG				
MI-r	0.1 ± 0.1 (6)	2.2 ± 1.0 (6)	4.4 ± 0.4 (6) ^{‡§¶}	21.4 ± 3.9 (3) ^{‡§¶}
MI-l	0.08 ± 0.03 (6)	0.08 ± 0.06 (6) [§]	0.05 ± 0.03 (6) [§]	0.60 ± 0.21 (3)
AI-r	0.3 ± 0.3 (6)	0.4 ± 0.2 (6)	1.8 ± 0.4 (6) ^{‡§¶ **}	3.6 ± 0.8 (3) ^{‡ **}
AI-l	0.01 ± 0.02 (6) [§]	0.04 ± 0.02 (6)	0.05 ± 0.03 (6)	0.20 ± 0.06 (3)
BrdU1h-r	1.5 ± 0.1 (3) ^{‡¶}	37.9 ± 5.1 (3) ^{‡§¶}	11.6 ± 0.3 (2) ^{‡§¶ **}	—
BrdU1h-l	0.8 ± 0.0 (3)	0.7 ± 0.2 (3)	0.6 ± 0.2 (2)	—
BrdU7d-r	48.9 ± 28.2 (3)	271.8 ± 36.1 (3) ^{‡¶}	352.4 ± 11.8 (3) ^{‡§¶}	154.7 ± 40.1 (3) [‡]
BrdU7d-l	20.1 ± 6.6 (3)	11.7 ± 3.8 (3)	18.0 ± 0.8 (3)	21.4 ± 6.8 (3)
CG1				
MI-r	0.7 ± 0.5 (6)	1.2 ± 0.4 (6) [¶]	0.2 ± 0.2 (6) [¶]	—
MI-l	0.22 ± 0.13 (6)	0.32 ± 0.08 (6)	0.23 ± 0.02 (6)	—
AI-r	0.2 ± 0.2 (6)	0.2 ± 0.1 (6)	0.2 ± 0.2 (6)	—
AI-l	0.22 ± 0.17 (6)	0.1 ± 0.0 (6)	0.08 ± 0.03 (6)	—
BrdU1h-r	1.7 ± 0.2 (3) [¶]	3.9 ± 0.4 (3) ^{¶ ††}	1.7 ± 0.5 (3)	—
BrdU1h-l	1.1 ± 0.3 (3)	3.7 ± 1.8 (3)	1.6 ± 0.5 (3)	—
BrdU7d-r	36.2 ± 7.7 (3)	152.6 ± 26.1 (3) ^{¶ ††}	15.2 ± 0.3 (3) ^{**}	—
BrdU7d-l	32.3 ± 19.6 (3)	71.4 ± 9.9 (3)	24.2 ± 3.5 (3) ^{**}	—
CG2				
MI-r	0.10 ± 0.08 (6)	0.12 ± 0.07 (6)	0.02 ± 0.01 (6)	—
AI-r	0.05 ± 0.03 (6)	0.07 ± 0.03 (6)	0.07 ± 0.03 (6)	—
BrdU1h-r	0.8 ± 0.1 (3)	0.9 ± 0.3 (3)	0.3 ± 0.1 (3)	—
BrdU7d-r	19.0 ± 7.4 (3)	16.9 ± 6.2 (3)	6.2 ± 0.6 (3)	—

*MG, Main group (thyroidectomy and intrahepatic thyroid follicle transplantation); CG1, control group 1 (intrahepatic thyroid follicle transplantation and no thyroidectomy); CG2, control group 2 (thyroidectomy and no transplantation).

†Mean values ± SEM are shown. The number of animals (n) is shown in parentheses. MI and AI were calculated as the numbers of mitotic figures and apoptotic bodies per 1000 hepatocytic nuclei. If more than 10,000 nuclei were counted (left part of the liver (l) and CG2), two digits are shown after the digital point. BrdU-LI after either 1 hour of pulse labeling or after 7 days of continuous infusion via osmotic pumps is the number of BrdU-labeled nuclei per 1000 hepatocytic nuclei. For the MG and the CG1, all indices were separately calculated for the liver acini downstream of the transplanted thyroid follicles in the right part of the liver (r) and the left part of the liver (l) which was free of transplants (intraindividual control). When no alterations were identifiable downstream of the transplants (MG at 1 week; CG1 at all times), the indices for the right part of the liver were calculated with the hepatocytes in a distance of about 1 mm from the transplants. For CG2 the indices were calculated only for the right lobe.

‡Significantly different from the left part of the liver.

§Significantly different from CG1.

¶Significantly different from CG2.

||Significantly different from 1 week.

**Significantly different from 3 weeks.

††Significantly different from 3 months.

§§Wilcoxon-Mann-Whitney test $P < 0.05$.

viewed with dark-field illumination at a Zeiss Axiophot microscope (Oberkochen, Germany).

Serum Free T3, Free T4, and TSH

Serum samples were taken from aortal blood at sacrifice. Free T3 (fT3) and free T4 (fT4) were measured by an electrochemiluminescence immunoassay with an Eelec-sys analyzer (Boehringer Mannheim, Mannheim, Germany). TSH was measured with the rat TSH ¹²⁵I assay system from Amersham (Amersham, Buckinghamshire, UK).

Histochemistry of Enzymes and Metabolic Products

Pieces from snap-frozen liver tissue of five rats were frozen onto the same tissue holder, and serial sections of all pieces were cut simultaneously in a cryostat (Jung, Nussloch, Germany).¹⁴ The sections were mounted onto the same slide or the same membrane and incubated by the respective histochemical reaction. With this technique it was possible not only to produce sections of the

same thickness but also to treat them simultaneously under identical conditions for the specific histochemical assays. The liver and the thyroid gland of one completely untreated rat were included as a normal control. The following enzymes were investigated: glycogen synthase (SYN), glycogen phosphorylase (PHO), glucose-6-phosphatase (G6Pase), glucose-6-phosphate dehydrogenase (G6PDH), pyruvate kinase (PK), succinate dehydrogenase (SDH), malic enzyme (ME), mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH), cytochrome c-oxidase (COX), acid phosphatase (AP), and γ-glutamyltransferase (GGT). Incubation conditions were essentially as previously described.^{11,15–17} Furthermore, serial cryostat sections were stained for basophilia with toluidine blue, for the presence of neutral lipids with Fettrot B, and for the presence of glycogen with the PAS reaction.

The intensities of the histochemical parameters in the serial sections were estimated semiquantitatively using five grades (no change, increase, strong increase, decrease, strong decrease) as compared with the reaction in the unaltered tissue of the same specimen (internal control).

Figure 8. Electron micrographs show nonaltered (**a**) and altered (**b**) hepatocytes of the same liver at 3 months after transplantation. The altered hepatocytes exhibit a more condensed nuclear chromatin, a higher number of mitochondria, and an increase in rough endoplasmic reticulum when compared with the nonaltered hepatocytes. Glycogen and smooth endoplasmic reticulum are strongly decreased in the altered hepatocytes. The number of peroxisomes is not increased in the altered hepatocytes. Original magnification, $\times 16,000$.

Figure 9. Atypical mitotic figures were found in the hepatocytes of the altered liver acini at 3 months (**a**) and at 18 months (**b**) after thyroid follicle transplantation. **a:** A tripolar mitotic figure with three mitotic spindles. **b:** The mitotic figure on the right exhibits dislocated chromosomes. Paraffin sections, PAS stain (**a**), and H&E stain (**b**); original magnifications, $\times 735$ (**a**) and $\times 630$ (**b**).

Results

Weight Gain, *ft3*, *ft4*, and TSH

The body weight gains are shown in Table 1. At 1 week and 3 weeks after transplantation, MG and CG1 were not different from CG2, obviously because CG2 had no abdominal surgery. At 3 months, the weight gain of CG1 was higher compared with CG2. The histological examination of the heart, lung, kidney, adrenal gland, spleen, and pancreas did not reveal any unusual finding. The immunohistochemically TSH-positive cells of the hypophysis were larger in the thyroidectomized animals (MG and CG2) than in the nonthyroidectomized animals (CG1).

As expected, the MG and CG2 animals had significantly decreased *ft3* and *ft4* and significantly increased TSH serum values compared with the CG1 animals (Table 2). It was surprising that no animal of the CG2 had completely negative *ft3* or *ft4* values. We made four-step sections at the anatomical site of the excised thyroid, but we did not find a thyroid remnant in any case. Between 3 and 18 months after transplantation, the *ft3* and *ft4* values of the MG increased, and the TSH decreased. At 18 months, the serum values (and the hypophyses) of the MG did not differ from the CG1 at 3 months.

The Transplanted Follicles

Stereomicroscopic examination of the unstained liver slices was a great help for finding the transplanted follicles for light and electron microscopical investigations (Figure 1, a and b). The transplants were vascularized (Figure 1, c and d) during the first 3 weeks, after which the follicles were surrounded by regularly fenestrated capillaries (Figure 2). In contrast, the transplants of the CG1 rats fibrosed during the first 3 weeks (Figure 1, e and f), probably due to low ("normal") TSH serum levels.

In the MG, the epithelial cells of the transplants formed typical follicles and showed a well-developed cytoplasm, regular organelles and microvilli at the electron microscopical level (Figure 2), and positive immunohistochemical stainings for TG, T4, or T3 (Figure 3, A–C) during the whole experiment. Only a few calcitonin-positive C cells were found by means of immunohistochemistry. In many specimens investigated electron microscopically, no C cell was present. The autoradiography after ^{125}I application at 2 months after transplantation showed an iodine uptake by the intrahepatic thyroid follicles (Figure 4). The follicular epithelial cells of the MG exhibited a strong proliferative activity (Figure 5, a and b). A typical follicle transplant at 3 months is shown in Figure 6. At 18 months most of the transplants did not differ from those at 3 months, but single transplants grew up to 2 mm in size (Figure 7, a–c), and were then still showing mitotic figures.

Light and Electron Microscopy of Liver Acini Downstream of the Transplanted Follicles

At 3 days, 4 days, and 1 week after transplantation, no morphological alterations of the liver tissue surrounding the thyroid follicles were observed in the MG. However, in the MG and the CG1, the hepatocytes in the neighborhood of the transplants showed a slight increase in the MI and the BrdU-LI-1h as compared with the CG2. Furthermore the BrdU-LI-1h of the MG was increased in the right part of the liver compared with the left part (Table 3).

Three weeks after transplantation, the hepatocytes downstream of the transplanted follicles in the MG showed a slight decrease in the glycogen content and some alterations of the enzyme activities, which were strongly altered at later times (see below). The morphological alterations 3 weeks after transplantation represent an intermediate stage on the way to the later, more distinct alterations; but the BrdU-LI 1h and 7d at 3 weeks were already increased when compared not only with the 1-week values (25-fold for the 1h BrdU-LI), but also with the CG1 (10-fold) and CG2 (40-fold) values, and the individual values of the left liver part (50-fold; Table 3; Figure 5, b and c). The 10-fold increase in the AI mean value in relation to the mean value of the left part was not significant (due to a high SEM).

At 3 as well as at 18 months after transplantation, the liver acini downstream of the transplanted follicles of the MG were clearly demarcated from the surrounding unaffected acini by a strong increase in cytoplasmic basophilia and eosinophilia and a negative PAS staining (Fig-

Figure 10. Altered liver acinus at 18 months after transplantation shows a striped (tigroid) basophilia (due to highly organized rough endoplasmic reticulum) on an amphophilic background. This type of alteration is called amphophilic-tigroid cell focus. Nonaltered liver tissue is shown in the right third of the figure. A central vein is shown at the bottom. H&E stain; original magnification, $\times 740$.

ure 6; Figure 7, b-e and g). These altered light microscopic staining properties resulted from an increase in rough endoplasmic reticulum, a multiplication of mitochondria, and a depletion of glycogen, as shown by electron microscopy (Figure 8). The number of peroxisomes was not increased in the cytoplasm of the altered hepatocytes. Within some altered liver acini, single hepatocytes showed intracytoplasmic areas of glycogen accumulation, whereas in other areas of the same cells glycogen was depleted but mitochondria and rough endoplasmic reticulum were increased (Figure 7h). It is not clear whether these cells are intermediate cells between unaltered and altered hepatocytes or they result from secondary events in the altered-cell population. The nuclei of the hepatocytes downstream of the transplanted follicles were enlarged and hyperchromatic, and they showed multiple condensations of their chromatin and several nucleoli (Figure 7i). Many mitotic figures, some of which were pathological (Figure 9), but also many hepatocytic apoptotic bodies, were found in the same altered liver acini (Figure 7g), which is reflected by the fact that the MI, AI, and BrdU-LI (1h and 7d) at 3 months were strongly increased compared with 1) the left liver part of the same animals, 2) the MG at 1 week, and 3) CG1 and CG2. Furthermore the AI and the BrdU-LI 1h were significantly increased compared with the MG at 3 weeks. At 18 months, the MI was significantly higher than at 3 months (Table 3). Single altered acini at 18 months

showed an amphophilic cytoplasm with basophilic stripes (Figure 10). This was a result of a highly organized rough endoplasmic reticulum, whereas the number of mitochondria was increased, and glycogen was depleted. This type of alteration was called amphophilic-tigroid cell focus. The livers of the animals of CG1 and the CG2 did not show any of the alterations observed in the MG.

Histochemical Alterations of Liver Acini Downstream of the Transplanted Follicles

The enzyme activities of the transplanted follicles did not differ from the normal control gland. In the unaltered liver acini, the histochemical pattern of the liver parenchyma reflected the well-known metabolic zonation showing gradients in enzyme activities from zone 1 to zone 3 of the acini. Thus, the reactions of the histochemical assay for G6Pase, G6PDH, SDH, AP, mG3PDH, COX, PHO, and SYN were stronger in zone 1 than in zone 3, whereas those for PK and the PAS stain were more pronounced in zone 3. ME and mG3PDH were more intense in zones 1 and 3 and less intense in zone 2. GGT was positive in bile ducts and some hepatocytes of zone 1.

The histochemical patterns of the altered liver acini at 3 and 18 months after follicle transplantation are summarized in Table 4, and examples are shown in Figures 11

Table 4. Histochemical Pattern of Altered Liver Acini at 3 and 18 Months after Intraportal Thyroid Follicle Transplantation in Thyroidectomized Rats

Parameter	Pattern
Basophilia	↑↑
Glycogen	↓↓
Fat	NC
PHO	↓↓
SYN	↓↓
G6Pase	↑
G6PDH	↑
PK	NC
ME	↑↑
mG3PDH	↑↑
SDH	↑↑
COX	↑↑
AP	↑
GGT	NC
GST-P	NC
TGF α	NC

The intensity of the histochemical parameters in focal lesions was estimated semiquantitatively in comparison with the unaltered liver acini of the same tissue sections, using five grades: NC, no change; ↑, increase; ↑↑, strong increase; ↓, decrease; ↓↓, strong decrease.

and 12. The altered acini were characterized by increased activities of G6PDH, ME, G6Pase, mG3PDH, COX, AP (Figures 11, d–i; and 12, b, e, f) and SDH. The activities of PHO and SYN were strongly reduced (Figures 11c and 12, c and d). PK was not changed. GGT was seen orthotopically only in bile ducts, but was not expressed in the altered acini.

The immunostains for transforming growth factor- α and GST-P were negative in the altered acini. At 18 months after follicle transplantation, one animal exhibited a few very small foci of glycogen-storing hepatocytes, which were seen in paraffin sections of both parts of the liver and were not topographically related to the transplants. These foci were positive for transforming growth factor- α and GST-P. In the cryostat sections of the middle lobe of this animal, the incidental single glycogen-storing foci were not present.

Discussion

This is the first report on portal-embolic intrahepatic thyroid follicle transplantation in rats. The increase in serum TSH induced by thyroidectomy was a required precondition for the persistence and growth of the transplants. The elevated TSH serum levels persisted after follicle transplantation for at least 3 months and returned to “normal” levels 18 months after transplantation. The functional activity of the morphologically intact transplants was demonstrated by the immunohistochemical detection of T3, T4, TG, and calcitonin at 1 and 3 weeks, 3 months, and 18 months and by ability of transplanted thyroid tissue to incorporate ^{125}I at 2 months after transplantation. Compared with CG2, the values of fT3, fT4, and TSH did not change during the first 3 months after transplantation. As long as the transplants were small, the amounts of T3 and T4 secreted by the transplants were obviously absorbed by the liver acini downstream of the

transplanted follicles. Only when the transplants acquired a larger size (at 18 months) did the hormones reach the blood of the hepatic veins. The follicle epithelial cells of single large transplants were at this time still proliferating, and it requires further studies to answer the question whether single transplants could proceed to thyroid neoplasia. This would be analogous to the intrahepatic insulinomas that developed from hyperplastic islets after low-number pancreatic islet transplantation in diabetic rats.¹⁸

The hepatocytes of the liver acini downstream of the transplants were altered in their morphology, cell turnover, and enzymic pattern. The increase in hepatocellular proliferation and, at the same time, in their apoptotic elimination were the first alterations that could be detected after thyroid follicle transplantation. This supports earlier findings that T3 is a strong mitogen for hepatocytes inducing so-called direct hyperplasia and polyploidization *in vivo*.^{1,2,19–21} However, it cannot be ruled out that additional factors secreted by the thyroid tissue may contribute to the effects observed. Interestingly, the AI was simultaneously increased in the altered liver acini, which is a common phenomenon of preneoplastic liver foci including amphophilic cell foci^{22–24} that was also observed in glycogen-storing altered liver acini after pancreatic islet transplantation.⁴

The altered hepatocytes did not grow beyond the borders of their acini, where they would be out of the influence of the thyroid hormones. It is unlikely that the altered hepatocytes undergo a complete reversion to “normal” after their outgrowth of the acinar borders and the influence of the hormones because there was no indication of any transitional changes in the adjacent acini. An immediate change under the microenvironmental conditions of the adjacent acini is not plausible, because the development of the alterations needed such a long time.

An intriguing question is whether the altered acini represent clonal populations. Compared with alterations of the hepatocytes that occurred 2 days after islet transplantation and that were obviously not monoclonal, the slow onset of the alterations after thyroid follicle transplantation might argue for a monoclonal expansion. However, the observation that, 3 and 18 months after transplantation, all transplants induced amphophilic cell alterations in all animals of the MG is an argument against monoclonality. Moreover, no single transplant without a surrounding amphophilic cell population was found either 3 months or 18 months after transplantation. There was also no indication for the induction of a basically different phenotypic hepatocellular alteration. All alterations after transplantation were strictly confined to the acinar borders, this being a further argument against early clonal expansion of the amphophilic cell populations.

The increase in mitochondria and rough endoplasmic reticulum as well as the decrease in glycogen are in line with expected alterations induced by thyroid hormones.³ The resulting amphophilic H&E staining of the cytoplasm, however, is very similar to a well-known type of hepatocytic alteration in the amphophilic cell focus. This is a preneoplastic phenotype mainly induced by chemical hepatocarcinogens belonging to the group of peroxisome proliferators, eg, hypolipidemic drugs, and the ad-

Figure 11. Serial cryostat sections of altered liver acini downstream of a follicle transplant (**a**, arrow) at 3 months after transplantation. Central veins are indicated by arrowheads in **a**. Toluidine blue stain (**a**) shows the increase in basophilia of the altered acini, whereas the PAS stain (**b**) demonstrates the loss of glycogen. Activity of PHO (**c**) was decreased, whereas the activities of G6Pase (**d**), ME (**e**), G6PDH (**f**), mG3PDH (**g**), COX (**h**), and AP (**i**) were increased. The morphological aspect and the histochemical pattern of these altered liver acini resemble those of amphophilic preneoplastic liver foci. Original magnification, $\times 34$ (**a-i**).

Figure 12. Serial cryostat sections at 18 months after transplantation show altered liver parenchyma surrounding a proliferated transplant. PAS stain (a) demonstrates glycogen loss. The activities of ME (b), AP (e), and mG3PDH (f) were increased, whereas the activities of SYN (c) and PHO (d) were decreased in the altered liver tissue. The alterations of enzyme activities were even more pronounced when compared with the altered acini at 3 months after transplantation (Figure 11). Original magnification, $\times 39$.

renal gland hormone dehydroepiandrosterone.^{7,9,11,25} Peroxisome proliferators and T3 are both so-called direct mitogens, which may induce hepatocyte proliferation without preceding cell loss.^{1,2,19} They both act via nuclear receptors on some common target genes, eg, ME and COX.^{26,27} Thyroid hormone receptor and peroxisome proliferator-activated receptor belong to a nuclear receptor superfamily, together with the receptors of sexual steroids and retinoic acid.^{2,28,29} Alterations induced by T3, such as the increase in activities of COX, mG3PDH, ME, G6Pase, G6PDH, SDH, and AP and the decrease of PHO and SYN have also been observed in

preneoplastic amphophilic-cell foci induced by peroxisome proliferators.^{7,10,11} However, peroxisome proliferation was not present after follicle transplantation.

It is also interesting that both the amphophilic cell foci and the amphophilic liver acini presented here exhibit neither an increase in GGT nor an expression of transforming growth factor- α or GST-P, which are often involved in the glycogenotic-basophilic progression in many models of hepatocarcinogenesis.^{7,30} The progression from amphophilic cell foci to hepatocellular neoplasia usually needs more time than the neoplastic progression of glycogenotic cell populations. Therefore, the fact that the

three animals of the MG at 18 months did not develop a hepatocellular tumor does not exclude that these altered liver acini are preneoplastic.

The nonparenchymal cells in the altered liver acini after thyroid follicle transplantation have not yet been studied; nevertheless, they might play an important mediator role between the thyroid follicles, their hormones, and the hepatocytes.³¹

In conclusion, we have demonstrated a new transplantation model for the study of the effects of thyroid hormones on hepatocytes. Long-term studies have been started to investigate whether the altered liver acini downstream of the transplanted follicles not only showed morphological and enzymic alterations similar to preneoplastic amphophilic cell foci but also may proceed to neoplasia.

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