Large Induction of Type III Deiodinase Expression After Partial Hepatectomy in the Regenerating Mouse and Rat Liver

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The deiodinase types 1 (D1) and 2 (D2) catalyze the activation of T_4 to T_3, whereas type 3 deiodinase (D3) catalyzes the inactivation of T_3 and T_4. D3 plays a key role in controlling thyroid hormone bioavailability. It is highly expressed during fetal development, but also in other processes with increased cell proliferation, *e.g.* in vascular tumors. Because tissue regeneration is dependent on cellular proliferation and is associated with activation of fetal genes, we evaluated deiodinase activities and mRNA expression in rat and mouse liver, as well as the local and systemic thyroid hormone status after partial hepatectomy (PH). We observed that in rats, D3 activity was increased 10-fold at 20 h and 3-fold at 48 h after PH; D3 mRNA expression was increased 3-fold at 20 h. The increase in D3 expression was associated with maximum 2- to 3-fold decreases of serum and liver T_3 and T_4 levels at 20 to 24 h after PH. In mice, D3 activity was increased 5-fold at 12 h, 8-fold at 24 h, 40-fold at 36 h, 15-fold at 48 h, and 7-fold at 72 h after PH. In correlation with this, D3 mRNA was highest (6-fold increase), and serum T_3 and T_4 were lowest at 36 h. Furthermore, as a measure for cell proliferation, 5-bromo-2′-deoxyuridine incorporation peaked at 20–24 h after PH in rats and at 36 h in mice. No significant effect on D1 activity or mRNA expression was found after PH. D2 activity was always undetectable. In conclusion, we found a large induction of hepatic D3 expression after PH that was correlated with an increased cellular proliferation and decreased serum and liver T_3 and T_4 levels. Our data suggest that D3 is important in the modulation of thyroid hormone levels in the regenerating liver, in which a decrease in cellular T_3 permits an increase in proliferation. **(*Endocrinology* 150: 540–545, 2009)**

A strict regulation of thyroid hormone levels is essential for normal development of different tissues, as well as for the metabolic function of these tissues. The bioavailability of thyroid hormone is regulated by three iodothyronine deiodinases (D1, D2, and D3). D1 and D2 are thyroid hormone-activating enzymes that catalyze the conversion of the prohormone T_4 to the active hormone T_3. D1 is expressed in the liver, kidney, thyroid, and pituitary, and plays an important role in the production of serum T_3 (1). D2 is mainly expressed in the brain, pituitary, and brown adipose tissue. In tissues such as brain, this enzyme is important for the local activation of T_3. D3 is the major inactivating pathway, catalyzing the degradation of T_3 to 3,3′-diiodothyronine and the conversion of T_4 to the inactive metabolite reverse T_3 (rT_3). It is abundantly present in fetal tissues such as liver and brain, and in placenta, pregnant uterus and umbilical arteries and vein (1, 2). The high expression in the fetal compartment suggests that D3 plays an essential role in the regulation of fetal T_3 levels, as a mechanism to protect the fetus from excessive exposure to active thyroid hormone. For instance, in the fetal brain, D3 expression is highly regulated in different regions, protecting the brain from excessive T_3 until differentiation is required (3).

In contrast to the high D3 activities in the fetus, its activities are normally undetectable in most adult tissues. This is probably

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; Ct, cycle at threshold; D1, deiodinase type 1; DTT, dithiothreitol; HIF, hypoxia inducible factor; HPRT, hypoxanthine-guanine phosphoribosyltransferase; PH, partial hepatectomy; rT_3, reverse T_3.

why D3 has long been neglected in studies on the regulation of thyroid hormone bioavailability in the adult. However, recent studies have revealed the reexpression of D3 in different pathophysiological conditions, among which cancer, cardiac hypertrophy, myocardial infarction, chronic inflammation, and critical illness (4–9). Just like fetal growth, most of these are processes in which proliferation or cell growth takes place.

It is generally believed that T_3 stimulates the differentiation of cells and decreases their proliferation. Hernandez *et al.* (10) recently showed that differentiation of precursor cells to adipocytes was associated with a decrease in D3 expression, and Dentice *et al.* (11) demonstrated that D3 is increased in proliferating keratinocytes. In addition, these findings confirm the concept that in general T_3 stimulates cellular differentiation, and suggest a role for D3-mediated low intracellular T_3 concentrations in cellular proliferation.

Liver regeneration is also a process dependent on cellular proliferation. After hepatectomy of 70% of the liver, liver cells switch from a quiescent state to a proliferative state and reenter the cell cycle (12). In rodents, during the first 4 h after partial hepatectomy (PH), quiescent hepatocytes enter the cell cycle (G1 phase). This proliferative phase is rather short in rats but is prolonged in mice, the peak of DNA synthesis being 40–44 h after PH (12, 13). During these processes, many fetal genes, which are not expressed in normal adult liver, are reactivated (12, 14). In the present study, we investigated the possible reexpression of D3 in regenerating liver, by determining deiodinase activities and mRNA expression in rat and mouse liver after PH.

Materials and Methods

Materials

[3′-^{125}I]T_3 and [3′,5′-^{125}I]T_4 were obtained from GE Healthcare (Amersham, UK), and [3′,5′-^{125}I]rT_3 was prepared by radioiodination of 3,3′-diiodothyronine, as described previously (15). Nonradioactive iodothyronines were purchased from Henning Berlin GmbH (Berlin, Germany) and dithiothreitol (DTT) from ICN Biochemicals Inc. (Costa Mesa, CA). 5-Bromo-2′-deoxyuridine (BrdU) and real-time PCR probes and primers were obtained from Sigma-Aldrich Corp. (St. Louis, MO), and Eukitt mounting medium from O. Kindler GmbH & Co. (Freiburg, Germany).

PH model

Wistar rats were obtained from the Hubrecht Laboratory (Utrecht, The Netherlands). Mice on the FVB background were obtained from Harlan (Horst, The Netherlands). The animals were allowed free access to food and water. Mice and rats were anesthetized by buprenorphine and then underwent PH by removing 70% of the liver, or only opening of the abdominal skin in the case of sham-treated control animals. Serum and liver tissues were collected at PH (t = 0) and at different time periods after PH. The same region of the liver was isolated from sham-treated controls. All liver samples were immediately frozen in liquid nitrogen and stored at −80 C until further analysis; serum was stored at −20 C. All procedures performed on the animals were approved by the Committee on Animal Experiments of the Utrecht University (protocols 102648-1 and 102648-2).

Determination of BrdU incorporation

Two hours before ending the experiment, animals received an ip injection with BrdU in PBS, using 30 μg/g body weight BrdU for mice or 50 μg/g body weight BrdU for rats. After the animals were killed, liver tissues were fixed in 10% formalin and embedded in paraffin. Slides were stained overnight with the primary mouse anti-BrdU antibody M0744, clone Bu20a (Dako, Heverlee, Belgium), diluted 1:50 for rats and 1:100 for mice. Subsequently, peroxidase-conjugated rabbit antimouse IgG was applied, and immunoreactivity was detected using the DakoCytomation Envision+ System-HRP (Dako) according to the manufacturer's guidelines. Slides were counterstained with hematoxylin for 30 sec and mounted in Eukitt mounting medium. Percentages of BrdU positive cells per slide were calculated after counting 1000 nuclei per animal.

Determination of T_4 and T_3 concentrations in serum and liver samples

Serum T_4 and T_3 were measured by Vitros ECi technology (Immunodiagnostic System; Ortho-Clinical Diagnostics, Beerse, Belgium). The iodothyronine levels in the liver were determined by highly sensitive and specific RIAs after extraction and purification of the iodothyronines from the liver, as previously described (16).

Deiodinase activity assays

Tissues were homogenized on ice in 10 volumes of 0.1 M phosphate (pH 7.2), 2 mM EDTA, containing 1 mm DTT, using a Polytron (Kinematica, Lucerne, Switzerland). The tissue homogenates were stored at −80 C until further analysis. Protein concentrations were determined using the method of Bradford (17), using BSA as standard. D1 activities were determined by incubation of 0.1 μM rT_3 (including 200,000 cpm [3′,5′-^{125}I]rT_3) for 30 min at 37 C with 10 μg protein/ml tissue homogenate in 0.1 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA, 10 mm DTT (PED10). D2 and D3 activities were determined by incubation of 1 nM [3′,5′-^{125}I]T_4 (200,000 cpm, D2) or 1 nM T_3 (including 200,000 cpm [3′-^{125}I]T_3, D3) for 60 min at 37 C with 5 mg protein/ml tissue homogenate in 0.1 M PED10. Reactions were stopped by the addition of 0.1 ml ice-cold methanol. After centrifugation, 0.1 ml supernatant was mixed with 0.1 ml 0.02 M ammonium acetate (pH 4.0), and 0.1 ml of the mixture was applied to a 4.6- × 250-mm Symmetry C18 column connected to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands), and eluted with a gradient of acetonitrile in 0.02 M ammonium acetate (pH 4.0) at a flow of 1.2 ml/min. The proportion of acetonitrile was increased linearly from 30–44% in 10 min. The radioactivity in the eluate was determined using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

RNA isolation and quantitative RT-PCR

RNA from rat liver was isolated from 25 mg liver tissue using the high-pure RNA tissue isolation kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's guidelines. RNA from

TABLE 1. Oligonucleotide primers used for real-time PCR

Gene	Sense primer (′–3′)	Antisense primer (′–3′)	Probe (′–3′)
D3	5′-TTCATGGCGCGGATGAG-3′	5′-GATGATAAGGAAGTCAACGTCGC-3′	5′-FAM-TTCCAGCGCCTGGTCACCAAGTACC-TAMRA-3′
D1	5′-ATTTGACCAGTTCAAGAGACTCGTAG-3′	5′-CCACGTTGTTCTTAAAAGCCCA-3′	5′-FAM-TCATTTACATTGAAGAAGCTCACGCCACAGAAGA-TAMRA-3′
HPRT	Rat 5′-TATCAGACTGAAGAGCTACTGTAATGACC-3′ Mouse 5′-TATCAGACTGAAGAGCTACTGTAATGATC-3′	5′-TTACCAGTGTCAATTATATCTTCAACAATC-3′	5′-FAM-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-TAMRA-3′

mouse liver was isolated using TRIZOL (Invitrogen Corp., Breda, The Netherlands). Subsequently, 10 μg RNA were treated with 10 U deoxyribonuclease (Promega Corp., Leiden, The Netherlands) for 30 min at 37 C, and purified using the RNeasy mini kit RNA cleanup protocol (QIAGEN, Inc., Valencia, CA). Five hundred nanograms of RNA were used for cDNA synthesis using TaqMan RT reagents (Roche Diagnostics). RNA samples were verified to be free from genomic DNA by performing negative control cDNA synthesis reactions of 500 ng total RNA using TaqMan RT reagents without reverse transcriptase.

Quantitative real-time PCR was performed using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Reactions were performed in 25 μl TaqMan universal PCR master mix (Roche Diagnostics), containing 20 ng cDNA. Per reaction, 200 nM primers and probe were used for D3, D1, and for the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Table 1 shows the sequences of the different primers and probes. Real-time PCRs were done for 2 min at 50 C and for 10 min at 95 C, followed by 40 cycles of 15 sec at 95 C and for 1 min at 60 C. Cycle at threshold (Ct) values represent the cycle numbers at which probe-derived absorbance reaches the calculated threshold value. ΔCt values represent the Ct values of the housekeeping gene minus the Ct values of the target gene. Data are expressed as $2^{\Delta Ct}$ or as $2^{\Delta Ct^*}1000$ (relative number of mRNA copies).

Statistics

Results are expressed as means ± SEM. The significance of differences between means was tested using the Student's *t* test for unpaired observations of the SPSS 10.1 statistical package (SPSS, Inc., Chicago, IL). *P* values 0.05 or less were considered significant.

Results

Effects of PH on liver deiodinase expression and BrdU incorporation

We first tested deiodinase activity in rat liver at different time periods after PH (t = 0). Figure 1A shows that D3 activity was increased 10-fold at 20 h and 3-fold at 48 h after PH, whereas D1 tended to decrease. D2 activity was absent in all rat liver samples. Next, we determined rat liver deiodinase mRNA levels. D3 mRNA expression tended to increase, whereas D1 mRNA expression tended to decrease at 20 h (Fig. 1B).

We also studied the effects of PH on deiodinase activity and mRNA levels in mice. In this model, D3 activity was increased 5-fold at 12 h, 8-fold at 24 h, 40-fold at 36 h, 15-fold at 48 h, and 7-fold at 72 h after PH compared with t = 0, whereas sham-treated animals showed only a 4-fold increase at 48 h after sham surgery (Fig. 2A). D1 activity decreased 2-fold compared with

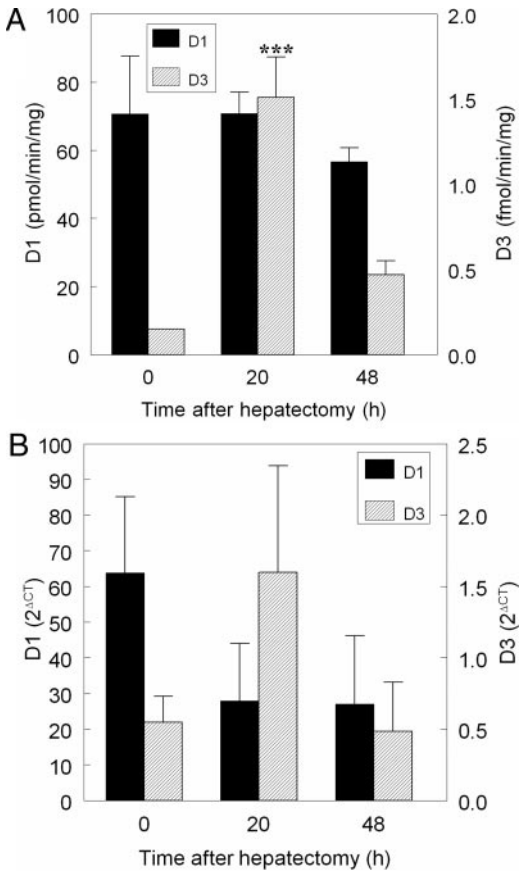

FIG. 1. Effect of PH in rats on D1 and D3 activity (A) and mRNA level relative to HPRT (B). Reaction conditions for the D3 activity assay were 1 nM ^{125}I-labeled T_3, 5 mg protein/ml tissue homogenate, and 1 h incubation. Reaction conditions for the D1 assay were 100 nM ^{125}I-labeled rT_3, 10 μg protein/ml tissue homogenate, and 1 h incubation. Results are the means ± SEM (n = 2–4). Significance of differences is indicated as follows: ***, $P < 0.001$ *vs.* control (t = 0).

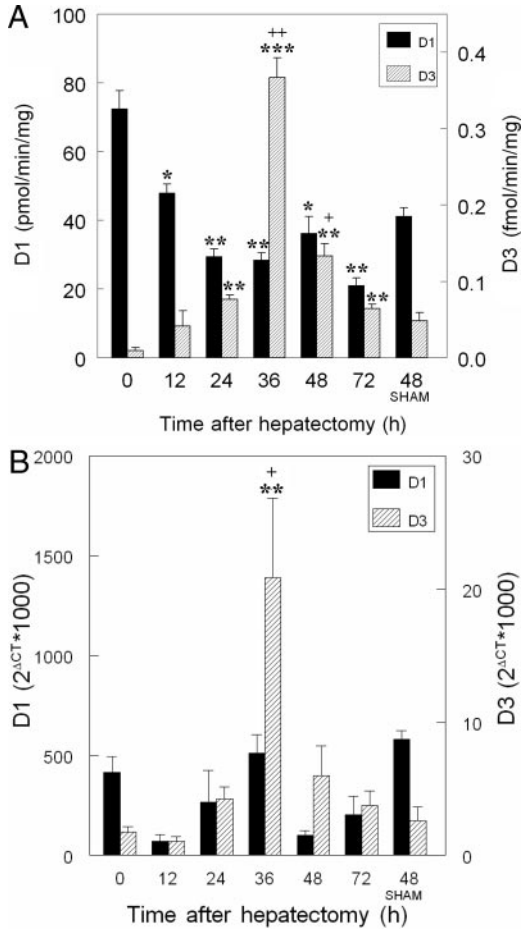

FIG. 2. Effect of PH in mice on D1 and D3 activity (A) and mRNA level relative to HPRT (B). Reaction conditions for the D3 activity assay were 1 nM ^{125}I-labeled T_3, 5 mg protein/ml tissue homogenate, and 1 h incubation. Reaction conditions for the D1 activity assay were 100 nM ^{125}I-labeled rT_3, 10 μg protein/ml tissue homogenate, and 1 h incubation. Results are the means ± SEM (n = 3). Significance of differences is indicated as follows: ***, $P < 0.001$ *vs.* control (t = 0); **, $P < 0.01$ *vs.* control (t = 0); *, $P < 0.05$ *vs.* control (t = 0); ++, $P < 0.01$ *vs.* control (t = 48 sham); +, $P < 0.05$ *vs.* control (t = 48 sham).

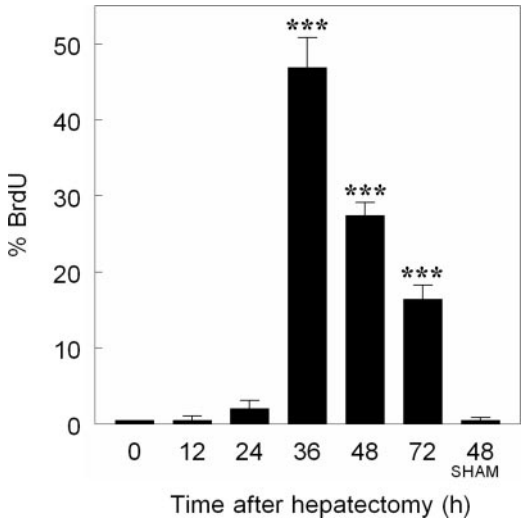

FIG. 3. Effect of PH in mice on BrdU incorporation in mice. Results are the means ± SEM (n = 3). Significance of differences is indicated as follows: ***, $P <$ 0.001 *vs.* control (t = 0).

t = 0 but was not affected compared with sham-treated animals 48 h after sham surgery. In all mouse livers, D2 activity was absent. Figure 2B shows a 6-fold increase of D3 mRNA at 36 h after PH compared with t = 0, and no significant difference in D1 mRNA levels between time periods after PH.

Figure 3 depicts the levels of BrdU incorporation as a measure of cellular proliferation status at different time periods after PH. In correlation with D3 activity, the BrdU level peaked at 20–24 h in rats (data not shown), and at 36 h after PH in mice.

Effects of PH on systemic and local thyroid hormone levels

Finally, we analyzed local and systemic thyroid hormone levels at PH and at different time periods thereafter. In rats, liver and serum T_3 and T_4 levels decreased markedly, with maximum 2- to 3-fold decreases at 20–24 h after PH (Fig. 4). This correlated well with the peak in D3 activity at 20 h after PH in rats (Fig. 1A). In mice, serum T_3 and T_4 decreased until minimum levels at 36 h after PH (Fig. 5), coinciding with the peak in D3 activity and BrdU incorporation at 36 h after PH (Figs. 2A and 3).

Discussion

D3 is highly expressed in tissues of the fetoplacental unit, and has, therefore, long been considered to be mainly important in the regulation of the fetal thyroid hormone status. By inactivating the active thyroid hormone T_3 and the prohormone T_4, D3 is thought to protect developing organs against excessive exposure to thyroid hormone (18). Although D3 is not expressed in most normal adult tissues, recent studies have demonstrated the reexpression of the enzyme in various disease states. For instance, D3 is induced in liver and skeletal muscle of critically ill patients (6), and high levels of D3 are found in vascular tumors (4, 5). Furthermore, in different animal models, D3 was found to be induced in cardiac hypertrophy, myocardial infarction, and chronic inflammation (7–9). The finding that in some of these

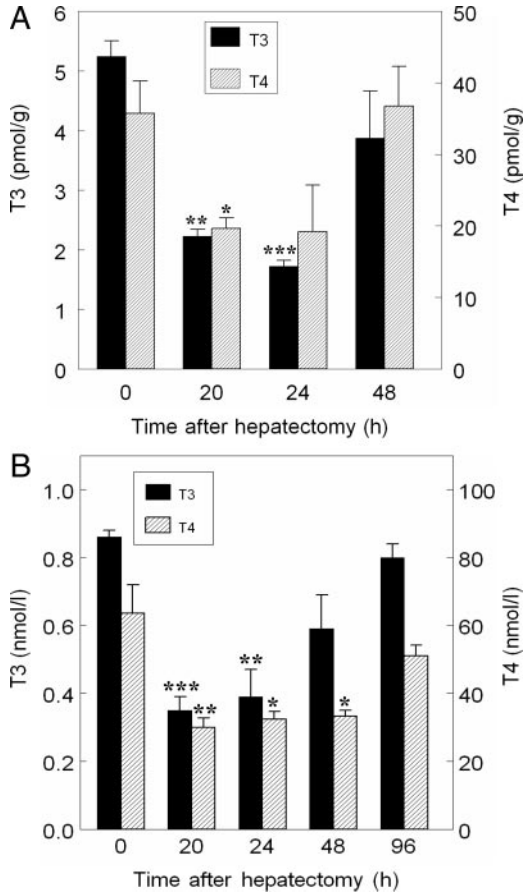

FIG. 4. Effect of PH on liver T_3 and T_4 levels in rats (A) or on serum T_3 and T_4 levels in rats (B). Results are means ± SEM (n = 2–5). Significance of differences is indicated as follows: ***, $P < 0.01$ *vs.* control; **, $P < 0.01$ *vs.* control (t = 0); *, $P < 0.05$ *vs.* control (t = 0).

conditions the accelerated degradation of thyroid hormone causes low serum T_3 (6) or even severe hypothyroidism (4, 5) indicates the clinical importance of D3 in the regulation of local and systemic thyroid hormone status.

Not only fetal growth but also the aforementioned pathophysiological conditions are processes in which cellular growth or proliferation takes place. Therefore, we hypothesized that

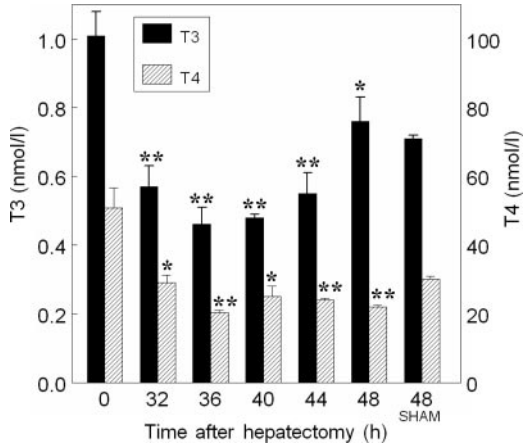

FIG. 5. Effect of PH on serum T_3 and T_4 levels in mice. Results are the means ± SEM (n = 3–5). Significance of differences is indicated as follows: **, $P < 0.01$ *vs.* control (0); *, $P < 0.05$ *vs.* control (t = 0).

D3-mediated low intracellular T_3 levels are needed for cellular proliferation. Interestingly, Hernandez *et al.* (10) recently showed high D3 expression in proliferating preadipocytes from brown adipose tissue, and a marked decrease in D3 expression when the cells differentiate into mature adipocytes. In addition, by knocking down D3 in basal cell carcinomas, Dentice *et al.* (11) recently proved that D3 promotes the proliferation of malignant keratinocytes by inhibiting the differentiating action of T_3. Liver regeneration is also dependent on cellular proliferation and involves induction of various fetal genes (14). Therefore, in the present study, we investigated deiodinase expression and local and systemic thyroid hormone status in rat and mouse liver after PH. We found a 10- to 40-fold induction of hepatic D3 activity after PH that was associated with an increased cellular proliferation and decreased local and circulating T_3 and T_4 levels. Remarkable in this respect is the tendency to a decrease of D1 in rats at 48 h after PH and the 2-fold decrease of D1 in mice at 48 and 72 h after PH. Because D1 is positively regulated by T_3 (1), this may indicate that the slight decrease in D1 is secondary to the decrease in the systemic T_3 level.

It should be noted that D1 activity was 2-fold decreased and D3 activity was 4-fold increased in sham-treated mice compared with t = 0. Therefore, although D1 activity after PH is 2-fold decreased compared with t = 0, it is not significantly altered compared with sham-treated controls. However, when D3 activity after PH is compared with sham-treated controls, it remains highly induced after PH. The mechanisms involved in the decrease of D1 and the increase of D3 activity after sham treatment are unknown. The combined up-regulation of D3 and down-regulation of D1 by the sham operation may be caused by illness (6) and/or inflammation (9). In addition, the possible influence of the used anesthetics on deiodinase expression should be considered because it is well known that some anesthetics alter thyroid function. We used the opiate buprenorphine. Baumgartner *et al.* (19) showed an acute down-regulation of D2 and up-regulation of D3 in rat brain after administration of the μ-opiate agonist etonitazene.

We measured tissue and serum T_3 and T_4 levels, and found a significant decrease of local and systemic thyroid hormone levels at 20 h after PH in rat and at 36 h after PH in mice, which correlated well with the increase in D3 at these time periods. Tien *et al.* (20) also studied rT_3 levels in mouse liver after PH, and reported on a significant increase in the rT_3 level after PH compared with sham-treated controls at 36 h after PH, which was correlated to a significant decrease in the T_3-regulated genes tyrosine aminotransferase and basic transcription element binding protein. In agreement with our data, they found 50% decreased D1 at 36 h after PH compared with sham-treated controls. However, because of nearly undetectable D3 mRNA expression levels, they did not determine D3 activities (20).

Previous cell culture studies have shown that 12-*O*-tetradecanoylphorbol-13-acetate, growth factors such as epidermal growth factor, fibroblast growth factor and TGFβ, cyclic AMP, T_3, and retinoids are able to induce D3 activity (21–28). However, the molecular mechanisms responsible for the reactivation of D3 in the regenerating liver are still poorly understood. In the proliferating skin, hedgehog signaling is involved in the induction of D3 expression (11). However, gene expression microarray analyses of regenerating liver after PH do not indicate that the sonic hedgehog/gli protein pathway is involved in this process (29–32).

As for the TGFβ/Smad signaling pathway, TGFβ1 expression does increase after PH, with a peak level 24–48 h after operation (33–35). However, this increase in TGFβ is accompanied by a down-regulation of the TGFβ receptors, and by an increase of the transcriptional repressors SnoN and Ski (33–35), and TGFβ does not increase but suppresses hepatocyte proliferation through direct effects on the hepatocytes (36).

Another mechanism involved in the induction of D3 after PH might be cellular hypoxia. Interestingly, Simonides *et al.* (37) recently showed that hypoxia induces D3 expression via a hypoxia inducible factor (HIF)-dependent pathway. Because PH is well recognized as a hypoxic-ischemic injury, and HIF-1α is induced after PH (38), the HIF-dependent pathway may well contribute to the induction of D3 after PH at the transcriptional level.

Regulation of D3 expression is complex because the *Dio3* gene is imprinted, with preferential expression from the paternal allele (28). The gene is located in an imprinted region on human chromosome 14, mouse chromosome 12, and rat chromosome 6. One of the other imprinted genes in this region is called *Dio3os*, and is transcribed from the opposite DNA strand compared with *Dio3* (28). Interestingly, Hernandez *et al.* (10) demonstrated an inverse correlation of the paternally imprinted *Dio3* gene with the maternally imprinted *Dio3os* gene. This *Dio3os* gene partially overlaps the coding region of the *Dio3* gene, and may thus interfere with the translation of the Dio3 transcript.

In summary, we found a large induction of D3 expression in regenerating liver that was correlated with an increased cellular proliferation and decreased local and systemic T_3 and T_4 levels. Future research should elucidate the roles of hypoxia and *Dio3os* in the regulation of Dio3 transcription and translation. Our findings support the concept that D3-mediated low cellular T_3 is needed for cellular proliferation. Future studies, *e.g.* using conditional D3 knockout mice, should further unravel the role of D3 in this process.

Acknowledgments

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