

Triiodothyronine Enhances the Regenerative Capacity of the Liver Following Partial Hepatectomy

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This study investigates the effects of administering a primary mitogen, triiodothyronine (T₃), at the time of 70% partial hepatectomy (PH) in the rat, thus combining the 2 distinct pathways of liver growth: direct hyperplasia and compensatory regeneration. T₃ enhances the proliferative response of hepatocytes within the liver following PH. Flash bromodeoxyuridine (BrdU) labeling showed a cell proliferation index 24 hours after PH alone of 26.5% ± 2.8%; when T₃ was administered at PH, it increased to 39.5% ± 5.0% (*P* < .01 compared with PH alone). Continuous BrdU labeling performed every 6 hours between 15 and 72 hours following surgery showed an index of 84.0% ± 4.0% when T₃ was administered at PH compared with 71.0% ± 4.0% with PH alone (*P* < .01 compared with PH alone). This increase in cell proliferation resulted in a larger liver mass at 4 days in rats receiving T₃ at PH compared with PH alone (*P* < .05 compared with PH alone). The difference in liver mass was matched with corresponding increases in total DNA and total protein levels as well as cell division, as confirmed by the frequent demonstration of twin daughter cells on histology. In conclusion, this study shows that a single dose of T₃ enhances the regenerative capacity of the liver following PH. The ability to enhance cell proliferation during compensatory hyperplasia following PH could be therapeutically valuable if applicable to humans. (HEPATOLOGY 2003;37:79-86.)

Liver cell proliferation can be induced in 2 distinct patterns: compensatory regeneration and direct hyperplasia.¹ In compensatory regeneration, the initial event is hepatic injury (chemical/viral) or surgical resection with a reduction in liver mass. These events are followed by proliferation, initially of hepatocytes and then other cell populations of the liver, to rapidly restore the normal precisely regulated liver mass.² The best recognized experimental model of liver regeneration is 70% partial hepatectomy (PH) in the rat,³ in which liver cell proliferation is a secondary event in response to surgical resection and the liver mass is restored to its original value

within 7 to 10 days. PH is generally regarded as the strongest reproducible stimulus to hepatocyte proliferation, and the molecular mechanisms that regulate it have been well described.⁴

In contrast, in direct hyperplasia, hepatocytes are stimulated to proliferate by primary mitogens in the absence of liver injury or resection, resulting in an increase in liver mass to greater than the normal value.⁵ The liver mass subsequently returns to its normal value following removal of the mitogenic stimulus through apoptotic deletion of excess cells.^{6,7} A variety of agents can act as primary mitogens (lead nitrate, retinoic acid, peroxisome proliferators including), and the processes by which they induce DNA synthesis and cell division are not clear. This reflects in particular the fact that different primary mitogens may act in different ways to stimulate the G₁ to S transition and that the effects of many primary mitogens cannot be mimicked *in vitro*.⁸

This study investigated the effects of administering the primary mitogen triiodothyronine (T₃)⁹ at the time of PH, thus combining the 2 distinct pathways of liver growth (direct hyperplasia and compensatory regeneration). T₃ is of particular interest as a primary mitogen in view of its potential exploitation as a pharmaceutically available hormone that has been critically appraised in humans.

Abbreviations: PH, 70% partial hepatectomy; T₃, triiodothyronine; BrdU, bromodeoxyuridine.

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R.M. is a Medical Research Council research training fellow; he was previously a Wellcome Trust introductory fellow. Additional support was provided by the Liver Group Charity (U.K.).

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0270-9139/03/3701-0015\$35.00/0

doi:10.1053/jhep.2003.50001

Materials and Methods

Male Sprague-Dawley rats (250-300 g) were housed in a temperature- and light-controlled room (12-hour light/dark cycle) with free access to food and water. Animal care and all procedures were compatible with the Animals (scientific procedures) Act 1986, UK Home Office.

T₃ was obtained from Sigma (Poole, England) and dissolved in 0.01 mol/L NaOH at a concentration of 5 mg/mL. It was administered to rats routinely at 8 AM by subcutaneous injection at a dose of 4 mg/kg body wt. PH was performed between 8 AM and 12 PM by a single operator as previously described,³ with removal of the median and left lateral lobes of the liver.

The following protocols were used. (1) Rats were injected with a single dose of T₃ and killed at 24, 48, 72, and 96 hours. (2) Control rats received vehicle only and were killed at 24 or 96 hours. (3) Rats were injected with a single dose of T₃ 24 hours before PH and killed 24, 72, or 96 hours after PH. (4) Rats were injected with a single dose of T₃ at PH and killed 24, 72, or 96 hours after PH. (5) PH controls received vehicle only at PH and were killed 24, 72, or 96 hours after PH.

Serum Analysis

Blood was taken from rats receiving T₃ at 0, 8, 24, 48, and 96 hours for serum analysis and compared with control samples taken from rats receiving vehicle only. Standard laboratory liver tests were performed, and a radioimmunoassay (Harbor-UCLA, Los Angeles, CA) was used to determine serum thyroid-stimulating hormone levels.

Tissue Harvest

Animals were killed by exsanguination under isoflurane anesthesia, and both the liver and body mass were measured for calculation of the liver/body mass ratio, the most sensitive index of liver mass.¹⁰

Protein Estimation

A total of 200 to 300 mg of rat liver was weighed, suspended in 5 mL of 1% phosphate-buffered saline, and homogenized in 5 mL 0.6 mol/L perchloric acid. The homogenate was centrifuged at 2,200g for 20 minutes and the supernatant removed. The pellet was dissolved in 5 mL of 0.3 mol/L NaOH/1% sodium dodecyl sulfate. A 50- μ L sample was assayed using a micromodification of the Lowry method.¹¹

DNA Estimation

DNA was measured using a quantification DNA analysis kit (DNeasy kit [no. 69504]; Qiagen, London,

United Kingdom). A 15- to 25-mg liver portion was weighed, dissolved in solubilization buffer, and loaded onto a DNA column. The DNA content was determined by measuring absorbance at 260 nm in a UV spectrophotometer.

Measurement of Hepatocyte Proliferation

Cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation into nuclei and immunohistochemical recognition.¹² Two BrdU labeling methods were used. (1) For flash labeling, BrdU was administered intraperitoneally at a dose of 50 mg/kg 1 hour before the animals were killed to estimate the proportion of cells in DNA synthesis at the time of sacrifice. (2) For continuous labeling, BrdU was injected intraperitoneally at a dose of 50 mg/kg every 6 hours between 15 and 72 hours following surgery or injection. The pharmacokinetics of repeated injections of BrdU are such that the compound will remain in the body for up to 24 hours after the final injection.¹³ Thus, with an S-phase duration of approximately 8 hours, this technique ensures that all cells that have undergone DNA synthesis from 15 hours to killing at 4 days will be labeled.

Immunohistochemistry

For immunohistochemical analysis, 4- μ m-thick sections were used. Incorporation of BrdU into hepatic nuclei was detected using a mouse monoclonal antibody (Dako Ltd., Cambridge, England) followed by a biotinylated rabbit anti-mouse secondary antibody (Dako Ltd.). Visualization was achieved by the indirect peroxidase technique using 3,3'-diaminobenzidine (Sigma) as a substrate.

Quantification Procedures

The BrdU labeling index was determined by counting 4,000 hepatocyte nuclei per section from consecutive light-microscopy fields. The immunolabeled sections were evaluated blindly to avoid bias, with the number of positively stained nuclei expressed as a percentage. Specificity of staining was confirmed by examining serial sections.

Statistical Analysis

Results are shown as the mean \pm SD of the sample ($\mu \pm SD_{n-1}$). Statistical differences were determined using the 2-tailed *t* test and reported if *P* < .05 compared with controls.

Results

Effect of T₃ and PH on Hepatocyte Proliferation

Flash Labeling. Hepatocytes are mitotically quiescent in intact adult liver, as reflected by a flash BrdU

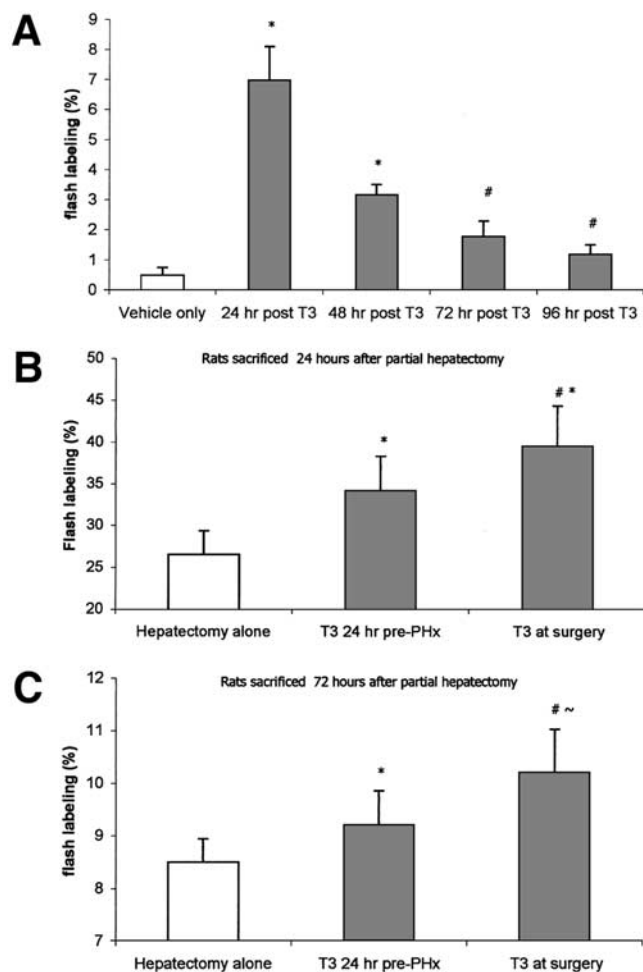


Fig. 1. (A) The flash BrdU labeling index *in vivo* showing the effects of T_3 on the adult rat liver, comparing vehicle only with the effects of T_3 at 24, 48, 72, and 96 hours. The administration of BrdU 1 hour before the animals were killed allowed a cell proliferation index within the liver to be generated. Results are expressed as means \pm SD ($n = 6$). * $P < .01$ compared with vehicle alone; # $P < .05$ compared with vehicle alone. (B) The flash BrdU labeling index 24 hours after surgery *in vivo* in adult rat liver comparing PH alone, T_3 administered 24 hours before PH (combination of the 24-hour effects of PH and the residual effects 48 hours after T_3), and T_3 administered at PH (combination of the 24-hour effects of PH and T_3). Results are expressed as means \pm SD ($n = 9$). * $P < .01$ compared with PH alone; # $P < .05$ compared with T_3 administered 24 hours before PH. (C) The flash BrdU labeling index 72 hours after surgery *in vivo* in adult rat liver comparing PH alone, T_3 administered 24 hours before PH (combination of the 72-hour effects of PH and the residual effects 96 hours after T_3), and T_3 administered at PH (combination of the 72-hour effects of PH and T_3). Results are expressed as means \pm SD ($n = 6$). * $P < .05$ compared with PH; # $P < .05$ compared with T_3 administered 24 hours before PH; ~ $P < .01$ compared with PH alone.

labeling index of less than 1% ($0.45\% \pm 0.25\%$) in control rats. A single injection of T_3 induced a proliferative response within the intact liver, resulting in a peak in cell proliferation at 24 hours followed by a gradual decline. The flash labeling index was $7.0\% \pm 1.1\%$ at 24 hours, $3.2\% \pm 0.3\%$ at 48 hours, $1.8\% \pm 0.5\%$ at 72 hours, and $1.2\% \pm 0.3\%$ at 96 hours (Fig. 1A).

The flash labeling index 24 hours after PH alone showed that $26.5\% \pm 2.8\%$ hepatocytes were in S phase (Fig. 1B), confirming the major effect of PH on hepatocyte proliferation. By 96 hours after surgery, the labeling index had decreased to $8.5\% \pm 0.4\%$ (Fig. 1C).

When T_3 was administered at the time of PH, the flash labeling index 24 hours after PH reached a maximum at $39.5\% \pm 5.0\%$ (Fig. 1B), representing the combined 24-hour effects of PH and T_3 ($P < .01$ compared with PH alone). When T_3 was administered 24 hours before PH, the flash labeling index was $34.2\% \pm 4.1\%$ (Fig. 1B), representing a combination of the 24-hour effects of PH and the residual effects 48 hours after T_3 ($P < .01$ compared with PH alone). This index after combined T_3 and PH was significantly greater when T_3 was administered at the time of PH than when administered 24 hours before surgery ($P < .05$). Intriguingly, when PH was combined with T_3 (either 24 hours before or at the time of PH) and flash labeling was performed 24 hours after surgery, the 2 stimuli seem to be synergistic. Thus, with the combination, the flash labeling index 24 hours after surgery was greater than the sum of the effects of PH alone and T_3 alone ($P < .01$ in each case).

At the later time point, 72 hours after PH, the addition of T_3 (either at surgery or 24 hours before surgery) also showed enhanced flash labeling compared with PH alone. At 72 hours after PH alone, $8.5\% \pm 0.4\%$ hepatocytes were in S phase (Fig. 1C). When T_3 was also administered at the time of PH, the flash labeling index 72 hours after surgery was $10.2\% \pm 0.8\%$ (Fig. 1C) ($P < .01$ compared with PH alone). When T_3 was administered 24 hours before PH, the flash labeling index at the same time point was $9.2\% \pm 0.6\%$ (Fig. 1C) ($P < .05$ compared with PH alone). The 72-hour postsurgical effect of T_3 was greater when T_3 was administered at the time of PH than when administered 24 hours before surgery ($P < .05$).

Thus, the flash labeling index 72 hours after PH showed that T_3 had an enhancing effect on cell proliferation, although at this point synergy was not apparent.

Continuous Labeling. To further investigate the effects of T_3 on PH, continuous labeling experiments were performed to determine whether a greater proportion of cells went through S phase with the combined stimulation. In the intact liver, the continuous BrdU labeling index following vehicle alone was less than 1%, reflecting the quiescent nature of the hepatocyte (Fig. 2). The continuous labeling index following T_3 alone was $31.0\% \pm 3.2\%$ (Fig. 2), confirming that a significant proportion of cells had undergone a semisynchronous proliferative response over the 4-day period. The continuous labeling index following PH alone was $71.0\% \pm 4\%$ (Fig. 2). When the 3 stimuli were combined, the index was 78.0%

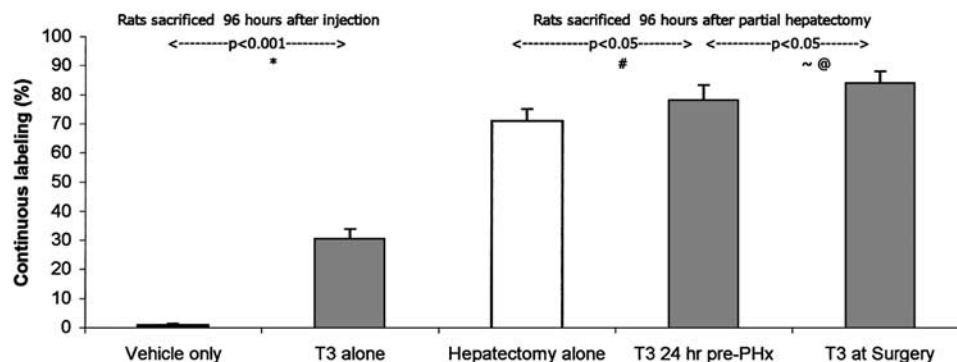


Fig. 2. The continuous BrdU labeling index *in vivo* in adult rat liver comparing vehicle only, T₃ alone, PH alone, T₃ administered 24 hours before PH, and T₃ administered at PH. The continuous administration of BrdU ensured that the total number of cells entering S phase from 15 hours to 4 days could be quantified. Results are expressed as means \pm SD. * $P < .001$ compared with vehicle alone ($n = 6$); # $P < .05$ compared with PH ($n = 9$); ~ $P < .05$ compared with T₃ administered 24 hours before PH ($n = 9$); @ $P < .01$ compared with PH ($n = 9$).

$\pm 5.2\%$ (Fig. 2) when T₃ was administered 24 hours before PH ($P < .05$ compared with PH alone) and $84.0\% \pm 4.0\%$ (Fig. 2) when T₃ was administered at the time of PH ($P < .01$ compared with PH alone). The effect of the 2 stimuli simultaneously was significantly greater than when T₃ preceded surgery by 24 hours ($P < .05$).

Thus, combining T₃ with PH increased the number of cells undergoing DNA synthesis greater than PH alone, though not in a simple summative manner. The synergistic effects of T₃ and PH on cells in S phase at 24 hours (flash labeling) compared with the enhancement of effect at 72 hours (flash labeling) and 4 days (continuous labeling) prompted us to analyze the histologic distribution of cells entering S phase.

Distribution of BrdU Labeling

Flash Labeling. The cells proliferating at 24 hours after T₃ alone were predominantly in the midzonal region of the hepatic lobule (Fig. 3A), whereas those after PH alone were predominantly in the periportal region of the liver (Fig. 3B). When T₃ was administered at PH, the labeled cells occupied both the midzonal and periportal regions of the liver (Fig. 3C). Thus, within the first 24 hours of T₃ administration or PH, cells in different parts of the hepatic lobule (midzonal, T₃; periportal, PH) are recruited into the cell cycle to induce proliferation. The cells proliferating at 72 hours after PH alone, T₃ alone, or the combination were predominantly in the midzonal region.

Continuous Labeling. The continuous labeling pattern over 4 days after administration of T₃ again showed labeled cells predominantly in the midzonal region of the hepatic lobule (Fig. 3D). The distribution with PH alone showed labeling of cells in both the periportal and midzonal regions of the liver (Fig. 3E). The pattern when T₃ was administered at PH showed labeling in both the periportal and midzonal regions of the liver; however, the

labeling was more complete in the midzonal region than with PH alone (Fig. 3F). In all cases, the pericentral population of hepatocytes did not show a significant amount of BrdU staining.

Confirmation that the BrdU incorporation in these continuous labeling experiments was a direct result of DNA synthesis within the context of cell proliferation, rather than DNA synthesis as part of a DNA repair mechanism within the hepatocyte, was attained through the frequent demonstration of labeled twin daughter cells (Fig. 3C). These were BrdU-labeled cells occurring in pairs, separated by a visible plasma membrane.

Effect of T₃ and PH on Hepatic Mass at 24 Hours and 4 Days

We investigated whether the increase in the proliferative response with simultaneous costimulation resulted in an increase in liver mass as well as total DNA and protein levels. There was no significant difference in liver mass or total DNA or liver protein levels between the T₃ at PH group and PH-alone group when animals were killed 24 hours after surgery. However, there was a significant difference between the groups 4 days after surgery, where the liver mass was increased in the group receiving T₃ at PH compared to the PH-alone group ($P < .05$ compared with PH alone; Fig. 4A and B). There were corresponding increases in total DNA ($P < .05$ compared with PH alone; Fig. 4C) and total protein levels ($P < .05$ compared with PH alone; Fig. 4D), confirming that the increase in liver mass was cellular and not a result of intracellular/extracellular fluid retention in the liver.

Investigating the Toxic and Systemic Endocrine Effects of T₃

We investigated the effects of T₃ on serum levels of bilirubin and alanine aminotransferase and inspected his-

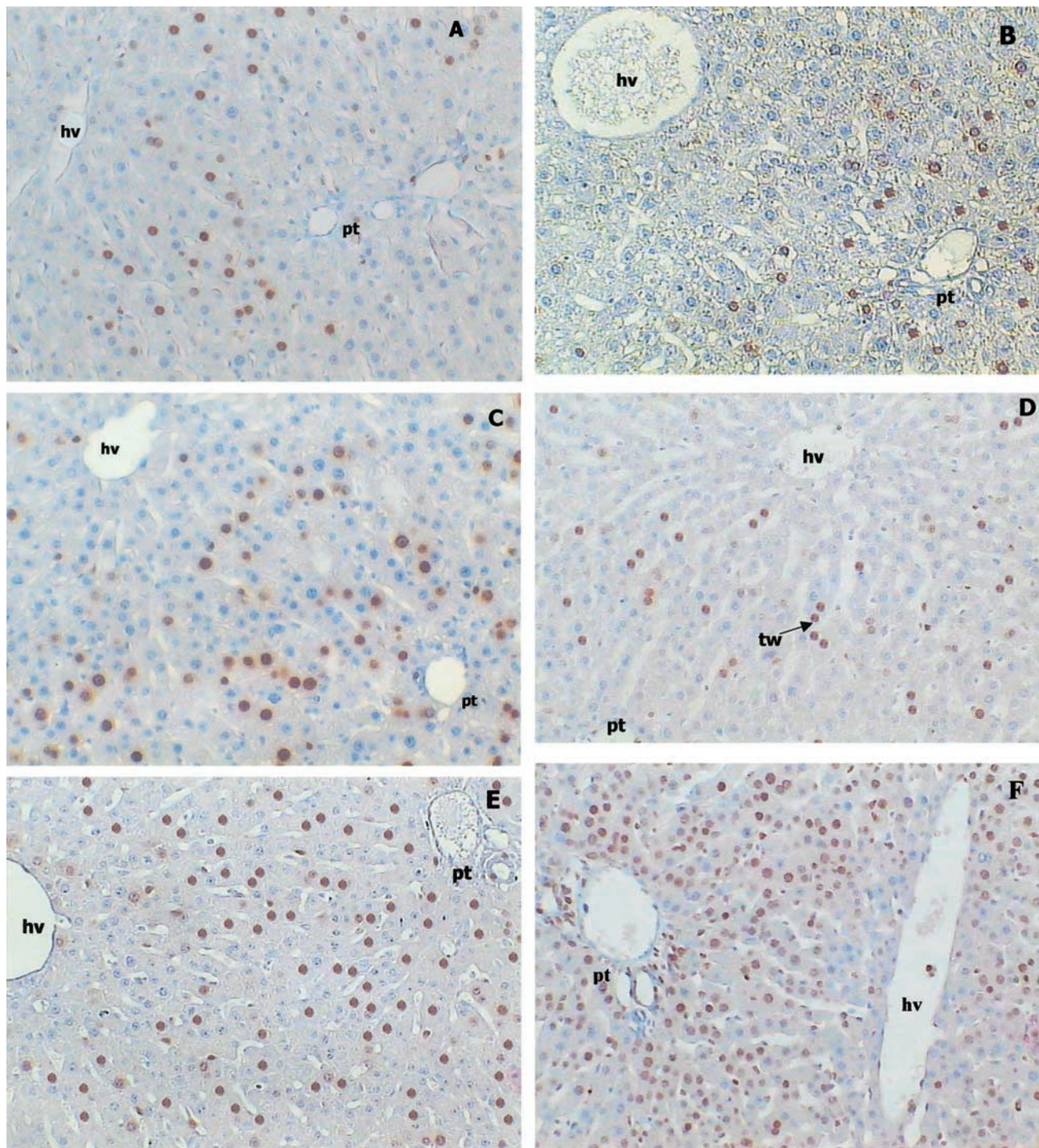


Fig. 3. Immunostaining for BrdU performed with the following. (A) T_3 alone; this flash-labeled section shows the characteristic midzonal staining of hepatocytes. (B) PH; this flash-labeled section shows the characteristic periportal staining of hepatocytes. (C) T_3 administered at the time of PH; this flash-labeled section shows a combination of periportal and midzonal staining. (D) T_3 alone; this continuous labeled section shows the characteristic midzonal staining and the twin daughter cell appearance of true hyperplasia. (E) PH alone; this continuous labeled section shows a combination of midzonal and periportal staining. (F) T_3 administered at the time of PH; this continuous labeled section shows a combination of midzonal (more complete than in E) and periportal staining. In all cases, the pericentral population of hepatocytes does not show a significant amount of BrdU staining. hv, hepatic vein; pt, portal tract; tw, twin daughter cell labeling.

tologic sections of the liver. There were no differences in serum bilirubin and alanine aminotransferase levels following T_3 stimulation at 8, 24, 48, and 96 hours compared with the vehicle-only group (Table 1).

Histologic examination of the livers of rats receiving T_3 showed no evidence of a necrogenic liver injury. There was no centrilobular necrosis, a characteristic feature of thyrotoxic liver injury in humans,¹⁴ probably reflecting the

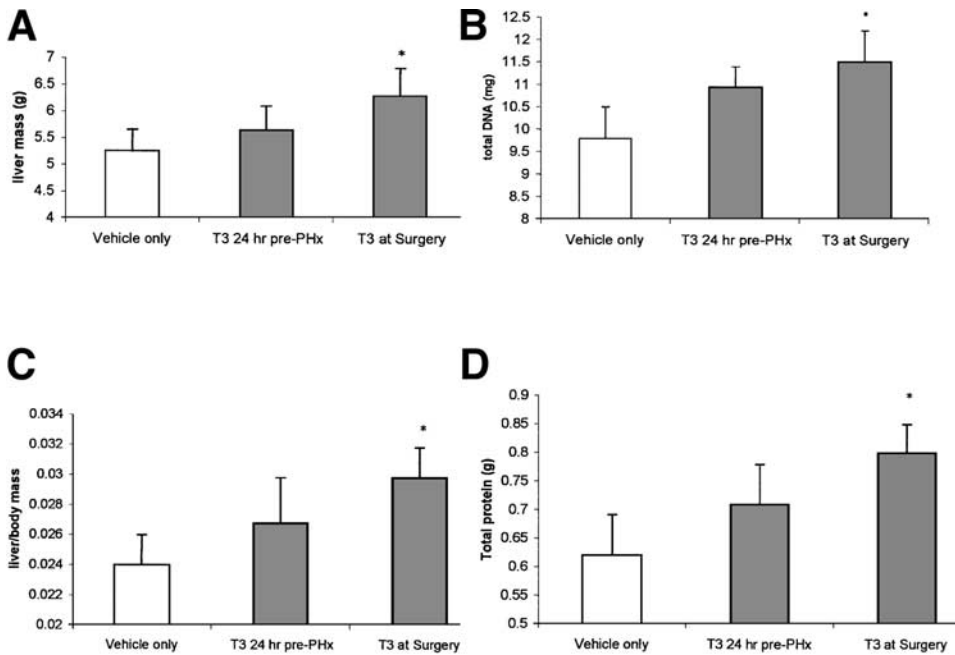


Fig. 4. The effect of T_3 administration 1 day before and at the time of PH on (A) liver mass, (B) total DNA levels, (C) liver/body mass ratio, and (D) total protein levels 4 days after surgery. Administration of T_3 at the time of PH resulted in a larger liver mass, larger liver/body mass index, and corresponding increase in total DNA and protein levels at 4 days compared with PH alone. Results are expressed as means \pm SD ($n = 9$). * $P < .05$ compared with PH alone.

temporal difference between a single high dose of T_3 in a rat and the sustained elevated levels of T_3 in hyperthyroidism in humans.^{15,16} This confirms that the mitogenic doses of T_3 used induced cell proliferation in the absence of liver injury. This, in combination with the absence of necroinflammatory changes on sections with costimulation of T_3 and PH, suggest that the mechanism of enhanced regeneration was not the result of a superimposed necrogenic liver injury but a result of true hyperplasia.

The single subcutaneous injection of T_3 at 4 mg/kg was well tolerated by recipient rats, with maintenance of an adequate appetite and body weight (Table 1) as well as no unexpected mortality over the 4-day period. As anticipated, the mitogenic doses of T_3 resulted in hyperthyroidism in recipient rats, shown by the suppression of thyroid-stimulating hormone secretion, with the trough level at 48 hours less than 1 ng/mL and the levels returning to near normal at 96 hours at 2.6 ng/mL (Fig. 5).

Discussion

This study shows that T_3 increases the proliferative response of hepatocytes following PH and thus enhances

regeneration during one of the strongest known stimuli to hepatocyte proliferation.¹⁷

The cells proliferating 24 hours after PH were predominantly in the periportal area of the liver,² whereas those proliferating 24 hours after administration of T_3 were predominantly in the midzonal area. The histologic pattern at 24 hours with combined stimulation shows a combination of both periportal and midzonal staining. However, recruitment of cells from different regions of the hepatic lobule by each stimuli seems to be only a partial explanation for the flash labeling result, because the effect of combined stimulation is greater than the sum of the individual stimuli at 24 hours. This is likely to represent the recruitment of a population of cells at this time point within the midzonal or periportal regions of the liver (the predominant areas in which labeling is occurring) that are not recruited by each of the individual stimuli.

This explanation seems plausible because each stimulus initiates DNA synthesis in hepatocytes via different pathways that ultimately converge. The initial event in compensatory regeneration after PH seems to be an in-

Table 1. Effect of Thyroid Hormone on Body Weight, Serum Bilirubin, and Alanine Aminotransferase Levels ($n = 6$)

	Vehicle Only	T_3 Group (hr)			
		8	24	48	96
Body wt (g)	277 \pm 13	NA	269 \pm 19	266 \pm 11	270 \pm 10
Bilirubin (μ mol/L)	1.4 \pm 0.9	1.4 \pm 0.5	1.2 \pm 0.4	1.6 \pm 0.5	1.4 \pm 0.5
Alanine aminotransferase (U/L)	83 \pm 9.1	85 \pm 6.2	84 \pm 4.2	82 \pm 7.6	81 \pm 7.5

Abbreviation: NA, not applicable.

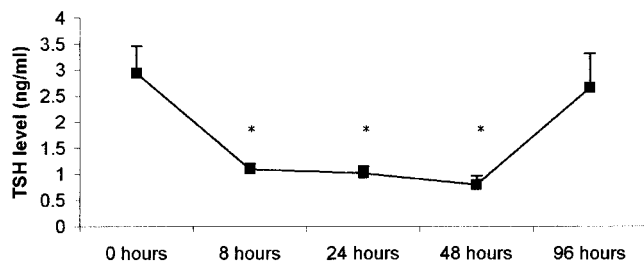


Fig. 5. Serum thyroid-stimulating hormone levels following administration of a single dose (4 mg/kg) of T_3 to rats. Results are expressed as means \pm SD ($n = 6$). * $P < .01$ compared with time 0.

crease in tumor necrosis factor α levels,^{18,19} leading to several sequelae, including (1) the priming of liver cells through the activation of the tumor necrosis factor $\alpha 1$ receptor to generate nuclear factor κB ²⁰ followed by increases in interleukin-6 levels²¹ to activate the transcription factor STAT 3; (2) activation of immediate-early genes such as *c-fos*, *c-jun*, and *c-myc* (AT-1 complex)²²; and (3) the release and activation of hepatocyte growth factor (and other factors) in serum, leading to the occupation of their corresponding receptors (tyrosine kinases) on liver cells.^{23,24}

A combination of growth factor stimulation and delayed gene expression results in cyclin D1 expression within liver cells,¹⁰ which allows the hepatocyte to pass beyond the G1 restriction point, initiate DNA synthesis, and become irreversibly committed to cell division.

In contrast, the mitogenic effects of T_3 on the liver are independent of activation of the transcription factors nuclear factor κB and STAT 3.²⁵ Pibiri et al. identified an earlier onset of expression of cyclin D1 when compared with PH, suggesting that there were different mechanisms regulating T_3 -induced proliferation than those seen after PH.²⁵ Thus, the synergistic effect at 24 hours is likely to represent the activation of different pathways within the same cell. We hypothesize that this simultaneous activation increases the chances for individual cells to have made the critical G₁ to S transition through the cell cycle and initiate DNA synthesis at the 24-hour time point. This is supported by our previous observations that exogenous administration of T_3 and hepatocyte growth factor, a major component of the compensatory hyperplasia pathway after PH, is synergistic to hepatocyte proliferation *in vivo* in rats.²⁶

After 4 days, the continuous labeling data showed that proliferation after T_3 administration remained confined to the midzonal region, whereas cells were recruited from both the periportal and midzonal areas after PH.²⁷ With the addition of T_3 at PH, there was an enhancement of the number of midzonal cells recruited when compared with PH alone (a similar finding to flash labeling at 72 hours).

The enhancement is less striking than that seen in the flash labeling experiments at 24 hours, without the synergy noted in those experiments but simple enhancement. We suggest that this is because there is an overlap of the 2 stimuli for many cells in the midzone. It is noteworthy that cells in the pericentral area of the lobule remain predominantly unrecruited into DNA synthesis at 4 days, even by the combination of the 2 stimuli. This centrilobular population of hepatocytes receives the lowest concentrations of oxygen, nutrients, hormones, and growth factors from the circulation, which may in part explain their lack of recruitment into the cell cycle.

The increase in the proliferative response with co-stimulation resulted in a larger postoperative liver mass at 4 days in rats receiving T_3 at PH compared with PH alone. The differences in liver mass were matched with corresponding increases in both total DNA and total protein levels, confirming that the increase in liver mass was cellular. The frequent appearance of twin labeled daughter cells histologically indicated that BrdU incorporation was a direct result of DNA synthesis within the context of cell proliferation, confirming that cell division had occurred within the previous 4 days and that the increase in liver mass included true hyperplasia. However, because T_3 is known to induce hypertrophic changes within the hepatocyte as well,¹⁴ hypertrophy may also contribute to this mass effect. The doses of T_3 administered were well tolerated and not associated with any features of hepatocellular damage, as assessed by circulating hepatic enzyme levels and microscopic examination for hepatocellular necrosis on histology.

The ability to enhance proliferation during compensatory hyperplasia following PH could be very valuable therapeutically.²⁸ Increased liver mass before and after surgery could enhance hepatic reserve and prevent decompensation. In transplant surgery, small-for-patient liver grafts could be encouraged to grow more rapidly to the appropriate mass and thereby prevent the onset of portal hypertension in the graft,²⁹ and indeed the principle may be applied before donation in living related transplantation. Whether T_3 could play this role in humans remains to be determined, but this study indicates that primary mitogens that induce direct hyperplasia could be valuable adjuncts therapeutically.

Acknowledgment: The authors thank Dr. Korsia Khan in the Histopathology Department and the Comparative Biology Unit at the Royal Free Campus of the Royal Free and University College Medical School.

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