

TGF- β 1 MEDIATES CELL PROLIFERATION AND DEVELOPMENT OF HEPATOCARCINOGENESIS BY DOWNREGULATING DEIODINASE 1 EXPRESSION

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Abstract Hepatocellular carcinoma (HCC) is the most common primary liver tumor. Hexachlorobenzene (HCB) is an endocrine disruptor and a liver tumor promoter. Deregulation of thyroid hormone (TH) homeostasis may play a significant role in early neoplastic transformation. The aim of this study was to evaluate the relation between TH metabolism and the regulation of cell growth in an *in vivo* and *in vitro* model. We examined the role of transforming growth factor- β 1 (TGF- β 1) on TH deiodinase expression and hepatocyte proliferation. An initiation (DEN)/promotion (HCB) tumor model from rat liver and HepG2 cells were used. We evaluated PCNA, p21, p27, SMAD2/3, TGF- β 1, deiodinase 1 (D1), D3, protein expression levels; D1 and D3 mRNA expression; TH and TGF- β 1, D1, D3, and GST-P protein levels in focal/non-focal areas. *In vivo*, HCB decreased triiodothyronine (T3) and D1 mRNA levels and increased thyroxine (T4) and D3 mRNA levels in liver from DEN+HCB vs. DEN group. HCB increased protein levels from D3, TGF- β 1, and PCNA and decreased D1 in focal-areas. *In vitro*, HCB increased PCNA, pSMAD 2/3, and TGF- β 1 protein levels and mRNA expression and decreased p21 and p27 protein levels. Exogenous T3 treatment prevent HCB induced molecular alterations related to hepatocyte proliferation whereas T4 did not have any effect. These effects were prevented by using a TGF- β 1 receptor II inhibitor. Results suggest that alteration of TH homeostasis, through D1 function, play a key role in hepatocyte proliferation and that TGF- β 1-SMAD pathway is involved in this process confirming their role in early neoplastic transformation in HCC.

Key words: TGF- β 1, hepatocellular carcinoma, thyroid hormones, deiodinases, hexachlorobenzene

Resumen *La desregulación de la deiodinasa 1, mediada por TGF- β 1, impulsa la proliferación celular y el desarrollo de hepatogénesis.* El hepatocarcinoma (HCC) es un tumor hepático primario. El hexaclorobenceno (HCB) es un disruptor endocrino y un promotor de tumores hepáticos. La desregulación de la homeostasis de las hormonas tiroideas (HT) puede ser un proceso importante para la transformación neoplásica temprana. Nuestro objetivo fue evaluar la relación entre el metabolismo de las HT y la regulación de la proliferación celular. Se utilizó un modelo tumoral de iniciación (DEN)/promoción (HCB) de hígado de rata (*in vivo*) (DEN/HCB) y células HepG2 (*in vitro*). Evaluamos los niveles de PCNA, p21, p27, SMAD2/3, TGF- β 1, D1, D3, ARNm de D1 y D3, HT y los niveles de TGF- β 1, D1, D3 y GST-P en áreas focales/no focales. *In vivo*, HCB disminuyó los niveles de T3 y ARNm de la D1 y aumentó los niveles de T4 y ARNm de D3 del grupo DEN + HCB frente al grupo DEN. El HCB aumentó los niveles de D3, TGF- β 1 y PCNA y disminuyó el D1 en las áreas focales. *In vitro*, HCB aumentó los niveles de PCNA, pSMAD 2/3 y TGF- β 1 y la expresión de ARNm mientras que disminuyó los niveles de p21 y p27. El tratamiento con T3 exógeno previno las alteraciones moleculares relacionadas con la proliferación hepatocitaria. Estos efectos se evitaron utilizando un inhibidor del receptor II de TGF- β 1. Los resultados sugieren que la alteración de la homeostasis de HT, a través de la D1 y la vía TGF- β 1-SMAD, juega un papel clave en la proliferación celular y en las transformaciones neoplásicas tempranas en el HCC.

Palabras clave: TGF- β 1; hepatocarcinoma, hormonas tiroideas, deiodinasas, hexaclorobenceno

KEY POINTS

- In the present study we examined the key role of TGF- β 1, deiodinases and thyroid hormones in the development of hepatocellular carcinoma in a rat model
- There is little information available about deiodinase D1 involvement in hepatocarcinogenesis
- TH may have a potential therapeutic role in HCC and this kind of studies is the translational way to evaluate their effect in preclinical models
- Further studies are ongoing to evaluate the effect of TH in HCC progression.

Primary liver cancer is the fifth most diagnosed malignancy in adult men and remains the third leading cause of cancer death in the world¹⁻³. Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and accounts for 70% to 85% of total liver cancer⁴. HCC is a complex multistep process in which several signaling cascades related to cell survival and proliferation are activated. There is growing evidence suggesting that gradual accumulation of mutations and genetic changes in preneoplastic hepatocytes cause malignant transformation and lead to the development of HCC. Several rodent models have been used over the years to study HCC pathogenesis⁵. The model described by Ito et al. (199) uses medium-term bioassays for the development of preneoplastic foci in rat liver; in this model, the use of diethylnitrosamine (DEN) as an initiator in hepatocarcinogenesis and hexachlorobenzene (HCB) as a promoter of proliferation results in the carcinogenic transformation of hepatocytes by similar pathways than those reported in human HCC⁵⁻⁷.

HCB is a widespread environmental pollutant and a liver tumor promoter in rodent medium-term bioassay study designs⁸. We have previously reported that HCB induces cell proliferation in hepatic preneoplastic foci and expression of hepatic growth regulators, such as transforming growth factor beta-1 (TGF- β 1), in rat liver^{9,10}. We have also demonstrated that HCB is an endocrine disruptor that alters thyroid hormones (TH) homeostasis in several rat tissues¹¹. TH deiodinase activity is important in the metabolism, signaling, and regulation of TH status. Both type 1 and 2 deiodinases (D1 and D2) can convert T4 into T3 via outer-ring (5') monodeiodination. The primary role of D1 is to provide circulating T3 and it is highly expressed in the liver, kidney, and thyroid gland¹². In contrast, type 3 deiodinase (D3) has inner ring deiodinase activity and catalyzes the conversion of T4 and T3 to the inactive metabolites, reverse T3 (rT3) and 3, 3'-diiodothyronine (T2), respectively. So far, this enzyme controls local TH homeostasis and protects tissues from TH excess¹³. It has been shown that local hypothyroidism favors the progres-

sion of preneoplastic lesions to HCC in rats¹⁴. Recent findings demonstrated that increased expression of D3 enhance tumor proliferation¹⁵, while decreased D1 activity has been reported in the non-thyroid illness syndrome and in several human neoplasms¹⁶. These data support the idea that deiodinating enzymes may influence cancer development. Cytokines, such as TGF- β 1, are involved in the regulation of cell differentiation and neoplastic transformation through alterations in the transcription of their target genes¹⁷. TGF- β 1 induces endogenous D3 in diverse human cell types, including fetal and adult fibroblasts from several tissues¹⁸.

The aim of this study was to investigate alterations in D1, TGF- β 1, p27, and p21 -key molecules involved in cell growth regulation- and its relation to TH metabolism in a rat liver tumor promotion model and in HepG2 cells treated with HCB.

Material and methods

HCB, anti- β -actin, immunoglobulin, anti-proliferating cell nuclear antigen (PCNA), phosphorylated protein moths against decapentaplegic homolog 2/3 (pSMAD 2/3), triiodothyronine, thyroxine, and Modified Eagle's Medium (MEM) were purchased from Sigma-Aldrich Co. (St Louis, MO). Anti-Deiodinase 1, 3, anti-p21, and anti-p27 were purchased from Santa Cruz, Argentina. TRI-Reagent used for the extraction of mRNA was purchased from MRC (Molecular Research Center, Inc. Montgomery Road, Cincinnati, OH). The reagents used for cDNA synthesis, reverse transcriptase, molecular weight markers, random primers and dNTPs, and Taq enzyme polymerase were purchased from SRL Biodynamics, (Buenos Aires, Argentina). Polyvinyl difluoride membranes (PVDF) for immuno-blotting, secondary goat anti-mouse IgG, and anti-rabbit IgG were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). The CP-BU plates were purchased from AGFA-Gevaert Argentina SA. Monoclonal anti-TGF- β 1 was obtained from Abcam Inc. (Cambridge, MA) and polyclonal anti GST-P+ was from Assay Designs Inc. (New York, NY). Both the Texas Red dye conjugated IgG and the IgG conjugated with Alexa dye were acquired from Jackson Immuno Research Laboratories (West Grove, PA). Specific primers for D1, D3, TGF- β 1, GADPH, and L19 were purchased from Invitrogen Life Technologies (Carlsbad, CA). Heat-inactivated fetal bovine serum (FBS, Natocor, Cordoba, Argentina). All reagents were of analytical grade.

Female Wistar rats (100g) were acquired from the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina. Rats were fed Purine 3 rat chow (Cabeca S.C.A, Argentina) and water *ad libitum*. The animal protocol was designed to minimize pain or discomfort to the animals. The general health of the animals was not affected by the employed doses of HCB or DEN as evaluated by the behavior and appearance of the rats including examination of the coat, mucous membranes, body weight, and food and water consumption. All experiments were performed in compliance with institutional and international guidelines for laboratory animal care. Ethical approval was obtained from the ethics committee of the School of Medicine, Universidad de Buenos Aires. The animals were kept in environmental conditions that

consisted of a 12 h light-dark cycle, 20-24 °C, and 45-75% humidity. Thirteen-day-old animals, following a 7-day acclimation period, were randomly divided into 4 groups, containing 3-5 animals per group and treated (Fig. 1) with the following schedule: Control group received an i.p injection of saline solution (9%) on day 0; DEN group received an i.p injection of DEN (100 mg/kg dissolved in 0.9% saline) on day 0 and, on day 45, saline solution by gastric tube; HCB group received i.p. saline solution on day 0 and, on day 45, HCB (100 mg/kg/day) by gastric tube; finally, DEN + HCB group received i.p. DEN (100 mg/kg dissolved in 0.9% saline) on day 0 and, on day 45, HCB (100 mg/kg/day) by gastric tube. All groups underwent partial hepatectomy on day 52 and were sacrificed on day 100 of treatment. At this time, all animals were euthanized by i.p. ketamine and xylazine overdose injection for tissue collection; complete necropsies were done, and about one-half of the left and right lateral liver lobes were immediately frozen by immersion in liquid nitrogen for subsequent western blotting of different proteins. The remaining parts of the left and right lateral lobes were fixed overnight in freshly prepared 4% paraformaldehyde, and embedded in paraffin for light microscopy. Small portions of the tissue were also frozen at -70 °C for mRNA extraction. Serum was obtained for hormone analyses.

In vitro studies were performed in the human liver cancer cell lines HepG2 and Huh-7 obtained from the American Type Culture Collection. The cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 mg/ml) in cell culture flasks at 37°C, under 5% CO₂. Cells

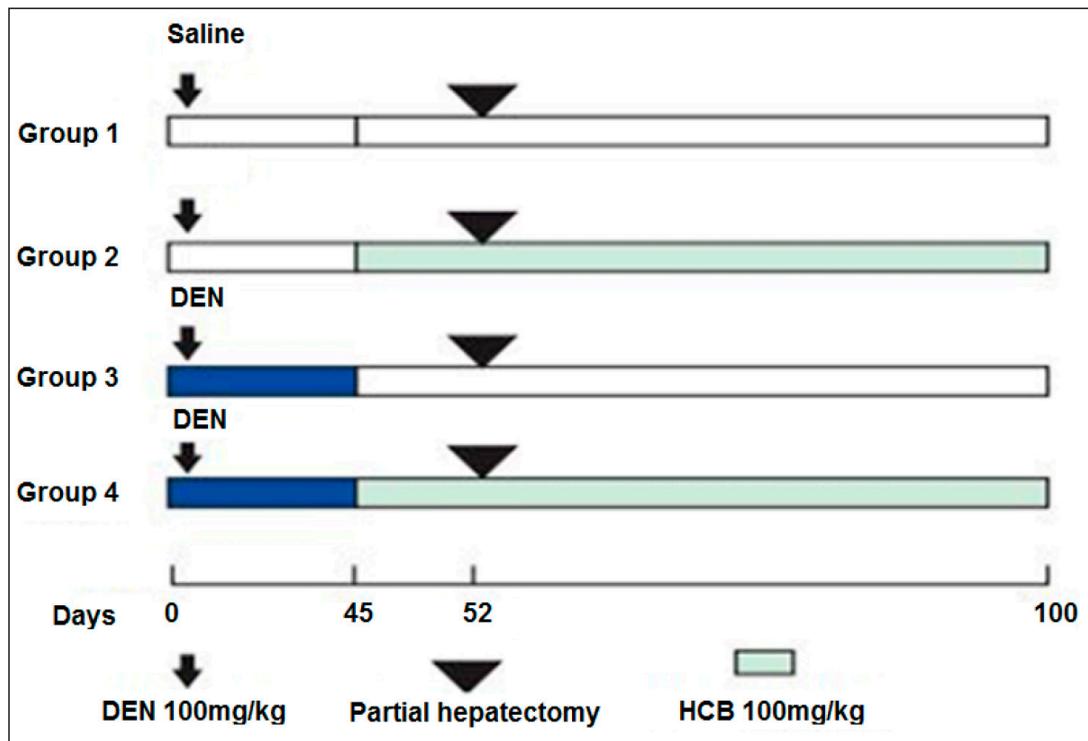
in exponential growth with 80% confluence were washed with phosphate buffer solution (PBS) and then treated with 0.25% trypsin-0.03% EDTA for 1-5 minutes at 37 °C. Cells were seeded in 6-well plates at a density of 5 x 10³ cells per well. The plates were preincubated for 24h to allow cell adherence and then MEM or HCB were added. HCB was dissolved in ethanol (ETOH) and final concentrations in the incubation medium were 0.005, 0.05, 0.5, and 5 µM. Cells were treated for 24h. Control cells were treated with the same volumes of ETOH.

For experiments performed with exogenous T3 or T4, HepG2 cells were pre-treated with HCB (5 µM) for 24h, and then received T3 (10⁻⁹, 10⁻⁷, and 10⁻⁵ M), T4 (10⁻⁸, 10⁻⁶, and 10⁻⁴ M), or dimethyl sulfoxide (DMSO) vehicle for T3, for 24h. Then we evaluated the expression of PCNA levels. T3 or T4 were selected based on our previous experience and that from other authors.

To analyze the role of TGF-β1/SMAD pathway in the proliferative effect of HCB as well as in the involvement of D1, p21, and p27, HepG2 cells were pretreated for 2h with SB431542, a specific inhibitor of TGF-β1 receptor 1 tyrosine kinase. To determine the effective inhibitory dose of SB431542, HepG2 cells were pretreated for 2h with SB431542 (0.5 and 1 µM), followed by 24h treatment with 5 µM HCB. Then we evaluated the expression of PCNA, pSMAD, D1, p21, and p27.

The expression of glutathione S-transferase placental type (GST-P) in three or more contiguous cells is used as an early marker of preneoplastic foci in the hepatic carcinogenic process¹⁹. Histological sections of rat liver were incubated

Fig. 1.– Experimental design for the initiation/promotion study. The animals were treated as indicated in M&M



DEN: diethylnitrosamine; HCB: hexachlorobenzene

with primary antibodies anti-GST-P+ and anti-D1 or with anti-D3 (which are expressed in the cytoplasm) and anti-TGF- β 1 (which is externally bound to the cell membrane). Slides were stained with a double system of secondary antibodies conjugated to Alexa or Texas Red fluorophores. Each of the D1, D3, and TGF-B1 proteins were quantified as the number of labeled cells relative to total cells, for the focal and non-focal areas. One thousand cells were analyzed, for focal and non-focal areas, and the staining was quantified and expressed as the number of stained cells per total cells analyzed (1000). The value obtained in the control group was normalized to value of "1". Proteins were analyzed and quantified using a fluorescence Nikon C1 (Plan Apo 40/0.95) confocal microscope.

Rat liver, HepG2, or Huh-7 cell protein lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, and immunodetected with anti-PCNA (1:500), anti-D1 or D3 (1:500), anti-pSMAD (2/3) (1:500), anti-TGF- β 1 (1:500), anti-p21(1:500), anti-p27 (1:500), or anti- β -actin (1:1000) antibodies. The suitable horseradish peroxidase-conjugated anti-species antibodies (1:1000) were used for protein detection, visualized by an enzyme-linked enhanced chemoluminescence kit, and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.

Total RNA was extracted with a phenol-isothiocyanate of guanidine (TRI-Reagent) solution²⁰. An aliquot of 2 μ g of total RNA was used to synthesize the first-strand cDNA with random primers, deoxynucleotide triphosphates, reverse transcriptase (RT), and RT buffer. The reaction mixture for TGF- β 1, GAPDH, and L19 amplification contained specific GoTaq reaction buffer (1.25 U GoTaq DNA polymerase, 0.5 μ M of each forward and reverse primers, and 1 μ l of RT products). PCR was performed as follows: reaction mixture for TGF- β 1 amplification was incubated at 94 °C for 5 min and then amplified at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Reactions were repeated for 27 cycles. Reaction mixture for glyceraldehyde 3-phosphate dehydrogenase (GPDH) amplification was incubated at 94 °C for 5 min, and then amplified at 94°C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Reactions were repeated for 27 cycles. The sequences of forward and reverse primers were as follows:

TGF- β 1: 5'-CTGCTGGCAATAGCTTCCTA-3'

5'-CGAGCCTTAGTTGGACAGGAT-3'.

L19: 5'-TGAACGGGAAGCTCACTGG-3'

5'-TCCACCACCCTGTTGCTGTA-3'.

GAPDH: 5' CCTGGTAGACAAGTTCCCTTGTTTCATG-GC-3'

5'-GGCCCTCGTAGCACACCTT-3'.

L19 and GAPDH cDNAs were used as loading controls. PCR products were detected as a single band on 2% agarose gel, containing 0.05% (v/v) ethidium bromide. Bands were detected and intensity was quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

Thyroid hormone concentrations were determined in liver extracts using specific and sensitive radioimmunoassays (RIAs), as previously described, using methanol-chloroform extraction, back-extraction into an aqueous phase, and purification of tissue extracts using Dowex columns²¹. TH were determined in the purified liver extracts using sensitive RIAs. The sensitivity of the RIAs is 2.5 pg T4/tube and 0.7 pg T3/tube and cross-reactivity of the RIAs have been described previously²¹. Samples were processed in duplicate.

Data are expressed as mean \pm SEM. Differences between treated and control groups were analyzed by one-way ANOVA, at a 95% confidence interval, followed by Tukey *post hoc*

test to identify significant differences between the means of samples and controls, after testing homogeneity of variance using Barlett's procedure. Differences between control and treated animals were considered significant when p values were < 0.05.

Results

HCB induces cell proliferation in rat liver preneoplastic foci and is a disruptor of TH status. DEN is an extremely potent liver tumor initiator in rats. When combined, DEN and HCB act synergistically⁹⁻¹¹. Since TH regulates key cellular processes including proliferation, differentiation, and apoptosis in virtually all human cells, we evaluated T4 and T3 rat liver concentrations in a medium-term carcinogenicity bioassay. Our results showed that in the DEN + HCB group, liver T3 levels were significantly decreased (45 and 50%), and T4 concentration increased (64 and 77%) compared to the DEN or HCB group, respectively (Fig. 2).

Since benign or malignant neoplastic transformation of different cells can be associated with hormonal changes²², we evaluated the mRNA expression of iodothyronine deiodinase enzymes D1 and D3, involved in TH metabolism.

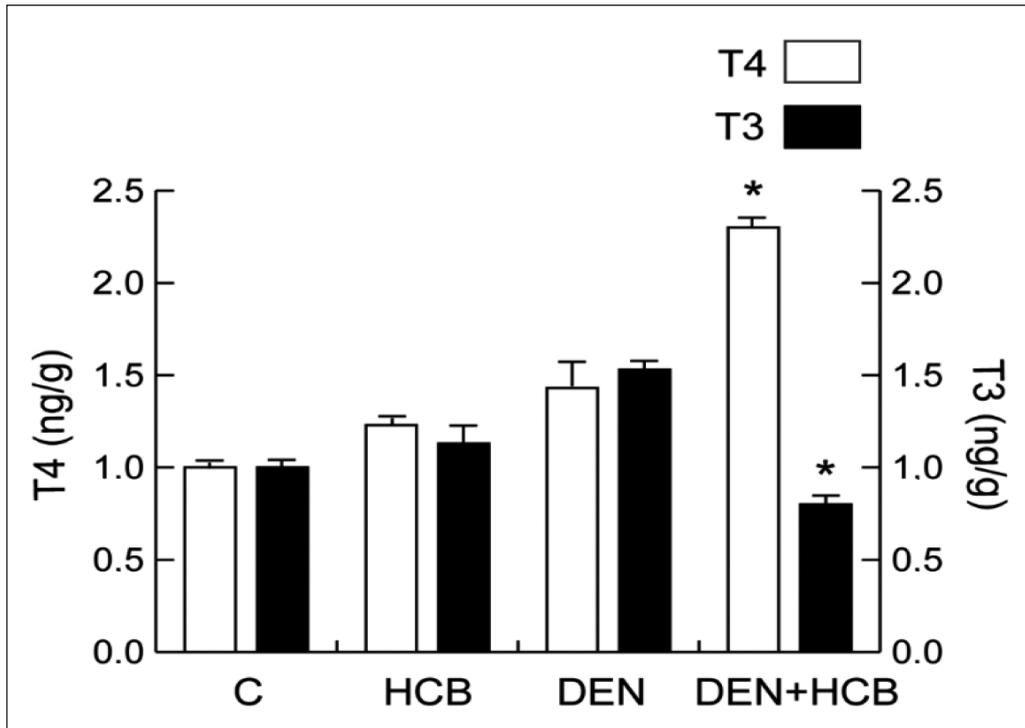
Our results showed a significant decrease in D1 mRNA expression in the DEN + HCB group (46 and 43%) compared to DEN or HCB, respectively (Fig. 3A). D3 is undetectable in most mature tissues, including the liver; however, D3 robust reactivation has been documented in many disease states¹⁵. Figure 3B shows an increase in D3 mRNA levels in the DEN + HCB group (51 %) compared to DEN or HCB groups. These observations were corroborated in rat liver by immunohistochemistry.

The expression of D1 protein levels showed a decrease in the HCB group (60%) compared with the control group. (Fig. 3C, photo A and B).

In the same way, the expression of D3 protein levels showed an increase in the HCB group (41%) compared with the control group (Fig. 3D, photo A and B). When analyzing the expression of D1 and D3 between focal and non-focal areas, it was observed that D3 increased in the focal zone (Figure 3D, Photos C and D) while D1 decreased in both focal and non-focal zones (Fig. 3C, Photos C and D).

On the other hand, the expression of D1 and D3 mRNA in the HCB group in rat liver did not show significant differences with respect to control groups. Contrarily, at the protein level, we found significant differences between these groups. D1 expression decreased (33%) while D3 increased (35%) compared to the control, suggesting a differential regulation of transcription and translation (Figs. 3, A and B).

Fig. 2.– Liver TH concentrations. T4 and T3 tissue levels were estimated as described in M&M. The results are the mean \pm SEM of three independent experiments. Significantly different from DEN or HCB values (* $p < 0.05$)



DEN: diethylnitrosamine; HCB: hexachlorobenzene; T4: thyroxine; T3: triiodothyronine

TGF- β 1 is one of the most important cytokines in liver cell growth maintenance and it induces D3 expression¹⁸. We evaluated TGF- β 1 protein levels (red staining) in liver focal (three or more contiguous green staining) and non-focal areas from DEN and DEN+HCB groups (Figure 4). Immunohistochemical staining showed that HCB induced a significant increase of TGF- β 1 protein levels in HCB (40%) and DEN (50%) groups compared to the control group (Figure 4, Photos C and D). On the other hand, in focal areas, HCB increased TGF- β 1 protein levels in the DEN+HCB group compared to the DEN group (156%) (Figure 4E). We also found that in the DEN+HCB group the number of preneoplastic foci (Figure 4G) and the foci size [(cell number/foci, (Figure 4H)] were increased (110 and 48%, respectively) when compared to the DEN group.

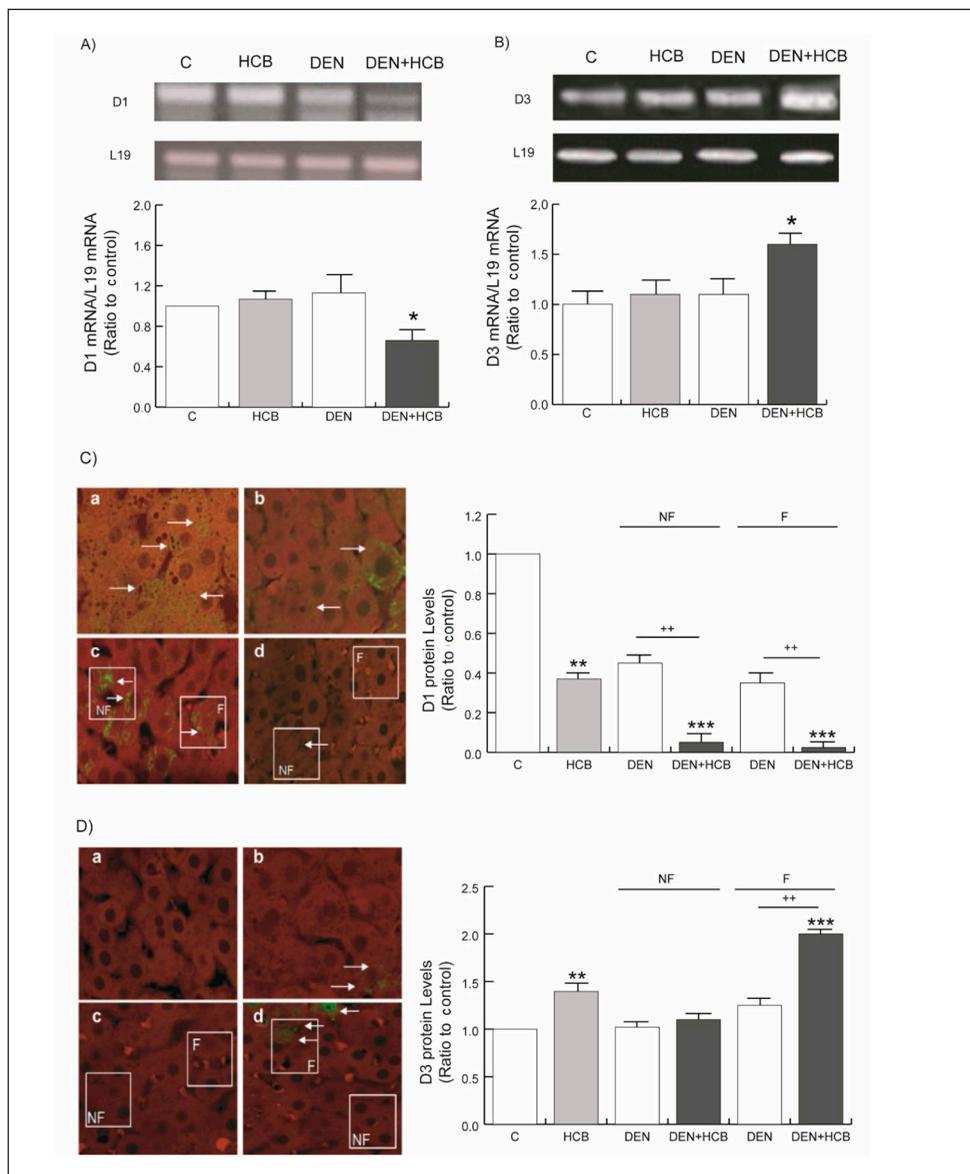
HepG2 is a well-differentiated human HCC cell line that preserves numerous liver-specific functions and thus can serve as an *in vitro* model. In addition, this cell line is characterized by presenting high levels of D1²³. We observed that the treatment of the HepG2 cell line with increasing concentrations of HCB induced a reduction of D1 mRNA

and protein levels (Fig. 5A). Similar results were obtained with the Huh-7 cell line (Fig. 5C). Regarding protein levels, the enzyme presented a slightly different profile than the mRNA (Fig. 5B). Similar results were obtained with the Huh-7 cell line (Fig. 5D).

Next, we hypothesized that the effect of HCB on the proliferation observed in rat liver, as well as in the HepG2 cell line⁸, is probably due to a reduction of T3 levels because of HCB reducing D1 expression levels.

Thus, HepG2 cells were pre-treated with HCB (5 μ M) for 24h and then received increasing concentrations of T3. HCB treatment increased PCNA protein levels (172% compared to ETOH) as it has been previously reported⁹. When HCB-treated cells were administered 10⁻⁵ M T3, PCNA levels returned to ETOH values. However, lower concentrations of T3 (10⁻⁷ and 10⁻⁹ M) resulted in a decrease of HCB-induced proliferative effect without reaching basal levels (Fig. 6A). When adding exogenous T4, no effect was observed on the increased PCNA levels by HCB. This result coincides with the observed decrease in D1 expression (Fig. 6B).

Fig. 3.– Expression of D1 and D3 mRNAs and protein in liver tissue of HCB-treated rats. Rats were treated as indicated in M&M. The upper panels show (A) representative pattern of RT-PCR amplification of D1 cDNA and (B) Representative pattern of RT-PCR amplification of D3 cDNA. Quantification of D1 mRNA/ L19 mRNA or D3 mRNA/ L19 mRNA is shown in the lower panels. Values are the mean ± SEM of three independent experiments. Significant differences (*p<0.05) compared to DEN as well as HCB group. Immunolocalization of D1 (C) and D3 (D) expression in rat liver histological sections. (a) control, (b) HCB, (c) DEN and (d) DEN/HCB. Magnification: 400x. The white squares indicate the focal and non-focal areas of the DEN and DEN + HCB groups. Arrows show D1 and D3 expression (green staining). The red staining corresponds to GST-P positive cells. The D1 and D3 protein quantification levels are shown in the right panels. Values are means ± SEM of three independent experiments. Significantly different (**p< 0.01 and ***p< 0.001) compared to C, (++) compared to DEN group



DEN: diethylnitrosamine; HCB: hexachlorobenzene; D1: deiodinase 1; D3: deiodinase 3; NF: non-focal; F: focal

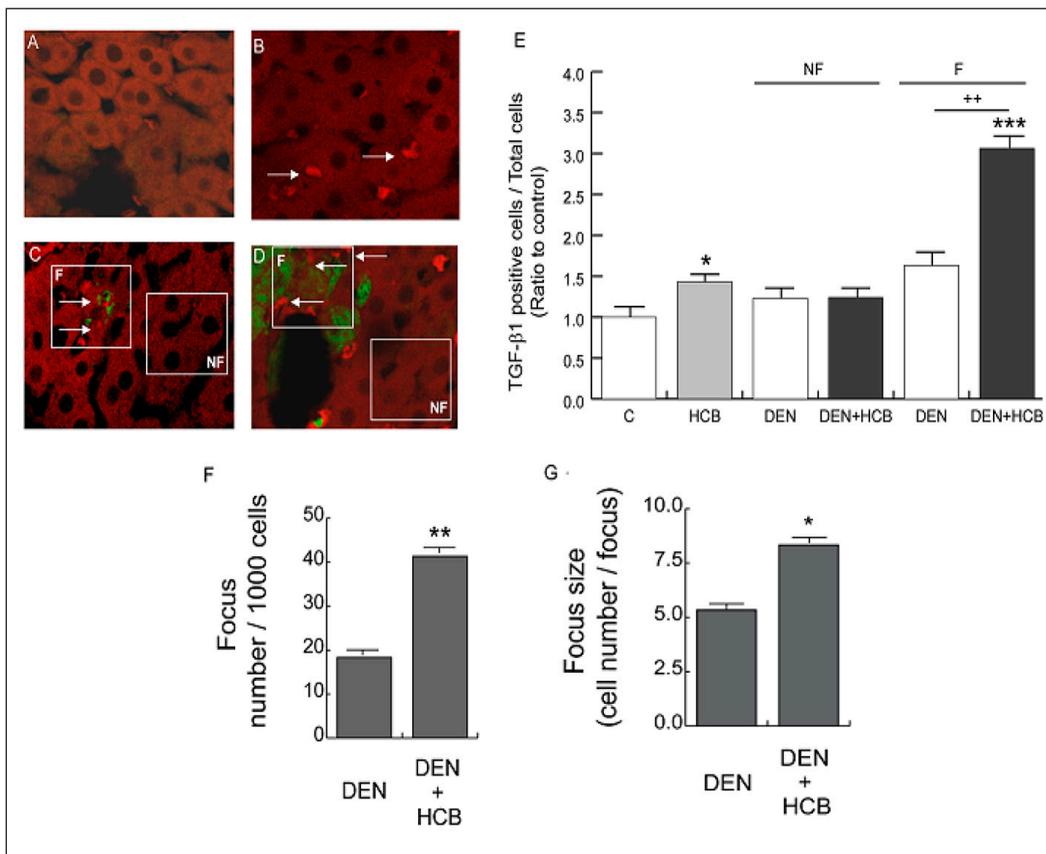
Since TGF-β1 is a positive regulator of cell growth in HepG2 cells, its mRNA levels were evaluated in these cells treated with different doses of HCB. Our results showed that HCB increased TGF-β1 mRNA expression in a dose-dependent manner (50, 102, 150, and 145%), when compared to ETOH (Fig. 7A). On the other hand, HCB treatment increased TGF-β1 protein levels only at the two highest concentrations compared to the ETOH group (180% and 270%, respectively) (Fig. 7B).

We evaluated whether HCB-induced cell proliferation was dependent on TGF-β1/SMAD pathway. As shown in Figure 8A, the proliferative effect of HCB (measured as PCNA levels) was decreased 25% with 0.5 μM SB431542 and returned to basal levels after 1 μM SB431542-treatment when compared to values without the inhibitor.

To analyze if pSMAD 2/3 is involved on the proliferative effect of TGF-β1 induced by HCB, we evaluated pSMAD 2/3 levels in HCB-treated cells as well as after inhibiting TGF-β1 type II receptor. In a similar manner, pSMAD 2/3 protein levels were increased by HCB 5 μM (83%) and decreased when treating HepG2 cells with the TGF-β1 inhibitor. The 0.5 μM dose had no effect while the 1 μM dose reversed it completely (Fig. 8B).

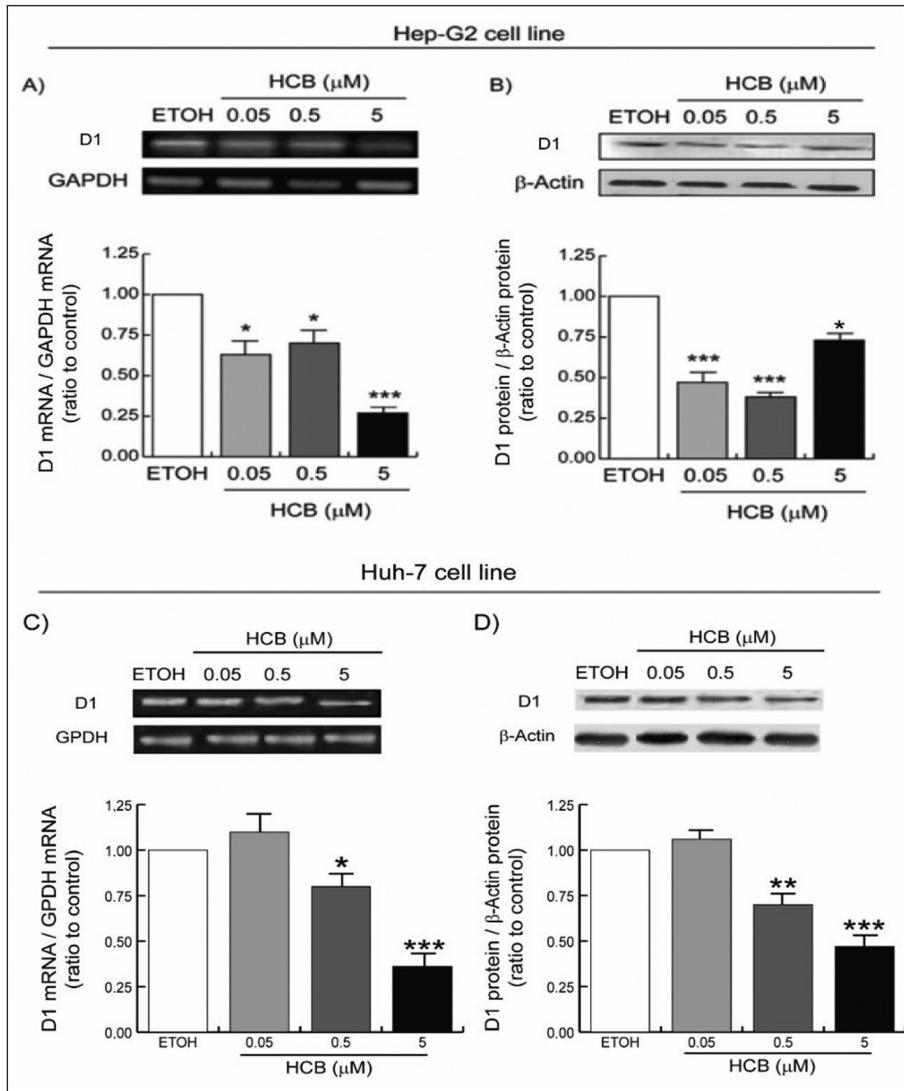
We further addressed whether TGF-β1 signaling contributes to HCB effects on D1 mRNA expression. Our results showed that the TGF-β1 inhibitor prevents the HCB-induced decrease on D1 mRNA (Figure 8C). Finally, we investigated the effect of TGF-β1 signaling on the expression of the cell cycle regulatory proteins, p21 (Fig. 8D) and p27 (Fig. 8E). The results showed a

Fig. 4.– Immunolocalization of TGF-β1 in liver histological sections. Representative images are shown in the upper panel; (A) Control, (B) HCB, (C) DEN, and (D) DEN + HCB. Magnification: × 400. The white squares indicate the focal and non-focal areas of the DEN and DEN + HCB groups. Arrows point to TGF-β1 positive cells. Green staining corresponds to GST-P+, and red staining to TGF-β1+ cells. Quantitative analyses of TGF-β1 per 1000 cells for the different treatments are shown on the side panel (E). The control value of TGF-β1 positive cells / total cells was normalized to 1. Significantly different (*p<0.01) and (**p < 0.001) compared to control. Focus number/1000 cells is shown on the panel (F). Focus size (cell number/focus) is shown in panel (G). Values are means ± SEM of three independent experiments. Significantly different (*p<0.01) and (**p<0.001) compared to DEN group



DEN: diethylnitrosamine; HCB: hexachlorobenzene; D1: deiodinase 1; D3: deiodinase 3; NF: non-focal; F: focal

Fig. 5.— D1 mRNA and protein expression in HCB treated HepG2 and Huh-7 cell lines. Cells were treated as indicated in M&M. In the upper panel (A)(C) representative pattern of RT-PCR amplification of D1 cDNA and (B)(D) representative western blot of D1 protein expression are depicted. Quantification of D1 mRNA/GAPDH or D1/β-actin is shown in the lower panel. Values are the mean ± SEM of three independent experiments. Significant differences (*p < 0.05; **p < 0.01; ***p < 0.01) compared to ETOH



HCB: hexachlorobenzene; D1: deiodinase 1

decrease in the expression of both proteins upon HCB treatment with respect to ETOH (46 and 48%, respectively) and that such effect was mediated by TGF-β1.

Discussion

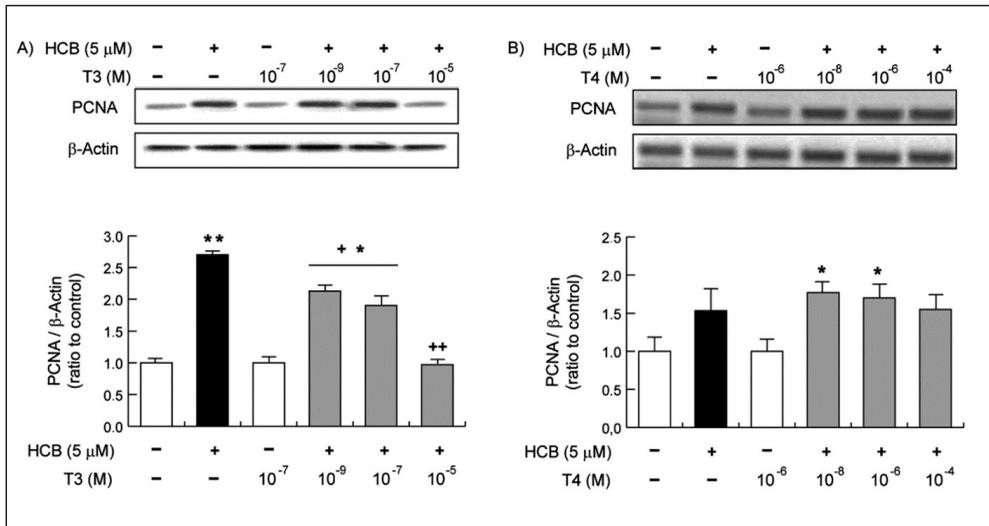
HCC becomes a global health problem as its incidence increases. Given the limited therapeutic options for the treatment of HCC, it is mandatory to find effective therapies

for this tumor. To do this, the elucidation of mechanisms involved in its pathogenesis is a priority.

There is a clear relationship between liver pathologies and endocrine disruptors. The environmental pollutant HCB is an endocrine disruptor. Its tumoral promotor effect on liver of rats and mice has been demonstrated using chronic treatment trials; however, the molecular mechanisms that trigger these events are still unclear²⁵.

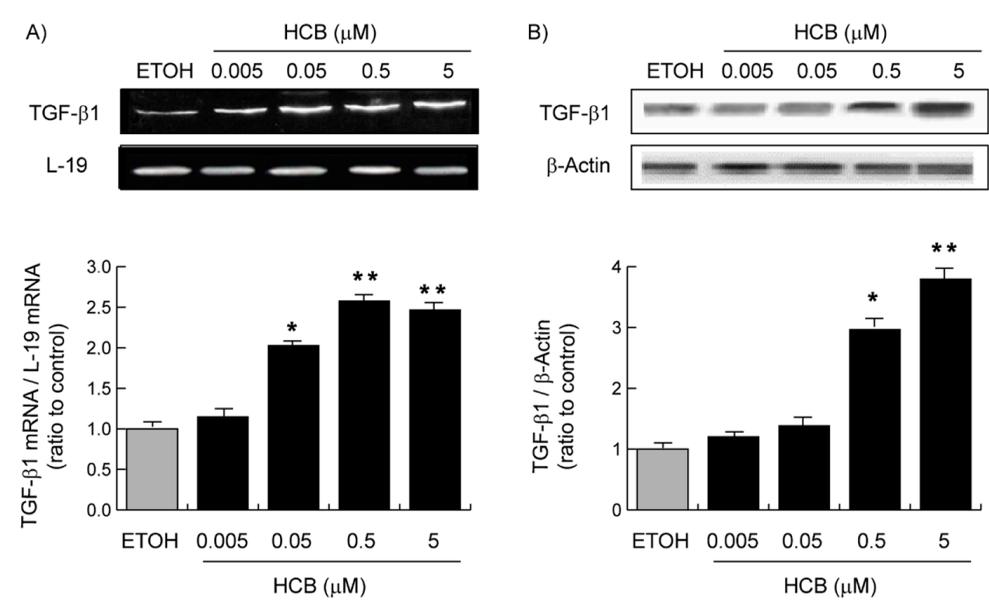
The objective of this work was to investigate the alterations in key molecules involved in the regulation

Fig. 6.– Dose-dependent effect of T3 (A) or T4 (B) on PCNA protein content in HCB-treated HepG2 cells. Cells were treated as indicated in M&M. PCNA protein levels were analyzed by Western blot in the upper panel. Quantification of PCNA/β-actin ratio to ETOH is shown in the lower panel. Values are means ± SEM of three independent experiments. Significant differences (*p < 0.05; **p < 0.01) compared to ETOH, and (+p < 0.05; ++p < 0.01) compared to 5 μM HCB



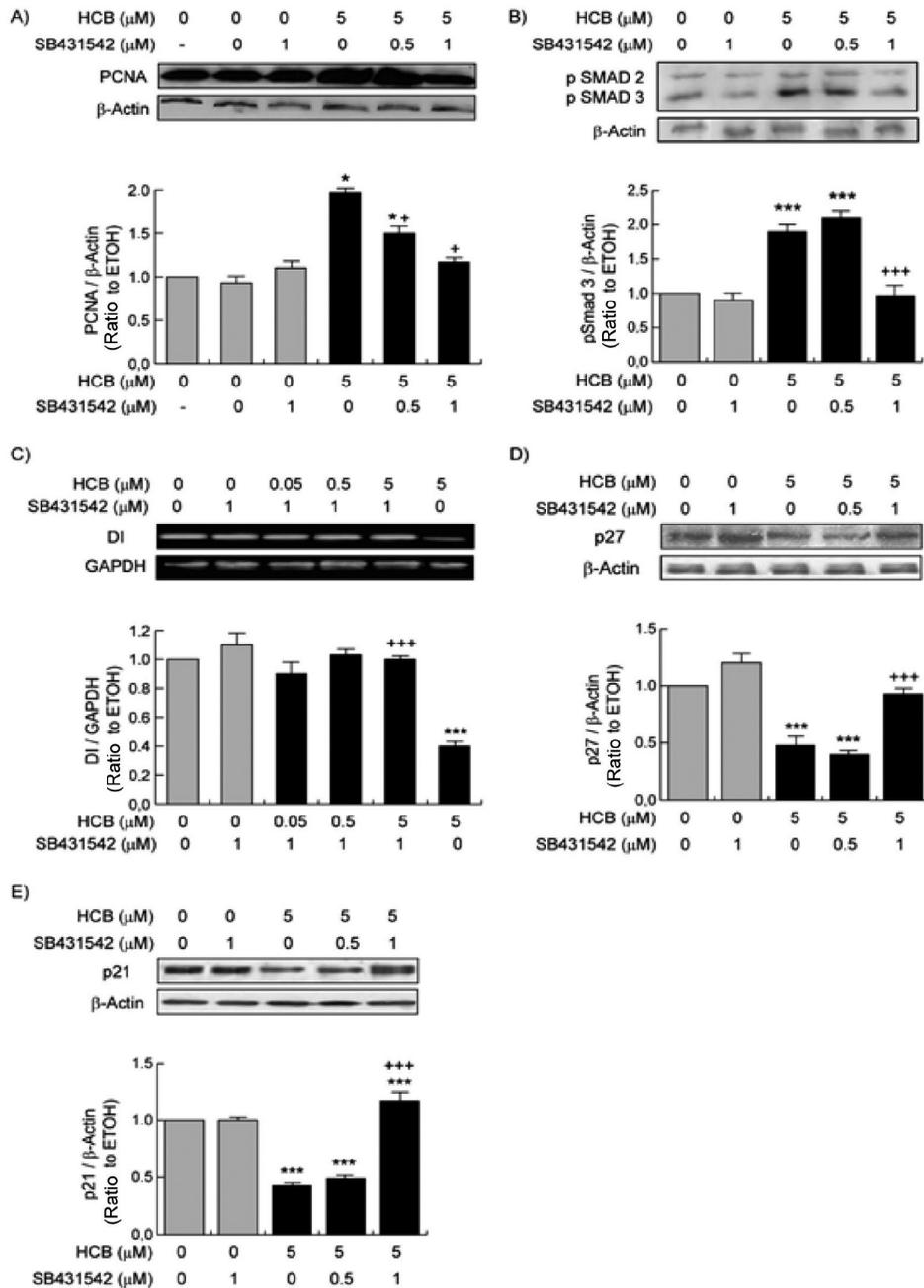
HCB: hexachlorobenzene; T4: thyroxine; T3: triiodotironine

Fig. 7.– HCB effect on TGF-β1 mRNA and protein expression in HepG2 cell line. Cells were treated as indicated in M&M. (A) A representative pattern of RT-PCR amplification of TGF-β1 cDNA from control and HCB-treated cells, synthesized from total RNA, and (B) TGF-β1 protein levels were analyzed by Western blot as shown in the upper panel. Quantification of TGF-β1 mRNA/ L19 mRNA or TGF-β1 protein levels are shown in the lower panel. Values are means ± SEM of three independent experiments. Significantly different (*p < 0.05; **p < 0.01; ***p < 0.01) compared to ETOH



HCB: hexachlorobenzene

Fig. 8.– Dependent effect of TGF-B1 on PCNA, pSMAD, p21, p27 protein levels and D1 mRNA expression in HCB-treated HepG2 cells. Cells were pre-treated as indicated in M&M. The upper panels show (A) PCNA, (B) pSMAD2/3, (D) p27, and (E) p21 analyzed by Western blot and (C) D1 analyzed by RT-PCR. Quantification of protein/β-actin or protein/GAPDH is shown in the lower panels. Values are means ± SEM of three independent experiments. Significant differences (*p < 0.05; ***p < 0.01), compared to ETOH, and (+p < 0.05; +++p < 0.001) compared to 5 μM HCB.



HCB: hexachlorobenzene; D1: deiodinase 1

of cell growth, in a model of induction/promotion of rat liver tumors and in HepG2 and Huh-7 cells treated with the endocrine disruptor HCB. Deregulation of

TH homeostasis has been proposed as an important initial step in the neoplastic transformation process and subsequent tumor progression²⁶. However, there

is not enough data for a complete understanding of this process.

Assuming that HCB is an endocrine disruptor, we speculated that dysregulation of TH homeostasis in the liver may play an important role in the generation of hepatic preneoplastic foci²⁷. In this study, we have shown that HCB decreased T3 and increased T4 concentrations in the liver. These findings are consistent with other authors that demonstrated that treatment with T3 or TH receptor agonists reduce the number of preneoplastic foci in rat livers²⁸. These results and other observations made by other authors show the way T3 leads to a more normal phenotype. However, in this work we show that a decrease in D1 expression and an increase in D3 expression is the origin of this decrease in T3 tissue levels by regulating its intracellular concentration. Sabatino et al. reported in 2000 low levels of D1 expression in HCC compared to normal tissue²⁸. Local control of TH signaling provided by regulation of D3 activity has also been shown to be associated with the development, progression, and recurrence of cancer in other tissue types²².

D3 is undetectable in most mature tissues including the liver; however, robust D3 reactivation has been documented in many disease states¹⁵. In the present study, we demonstrated that D1 protein and mRNA expression were decreased, while D3 protein and mRNA increased in rat liver, consistent with the reduction in T3 tissue concentration observed in this group. Similarly, we have also shown that D1 expression levels are decreased in HepG2 cells. It should be noted that although HCB decreases D1 mRNA levels as well as protein levels, the degree of significance in the dose of 5 μ M of HCB varies between both expression levels. This suggests an effective but differential action between mRNA and protein levels consistent with the expected actions of toxic endocrine disruptors. When we analyzed the effect of toxics on D1 expression in another cell type, Huh-7, we determined that the expression also decreases although the level of sensitivity for this cell line is lower. Only the two highest concentrations produced this decrease, presenting similar impact both at mRNA and protein levels. These findings suggest a link between deiodinase-mediated TH metabolism and HCB-induced cell proliferation. Other authors have also reported that with local hypothyroidism, a regulation of T3/TR β receptor expression may contribute to the progression of preneoplastic lesions to HCC^{14,30}. However, our results show that adding exogenous T3 to HCB treated HepG2 cells may prevent the increase observed in PCNA levels by HCB, unlike adding exogenous T4, which appears to have no impact. This is probably due to the decrease in the expression of mRNA as well as of the protein levels of D1 responsible for generating T3, a biologically active hormone, which negatively regulates cell proliferation.

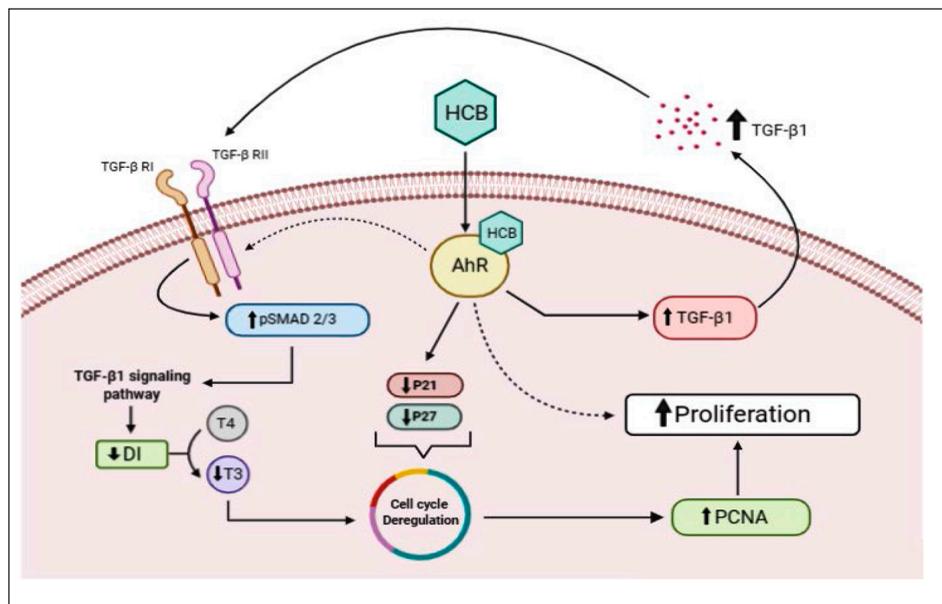
This appears to confirm the involvement of TH in the regulation of cell proliferation. Even though most studies showed a protective action of TH on HCC progression, other studies showed that T3 action on TR may increase HCC aggressiveness and that somatic point mutations of TR α and TR β may increase its frequency³¹.

Unfortunately, most of the studies were performed in rats, mice or *in vitro* and human studies are very few. As far as we know, only one study reported human data showing that D1 expression was significantly lower and that D1 mRNA levels were reduced in human HCC patients compared with normal liver samples³². In the same study, serum T3 levels were decreased in HCC patients when compared with healthy individuals³².

Extensive evidence shows that endogenous or exogenous cytokines appear to block hepatic D1 activity in the HepG2 cell line³³. Members of the TGF- β family control cell growth and regulate TH signaling. In this study, we have shown that HCB induces TGF- β 1 expression in focal areas of rat liver from the DEN+HCB-treated group, as well as in HepG2 cells. It should be noted that the number of foci, as well as their size, increases significantly with HCB and this correlates with the increase in TGF- β 1 expression in the foci. Other authors have also reported the involvement of TGF- β 1 in hepatic carcinogenesis, but none so far has linked this cytokine with deiodinases³⁴. Our results showing that TGF- β 1 inhibition prevents HCB-induced PCNA protein and pSMAD expression in HepG2 cells, demonstrate that the TGF- β 1/SMAD signaling pathway is implicated in cell proliferation in our model. On the other hand, we demonstrated an increase in TGF- β 1 and a decrease in D1 expression at the same treatment concentrations, and the inhibition of TGF- β 1 impairs the effect on the expression of D1 mRNA. These results, together with the T3 reversal of induced cell proliferation, suggest that D1 might be involved in this cell proliferation process. To advance the probable mechanism of action of the downstream toxic, we studied the role of TGF- β 1 at the levels of two of the negative cell cycle regulatory proteins p21 and p27. Inhibition of TGF- β 1 reversed the decrease generated by HCB in p21 as well as in p27 at the same inhibitor concentrations that reversed the effect on PCNA, pSMAD, and D1 levels. These observations agree with those of Huang *et al.*, confirming a link between TGF- β 1, T3 and cell proliferation³⁵.

In summary, in this study we have shown a relation between the expression of TGF- β 1, D1, and D3 as well as local TH concentration in hepatocarcinogenesis in rat liver. Furthermore, we have shown that TGF- β 1/pSMAD is involved in cell proliferation as well as D1 mRNA, p21, and p27 expression in HepG2 cells (Figure 9). These results suggest that TH metabolism plays a critical role in hepatocarcinogenesis involving D1 and TGF- β 1/pSMAD molecules.

Fig. 9.— Role of p21, p27, TGF- β 1, pSMAD, D1, and T3 in the proliferative effect generated by HCB. HCB alters cell cycle regulatory proteins, decreases the expression of p21 and p27. On the other hand, it activates TGF- β 1 / pSMAD 2/3 signaling, decreasing the expression of D1, which generates a decrease in the T3 pool. In this way, the negative regulation of T3 on the cell cycle is eliminated. Together, these processes favor the increase in cell proliferation evidenced by the increase in the expression of the PCNA protein



HCB: hexachlorobenzene; AhR: aryl hydrocarbon receptor; D1: deiodinase 1; T4: thyroxine; T3: triiodotironine

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