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Cyclin D1 is up-regulated in hepatocytes in vivo following cell-cycle block induced by retrorsine

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***Background/Aims*: We reported massive liver repopulation by transplanted hepatocytes in rats given retrorsine (RS), a pyrrolizidine alkaloid which blocks proliferation of resident cells. In these studies, molecular alterations induced by RS on hepatocyte cell cycle were investigated.**

***Methods*: Animals were treated according to the protocol for liver repopulation, i.e. two injections of RS (30 mg/kg) followed by two-thirds partial hepatectomy (PH) and were sacrificed at various time points thereafter. Livers were analyzed for the expression of cell cycle-related genes.**

***Results*: Prior to PH, increased cyclin D1 mRNA and protein levels were found in livers of RS-treated rats. Expression of PCNA was also increased; however, DNA synthesis was not significantly changed. Other cyclins, including cyclin B and cyclin E, were not induced. Cyclin D1 expression increased in controls post-PH and then declined by 48 h, as expected. By contrast, no such modulation of cyclin D1 levels was seen in RS group receiving PH and expression remained high at 48 h, without mitotic division.**

***Conclusions*: Exposure to RS is able to block cell cycle progression after cyclin D1 and PCNA induction, but prior to S phase. Such persistent block outside the resting phase may contribute to the selective replacement of resident cells during liver repopulation.**

***Keywords*: Retrorsine; Liver repopulation; Cyclin D1; Hepatocyte; Cell cycle block; G1 phase; Cell senescence**

1. Introduction

During the past several years we have developed a novel strategy for massive repopulation of a host liver via hepatocyte transplantation [1–3]. In this model strategy, both donor hepatocytes and the recipient animals are normal; however, the latter are pretreated with retrorsine (RS), a naturally occurring pyrrolizidine alkaloid which inhibits the proliferative capacity of resident hepatocytes. Under these conditions, selective proliferation of injected cells is observed, with near total replacement of the recipient organ [1,2].

The precise mechanism(s) underlying this phenomenon remain(s) to be elucidated. The available evidence suggests that RS sets the stage for the selective growth of transplanted cells by virtue of its ability to impose a persistent block on endogenous hepatocyte cell cycle [3,4]. Consistent with this, we found that the protocol associated with liver repopulation is able to completely abolish the regenerative response of the liver after two-thirds partial hepatectomy (PH), for at least 2 weeks [5].

The block imposed by pyrrolizidine alkaloids, including RS, has long been associated to the G2-M phases of the cell cycle [6,7]. This was mostly based on the presence of enlarged hepatocytes (megalocytes) in the liver exposed to these naturally occurring chemicals [8–10]. Megalocytes are in fact interpreted as the results of one or more cycles of endoreduplication, with DNA synthesis occurring without subsequent cell division [6–10]. However, data from our

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laboratory have indicated that the S-phase of the cell cycle is also affected following exposure to RS. Incorporation of 5′-bromo-deoxyuridine (BrdU) into DNA was in fact greatly reduced in response to two-thirds partial hepatectomy (PH) in rat liver treated with RS [5]. Similar findings were later reported by Picard et al. [11], using the portal branch ligation model to stimulate liver cell proliferation in RS-treated rats.

The aim of the present study is to better define the location and to investigate the molecular bases of the cell cycle block induced by RS on rat hepatocytes, using the experimental conditions associated with liver repopulation in the RS model. Expression of specific cyclins was analyzed because of their established regulatory role during transition from G1 to S phase. Results indicated that a large proportion of hepatocytes exposed to RS overexpress cyclin D1 and PCNA; however, cyclin E was not induced and cyclin D1 and/or PCNA-positive hepatocytes were largely unable to enter DNA synthesis.

2. Materials and methods

2.1. Animals and treatments

Six-week-old male Fisher 344 (F344) (80±20 g) were purchased from Charles River, Milan (Italy). They were maintained on alternating 12 h light/dark daily cycles, with food and water available ad libitum. They were fed Purina Rodent Lab Chow diet (Ditta Piccioni, Italy) throughout the experiments.

After 1 week of acclimatization, rats were treated according to a standard RS-based protocol for liver repopulation [1,2]. Briefly, one group of animals received two injections of RS (Sigma Chemical Co., St Louis, MO), 30 mg/kg each, i.p., 2 weeks apart, while controls were given saline instead of RS. Two-thirds partial hepatectomy (PH) was performed 4 weeks after the last injection of RS and animals from both groups were sacrificed at time 0 and at 2, 4, 8, 16, 24, 36 and 48 h post-surgery. When this protocol is coupled with hepatocyte transplantation, massive liver repopulation occurs within 2 months [1]. In groups killed at time 0, rats were injected with 5-bromo-deoxyuridine (BrdU, 50 mg/kg, i.p., Sigma-Aldrich) 2 h before killing. Livers were excised and samples were taken and fixed in 4% buffered formaldehyde for histological and immunohistochemical analysis; the remaining part was snap-frozen in liquid nitrogen and stored at −80 °C for molecular analysis. Three/five animals per group per time point were included. Each experiment was repeated twice (response to PH) or three times (all others), using different animals, with similar pattern of results. All animals received human care and studies were conducted in accordance with the University of Cagliari guidelines.

Liver DNA content was measured according to Burton's method [13].

2.2. Histology and immunohistochemistry

Formalin-fixed, paraffin-embedded liver tissue was sectioned at 6 μm and stained with hematoxylin and eosin (H&E) according to standard procedures.

Immunohistochemistry for BrdU, PCNA and cyclin D1 were performed using specific monoclonal antibodies (anti-BrdU was purchased from Dako, while anti-PCNA and cyclin D1 were from Sigma). Detection of primary antibodies was accomplished using the avidin/biotin peroxidase system (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Labeling index (LI) was obtained by scoring at least 6000 hepatocytes for each sample, in high power random fields and was defined as the percent ratio between labeled cells and total counted cells. Similarly, mitotic index (MI) was scored as the percent ratio between mitotic figures and total cells counted.

2.3. RT-PCR analysis

Total RNA was isolated from liver tissue using the Trizol reagent (Gibco BRL). Integrity of RNA was evaluated by agarose gel electrophoresis, RNA yield was quantified spectrophotometrically, and A260/A280 ratios were determined. Equal amounts of total RNA (1 μg) were reverse transcribed into cDNA using the random hexamer method [12]. cDNA was subsequently amplified by the polymerase chain reaction (PCR) in the presence of specific primers (Table 1). During PCR reaction the non-radioactive label digoxigenin-11-dUTP (Roche Molecular Biochemicals) was incorporated into the amplicons. These were resolved by agarose gel electrophoresis and then blotted into a nylon membrane for 16 h in 20× SSC. The blot was exposed to a light-sensitive film for 2–10 min. The housekeeping gene hypoxantin-guanine phosphoryltransferase (HPRT) was taken as control.

2.4. Cellular extract and liver protein content

Whole cell lysates were obtained by incubating 100 mg of tissue at 4 °C for 30 min in ice cold RIPA buffer consisting of 50 mM Tris–HCl pH 7.6, 1% Triton X-100, 1 mM EDTA, 0.1% SDS, 150 mM NaCl, 0.5% Na-deoxycholate, 100 μM PMSF and protease inhibitors cocktail (Complete Mini-Roche cat. No. 183653). After 10 min centrifugation at 10,000×*g* at 4 °C, the supernatant was collected and frozen in aliquots at −80 °C. Protein content in liver extracts was assessed using Bradford Assay with serum albumin as a standard [14].

2.5. Western blotting

Aliquots of protein extracts (between 30 and 60 μg) were resolved by SDS-PAGE (10–15%) and electrophoretically transferred to nitrocellulose membranes (Hybond ECL, Amersham) for 30 min at 100 V [15]. Membranes were preincubated in blocking solution containing Tris-buffered saline with 0.1% Tween-20 (TBS-T) with 5% non-fat dry milk for 1 h at room temperature. The following antibodies and incubation conditions were used: mouse monoclonal Anti-Actin antidody (clone C-2 Santa Cruz biotechnology, Santa Cruz, CA, USA) 1:1500 overnight 4 °C, rabbit polyclonal Anti-cdk4 antibody (clone C-22 Santa Cruz biotechnology, Santa Cruz, CA, USA) 1:2000 2 h at room temperature, rabbit polyclonal Anti-cdk2 antibody (clone M-2 Santa Cruz biotechnology, Santa Cruz, CA, USA) 1:2000 overnight 4 °C, mouse monoclonal Anti-PCNA antibody (Clone PC-10, Sigma) 1:3000 overnight 4 °C, rabbit polyclonal Anti-Cyclin E antibody (Clone M-20, Santa Cruz biotechnology, Santa Cruz, CA, USA) 1:1500 2 h at room temperature, mouse monoclonal Anti-Cyclin B antibody (Clone GNS-1, Santa Cruz biotechnology, Santa Cruz, CA, USA) 1:2500 2 h at 7 °C, mouse monoclonal Anti-D_1 antibody (Clone DCS-6, Sigma) 1:7000 overnight 4 °C and horseradish peroxidase conjugated goat anti-mouse and anti-rabbit IgGs (Amersham) 1:1000–1:5000 for 2 h at room temperature. All incubations were carried out in TBS-T containing 3% skimmed milk. The antigen-antibody reaction was visualized using ECL reagent (Amersham) and membranes exposed to

Table 1
Specific primers used for different gene products in PCR analysis

Genes	Primers	Length
CYC B	5′ TCAGGGTCACTAGGAACACG 3′ 5′ TACATGGTCTCCTGCAGCAGC 3′	634 bp
CYC D_1	5′ TGGAGCCCCTGAAGAG 3′ 5′ AAGTGCGTTGTGCGGTAGC 3′	424 bp
CYC E	5′ CTGGCTGAATGTTTATGTCC 3′ 5′ TCTTTGCTTGGGCTTTGTCC 3′	386 bp
PCNA	5′ GCCCTCAAAGACCTCATCAA 3′ 5′ GCTCCCCACTCGCAGAAAAC 3′	472 bp
HPRT	5′ CGGGGGACATAAAAGTTAT 3′ 5′ GGCGCAGCAACAGACATT 3′	484 bp