Cell Transplantation, Vol. 17, pp. 1415–1421, 2008
Printed in the USA.

0963-6897/08 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Brief Communication

Repopulation by Endogenous Hepatocytes Does Not Reconstitute Liver Mass in Rats Treated With Retrorsine

Sergio Laconi, Silvia Doratiotto, Stefania Montisci, Paolo Pani, and Ezio Laconi

Department of Biomedical Sciences and Biotechnology, Section of Experimental Pathology, University of Cagliari, Cagliari, Italy

The retrorsine (RS)-based model for massive liver repopulation was laid on the hypothesis that transplanted cells can proliferate in the recipient liver if the growth capacity of endogenous hepatocytes is persistently impaired. In order to directly test this hypothesis, we examined the long-term response to 2/3 partial hepatectomy (PH) in rats pretreated with RS, according to the protocol for liver repopulation. Rats were given RS or saline and 4 weeks later they underwent PH; they were killed up to 16 weeks thereafter. Liver weights, liver DNA, and protein content were significantly lower in the RS group throughout the experimental time considered (e.g., at 16 weeks post-PH relative liver weight was $1.99 \pm 0.30\%$ in RS group vs. $3.06 \pm 0.5\%$ in controls). Regenerative nodules were present in RS-treated livers; they occupied about 3% of the liver at 2 weeks post-PH and this value increased to nearly 50% at 8 weeks and to >95% at 16 weeks. In conclusion, RS-treated rat liver is unable to recover its original mass for several months following PH, despite the development of regenerative nodules. This long-lasting effect is likely to contribute to the growth of transplanted hepatocytes, leading to massive liver repopulation.

Key words: Retrorsine; Cell cycle block; Regenerative nodules; Cyclin D1

INTRODUCTION

Among solid organs, the liver is one of the sites for which effective cell transplantation and tissue repopulation is being most actively pursued (14,19). During the past several years we have developed a novel strategy for massive repopulation of a host liver via isolated hepatocyte transplantation (7–9). In this approach, both donor hepatocytes and the recipient animals are normal; however, the latter are pretreated with retrorsine (RS), a naturally occurring pyrrolizidine alkaloid that inhibits the proliferative capacity of resident hepatocytes (3,11, 16–18). Under these conditions, selective proliferation of injected cells is observed, with near total replacement of the recipient organ (7,8). Similar results have also been obtained using monocrotaline (6,21), an RS-related compound.

The precise mechanism(s) underlying this phenomenon remain(s) to be elucidated. Based on the available data, we have proposed the hypothesis 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 (3,11,17,18). However, this issue has not been directly addressed so far. Given that the process of liver repopulation takes place slowly, over a period of at least several weeks, we conducted a long-term study to analyze the regenerative response to 2/3 partial hepatectomy (PH) in rat liver exposed to RS. The experimental conditions were similar to those associated with liver repopulation. Our data indicated that, following PH, RS-treated rat liver is unable to recover its original mass for at least 16 weeks postsurgery, a time frame that is compatible with the kinetics of liver repopulation in this system.

MATERIALS AND METHODS

Animals and Treatments

Four-week-old male Fisher 344 (F344) (70 ± 20 g) were purchased from Charles River, Milan (Italy). They

Received April 8, 2008; final acceptance July 3, 2008.
Address correspondence to Ezio Laconi, Department of Biomedical Sciences and Technologies, Section of Experimental Pathology, University of Cagliari, Via Porcell, 4, 09125-Cagliari, Italy. Tel: +39-070-675-8682; Fax: +39-070-662574; E-mail: elaconi@unica.it

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 10 days of acclimatization, rats were treated according to a standard RS-based protocol for liver repopulation (7,8). Briefly, one group of animals received two injections of RS (Sigma Chemical Co., St. Louis, MO), 30 mg/kg each, IP, 2 weeks apart, while controls were given saline instead of RS. Two/thirds PH was performed 4 weeks after the last injection of RS and animals from both groups were killed at 2, 5, 8, and 16 weeks postsurgery. When this protocol is coupled with hepatocyte transplantation, massive liver repopulation occurs within 2 months (7). Where indicated, animals received four injections of 5-bromo-2-deoxy-uridine (BrdU, 50 mg/kg each, 8 h apart), starting 26 h before killing. Livers were excised and samples were taken and fixed in 4% buffered formaldehyde for histological and immunohistochemical analysis. Four/six animals per group per time point were included. All animals received human care and studies were conducted in accordance with the University of Cagliari guidelines. Liver DNA and protein content were measured according standard methods (1,2).

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 PCNA, cyclin D1, and BrdU was performed using specific monoclonal antibodies (from Sigma) (17). Detection of primary antibodies was accomplished using the avidin/biotin peroxidase system (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Labeling index (L.I.) was obtained by scoring at least 6,000 hepatocytes for each sample, in high-power random fields, and was defined as the percent ratio between labeled cells and total counted cells.

Statistical Analysis

All data are expressed as mean ± SEM. Two-tailed Student's *t*-test was used and differences were considered significant for values of $p < 0.05$.

RESULTS

Figure 1 reports results on relative liver weight, liver DNA, and protein content in animals killed at various time points after PH. At 2 weeks post-PH all parameters were significantly lower in rats pretreated with RS compared to controls, confirming previous results from our laboratory (17). Furthermore, the inability of RS group to recover liver mass was still evident at 5 and 8 weeks, and persisted at 16 weeks post-PH (Fig 1). For example, at the 16 weeks time point relative liver weight was 1.99 ± 0.30% in the RS group versus 3.06 ± 0.5% in controls (Fig. 1a).

Histological analysis revealed typical alterations associated with exposure to pyrrolizidine alkaloids and already described in the literature (3,10–12,15,18). They included extensive megalocytosis (enlarged hepatocytes) and the emergence of regenerative nodules (Fig. 2a–c). The latter were composed of normal appearing small hepatocytes, arranged in single cell plates, and occupied progressively larger proportions of the total liver: from about 3% at 2 weeks post-PH this value increased to nearly 50% at 8 weeks and was >95% at 16 weeks. Mitotic figures were common in regenerative nodules at 2 weeks post-PH (Fig. 2a), and they were occasionally found at later time points also (Fig. 2b). Rare, scattered areas of enlarged hepatocytes were still observed at the end of the follow-up period (i.e., 16 weeks after PH) (see Fig. 4e, f).

We further analyzed in situ expression of cyclin D1 and PCNA in RS-blocked megalocytes at various time points after PH. This was based on our previous study, indicating that RS-blocked megalocytes express high levels of these cell cycle-related gene products, both under resting conditions (i.e., in the absence of growth stimuli) and at early time points after PH (17). Results are presented in Table 1 and Figures 3 and 4. At 2 weeks post-PH a significant proportion (28.0 ± 2.3%) of RS-induced megalocytes displayed positive nuclear staining for cyclin D1 (Table 1, Fig. 3a); in addition, PCNA positive megalocytes were 22.4 ± 5.1%, while DNA synthesis was virtually absent (BrdU-positive hepatocyte nuclei were <0.1%) (Fig. 3e). Furthermore, both cyclin D1 and PCNA nuclear positivity were still present in megalocytic areas at 5, 8, and 16 weeks post-PH (Table 1, Fig. 4). Overall, the above data confirm and extend our previous observations indicating that RS-induced cell cycle block is associated with upregulation of cyclin D1 and PCNA in the absence of DNA synthesis. An additional finding of this study was the presence of megalocytes in RS-treated livers with positive cytoplasmic staining for cyclin D1 (Fig. 3a, Fig. 4a and c), associated with either positive or negative nuclear staining (Fig. 4a). Interestingly, rare hepatocytes with cyclin D1-positive cytoplasm were also found in control rat liver (Fig. 3b).

DISCUSSION

The present findings indicate that when rats are exposed to RS according to the protocol associated with liver repopulation, their ability to recover liver mass following PH is severely impaired for several months. At 2, 5, 8, and 16 weeks after PH, liver weight, liver DNA,

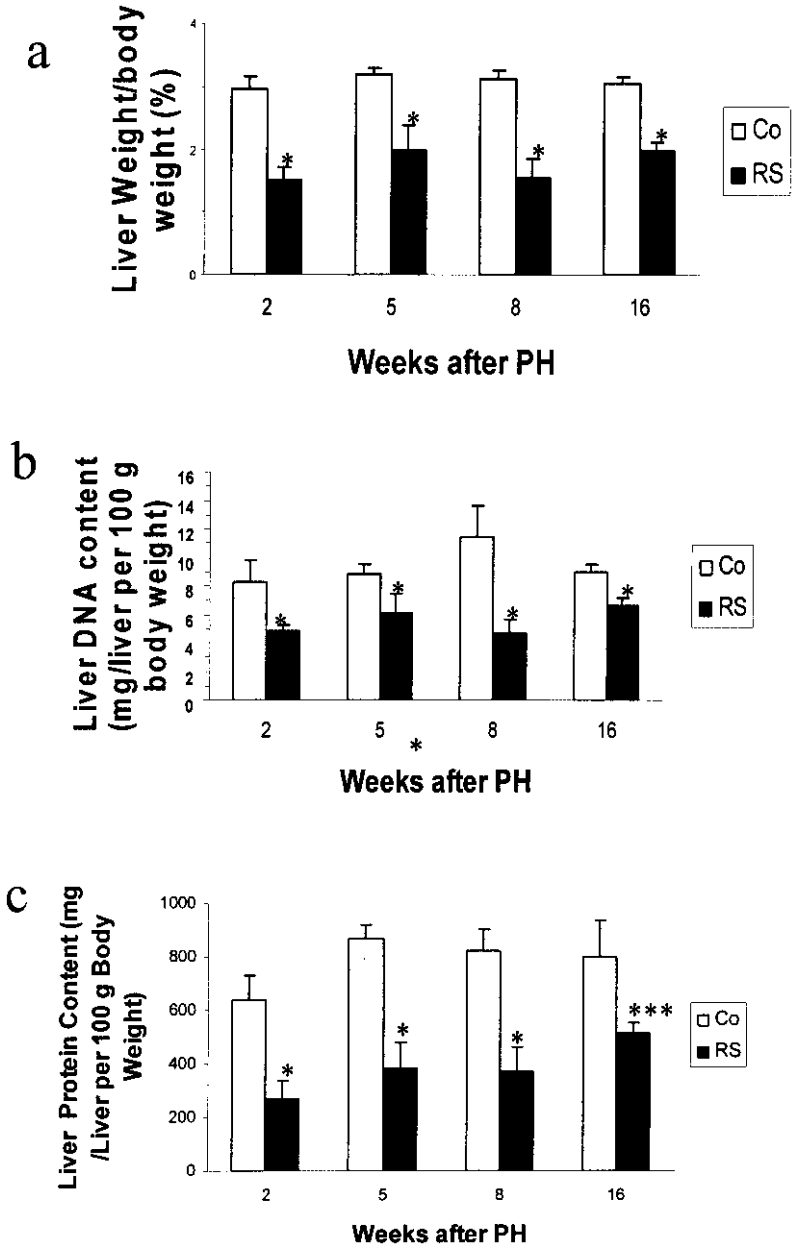

Figure 1. Relative liver weight (a), liver DNA content (b), and liver protein content (c) in rats treated with RS, followed by PH, and killed at various time points after surgery. Experimental details are given in the text (see Materials and Methods). Results are means ± SE of five to six animal per group. Significantly different from control: $*p < 0.001$; $**p < 0.01$; $***p < 0.05$.

and protein content were significantly reduced in animals pretreated with RS compared to controls. In fact, there was only a marginal increase in relative liver weight between groups killed at 2 or 16 weeks after operation (1.50 ± 0.21% vs. 1.99 ± 0.13%). A similar trend was also seen for both liver DNA and protein content (Fig. 1).

Typical alterations already described in the literature were seen in the liver of RS-treated rats. They included extensive megalocytosis (enlarged hepatocytes) and the emergence of regenerative nodules. The latter were composed of normal appearing small hepatocytes, arranged in single cell plates, and occupied progressively larger proportions of the total liver. However, areas of residual

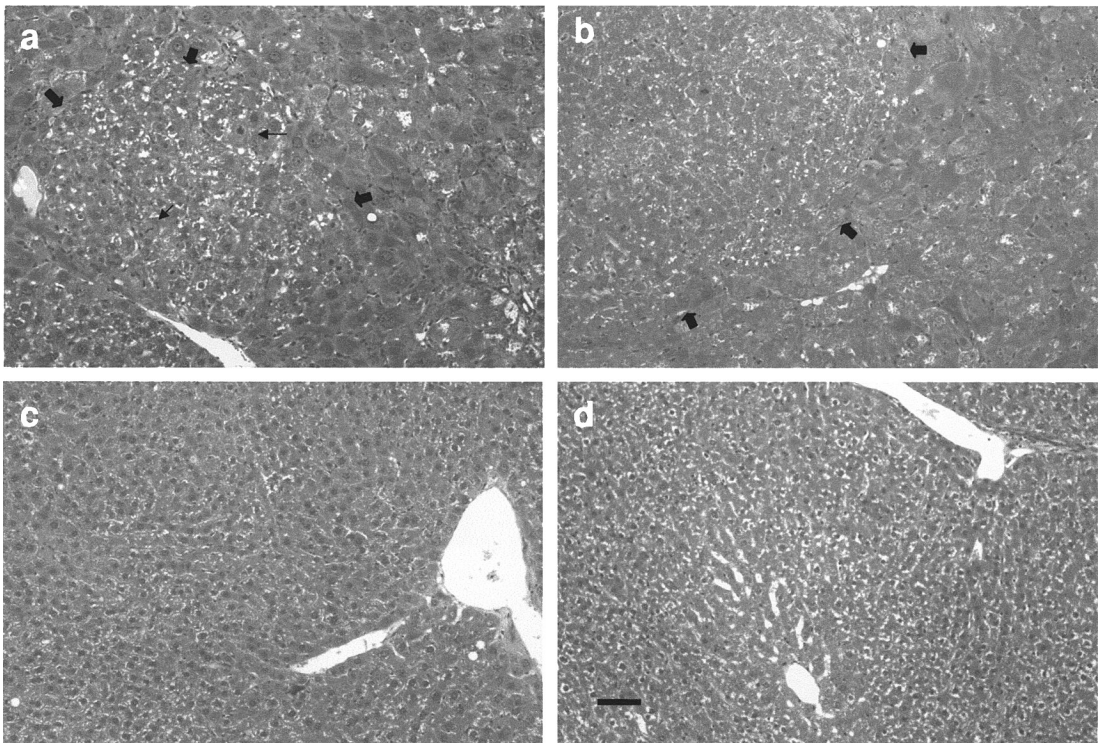

Figure 2. Liver histology in rats treated with RS, followed by PH, and killed at various time points after surgery. (a) RS-treated rat killed 2 weeks after PH; a regenerative nodule (large arrows) with mitotic figures (small arrows) is present, surrounded by extensive megalocytosis and proliferation of small bile ductular cells (open arrow). (b) RS-treated rat killed 8 weeks after PH; numerous regenerative nodules were detected (arrows), occupying about 50% of total liver section. (c) RS-treated rat killed 16 weeks after PH; >95% of the liver was composed of normal sized hepatocytes. (d) Normal liver histology in a control rat killed 16 weeks after PH. Experimental details are given in the text (see Materials and Methods). H&E staining; scale bar: 50 μm.

Table 1. Cyclin D1 and PCNA Labeling Indexes in Control and RS-Treated Rats Killed at Various Time Points After 2/3 Partial Hepatectomy

Treatment*	Cyclin D1 Labeling (%)	PCNA Labeling (%)
2 weeks		
Control	<0.05%	0.5 ± 0.2%
RS	28.0 ± 2.3%	22.4 ± 5.1%
5 weeks		
Control	<0.05%	1.1 ± 0.5%
RS	19.1 ± 3.5%	26.2 ± 4.3%
8 weeks		
Control	<0.05%	0.3 ± 0.2%
RS	22.5 ± 4.2%	27.1 ± 3.8%

*Experimental details are given in the text (see Materials and Methods). Data are mean ± SE of 5–6 samples per group.

megalocytes were still observed at the end of the follow-up period (i.e., 16 weeks after PH) (Fig. 4). It is noteworthy that such progressive expansion of regenerative nodules did not translate into any significant increase in liver mass over the same period (Fig. 1).

The RS-based model of liver repopulation via hepatocyte transplantation was laid on the hypothesis that the persistent block in the cell cycle imposed by the alkaloid on endogenous parenchymal cells would favor the selective growth of donor-derived cells, if appropriate stimuli were provided (9). Experimental evidence was in line with such proposition. When rats pretreated with RS were delivered normal hepatocytes in conjunction with PH, massive repopulation of the host liver occurred within 2 months (7). However, in the absence of PH, transplanted cells expanded at a much slower rate and repopulated the liver in 5–6 months (8). The results of the present study support our initial hypothesis: it was found that the regenerative capacity of the liver following PH is severely impaired for at least 4 months in rats treated with RS according to the protocol for hepatocyte

transplantation. Relevant to the point, a 4-month period is largely sufficient for near-complete liver repopulation to occur in this model (7).

Our findings are at variance with those reported by Gordon et al., who described complete regeneration of RS-treated rat liver within 4 weeks after PH (5). The reason(s) for such discrepancy is/are not immediately apparent. Possible differences include the age of the animals at the time of initial treatment, in that metabolism of RS is exquisitely age dependent (10), and/or breeding of the rat strain. Irrespective of the specific explanation, it would be important to determine whether the experimental conditions reported by Gordon et al. (5) are also associated with liver repopulation following transplantation of normal hepatocytes. If in fact hepatocytes from endogenous origin can proliferate and recover liver mass within a month post-PH, there should be no need nor opportunity for transplanted cells to expand and repopulate the entire organ under these conditions. Should the latter occur, alternative mechanisms for liver replacement in this system must be considered.

We have recently reported that a significant proportion of megalocytes in RS-treated rat liver expresses high levels of cyclin D1 and PCNA, in the absence of

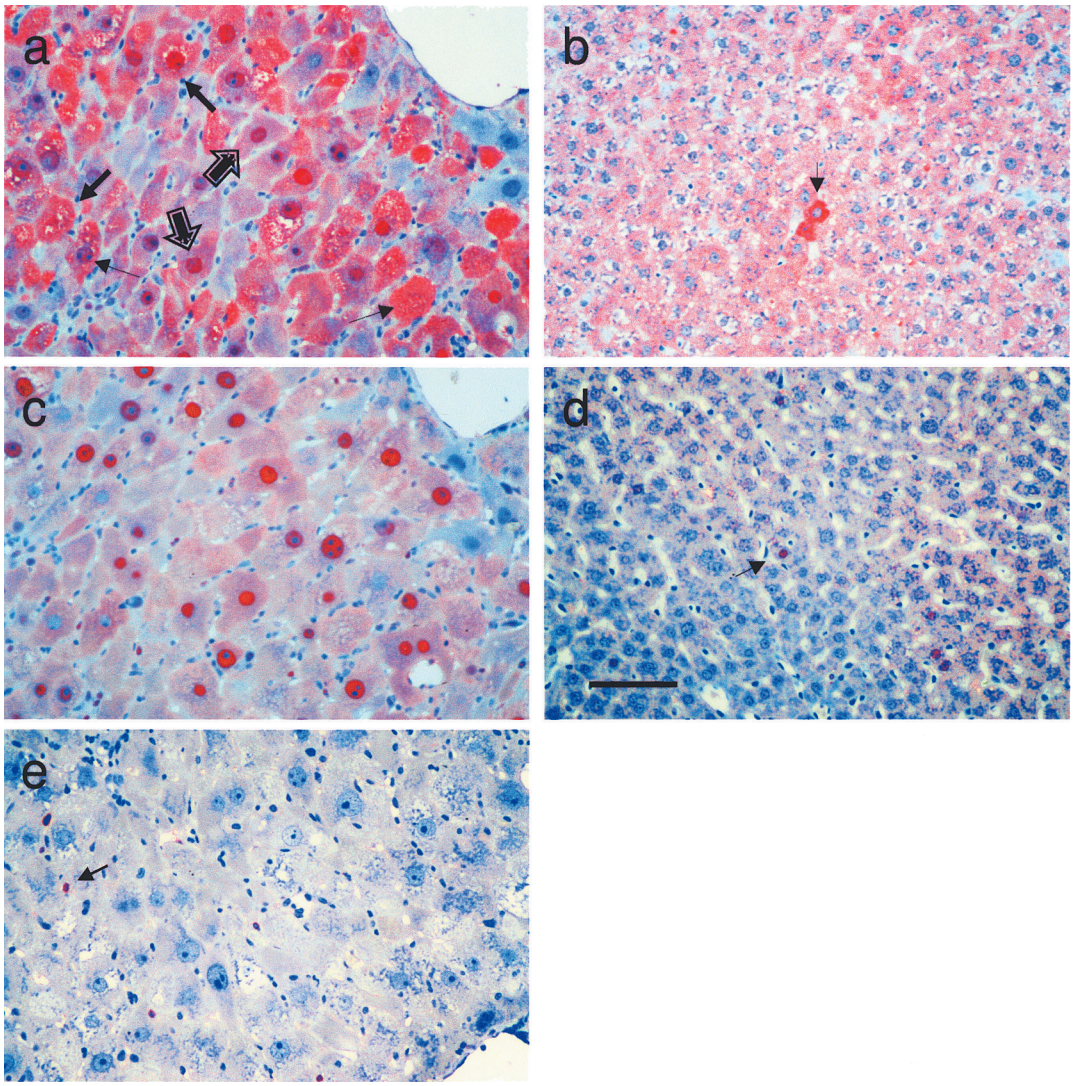

Figure 3. Immunohistochemical detection of cyclin D1, PCNA, and BrdU in RS-treated or control rats killed 2 weeks after PH. (a) Detection of cyclin D1 in RS-treated rats: numerous enlarged hepatocytes showed positive staining in the nucleus (open arrows), or in the cytoplasm (smaller arrows) or both (larger arrows). (b) Detection of cyclin D1 in control liver; rare isolated hepatocytes showed positive cytoplasmic staining (arrow). (c) Detection of PCNA in RS-treated rats: numerous enlarged hepatocytes showed nuclear staining. (d) Detection of PCNA in control liver; scattered hepatocytes showed positive nuclear staining. (e) Detection of BrdU incorporation in RS-treated rats: only rare nonparenchymal cells were positive for BrdU uptake. Scale bar: 50 μm.

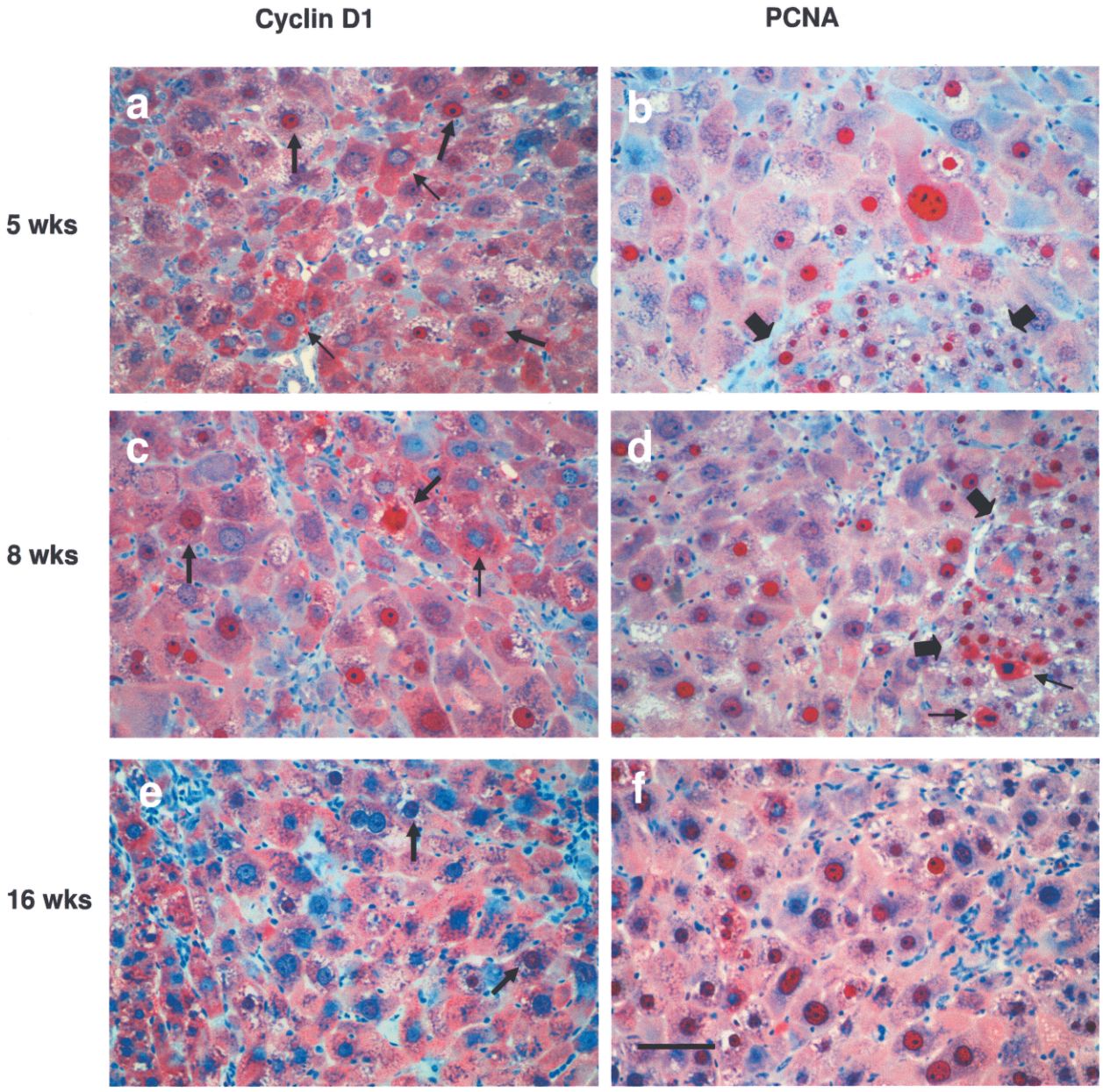

Figure 4. Immunohistochemical detection of cyclin D1 and PCNA in rats treated with RS followed by PH and killed at 5, 8, or 16 weeks after surgery. (a, c, e) Detection of cyclin D1 in rats killed at 5, 8, or 16 weeks after PH, respectively: enlarged hepatocytes were present with either nuclear (bold arrows) or cytoplasmic (thin arrows) positive staining. (b, d, f) Detection of PCNA in rats killed at 5, 8, or 16 weeks after PH, respectively: numerous enlarged hepatocytes showed positive nuclear staining; in (b) and (d) a small regenerative nodule is also shown (large arrows), with intense PCNA labeling and the presence of mitotic figures (d, thin arrows); note that mitotic figures were very rare in megalocytic hepatocytes. Scale bar: 50 μm.

DNA synthesis (17). In the present study, we confirmed and extended those results. Both cyclin D1 and PCNA were highly expressed in nuclei of megalocytes at 2 weeks post-PH, while there was no evidence of DNA synthetic activity (Fig. 3). Furthermore, positive nuclear staining for cyclin D1 and PCNA persisted at 5 and 8 weeks and was still observed in a fraction of scattered megalocytes at 16 weeks after PH (Fig. 4). It is noteworthy that cyclin D1-positive nuclei were very rare (<0.05%) in the livers from control rats killed at any time point considered.

Interestingly, at 2 weeks after PH a large number of megalocytes in the liver of RS-treated rats displayed cytoplasmic staining for cyclin D1, either with or without

nuclear staining (Fig. 3a); this was also observed, at lower frequency, at later time points (Fig. 4a, c, e). In addition, hepatocytes with cytoplasmic positivity for cyclin D1 were also detected in control rat liver (Fig. 3b), although at very low frequency (<0.01%).

The significance of these finding is unclear at the moment. However, persistent increased expression of cyclin D1 has been associated with inhibition of cell proliferation through binding of PCNA and cyclin-dependent kinase 2 (Cdk2) (4,13). Furthermore, cytoplasmic accumulation of cyclin D1 has been related to cell senescence and protection from apoptotic cell death in neurons (20).

Liver repopulation in rat liver exposed to RS is a rather complex phenomenon. While our present data highlight the role of an altered homeostasis in the control of liver mass, alternative or complementary mechanisms might be at play. For example, it is clear that transplanted normal hepatocytes do not simply proliferate side-by-side endogenous cells blocked by RS: they actually *replace* RS-induced megalocytes, indicating that they are endowed with both growth advantage and also survival advantage compared to resident cells. We suggest that both properties could indeed be relevant to liver repopulation in this system.

ACKNOWLEDGMENTS: This work was supported in part by grants from Italian Ministry of University and Education (MIUR), Italy (to P.P. and to E.L.) and AIRC (Italian Association for Cancer Research, to E.L.). We thank M. Boi, R. Marras, T. Puxeddu, and G. Porqueddu for their excellent technical and secretarial assistance.

REFERENCES

1. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72: 246–256; 1976.
2. Burton, K. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 65:315–323; 1956.
3. Downing, D. T.; Peterson, J. E. Quantitative assessment of the persistent antimitotic effect of certain hepatoxic pyrrolizidine alkaloids on rat liver. Aust. J. Exp. Biol. Med. Sci. 46:493–502; 1968.
4. Fukami-Kobayashi, J.; Mitsui, Y. Cyclin D1 inhibits cell proliferation through binding to PCNA and cdk2. Exp. Cell Res. 246:338–347; 1999.
5. Gordon, G. J.; Coleman, W. B.; Hixson, D. C.; Grisham, J. W. Liver regeneration in rats with retrorsine-induced hepatocellular injury proceeds through a novel cellular response. Am. J. Pathol. 156:607–619; 2000.
6. Joseph, B.; Kumaran, V.; Berishvili, E.; Barghava, K. K.; Palestro, C. J.; Gupta, S. Monocrotaline promotes transplanted cell engraftment and advances liver repopulation in rats via liver conditioning. Hepatology 44:1411–1420; 2006.
7. Laconi, E.; Oren, R.; Mukhopadhyay, D. K.; Hurston, E.; Laconi, S.; Pani, P.; Dabeva, M. D.; Shafritz, D. A. Long term, near total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. Am. J. Pathol. 153:319–329; 1998.
8. Laconi, S.; Pillai, S.; Porcu, P. P.; Shafritz, D. A.; Pani, P.; Laconi, E. Massive liver replacement by transplanted hepatocytes in the absence of exogenous growth stimuli in rats treated with retrorsine. Am. J. Pathol. 158:771–777; 2001.
9. Marongiu, F.; Doratiotto, S.; Montisci, S.; Pani, P.; Laconi, E. Liver repopulation and carcinogenesis: Two sides of the same coin? Am. J. Pathol. 172:857–864; 2008.
10. Mattocks, A. R.; White, I. N. H. Toxic effects and pyrrolic metabolites in the liver of young rats given the pyrrolizidine alkaloid retrorsine. Chem. Biol. Interact. 6:297–306; 1973.
11. Mattocks, A. R.; Driver, H. E.; Barbour, R. H.; Robins, D. J. Metabolism and toxicity of synthetic analogues of macrocyclic diester pyrrolizidine alkaloids. Chem. Biol. Interact. 58:95–108; 1986.
12. McLean, E. The toxic actions of pyrrolizidine (senecio) alkaloids. Pharmacol. Rev. 22:429–483; 1970.
13. Pagano, M.; Theodoras, A. M.; Tam, S. W.; Draetta, G. F. Cyclin D1-mediated inhibition of repair and replicative DNA synthesis in human fibroblasts. Genes Dev. 8: 1627–1639; 1994.
14. Park, S. M.; Vo, K.; Lallier, M.; Cloutier, A. S.; Brochu, P.; Alvarez, F.; Martin, S. R. Hepatocyte transplantation in the Long Evans cinnamon rat model of Wilson's disease. Cell Transplant. 15:13–22; 2006.
15. Peterson, J. E. Effects of the pyrrolizidine alkaloid lasiocarpine-N-oxide on nuclear and cell division in the liver of rats. J. Pathol. Bacteriol. 89:153–171; 1965.
16. Picard, C.; Lambotte, L.; Starkel, P.; Sempoux, C.; Saliez, A.; Van Der Berge, V.; de Saeger, C.; Horsmans, Y. Retrorsine: A kinetic study of its influence on rat liver regeneration in the portal branch ligation model. J. Hepatol. 39: 99–105; 2003.
17. Pitzalis, S.; Doratiotto, S.; Greco, M.; Montisci, S.; Pasciu, D.; Porcu, G.; Pani, P.; Laconi, S.; Laconi, E. Cyclin D1 is up-regulated in hepatocytes in vivo following cell-cycle block induced by retrorsine. J. Hepatol. 43:485–490; 2005.
18. Samuel, A.; Jago, M. V. Localization in the cell cycle of the antimitotic action of the pyrrolizidine alkaloid, lasiocarpine and of its metabolite, dehydroheliotridine. Chem. Biol. Interact. 10:185–197; 1975.
19. Strom, S. C.; Bruzzone, P.; Cai, H.; Ellis, E.; Lehmann, T.; Mitamura, K.; Miki, T. Hepatocyte transplantation: Clinical experience and potential for future use. Cell Transplant. 15(Suppl. 1):S105–110; 2006.
20. Sumrejkanchanakij, P.; Tamamori-Adachi, M.; Matsunaga, Y.; Eto, K.; Ikeda, M.-A. Role of cyclin D1 cytoplasmic sequestration in the survival of post-mitotic neurons. Oncogene 22:8723–8730; 2003.
21. Witek, R. P.; Fisher, S. H.; Petersen, B. E. Monocrotaline, an alternative to retrorsine-based hepatocyte transplantation in rodents. Cell Transplant. 14:41–47; 2005.