Published in final edited form as:

Shock. 2011 November ; 36(5): 471–477. doi:10.1097/SHK.0b013e31822d60ff.

Impaired Hepatocellular Regeneration in Murine Sepsis is Dependent on Regulatory Protein Levels

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Abstract

Sepsis is a poorly understood syndrome. Therefore we examined the mechanisms underlying failed regeneration in sham operated (SO), mildly septic (CLP) and severely septic (2CLP) C57Bl6 mice. Relative to no operation (T0) or SO, CLP, but not 2CLP, increased the number of cells staining for proliferating cell nuclear antigen (PCNA), a marker for cell division. Levels of the retinoblastoma protein (pRb) was detected at T0 and after SO. CLP increased while 2CLP decreased pRb abundance. Changes in phosphorylated pRB (P-pRb) were similar but more profound. The abundance of the transcription factor E2F was unaltered by SO, CLP or 2CLP. However, E2F DNA binding activity, while unchanged after SO, increased after CLP and decreased after 2CLP. The abundance of cyclinD1 in nuclear fractions increased following CLP but decreased after 2CLP. Neither SO or 2CLP altered the abundance of the cyclin-dependant kinase cdk-4. However, cdk-4 abundance increased after CLP. Finally, CLP increased the steady-state abundance of the mRNAs encoding Thymidine Kinase (TK), DNA Polymerase α (DNAPol α) and dihydrofolate reductase (DHFR), all required for DNA replication. No changes was noted after 2CLP. We conclude that 2CLP impaired hepatocyte proliferation following 2CLP in part via impaired CyclinD1/cdk-4 induced phosphorylation of pRb, maintaining the association between pRB and E2F and inhibited E2F transcriptional activity.

Keywords

retinoblastoma protein; E2F; PCNA; Thymidine Kinase; DNA Polymerase α ; dihydrofolate reductase; cyclinD1; cdk-4

Introduction

Sepsis and related syndromes are leading causes of death in critically ill patients (1). They are diagnosed in approximately 750,000 patients each year in the US and account for in excess of 215,000 deaths annually. Sepsis typically presents with evidence of excessive inflammation and abnormalities of oxygen delivery and utilization (2). Treatment of the attendant abnormalities of oxygenation and circulation often is possible. However, some patients develop the multiple organ dysfunction syndrome (MODS), characterized by sequential, persistent and intractable dysfunction in most organ systems (3). While recent

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advances have made it possible to support patients with MODS, poor understanding of the underlying pathogenesis and pathophysiology limit our ability to reverse the disorder. Most patients succumb when exogenous support is discontinued.

MODS is defined by specific abnormalities in each organ system (3). The clinical hallmark of hepatic dysfunction is jaundice, indicating a failure to detoxify and/or eliminate endogenous toxins. Other abnormalities include pathologic changes in the metabolism of glucose, protein and fat, impaired biotransformation and clearance of exogenous toxins, failure to synthesize essential proteins, such as clotting factors, that are used outside the liver, an alteration in the hepatic immune competence and impairment of the hepatic contribution to gastrointestinal function. Using two clinically relevant models of sepsis, cecal ligation and single puncture (CLP, a model of mild, reversible sepsis) and CLP with two punctures (2CLP, which is more fulminant and highly lethal), we have identified some of the pathophysiologic mechanisms at work in sepsis/MODS (4-6). While initial responses are similar following CLP and 2CLP, between 16 and 24 hours these responses change. Many reactions that are activated during CLP and early 2CLP become significantly attenuated in late 2CLP. Understanding the factors that contribute to the divergent responses in CLP and 2CLP may help identify the pathogenesis of MODS and also may suggest novel therapeutic approaches.

One key difference in the hepatocellular response following CLP and 2CLP involves cell proliferation (6). Normal human and murine hepatic cells are subject to low levels of apoptosis and necrosis. Sepsis and a number of other insults accelerate this (7-13). The adult liver retains the ability to regenerate, with cellular replication replacing lost cells (14). The mechanisms by which this occurs have been extensively studied (14). However, we previously found that 2CLP did not activate hepatocellular regeneration despite cell loss (6). The pathobiology underlying this defect is the subject of this study.

Proliferation via the cell cycle is tightly regulated. Cells are committed to replicate only when the cycle has progressed beyond a specific point, the G1 restriction point (15). The reactions that allow G-phase progression involve the expression of proteins, such as DNA polymerase- α (DNAPol α), thymidine kinase (TK) and dihydrofolate reductase (DHFR), that govern DNA replication. Transcription of the genes encoding these proteins is tightly controlled by the transcription factor E2F (16). In the quiescent phase of the cell cycle, intranuclear E2F is inactive as a result of tight binding to the retinoblastoma protein (pRb). Hyper-phosphorylation permits pRb dissociation from E2F. This frees E2F to catalyze transcription. pRb is in part phosphorylated by the cyclin-dependent kinase (cdk) 4/6. Activation of cdk4/6 requires cyclinD1. The E2F/pRb/cdk-4/6/cyclinD1 system is phylogenetically conserved and is perhaps the most primitive, fundamental mechanism known to regulate cell division. In this study, we test the hypothesis that 2CLP, but not sham operation or CLP, alters the abundance and/or activity of E2F, pRb, cdk4/5 and/or cyclinD1. We further propose that 2CLP attenuates pRb phosphorylation. This defect would prevent progression beyond the G1 restriction point, explaining the defect in hepatocellular proliferation following severe sepsis.

Material and Methods

Induction of sepsis

All animal protocols were approved by the Penn Institutional Animal Care and Utilization Committee and adhere to NIH standards. Study animals were 6-8 week old male C57BL/6 mice (Jackson Labs, Bar Harbor, ME). Experimental animals, on arrival, were housed in a climate control facility and allowed free access to food and water. After acclimation, mice underwent CLP or 2CLP with a 23 gauge needle as previously described (5;17;18).

Unoperated mice and sham operated animals, subjected to anesthesia, laparotomy and intestinal manipulation but not CLP/2CLP, served as controls. Immediately following surgery and every 24 hours thereafter, animals were fluid resuscitated with 40 ml/kg saline injected subcutaneously. At time 0 (unoperated control) and 3, 6, 16, 24, 48 and 72 hours after surgery, mice were re-anesthetized with 50 mg/kg intraperitoneal pentobarbital. Based on prior statistical analysis samples from three surviving animals at each time point and for each intervention were studied. Animals were sacrificed by exsanguination and liver tissue was harvested. Some liver tissue was processed to obtain total RNA for Northern Blot Hybridization Analysis and nuclear protein extracts for immunoblotting and electrophoretic mobility shift assays (EMSA). These samples were stored at -80°C until assays could be run in a single batch. An additional tissue sample was preserved in 10% neutral buffered formalin, embedded in paraffin and sectioned for histologic and immunohistologic evaluation.

Isolation and preparation of nuclear extracts

All procedures were either performed on ice or at 4°C . After harvest, livers were rinsed with PBS and homogenized in a buffer containing 50 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl (pH 8), 2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na fluoride, 1 mM Na_2MoO_4 , 1 mM Na_3VO_4 , and 2 $\mu\text{g/mL}$ antipain, aprotinin, bestatin, and leupeptin. The homogenate was layered onto this buffer and ultracentrifuged at 26,000 rpm for 50 min. The supernatant containing cytoplasmic protein was reserved for immunoblotting. The pellet containing intact nuclei was lysed and nuclear protein isolated as previously described (19). These samples were stored at -80°C until assays could be run in a single batch.

Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemistry

At each time point following SO, CLP and 2CLP, fixed tissue from three animals was dewaxed, rehydrated and quenched for endogenous peroxidase activity as previously described (18). Tissue samples were blocked in 1.5% goat serum/PBS for 30 minutes at 37°C and then exposed to avidin-biotin (Vector Laboratories, Burlingame, CA) to block non-specific binding. Sections were incubated with a primary mouse monoclonal anti-PCNA (Chemicon, Temecula, CA) for 60 minutes at room temperature followed by a 30 minute incubation with a biotinylated goat anti-mouse secondary antibody at 37°C . Sections then were treated with VECTASTAIN Elite ABC reagent (Vector Laboratories, Burlingame, CA) for 30 minutes. Chromogenic staining was performed using metal enhanced immune-pure 3,3-diaminobenzidine. After washing, sections were dehydrated, dried and mounted and PCNA positive cells per 20X field counted. Only dark nuclear staining within hepatocytes was counted as positive staining for PCNA.

EMSA for E2F-1

Nuclear protein extracts (5 μg as determined using the Bradford method) were incubated at room temperature in an equal volume of reaction buffer (20mM HEPES/KOH pH 7.6, 40mM KCl, 1mM EDTA, 5mM MgCl_2 , 1mM DTT, 1mM PMSF, 1mM spermidine, 0.1% NP-40 and 10% glycerol) and 1 μL of poly (dI-dC) for 20 minutes. One microliter (50,000 cpm) of oligonucleotide probe (Santa Cruz Biotech Inc., Santa Cruz CA) was added to each reaction mixture and incubated at room temperature for 30 minutes. Samples were loaded on an 8% non-denaturing TBE-polyacrylamide gel for electrophoresis (300 V, 2 hours, 4°C). In the super shift assay, the nuclear protein extract was incubated on ice with 1 μL of a mouse monoclonal antibody to E2F-1 (Santa Cruz) for one hour prior to addition of the probe. Gels were dried and autoradiography was performed.

Immunoblotting

All procedures were carried out at room temperature. Nuclear protein extracts (50 µg as determined using the Bradford method) were subjected to electrophoresis on a 10% discontinuous SDS-polyacrylamide gel, transferred to polyvinylidene fluoride membranes and subjected to immunoblotting as previously described (17). Membranes were probed with primary antibodies (1:1000 in blocking solution) to retinoblastoma protein (pRb), phospho-retinoblastoma protein (P-pRb), E2F-1, cyclin D-1 or cyclin-dependent kinase (cdk)-4 (Santa Cruz) followed by species-specific HRP conjugated secondary antibodies (Santa Cruz). Detection was performed using an Enhanced Chemi-Luminescent detection kit (Amersham, Piscataway, NJ).

Northern Blot Hybridization Analysis for Thymidine Kinase (TK), DNA polymerase-α (DNAPolα) and dihydrofolate reductase (DHFR)

Total RNA was isolated, 20ml of each sample subjected to agarose-formaldehyde electrophoresis and transferred to polyvinyl chloride membranes. Northern blot Hybridization Analysis was performed as previously described using cDNA probes to TK, DNAPolα and DHFR (4).

Statistics

The intensity of bands on immunoblots was determined using densitometry. Densities for each band were normalized to T0. Statistical significance ($P < 0.05$) was determined using ANOVA for repeated measures with the Bonferroni test of post hoc significance.

Results

CLP, but not 2CLP, provokes hepatocellular proliferation

PCNA is a 36 kilo-Dalton accessory protein to DNA polymerase-δ whose abundance in the nucleus increases during late G1 and S-phase (20). Therefore increased PCNA accurately reflects cell proliferation. Figs. 1a and b depicts representative stained sections. Cell counts were performed on ten randomly-chosen 20 X fields on each of five different slides obtained from a single animal. Quantitative data are presented in Fig 1c. Minimal staining was noted at T0 (Fig 1a,c), a finding consistent with quiescent liver tissue. This also was the case at all time points following SO (Fig 1b,c). Relative to T0 and SO controls, a statistically significant increase in the number of PCNA – positive cells was noted 24, 48 and 72 hrs following CLP. In contrast, PCNA counts following 2CLP were significantly lower than observed following CLP and did not differ from values following SO. These data indicate that 2CLP did not provoke the same proliferative response as CLP.

CLP increased and 2CLP decreased the abundance and phosphorylation of pRB

Regulation of the cell cycle is most tightly controlled at the G1-restriction site. At this point, the transcription factor E2F is activated, committing the cell to expression of genes encoding proteins required for DNA duplication (15). Binding of pRb maintains E2F in an inactive state. Phosphorylation of pRB (Phospho-pRb) leads to dissociation. This frees E2F to bind to DNA and stimulate transcription. One possible explanation for the lack of PCNA abundance, and thus of cell replication, following 2CLP is impaired pRb phosphorylation. Therefore, we performed immunoblotting to investigate the relative abundance of free pRb and Phospho-pRb in the nuclear protein fraction. Data are depicted in Fig. 2. Relative to T0 (unoperated control) no statistically significant change in the abundance of pRb was noted following SO (Fig 2a). Following CLP, pRb abundance was increased relative to T0 at 16, 24, 24, 48 and 72 hours following CLP. In contrast, pRb abundance after 2CLP was unchanged. pRb abundance 16, 24, 48 and 72 hrs after CLP was significantly higher than

that observed following SO and was up to twice that observed at T0. Levels after 2CLP were not significantly different than those observed following SO. Importantly, the antibody used for these experiments was directed at a pRb epitope common to both pRb and Phospho-pRb. Therefore, both forms were detected. In order to determine the effects of CLP/2CLP on pRb phosphorylation we probed the same blots with an antibody specific to phospho-pRb (Fig 2b). In T0 samples little Phospho-pRb was detected. No statistically significant change was noted following SO, paralleling the data obtained for total pRb. There was a statistically significant increase in Phospho-pRb abundance 16, 24, 48 and 72 hours following CLP. Abundance following 2CLP was significantly lower than T0 at the 6, 48 and 72 hour time points. Values for CLP differed from those of both SO and 2CLP at 16, 24, 48 and 72 hours while 2CLP values differed from SO at 6, 48 and 72 hours. These data are similar to the effects of 2CLP on pRb abundance although the change following 2CLP was more profound. Our results suggest that CLP increased the abundance of free nuclear pRb and similarly but even more profoundly altered phosphorylation, decreasing 2CLP levels relative to SO. These changes likely reflected altered release of phospho-pRb from E2F. This suggests that the 2CLP-induced loss of hepatocellular regeneration resulted from impairment of pRb phosphorylation

Abundance of E2F is not altered by SO, CLP or 2CLP

In previous work, we found that 2CLP decreased the expression of key proteins in hepatocytes (4;5). A similar defect in E2F expression might explain altered hepatocyte replication. Therefore we examined the abundance of E2F following SO, CLP and 2CLP. SO, CLP and 2CLP did not significantly alter E2F abundance (data not shown).

E2F DNA binding activity is increased following CLP but decreased following 2CLP

If hepatocyte regeneration following 2CLP reflects failed E2F activation then E2F binding to hepatocyte DNA should be decreased. Therefore we used EMSA to determine E2F DNA binding following SO, CLP and 2CLP. Data are depicted in Fig 3. A small amount of cell turnover occurs in quiescent liver. Therefore, some E2F DNA binding was present in T0 samples. Similar results were noted at all time points following SO. E2F DNA binding increased 3, 24 and 72 hours after CLP. In contrast, E2F DNA binding activity decreased at 3, 24, 48 and 72 hours following 2CLP. The patterns after SO and 2CLP paralleled but antedated changes in pRb and P-pRb abundance and PCNA staining. E2F DNA binding activity decreased at 3, 6, 16, 24 and 48 hours after 2CLP. This paralleled changes phospho-pRb

CLP Increases and 2CLP Decreases Intra-nuclear Abundance of CyclinD1

cdk-4 is the primary kinase responsible for phosphorylation of pRb (21). Activation of this enzyme requires formation of a complex with cyclinD1. In previous studies on rats, we found a sepsis-induced decrease in cyclinD1 abundance (6). Therefore we examined the relative abundance of cyclinD1 in nuclear extracts isolated following SO, CLP and 2CLP. These data are depicted in Fig. 4. Relative to T0, nuclear levels of cyclin D1 following SO were unchanged. After CLP, Cyclin D1 abundance was increased at 6, 16, 48 and 72 hrs. In contrast, cyclinD1 levels decreased at 24, 48 and 72 hours following 2CLP.

CLP Increases and 2CLP Does Not Alter Intra-nuclear Abundance of cdk-4

In contrast to cyclinD1, cdk-4 in quiescent liver cells is primarily cytoplasmic. Activation during G1 phase stimulates cdk-4 migration to the nucleus and formation of complexes with cyclinD1 (21). Therefore we examined intra-nuclear levels of cdk-4 following SO, CLP and 2CLP. Data are depicted in Fig 5. Intra-nuclear cdk-4 was barely detectable at T0. A small increase was noted 6 and 16 hours after SO. More profound increases were observed at 48

and 72 hours after CLP. While abundance 16 and 24 hrs after 2CLP was decreased relative to T0, this change was only significant relative to SO at T₁₆.

CLP Increases and 2CLP Decreases the Abundance of TK, DHFR and DNAPol α mRNA

Northern blot analysis was performed to determine the effects of SO, CLP and 2CLP on the abundance of the mRNAs encoding TK, DHFR and DNAPol α . Expression of these genes is controlled by E2F. Data are depicted in Fig. 6. No change was noted following SO (data not shown). Expression of these genes was increased following CLP. However, there was no change in mRNA abundance at time points following 2CLP. There was a statistically significant difference between CLP and 2CLP at all time points.

Discussion

The data presented here confirm and extend our previous demonstration of a sepsis-induced deficiency in the replication/regeneration of hepatocytes during experimental sepsis (6). This likely occurred as the result of impaired pRb phosphorylation, maintained binding of pRb to E2F and decreased E2F transcriptional activity. Thus, we demonstrate that sepsis impairs one of the most basic and fundamental mechanisms controlling cell replication, progression past the G1 restriction point in the cell cycle (15;22). Impaired activation of the transcription factor E2F limits expression of the genes encoding enzymes involved in DNA replication. This suggests that the changes that accompany sepsis are profound, altering a process that is of primary importance in eukaryotic cell function and preservation.

Large-scale cell replication and tissue regeneration is a characteristic and unique feature of the adult liver (14). While most epithelial-derived organs are subject to some form of cell turnover or replacement following injury only the liver appears capable of large-scale regeneration. This process is tightly orchestrated, highly controlled and involves both pro-mitotic and anti-apoptotic mechanisms. It is well-known that human hepatic mass may be restored following surgical resection (23). Similarly, animal studies following extreme (up to 75%) hepatectomy or massive toxic insults, such as that which occurs following carbon tetrachloride (CCl₄) administration, substantiate this capacity for regeneration (10;13;24). In addition, hepatic regeneration is important in recovery following liver transplantation (25) and impairment of this process may be indicative of graft dysfunction (26). In general the degree of hepatocyte growth is directly proportional to the degree of injury and cell loss (14;23;27). Thus, some level of regeneration occurs even when tissue loss is not extensive. Conversely, when tissue loss exceeds a certain threshold, the capacity for regeneration is lost (28). This limits the extent of surgical resection for malignancy.

The significance of hepatic cell loss and repletion in sepsis has not been well-described. Our previous work with bromodeoxyuridine (BrdU) staining in septic rats demonstrated hepatic regeneration following CLP but not 2CLP, findings that were accompanied by similar changes in cyclinD1 expression (6). Those data are replicated here in mice using PCNA rather than BrdU as a marker of DNA replication. Data from both species indicate that DNA synthesis peaks 24 hours after partial hepatectomy (29) or CCl₄ administration (10;24) and is sustained until hepatic mass is restored. Peak effects following CLP appear to occur somewhat later. This may reflect the timing of tissue loss. In addition, studies using TNF α (12;30) and IL-6 (13) knockout mice indicate that inflammation is an important component of the regenerative response. Thus, the delay in the appearance of significant BrdU/PCNA staining after CLP may reflect the timing of the inflammatory response. Importantly, our previous studies demonstrated impaired IL-6 signaling and failed activation of the IL-6-dependent transcription factor Stat3 after 2CLP (5;17-19). This may help explain the lack of regeneration observed in this study, as IL-6 signaling is an important component of the hepatocellular regenerative pathway (23). Indeed, when Stat-3 activation is blocked

following partial hepatectomy, regeneration is impaired (31). Recent reviews have suggested that this effect is indirect and that cytokines such as IL-6 “prime” hepatocytes for proliferation in response to more direct mitogens (14). However, recent work has shown that Stat3 directly stimulates cyclinD1 and cyclinE gene expression (32). In combination with our data showing impaired IL-6 signaling and hepatocyte replication, it is logical to postulate a more direct IL-6 effect on hepatocyte proliferation.

Hepatic regeneration follows both apoptotic and necrotic cell loss (27). Studies have revealed some hepatocellular apoptosis following CLP/2CLP (33) while Hotchkiss and co-workers found both in liver tissue from recently expired human victims of sepsis (34). Our work has not demonstrated significant apoptosis (6) but we have consistently found necrosis (6;18). Further, we have reported that serum α -GST, ALT and AST levels increase more profoundly following 2CLP than after CLP (5-7). Therefore the regeneration following 2CLP should be robust. The absence of this response, along with depressed gene expression (4), impaired cytokine signaling (17;18), depressed mitochondrial function (35) and high mortality (19) is yet another indication of the profound hepatic dysfunction characteristic of sepsis.

BrdU/PCNA activity is characteristic of cells preparing to transition to the S-phase of the cell cycle (20). This indicates progression past the key rate-limiting step of early cell replication, the G1 restriction point, a finding that directed our attention to the transcription factor E2F, which controls expression of key proteins required for DNA replication, including PCNA, and pRb (16). While pRb phosphorylation is most prominently controlled by cdk-4 and its major activator, cyclinD1 (32), cdc2 and cyclinE also are involved and will be examined in future studies (36). Our data on cyclinD1 abundance is consistent with data from septic rats (6) and clearly indicates that the regenerative response following severe sepsis is impaired.

In a large database developed with results from this and our previous reports, mortality at 72hrs following CLP was 30% and following 2CLP 90%. This directly correlated with increased hepatocyte PCNA staining. We have previously proposed that the initial response of hepatocytes to a severe insult is a state of “metabolic quiescence” (35). Our view is that sepsis is characterized by an insult so profound that it causes many types of cells to reduce the activities that make them unique and, instead, engage only in functions that are essential for cell survival. Thus, in response to severe sepsis, hepatocytes respond with reduced transcription of genes encoding liver-specific proteins (4;18), impaired responses to cytokines (17-19) and attenuated biotransformation of potentially toxic substances (17). The data presented here suggest that impaired regeneration is similarly altered. There are no similar studies on other tissues capable of regeneration, for example, skin, and it may be difficult to separate the effects on a healing wound of nutrition from those of sepsis itself. It would be interesting to determine the importance of regenerative loss by blocking the process in septic animals and determining the effects on hepatic function and survival. Unfortunately, use of agents that might interfere with regeneration is problematic *in vivo*. These compounds are not specific to hepatocytes. We have shown, for example, that direct interference with 2CLP-induced pulmonary cell replication using HSP70 improves pathology and outcome (37). Others have demonstrated important consequences of genetically altering the expression of proteins that modulate apoptosis (38). There are no similar data on the liver. Thus, while the actual contribution of impaired regeneration to either mortality or hepatic dysfunction is unknown, its importance makes for interesting speculation. On the one hand, the low level of cell loss makes it unlikely that limited regeneration by-and-of itself is a significant contributor to sepsis-induced liver failure. Conversely, cell regeneration is a fundamental biologic property of hepatocytes. While subject to modulation on multiple levels, E2F-mediated activation of pRb and consequent

expression of the genes encoding proteins such as TK, DHFR and DNAPol α directly controls DNA replication. It is perhaps the most primitive of all processes controlling cell replication and is conserved both phylogenetically and in most highly differentiated cells. As such, the findings reported here add credence to our hypothesis that sepsis alters hepatocellular behavior at a profound and very fundamental level. .

In summary, the current study demonstrates activated hepatocyte proliferation in mild sepsis and a failure of this process in severe sepsis. The two responses correlate with mortality. Because intact cellular proliferation most likely depends on intact levels and activity of regulatory proteins, the lack of cell proliferation following 2CLP may be another manifestation of sepsis-induced hepatocellular dysfunction. Further studies evaluating other control mechanisms are warranted. Correction of this lost process during sepsis may allow for new therapeutic approaches.

Acknowledgments

Supported by NIH/NIGMS Grants 5R01GM059930 (CSD, NRR), 5T32GM007612-32 (KMA, CSD), The Medical Student Anesthesia Research Fellowship of the Foundation for Anesthesia Education and Research (AA) and the Stavropoulos Sepsis Research Program (CSD, AA). Presented in part at the Annual Meeting of the Association of University Anesthesiologists, Denver Colorado, April 2010

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Fig 1. Proliferative Cell Nuclear Antigen (PCNA) staining of fixed hepatic sections

a. Section from unoperated (T0) control.

b. Sections from SO – Sham Operation, CLP – CLP with a single puncture, 2CLP – CLP with two punctures mice. Primary antibody - mouse monoclonal anti-PCNA, secondary antibody - biotinylated goat anti-mouse IgG. 20 X magnification. Arrows – representative PCNA staining (dark nuclei). Note virtual absence of staining in 2CLP T48 and T72 sections.

c. Graphic depiction of numbers of PCNA – positive cells/20X field. X-axis – time after intervention in hours. Y-axis – counts/field. Counts from ten 20x fields/section, five sections/animal, three animals/time point – intervention. Mean $\pm$ SD. * = statistically different from T0, ^ = statistically different from SO at same time point, † = statistically different from 2CLP at same time point, # = statistically different than 2CLP at same time point

Fig 2. Immunoblot Analysis of Retinoblastoma Protein (pRb) and Phospho-Retinoblastoma Protein (Phospho-pRb) abundance in the Nucleus

a. pRb. b. Phospho-pRb. Left: representative immunoblots. SO – Sham Operation, CLP – single puncture CLP, 2CLP – double puncture CLP. Number after T indicates time after intervention. 50 μ g nuclear protein/lane. Right: Graphic representation of relative abundance. X-axis – time after intervention in hours. Y-axis – relative density (no units). Black squares – SO, Red diamonds – CLP, Green circles – 2CLP. Data (Mean $\pm$ SD) derived from densitometric signal intensity on autoradiogram. Data normalized to signal at T0. Each data point represents the mean and SD of 3 measurements. * = statistically different from T0, ^ = statistically different from SO at same time point, † = statistically different from 2CLP at same time point

Fig 3. Electrophoretic Mobility Shift Analysis (EMSA) of Transcription Factor E2F DNA Binding Activity

EMSA representative of studies from three animals/time point - intervention. SO – Sham Operation, CLP – single puncture CLP, 2CLP – double puncture CLP. Number after T indicates time after intervention.

Fig 4. Immunoblot Analysis of cyclinD1 abundance

Top: representative immunoblots. SO – Sham Operation, CLP – single puncture CLP, 2CLP – double puncture CLP. Number after T indicates time after intervention. Bottom: Graphic representation of relative abundance. 50 μ g nuclear protein/lane. X-axis – time after intervention in hours. Y-axis – relative density (no units). Data (Mean $\pm$ SD) derived from densitometric signal intensity on autoradiogram. Data normalized to signal at T0. Each data point represents the mean and SD of 3 measurements. Black squares – SO, Red diamonds – CLP, Green circle – 2CLP. * = statistically different from T0, ^ = statistically different from SO at same time point, † = statistically different from 2CLP at same time point.

Fig 5. Immunoblot Analysis of cyclin-dependent kinase (cdk) - 4 abundance

Top: representative immunoblots. SO – Sham Operation, CLP – CLP with a single puncture, 2CLP – CLP with two punctures mice. Number after T indicates time after intervention.

Bottom: Graphic representation of relative abundance. 50 μ g nuclear protein/lane. X-axis – time after intervention in hours. Y-axis – relative density (no units). Data (Mean $\pm$ SD) derived from densitometric signal intensity on autoradiogram. Data normalized to signal at T0. Each data point represents the mean and SD of 3 measurements. Black squares – SO, Red diamonds – CLP, Green circles – 2CLP. * = statistically different than T0, ^ = statistically different than SO at same time point, † = statistically different than CLP at same time point.

Fig. 6. Northern Blot Analysis of Abundance of mRNAs encoding Thymidine Kinase (TK), Dihydrofolate Reductase (DHFR) and DNA Polymerase B (DNA Pol)

a. Representative Northern Blots. 20 μ g mRNA/lane. 18S – 18S Ribosomal Subunit. b. Graphic representation of relative abundance of TK. c. Graphic representation of relative abundance of DHFR. d. Graphic representation of relative abundance of DNAPol. X-axis – time after intervention in hours. Y-axis – relative density (no units). Data (Mean $\pm$ SD) derived from densitometric signal intensity on autoradiogram. Data for each signal normalized to 18S signal on same autoradiogram at same time point and then to signal at T0. Each data point represents the mean and SD of 3 measurements. Black squares – CLP, Green circles – 2CLP. * = statistically different than T0, ^ = statistically different from 2