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Negligible Oval Cell Proliferation Following Ischemia-Reperfusion Injury With and Without Partial Hepatectomy

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Background: Hepatic oval cells proliferate to replace hepatocytes and restore liver function when hepatocyte proliferation is compromised or inadequate. Exposure to chemical carcinogens, severe liver steatosis, and partial hepatectomy has been used in animal models to demonstrate the role of oval cells in liver regeneration. Ischemia-reperfusion injury (IRI) causes hepatocellular damage and death in the absence of confounding chemical toxicity; however, oval cell induction by IRI has not been demonstrated in vivo. We examine oval cell induction following partial IRI.

Methods: Wistar rats were subjected to 2 IRI protocols: 70% warm liver ischemia for 30 minutes followed by reperfusion or 70% warm liver ischemia for 30 minutes with partial hepatectomy of the nonischemic lobes followed by reperfusion. Liver injury was monitored by serum alanine aminotransferase (ALT) at 1 day and 7 days of reperfusion. Oval cell proliferation was monitored by indirect immunofluorescence staining using the surface markers BD.2 and Thy-1. Cellular proliferation was quantified by 5-ethynyl-2′-deoxyuridine (EdU) incorporation in vivo.

Results: Serum ALT elevation was only observed at the 1-day time point in the IRI with partial hepatectomy model. Oval cell marker expression was restricted to the biliary structures in both the ischemic and the nonischemic control lobes. Oval cell induction, measured by changes in the frequency of BD.2 and Thy-1 expression and EdU incorporation, was not significantly altered by IRI.

Conclusion: In both mild and moderate IRI models, we did not find evidence of oval cell induction or proliferation. EdU staining was restricted to hepatocytes, suggesting that liver regeneration following IRI is mediated by hepatocyte proliferation.

Keywords: *Hepatocytes, ischemia, liver, reperfusion*

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INTRODUCTION

Ischemia-reperfusion injury (IRI) to the liver results from prolonged hypoxia followed by blood reperfusion. Significant IRI can occur during liver surgery, transplantation, trauma, and other clinical procedures. Studies have shown that activation of multiple inflammatory pathways, including the generation of reactive oxygen species, activation of lymphocytes, infiltration of the liver by neutrophils, and activation of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), leads to hepatocellular damage and cell death.[1-3] However, the mechanisms underlying hepatic regeneration following IRI are not well understood.

Hepatocytes respond to liver injury by proliferation and compensatory hypertrophy to achieve homeostasis. Impairment of this response by drugs, viral infection, toxins, or carcinogens leads to liver insufficiency and activation of hepatic progenitor cells, termed oval cells in the rodent.[4-6] Studies have shown that oval cells have the capacity to differentiate into hepatocytes and ductal cells to restore liver mass.[7,8] Oval cells represent a heterogeneous population of facultative stem cells with regard to phenotypic markers and regenerative capacity.[9,10] IRI has not been investigated as a model for oval cell induction.[11] We used 2 rat partial-liver IRI models that mirror a clinical scenario and do not involve toxic carcinogens to investigate whether oval cell responses are activated after IRI. Animals were subjected to warm ischemia in the left-lateral and median lobes of the liver for 30 minutes, followed by reperfusion for 1 day or 7 days. Liver injury was monitored by histology and liver enzyme levels in the serum. Oval cell induction was observed by staining for the markers Thy-1 and BD.2.

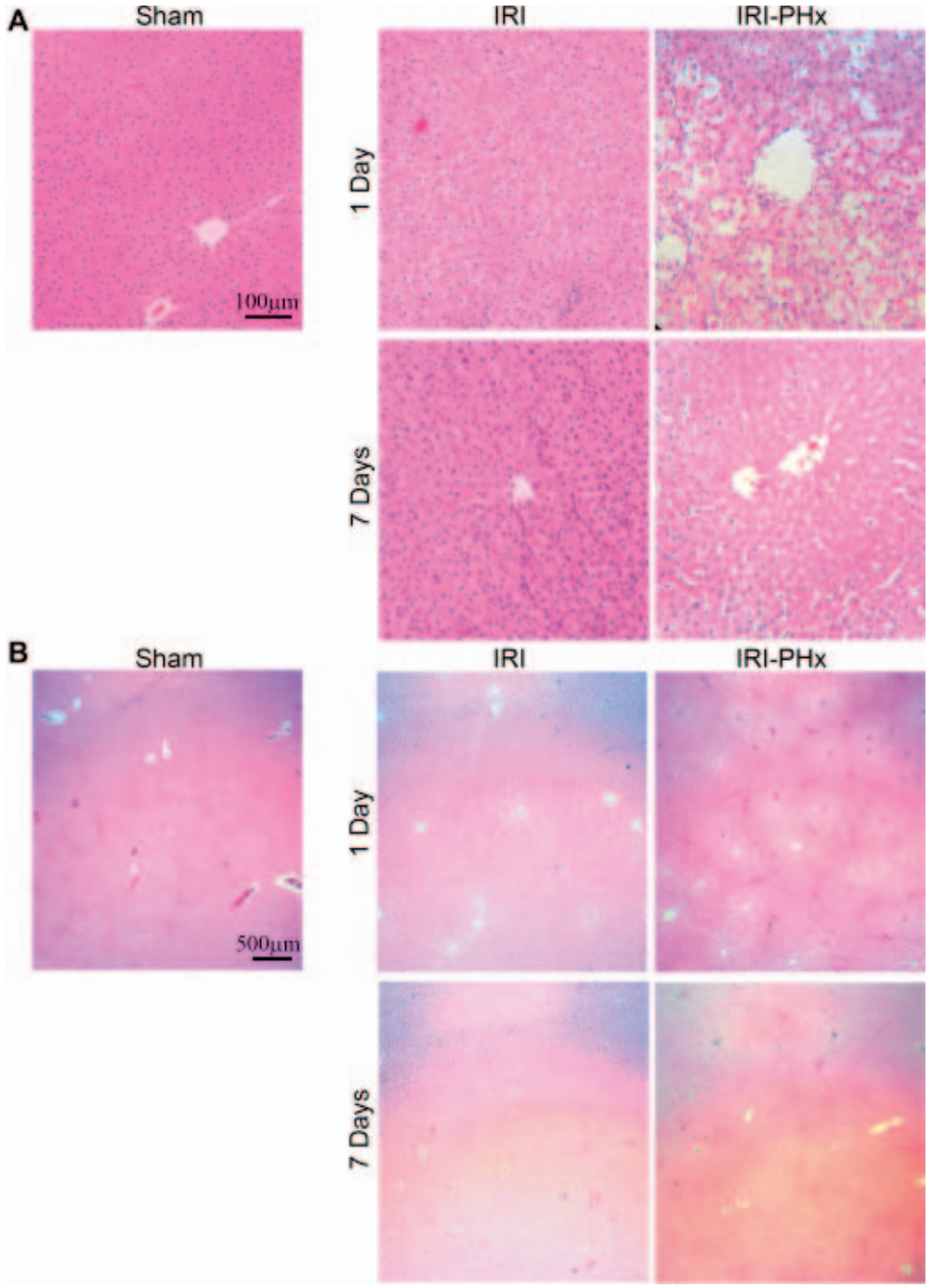

Figure 1. Hematoxylin and eosin staining of the left-lateral lobe of the liver in rats that underwent sham injury, ischemia-reperfusion injury (IRI), or IRI with partial hepatectomy (IRI-PHx) with reperfusion at 1 and 7 days at (A) 40× magnification and (B) 4× magnification. Histologic changes of sinusoidal congestion and hepatocyte coagulative necrosis were evident after 1 day of reperfusion with resolution by 7 days. (A color graphic is available at www.ochsnerjournal.org/toc/17/1 in the Focus on Transplantation section.)

METHODS

Animals

Male Wistar rats aged 5-7 weeks (Charles River Laboratories International, Inc.) with a mean weight of 189 ± 11 g were housed in a temperature- and humidity-controlled room with a 12-hour light-dark illumination cycle with ad libitum standard pellet chow and water access. Rats were fasted overnight prior to surgery with free access to water. Isoflurane was administered at 2.5% for induction and at 2.0% for maintenance anesthesia throughout the duration of surgery.

Twenty-four rats were used in the first injury model (IRI). Four rats per experimental group were subjected to IRI and evaluated at 1, 3, 5, and 7 days after reperfusion. For each

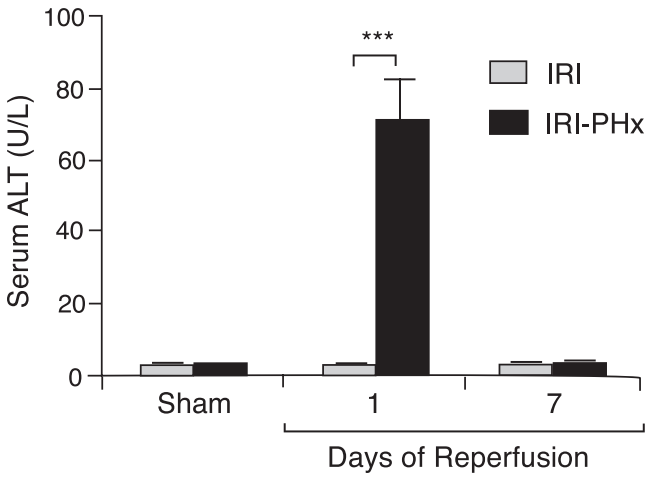

Figure 2. Alanine aminotransferase (ALT) values in serum samples obtained from blood drawn from the inferior vena cava prior to the endpoint at 1 or 7 days of reperfusion. Plots include a total of n=6 biological replicates from n=2 experimental format independently reproduced in 3 experiments. Asterisks indicate a $P<0.001$ significant Bonferroni posttest between treatment group time points after 2-way analysis of variance. IRI, ischemia-reperfusion injury; IRI-PHx, ischemia-reperfusion injury with partial hepatectomy.

time point, 2 rats served as controls and were subjected to sham surgery. Specifically, the sham surgery involved inducing anesthesia, performing a laparotomy, dissecting the major pedicles without induction of liver ischemia, and waiting 30 minutes before closing the abdomen and reversing anesthesia.

Utilizing an adaptive experimental design, we introduced a second injury model of IRI with partial hepatectomy (IRI-PHx),[12] with 1 rat in each experimental group that was evaluated at 1 and 7 days after reperfusion. The specimens in the IRI-PHx model were reproduced 3× per rat to provide a replicate size of 3. Four rats did not undergo any surgical procedure and served as negative controls. In total, 30 rats were used for this study.

The study was performed in strict accordance with recommendations provided in the *Guide for the Care and Use of Laboratory Animals*.[13] The study protocol was approved by the Institutional Animal Care and Use Committee of Ochsner Clinic Foundation.

Partial Ischemia-Reperfusion Injury Models

In the IRI group, a midline incision was performed to isolate the major pedicle supplying the left-lateral and median lobes of the liver, approximately 70% of the entire liver volume. An atraumatic vascular clamp was applied to render 30 minutes of warm ischemia to the left-lateral and median lobes. Gross alterations in the left-lateral and median lobes were noted over the entire course of ischemia, and reperfusion of these segments was confirmed upon release of the vascular clamp. Thereafter, the abdomen was closed. Clamp release was established as the reference time point for the start of reperfusion.

The IRI-PHx group was subjected to the same IRI to the left-lateral and median lobes as in the IRI group. Upon completion of 30 minutes of ischemia, the nonischemic segments (approximately 30% of total liver volume) were resected before the vascular clamp was removed.

Twenty-four hours prior to sacrifice, 50 μg per gram of bodyweight of 5-ethynyl-2′-deoxyuridine (EdU, Thermo Fisher Scientific, Inc.) was administered by intraperitoneal injection in a total volume of 1 mL of normal saline. For the 1-day experimental and sham groups, EdU was given during abdominal closure. At the designated endpoints, animals were anesthetized, and blood was collected from the inferior vena cava into sterile anticoagulant-free syringes. The animals were sacrificed by exsanguination. The blood samples were kept at room temperature for 2 hours and subsequently centrifuged at 3,000 RPM for 25 minutes to obtain serum that was stored at –80°C until assay. Liver tissues from both affected and nonaffected lobes were harvested separately, divided, and fixed in 10% neutral buffered formalin (Leica Microsystems Inc.) or embedded in CryoGel (Electron Microscopy Sciences) and stored at –80°C until sectioned. Biological samples collected at each time point were tested for the following.

Solt-Farber Model. Hepatocarcinogenesis was induced using the Solt-Farber model as originally described.[14] Briefly, rats received an intraperitoneal injection of diethylnitrosamine (DEN) 100 mg/mL in saline at a dose of 200 mg/kg. Two weeks after DEN administration, rats were placed on a diet containing 2-acetylaminofluorene for 1 week. After 1 week of feeding, rats underwent two-thirds PHx and were returned to the normal diet until endpoint (time point unknown).

Serum Alanine Aminotransferase, Tumor Necrosis Factor-α, and Interleukin-6. Serum alanine aminotransferase (ALT) levels were measured using the ALT Colorimetric Activity Assay Kit according to the manufacturer's instructions (Cayman Chemical Company). Serum TNF-α and interleukin-6 (IL-6) were measured using the Quantikine rat TNF-α ELISA immunoassay kit and M Rat IL-6 Quantikine ELISA Kit (R&D Systems, Inc.) as directed by the manufacturer. Optical densities were read using a microplate reader (Bio-Rad Laboratories, Inc.), and standard curves were generated using ReaderFit v.2.0 software (Hitachi Solutions America Ltd.).

Histology and Immunohistochemistry. Paraffin tissue samples were sectioned at 4 μm and stained with hematoxylin and eosin (H&E) for visualization by light microscopy. Frozen samples were sectioned at 10 μm and fixed for 5 minutes in 4°C acetone prior to staining. Immunohistochemistry was performed according to the antibody manufacturers' protocols. Primary antibodies used for the studies were Thy-1/CD90 (MRC OX-7) (Abcam, PLC), a marker for hematopoietic stem cells and oval cells; BD.2, a marker for oval cells and bile ducts;[15] and H4, a hepatocyte-specific marker.[15] For Thy-1 immunohistochemistry, we used a biotinylated rat-adsorbed secondary antibody kit (BA-2001, Vector Labs Inc.). Blocking was performed using an avidin/biotin blocking kit (SP-2001, Vector Labs Inc.) and 0.3% hydrogen peroxide in Tris-buffered saline. For negative controls, normal mouse immunoglobulin G (I-2000, Vector Laboratories, Inc.) was substituted for primary mouse antibodies. Slides were developed using a 3,3′-diaminobenzidine horseradish peroxidase substrate kit (SK-4100, Vector Laboratories, Inc.). Thy-1 staining of cells was quantified by counting small cells strongly staining for Thy-1 in 10 random portal tracts. Dual indirect immunofluorescent staining for the oval cell/cholangiocyte markers BD.2 and EdU and for the hepatocyte markers H4 and EdU was performed on cryosections as previously described.[16] The

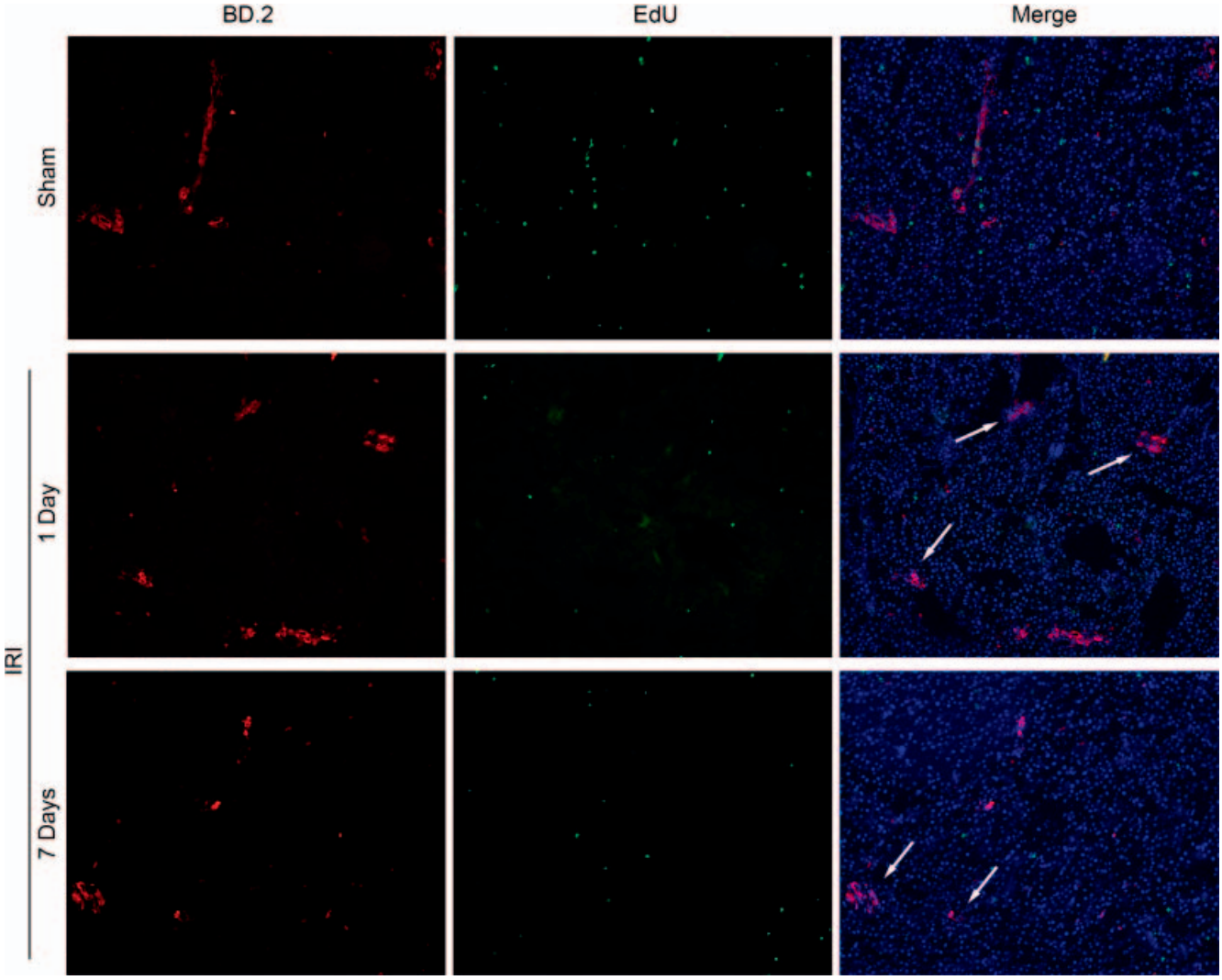

Figure 3. Indirect immunofluorescence staining of the oval cell marker BD.2 and proliferation marker 5-ethynyl-2′-deoxyuridine (EdU) in sham vs 1-day and 7-day reperfusion groups of rats that underwent ischemia-reperfusion injury (IRI). BD.2 staining in all groups was restricted to biliary structures, with diffuse parenchymal EdU staining not indicative of oval cell activation (white arrows). (A color graphic is available at www.ochsnerjournal.org/toc/17/1 in the Focus on Transplantation section.)

slides were counterstained with 4′,6-diamidino-2-phenylinade (Vector Laboratories, Inc.). Tissue sections were blindly read by the investigators and a pathologist.

Statistical Analysis

Statistical analyses were performed with SPSS v.20.0 (IBM). Continuous variables between groups were compared using *t* tests. We used the chi-square test for categorical variables. Two-way analysis of variance with Bonferroni posttest was performed on ALT values from the IRI and IRI-PHx groups at each time point using Prism (GraphPad Software, Inc.). All the *P* values reported were 2-sided, and we considered *P* values <0.05 statistically significant.

RESULTS

Liver Injury in the Two Ischemia-Reperfusion Injury Models

Two IRI models, differing in the degree of liver injury, were used to examine a potential role of oval cells during the reperfusion period. Lethality was not observed up to 7 days following injury in either the IRI or IRI-PHx models. H&E stains of the ischemic lobes in each model showed histologic changes consistent with IRI as early as 1 day after reperfusion (Figure 1A), with more prominent injury evident in the IRI-PHx model. Sinusoidal congestion and hepatocyte coagulative necrosis were evident after 1 day of reperfusion with resolution by 7 days (Figure 1B). Serum ALT was not significantly elevated in the IRI group vs sham group across time points of days 1, 3, 5, and 7 (data not shown). Elevated serum ALT was observed exclusively in the IRI-PHx group at 1 day (70.6 ± 11.4 U/L in the IRI-PHx group compared to 3.0 ± 0.3 U/L in the IRI group, $P<0.001$), with resolution to normal levels at 7 days of reperfusion (Figure 2). Serum levels of TNF-α and IL-6 were both below the limit of detection at all time points for both IRI and IRI-PHx models (data not shown). Thy-1 staining was seen in control and experimental livers along the portal tracts, but negligible change was seen across the time points to suggest an influx of leukocytes into the liver (data not shown).

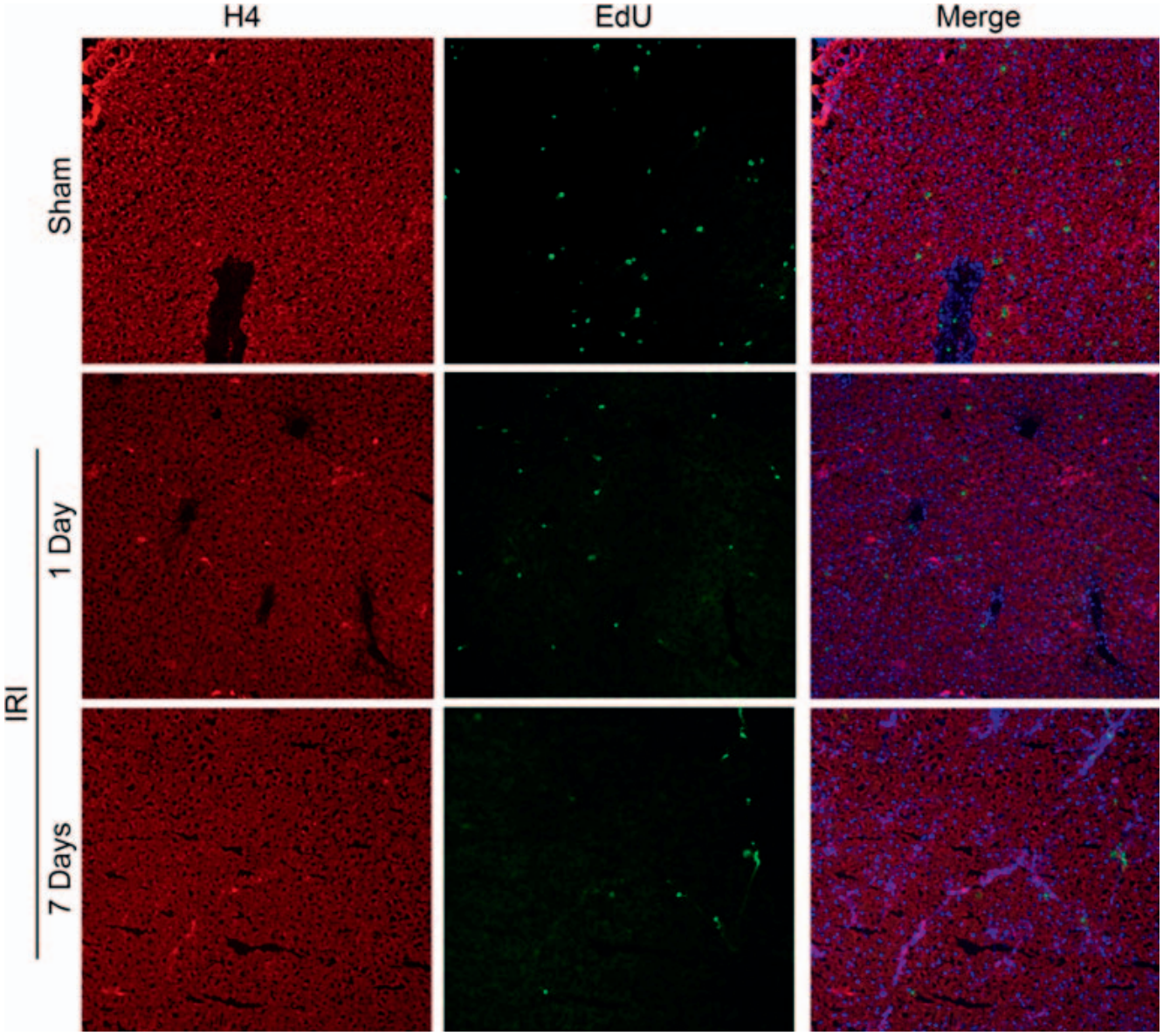

Figure 4. Indirect immunofluorescence staining of the hepatocyte marker H4 and proliferation marker 5-ethynyl-2′-deoxyuridine (EdU) in sham vs 1-day and 7-day reperfusion groups of rats that underwent ischemia-reperfusion injury (IRI). No apparent differences in the frequency or distribution of H4 and EdU coexpression were observed. (A color graphic is available at www.ochsnerjournal.org/toc/17/1 in the Focus on Transplantation section.)

Oval Cell Activation After Ischemia-Reperfusion Injury

We next examined the expression of the oval cell marker BD.2 to determine if IRI was sufficient to activate oval cells. In sham groups, expression of BD.2 was restricted to the bile ducts, consistent with BD.2 staining of mature cholangiocytes. To confirm this observation, dual staining for the oval cell/cholangiocyte marker BD.2[17] and EdU, a marker of cell proliferation, was performed (Figure 3). In the control animals and nonischemic lobes, BD.2-positive cells were confined to the bile ducts, indicating staining of mature cholangiocytes. The localization and total number of BD.2-positive cells in the affected lobes (subjected to IRI) were similar to the nonaffected lobes. We found no evidence of BD.2-positive cells extending away from the portal tracts into the liver parenchyma of either affected or unaffected lobes that would suggest proliferation of oval cells. The vast majority of EdU-positive cells were negative for BD.2. Dual staining for the hepatocyte marker H4 and the proliferation marker EdU revealed that the majority of proliferating cells were hepatocytes, suggesting that the regenerative response to IRI was confined to mature hepatocytes (Figure 4). As mentioned in the previous paragraph, negligible change in Thy-1 staining was seen across all time points to suggest the activation or presence of oval cells.

Because ALT values were not significantly elevated in the IRI model, indicative of a minor liver injury, we further examined whether oval cell activation could be achieved in the IRI-PHx model that was associated with greater

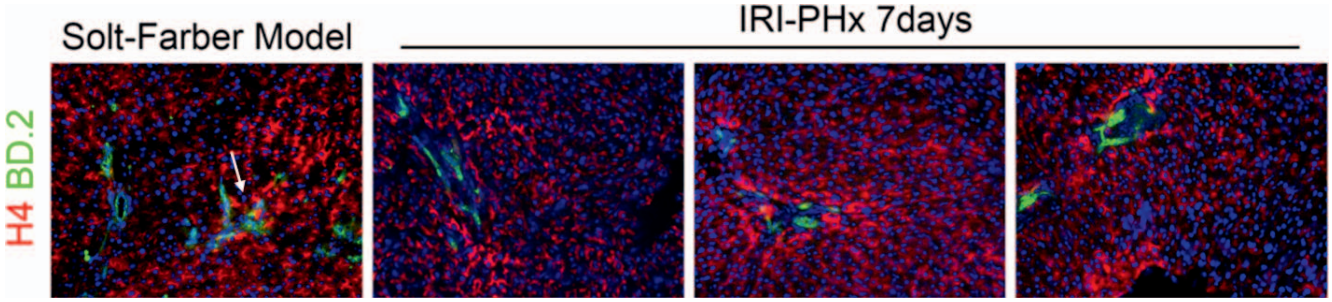

Figure 5. Indirect immunofluorescence staining for H4 and BD.2 coexpression in rats that underwent ischemia-reperfusion injury with partial hepatectomy (IRI-PHx) at the 7-day reperfusion time point. The Solt-Farber carcinogenesis model shows representative oval cell activation (white arrow) in the liver parenchyma, which is notably absent in the 3 independent IRI-PHx replicates. (A color graphic is available at www.ochsnerjournal.org/toc/17/1 in the Focus on Transplantation section.)

parenchymal injury. Tissue sections from IRI-PHx animals after 7 days of reperfusion were stained with H4 and BD.2 to visualize oval cells (Figure 5) and compared against a positive control for oval cell induction (Solt-Farber carcinogenesis model). Despite a greater IRI, no evidence of oval cell induction was observed in the liver parenchyma.

DISCUSSION

We were interested in determining whether IRI induces oval cell proliferation in vivo. The IRI and IRI-PHx models used here simulate an acute liver injury often seen during liver surgery. IRI can inflict significant hepatocellular injury, and, in contradistinction to chemical injury models, does not have mutagenic properties. We used a partial-liver IRI model, isolating the left-lateral and median lobes as the affected, ischemic lobes. The left-lateral and median lobes constitute approximately 70% of the total liver.[18] We have previously shown that systemic responses between partial and complete liver IRI rat models were similar (Cohen A, unpublished data). The partial ischemia model provided the added opportunity to compare differences between the affected and nonaffected lobes within the same animal.

The changes in serum ALT corresponded with histologic changes of injury seen on light microscopy after 1 day of reperfusion. We found that TNF-α levels were low and in a steady state in both control and experimental rats throughout all time points (data not shown). We previously used a whole liver IRI model in which TNF-α levels peaked at 6 hours after reperfusion, decreasing toward baseline after 24 hours.[19] This finding was consistent with the results in our IRI model that we tested for TNF-α levels at 1, 3, 5, and 7 days and did not detect measurable levels. Because TNF-α signaling is required, in part, for oval cell proliferation and TNF-α peaks at 6 hours of reperfusion, the TNF-α signaling may not have been available in our rats.[20]

We found no evidence for an oval cell response in the ischemic lobes. Staining for the oval cell markers BD.2 and Thy-1 throughout the measured time points was confined to the mature cholangiocytes lining the portal tracts. We observed no difference in the number of BD.2- or Thy-1–positive cells in the control animals and nonischemic lobes compared to ischemic tissue. Dual staining for hepatocyte marker H4 and proliferation marker EdU revealed minimal cellular proliferation that was confined to the hepatocytes. Thy-1, a hematopoietic stem cell marker, has been reported to be expressed in oval cells.[6,21,22] However, Thy-1 mRNA is not expressed in α-fetoprotein–positive oval cells. Myofibroblasts are immune-positive for Thy-1, suggesting that Thy-1 may be transiently expressed in oval cells.[23] Thy-1 expression was detected in portal tracts of control animals as well as experimental animals in the present study; however, none of the cells was EdU positive. Taken together, our data indicate that partial warm ischemia of 30 minutes to a normal liver followed by reperfusion of 1, 3, 5, or 7 days and partial warm ischemia of 30 minutes combined with hepatectomy of the nonischemic lobes followed by reperfusion of 1 and 7 days do not induce oval cell activation. Studies suggest that a more severe or chronic insult may be necessary to activate oval cell–mediated regeneration of the adult liver.[24] Because clinical scenarios involving IRI are acute by nature, further research may be directed toward delivering a near-lethal dose of IRI through a longer duration of vascular clamping or combining IRI with a fatty-liver or chronic hepatitis model in which regenerative responses are already impaired.

CONCLUSION

The IRI and IRI-PHx models in rats demonstrated biochemical injury and histologic injury in the affected lobes but did not result in further production of TNF-α beyond day 1 of reperfusion. Indirect immunofluorescence for BD.2 and immunohistochemistry for Thy-1 did not detect a distinct oval cell population in IRI lobes. EdU labeling indicated that the proliferation was confined to mature hepatocytes. Our studies suggest that the recovery of normal livers after being exposed to brief periods of IRI is most likely to be hepatocyte-mediated and does not involve oval cells.

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