GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Ischemic Preconditioning Affects Long-Term Cell Fate through DNA Damage—Related Molecular Signaling and Altered Proliferation

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Despite the potential of ischemic preconditioning for organ protection, long-term effects in terms of molecular processes and cell fates are ill defined. We determined consequences of hepatic ischemic preconditioning in rats, including cell transplantation assays. Ischemic preconditioning induced persistent alterations; for example, after 5 days liver histology was normal, but γ -glutamyl transpeptidase expression was observed, with altered antioxidant enzyme content, lipid peroxidation, and oxidative DNA adducts. Nonetheless, ischemic preconditioning partially protected from toxic liver injury. Similarly, primary hepatocytes from donor livers preconditioned with ischemia exhibited undesirably altered antioxidant enzyme content and lipid peroxidation, but better withstood insults. However, donor hepatocytes from livers preconditioned with ischemia did not engraft better than hepatocytes from control livers. Moreover, proliferation of hepatocytes from donor livers preconditioned with ischemia decreased under liver repopulation conditions. Hepatocytes from donor livers preconditioned with ischemia showed oxidative DNA damage with expression of genes involved in MAPK signaling that impose G1/S and G2/M checkpoint restrictions, including p38 MAPK—regulated or ERK-1/2—regulated cell-cycle genes such as *FOS*, *MAPK8*, *MYC*, various cyclins, *CDKN2A*, *CDKN2B*, *TP53*, and *RB1*. Thus, although ischemic preconditioning allowed hepatocytes to better withstand secondary insults, accompanying DNA damage and molecular events simultaneously impaired their proliferation capacity over the long term. Mitigation of ischemic preconditioning—induced DNA damage and deleterious molecular perturbations holds promise for advancing clinical applications. (*Am J Pathol* 2014, 184: 2779–2790; <http://dx.doi.org/10.1016/j.ajpath.2014.07.002>)

Organs are often exposed to ischemia and reperfusion (IR) under conditions such as thromboembolic events, vascular clamping during surgery, and organ procurement, preservation, or transplantation. However, molecular and cellular perturbations after IR are not fully defined, despite recognition of oxidative stress, inflammation, ion fluxes, and conditions as major contributors in IR-related events.¹ Although IR is generally deleterious, ischemic preconditioning (IP) (ie, a nonlethal period of IR followed by another period of ischemia) is tissue protective, as was initially reported for myocardium² and later for other organs, including liver, small intestine, kidneys, and pancreas.^{3–5} Mechanisms underlying early effects of IP have been elucidated to some

extent in terms of complex roles for multiple mediators (including adenosine, ATP, and nitric oxide) that may engage receptors and/or intracellular signaling pathways and transcription factors, such as p38 MAPK, NF- κ B, and mitogen-activated protein kinase 8 [MAPK8; alias c-Jun N-terminal kinase (JNK)].^{6,7} However, these studies have been limited to only the initial minutes or hours after IP, and thus the late effects of IP (including the long-term fate of cells exposed to

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IP) and potential mechanisms causing such effects are not understood. The need for such understanding becomes critical in terms of therapeutic implications of IP in tissue protection for organ transplantation and regeneration, and also for cell therapy.^{8–11} In many instances, attempts at organ protection by IP (eg, with liver allografts) have produced conflicting results, with both beneficial and nonbeneficial outcomes,^{9,10} perhaps because of differences in intracellular signaling or other reasons leading to varied cell fates.¹² The ability of IP to promote liver regeneration in a partial hepatectomy (PH) model¹¹ suggests that such benefits may extend to other conditions or organs, but this too requires analysis of cell fates in suitable systems.

Cell transplantation models have been helpful for defining biological mechanisms (eg, repopulation of liver with reporter hepatocytes) that elicit insight into gene expression, cell proliferation, stem cell differentiation, or disease correction.^{13–17} Because transplanted cells may be tracked individually or in groups, we reasoned that cell transplantation approaches for assaying cell fate would be particularly valuable for identifying effects of IP in given cell populations.

We hypothesized that if cells isolated from donor organs subjected to IP are more robust than without IP, then they should exhibit capacity for superior engraftment, proliferation, and organ replacement. IP induces antioxidant protection,^{6,7} and this should be relevant because intravascular injection of cells may cause IR, along with immediate blood-mediated responses, release of vasoactive peptides, cytokines, and chemokines from activated neutrophils, macrophages, and other cells, variously resulting in oxidative stress and clearance of transplanted cells.^{18,19} Similarly, oxidized extracellular matrix components impair cell survival through outside-in NF- κ B-related signaling, which could possibly be altered by IP.²⁰ On the other hand, if IP is only an adaptive tissue response without any long-term cytoprotection, this should culminate in varied fates of transplanted cells.

To examine these possibilities, we used rats deficient in dipeptidyl peptidase IV (DPPIV[−]) as a model for studies of liver injury induced by IP, carbon tetrachloride (CCl₄), or oxidative stress, along with assays of hepatocyte engraftment and proliferation,^{14,15,18,19} followed by dissection of relevant cell signaling pathways.

Materials and Methods

Animals

DPPIV[−] F344 rats, 8 to 10 weeks of age, were obtained from the Special Animal Core of the Albert Einstein College of Medicine Marion Bessin Liver Research Center. F344 rats were obtained from the NIH National Cancer Institute (Bethesda, MD). Rats were housed under a 14-hour light/10-hour dark cycle, with unrestricted food and water. Rats were anesthetized with ketamine–xylazine. The Animal

Care and Use Committee at the Albert Einstein College of Medicine approved protocols in conformity with current NIH guidelines.

For hepatic ischemia, venous and arterial blood flows were discontinued by an aneurysm clip on the hepatoduodenal ligament. For IR, the clip was maintained in place for 15 minutes before restoration of perfusion. For IP, liver was subjected to ischemia for 15 minutes, reperfusion for 15 minutes, ischemia for 60 minutes, and then ending with reperfusion. For additional injury, we administered 0.5 mL/kg i.p. CCl₄ in mineral oil (1:1, v/v) for 24 hours.¹⁴ Liver injury was graded in hematoxylin and eosin–stained sections on a five-point scale: uninjured normal (0) and minimal (1+), mild (2+), moderate (3+), or severe (4+) injury.²¹

Cell Isolation and Culture

Rat hepatocytes were isolated by collagenase perfusion.^{13,14} Cell viability was determined by trypan blue dye exclusion immediately after isolation and after suspension culture in RPMI 1640 medium with 10% fetal bovine serum and antibiotics for 6 or 12 hours at 4°C. For oxidative stress, 1×10^4 hepatocytes/cm² were cultured overnight in 24-well collagen-coated dishes in Dulbecco's minimal Eagle's medium, antibiotics, and fetal bovine serum, followed by addition for 1 hour of 50 to 100 μ mol/L *tert*-butylperoxide. Reduction of 0.5 mg/mL thiazolyl blue dye (MTT) over 90 minutes at 37°C was analyzed by lysing intracellular formazan in acid isopropanol with quantitation as described previously.¹⁸

Biochemical Assays

Total glutathione and catalase activity were measured in liver or cell samples homogenized in 6% salicylic acid or phosphate buffer, respectively, as described previously.¹⁷ The activity of Cu/Zn/Mn superoxide dismutase and of lipid peroxidation or malondialdehyde content was measured as thiobarbiturate acid-reacting substances (kits 706002 and 10009055, respectively; Cayman Chemical, Ann Arbor, MI).

RT-qPCR

For quantitative real-time reverse transcription PCR (RT-qPCR), total RNA was extracted with TRIzol reagent (Life Technologies, Carlsbad, CA) and was passed through Qiagen columns (RNeasy kit; Qiagen, Valencia, CA). After DNase treatment, 1 μ g RNA was reverse-transcribed using an RT² First Strand kit (Qiagen–SABiosciences, Frederick, MD) and amplified in an ABI Prism 7500 thermocycler (Life Technologies) using RT² real-time SYBR Green/ROX PCR master mix in an array of 84 genes (RT² Profiler PCR Array Rat MAP Kinase Signaling Pathway; PARN 061-A; Qiagen–SABiosciences), according to the manufacturer's recommendations. Each condition used three replicate samples. Data were analyzed by the $2^{-\Delta\Delta C_t}$ method. Relative

gene expression was normalized for all samples with housekeeping genes. Intergroup comparisons were made for gene expression differences. Gene expression ≥ 2 -fold greater than that of controls was considered of interest. Pathways of interest were examined with the Ingenuity Pathway Analysis (IPA) software system version 6.0 (Qiagen Silicon Valley, Redwood City, CA).

Western Blotting

Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). After blocking in 5% nonfat milk and Tris-buffered saline–Tween (20 mmol/L Tris, pH 7.5, 500 mmol/L NaCl, 0.5% Tween 20) for 1 hour, blots were probed for total or phosphorylated rat p38 MAPK, ERK (Cell Signaling Technology, Danvers, MA), JUN, MAPK8, or β -tubulin (Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C in Tris-buffered saline–Tween with 5% bovine serum albumin. For development, we used peroxidase-conjugated IgGs (dilution 1:10,000; KPL, Gaithersburg, MD) in Tris-buffered saline–Tween with bovine serum albumin for 1 hour at room temperature and chemiluminescence (Western Lightning Plus; PerkinElmer, Waltham, MA).

Tissue Staining Studies

Liver samples were frozen in methylbutane at -80°C , and 5- μm sections were prepared. For DPPIV and γ -glutamyl-transpeptidase (GGT), sections were fixed in chloroform–acetone and were stained as described previously.^{14,15} For heme oxygenase 1 (HMOX1), sections were stained with anti-rat HMOX1 antibody (Dr. Irving Listowsky, Albert Einstein College of Medicine). For apoptosis, terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) was used (ApopTag peroxidase *in situ* apoptosis kit; EMD Millipore, Billerica, MA).¹⁴ In negative controls, terminal deoxynucleotidyl transferase was omitted. Color was developed with diaminobenzidine (K3467; Dako North America, Carpinteria, CA). For Ki-67, sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), blocked in 3% goat serum for 1 hour, and incubated for 1 hour with mouse anti-Ki-67 (1:150; BD Pharmingen, San Diego, CA) and 1 hour with peroxidase-conjugated goat anti-mouse IgG (1:300; Sigma-Aldrich, St. Louis, MO). To assess oxidative DNA damage, sections or cells were ethanol-fixed and treated with RNase (100 $\mu\text{g}/\text{mL}$) for 1 hour at 37°C ; DNA was then hydrolyzed in 4 mol/L HCl for 7 minutes and neutralized in 50 mmol/L Tris base for 1 to 2 minutes, followed by blocking in 10% fetal bovine serum; finally, anti-8-oxo-dG (1:300; 4354-MC-050; Trevigen, Gaithersburg, MD) was applied overnight at 37°C . Tissues were quenched with 3% H_2O_2 in methanol for 30 minutes and incubated with peroxidase-conjugated goat anti-mouse IgG (A3682; Sigma-Aldrich). For Atm protein staining, sections were fixed in cold acetone–methanol (1:1,

v/v) for 2 minutes, washed in phosphate-buffered saline, incubated with 50 mmol/L ammonium chloride for 30 minutes, blocked with 3% goat serum for 30 minutes, and incubated for 1 hour with rabbit anti-rat Atm (1:200; AB3740; Chemicon, Temecula, CA) and for 1 hour with goat anti-rabbit IgG (1:1000, Sigma-Aldrich), using diaminobenzidine for color development.

For morphometric quantitations, 25 to 50 fields centered on portal radicles were examined under a microscope at $\times 100$ magnification for GGT⁺ areas or $\times 400$ magnification for Ki-67⁺ cells. For measuring the extent of changes in liver sections, ImageJ software version 1.39 (NIH, Bethesda, MD) was used.

Cell Engraftment and Liver Repopulation Assays

For cell engraftment, 2×10^7 F344 rat hepatocytes within 1 to 2 hours from isolation were transplanted intrasplenically in 0.25 mL serum-free RPMI 1640 medium.^{14,15,18,19} For liver repopulation, DPPIV[−] rats were preconditioned with 30 mg/kg retrorsine at 6 and 8 weeks of age, and two-thirds PH was performed 4 weeks later.²² At 1 week after PH, 5×10^6 hepatocytes were injected into spleen in 0.25 mL serum-free RPMI 1640 medium. Rats were sacrificed after 5 days for morphometric cell engraftment analysis with three sections per liver lobe per rat in 100 consecutive fields centered on portal areas under $\times 100$ magnification. Rats were sacrificed after 3 weeks for liver repopulation analysis. Multiple images of three sections per liver lobe per rat were captured at $\times 40$ magnification; ImageJ software (NIH) was used to analyze the liver area occupied by transplanted cells.

Serology

Serum samples were stored at -20°C . Alanine aminotransferase (ALT) was analyzed by an automated clinical system.

Statistical Analysis

Differences were analyzed by *t*-test, *U*-test, or analysis of variance (ANOVA) with Holm–Šidák post hoc test. $P < 0.05$ was considered significant. Data are expressed as means $\pm$ SEM.

Results

Hepatic ischemia for 15 minutes alone was without mortality, but ischemia for 30 minutes caused death in 50% of animals, and ischemia for 60 minutes caused death in 100% of animals ($n = 6$ per group). Nonlethal 15 minutes ischemia, 15 minutes reperfusion, and then 60 minutes ischemia was without mortality, indicating hepatoprotection, and we used this protocol for all subsequent IP studies. This protocol for IP was clinically relevant, because similar conditions would be expected with clamping of hepatic blood vessels during liver surgery.

Figure 2 Hepatic antioxidant systems 5 days after IR or IP. **A** and **B**: In whole liver extracts after IR and IP, biochemical assays for antioxidant defense mechanisms indicated no differences between groups for Cu/Zn/Mn superoxide dismutase (**A**) or catalase (**B**). **C**: Total glutathione levels were significantly greater (1.4-fold), compared with control rats, after IP but not after IR. **D**: Malondialdehyde content increased after IR and IP (**D**), which indicates lipid peroxidation, although malondialdehyde levels were lower after IP than after IR. Data are expressed as means $\pm$ SEM. $n \geq 3$ rats per condition. * $P < 0.05$ versus healthy controls, ANOVA with Holm–Šidák test. ANOVA, analysis of variance; Ctr, control.

well as in rats subjected to either IR or IP, although further differences were noted. For example, at 1 day after CCl_4 , control rats exhibited significant liver injury, with serum ALT of 3494 ± 872 U/L versus 65 ± 15 U/L in healthy rats. At this time point, in rats with prior IR and then CCl_4 , serum ALT was lower, 2760 ± 145 U/L (mean, 0.79-fold), but the difference did not reach statistical significance; in rats with prior IP and then CCl_4 , ALT levels were also lower, though far from normal, at 1728 ± 229 U/L (mean, 0.49-fold) ($P < 0.05$, ANOVA with Holm–Šidák test) (Figure 3B). Tissue from control rats at 1 day after CCl_4 showed perivenous necrosis and inflammation (grade 3 injury; range,

2.5 to 3) (Figure 3C). In rats with prior IR, liver necrosis and inflammation were evident (grade 3.3 injury; range, 3 to 3.5). By contrast, in rats with prior IP, liver necrosis was less (grade 2.3 injury; range, 2 to 3). The difference in CCl_4 –induced tissue injury was also noted at 2 days after CCl_4 ; mean tissue injury grades were 1.8, 2.0, and 1.2 in control, IR-treated, and IP-treated rats, respectively. In all animals, CCl_4 –induced liver injury was no longer obvious after 3 days, except for slight increases in inflammatory cell infiltrates in portal areas of control and IR-treated rats. This difference in liver injury was reflected in the extent of compensatory liver regeneration. In healthy control rats,

Figure 3 Effects of IR or IP on secondary CCl_4 –induced liver injury. **A**: Study protocol for CCl_4 –induced injury initiated 5 days after IR or IP, followed by analysis of liver tissue from rats after additional times of 1, 2, or 3 days. **B**: Elevated serum ALT levels indicated considerable liver injury 1 day after CCl_4 ; ALT levels were significantly lower in rats with prior IP, indicating partial hepatic protection. **C**: Significant perivenous necrosis was observed after 1 day in CCl_4 –treated healthy rats and in rats with prior IR, whereas liver damage was lessened in rats with prior IP. **D**: Onset of extensive compensatory liver regeneration was observed (Ki-67⁺ nuclei) in rats 1 day after CCl_4 with or without prior IR, whereas rats with prior IP exhibited much less liver regenerative activity (far fewer Ki-67⁺ cells). Data are expressed as means $\pm$ SEM. $n = 3$ rats per group for each time point. ALT, alanine aminotransferase.

Figure 4 Properties of hepatocytes isolated from healthy donor rats and from donors 5 days after IR or IP. **A–C:** In isolated hepatocytes from IR donors, levels of Cu/Zn/Mn superoxide dismutase (**A**), catalase (**B**), and total glutathione (**C**) were lower than in healthy donor cells; in cells from IP donors, enzyme levels declined less, remained normal, or were higher than in healthy donor cells. **D:** Malondialdehyde levels were higher in cells from IR donors, compared with healthy donor cells, and were normal in cells from IP donors. **E:** Viability in suspension culture declined less in cells from IP donors. **F:** Cells from IP donors better tolerated additional *tert*-butylperoxide–induced oxidative stress, compared with healthy donor cells or IR donor cells. Data are expressed as means $\pm$ SEM. * $P < 0.05$ versus control, ANOVA with Holm–Šidák test. $n = 3$.

only rare hepatocytes (<1 in 1000) were Ki-67⁺ (data not shown), whereas $32 \pm 4\%$ hepatocytes were Ki-67⁺ in CCl₄–treated rats at 1 day, and $30 \pm 8\%$ hepatocytes were Ki-67⁺ in rats treated with CCl₄ after IR (Figure 3C). By contrast, at 1 day after prior IP plus CCl₄, $16 \pm 3\%$ Ki-67⁺ hepatocytes were observed, which is approximately twofold lower than was observed in control or IR-treated rats ($P < 0.05$, ANOVA with Holm–Šidák test), indicating less need for liver regeneration because of lower CCl₄ hepatotoxicity.

The extent of liver regeneration and prevalence of Ki-67⁺ cells was lower at 2 days after CCl₄, compared with 1 day, in control or IR-treated rats, and was even lower in IP-treated rats, indicating that differences in liver regeneration inversely correlated with the extent of liver injury in these animals.

Characterization of Hepatocytes Isolated after IR or IP

Donor healthy rats and rats at 5 days after IR or IP ($n = 3$ per group) yielded similar numbers of cells (mean $300 \times 10^6 \pm 92 \times 10^6$ hepatocytes per liver). Immediately after isolation, cell viability as assessed by trypan blue staining was also similar ($83 \pm 2\%$). However, in hepatocytes from IR donors ($n = 3$) levels of superoxide dismutase, catalase, and

glutathione were lower, but malondialdehyde levels were higher, indicating oxidative stress (Figure 4, A–D). In IP donor cells ($n = 3$), levels of superoxide dismutase, catalase, and glutathione were higher, and malondialdehyde levels were normal, indicating less oxidative stress. Because overall levels of these biochemical activities were greater in isolated cells than in tissues, we concluded that isolation of cells induces further oxidative stress. Nonetheless, IP donor hepatocytes were more viable in suspension cultures over 6 hours and 12 hours (Figure 4E). Moreover, IP donor hepatocytes better withstood *tert*-butylperoxide–induced oxidative stress than did hepatocytes from healthy or IR donors ($P < 0.05$, ANOVA with Holm–Šidák test) (Figure 4F). Taken together, these findings indicated that IP is protective *in vivo* as well as *in vitro*. We therefore proceeded to cell transplantation studies.

Hepatocyte Engraftment and Repopulation

We used established protocols for cell engraftment and liver repopulation analysis in multiple recipients, with several donors per condition (Figure 5A).^{18,19} Transplantation of healthy donor hepatocytes into DPPIV[−] rats resulted in engraftment of 196 ± 26 transplanted cells per 100 portal

Figure 5 Engraftment and proliferation of transplanted cells from donors 5 days after IR or IP. **A:** Protocols for studies, including use of multiple donors 5 days after IR or IP, or healthy controls, for cell isolation. $n = 3$ rats per group. Cells were transplanted within 1 to 2 hours after isolation into multiple recipients for cell engraftment analysis after 5 days. $n = 3$ to 6 rats per group. For cell proliferation, animals were preconditioned with retrorsine (two treatments, 2 weeks apart) and PH (6 weeks after the second retrorsine treatment), and liver repopulation analysis was performed 3 weeks after cell transplantation ($n = 6$ per group). **B:** Morphological identification of DPPIV⁺ transplanted cells in liver of recipients. Cumulative data for differences in cell engraftment indicate that the cells from IR donors engraft less well than either healthy donor cells or IP donor cells. **C:** Clusters of proliferating transplanted cells in retrorsine–PH preconditioned rats. Morphometric quantitations indicate the extent of liver repopulation. Data are expressed as means $\pm$ SEM. * $P < 0.05$ versus recipients of healthy donor cells. PH, partial hepatectomy; Ret, retrorsine.

areas (Figure 5B). However, IR donor hepatocytes engrafted less well, with $33 \pm 6\%$ fewer cells in liver ($P < 0.05$). Engraftment of hepatocytes from IP donors was similar to but not superior to that of healthy donor hepatocytes. In retrorsine–PH preconditioned rats, multiple clusters of proliferating transplanted cells were observed with healthy control donors (Figure 5C). By contrast, fewer transplanted cell clusters were found in recipients of IR or IP donor cells. In recipients of healthy donor cells, liver repopulation was $17 \pm 10\%$, which was greater than the liver repopulation of $9 \pm 8\%$ from IR donor cells or $8 \pm 7\%$ from IP donor cells ($P < 0.05$, ANOVA with Holm–Šidák test). To control for differences in numbers of engrafted cells, we further analyzed areas occupied by individual transplanted cell clusters. These data were nonparametrically distributed; transplanted cell clusters variously contained hundreds of cells or only few cells. Compared with the median size of transplanted cell clusters in recipients of healthy donor cells (100% size), relative median cluster size in recipients of IR or IP donor cells was smaller ($46 \pm 8\%$ and $28 \pm 10\%$, respectively) ($P < 0.05$, ANOVA with Holm–Šidák test). Therefore, despite the superior ability of IP donor liver cells to withstand subsequent injuries, isolated cells from IP donors were unable to proliferate as efficiently as healthy donor cells.

Mechanisms Regulating Proliferation

To probe for signaling pathways regulated by IP, we examined 84 MAPK signaling and related genes (Supplemental Table S1). Comparison of hepatocytes from IP donors and healthy donors revealed significant differences; 31/84 (37%) genes were up-regulated by ≥ 2 -fold in hepatocytes from IP donors (Table 1). Differentially expressed genes represented MAPK, MKKK, and MEKK-interacting proteins, activated transcription factors, cell anchoring and scaffolding proteins, as well as ERK-1/2-regulated cell-cycle genes. Previously identified IP-induced regulators (eg, p38 MAPK^{6,7}) were present. To obtain insight into how this gene set might (despite the noncomprehensive nature of our analysis) possess the potential to regulate relevant hepatic processes (particularly cell cycling) because of less efficient liver repopulation by IP donor cells, we queried gene expression pathway profiles. This analysis focused on representation of differentially regulated genes in our RT-qPCR data set by experimentally validated or predicted pathways using Qiagen's IPA tools. Through the IPA method, we elicited within our relatively small data set of IP cells, representation of 228 canonical pathways that are normally active in the liver (Supplemental Table S2). This indicated that IP could have had broad effects on many hepatic processes and functions.

Table 1 Genes Up-Regulated in Isolated Hepatocytes from IP Donor Rats

Gene name*	Gene symbol*	Fold increase
MKKK signaling		
delta-like 1 homolog (<i>Drosophila</i>)	<i>DLK1</i>	3
mitogen-activated protein kinase kinase kinase 1	<i>MAP4K1</i>	5
v-mos Moloney murine sarcoma viral oncogene homolog	<i>MOS</i>	3
MAPK signaling		
mitogen-activated protein kinase 11	<i>MAPK11</i>	6
mitogen-activated protein kinase 12	<i>MAPK12</i>	10
mitogen-activated protein kinase 14	<i>MAPK14</i>	3
Activated transcription factors		
CREB binding protein	<i>CREBBP</i>	2
v-ets erythroblastosis virus E26 oncogene homolog 1	<i>ETS1</i>	6
v-ets erythroblastosis virus E26 oncogene homolog 2	<i>ETS2</i>	3
jun proto-oncogene	<i>JUN</i>	2
potassium voltage-gated channel, subfamily H (eag-related), member 8 (Elk1)	<i>KCNH8</i>	4
mitogen-activated protein kinase-activated protein kinase 5	<i>MAPKAPK5</i>	4
myocyte enhancer factor 2C	<i>MEF2C</i>	3
v-myc avian myelocytomatosis viral oncogene homolog	<i>MYC</i>	4
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	<i>NFATC4</i>	10
tumor protein p53	<i>TP53</i>	3
Genes with induced expression		
collagen, type I, alpha 1	<i>COL1A1</i>	40
FBJ murine osteosarcoma viral oncogene homolog	<i>FOS</i>	34
jun proto-oncogene	<i>JUN</i>	2
v-myc avian myelocytomatosis viral oncogene homolog	<i>MYC</i>	4
tumor protein p53	<i>TP53</i>	3
MEKK1-interacting proteins		
cell division cycle 42	<i>CDC42</i>	3
mitogen-activated protein kinase kinase kinase 1	<i>MAP4K1</i>	5
stratifin	<i>SFN</i>	3
Scaffolding and anchoring		
late endosomal/lysosomal adaptor, MAPK and MTOR activator 3 (alias mitogen-activated protein kinase kinase 1 interacting protein 1)	<i>LAMTOR3</i> (alias <i>MAP2K1IP1</i>)	4
mitogen-activated protein kinase 8 interacting protein 2	<i>MAPK8IP2</i>	4
Cell cycle proteins regulated by the ERK-1/2 pathway		
cyclin A1	<i>CCNA1</i>	3
cyclin B1	<i>CCNB1</i>	3
cyclin D2	<i>CCND2</i>	3
cyclin E	<i>CCNE1</i>	3
cyclin-dependent kinase 6	<i>CDK6</i>	2
cyclin-dependent kinase inhibitor 1C (p57, Kip2)	<i>CDKN1C</i>	3
cyclin-dependent kinase inhibitor 2A	<i>CDKN2A</i>	32
cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	<i>CDKN2B</i>	7
retinoblastoma 1	<i>RB1</i>	3

Gene expression was normalized in individual samples against housekeeping genes and then subjected to intergroup comparisons. Listed are genes with ≥ 2 -fold increased expression in IP donor cells, relative to healthy donors, out of an array of 84 genes (RT² Profiler PCR Array; Qiagen—SABiosciences).

*Human homologs. The rat symbols are identical to the human, except for capitalization.

Moreover, this offered opportunities to probe whether, after IP, individual pathway members within our data set of differentially expressed genes could have affected discrete processes, such as cell-cycle regulation.

Among annotated pathways, such as the p38 MAPK pathway, intersecting genes identified by IPA included both intracytoplasmic and nuclear signaling members (Supplemental Figure S1). Similarly, multiple cyclins regulating cell-cycle progression were also represented (Supplemental Figure S2).

Moreover, this list included genes in G1/S checkpoint control (Supplemental Figure S3) and G2/M checkpoint control (Supplemental Figure S4). These findings were generally in agreement with IP-induced alterations in the long-term fate of cells.

To substantiate correlates of our gene expression pathway analysis, we examined expression of selected genes in the experimental samples. Western blotting validated results of the RT-qPCR analysis at the protein level (Figure 6A). For

Figure 6 Changes in hepatic cell signaling 5 days after IP. **A:** Western blots from freshly isolated hepatocytes from three normal donor rats and from three donors at 5 days after IP. β -Tubulin was used as the loading control. The blots confirmed increased expression in hepatocytes from IP donors of p38 MAPK, including p-p38, p-ERK-1/2, JUN, and p-MAPK8 (p-JNK). **B:** Compared with healthy normal donors, liver from donors 5 days after IP exhibited widespread 8-oxo-dG adducts (brown nuclei, **top row**, and **arrow** in **inset**), as well as absence of Atm protein expression (brown nuclei, **middle row**, and **arrow** in **inset**). Freshly isolated cells in IP donors exhibited widespread prevalence of 8-oxo-dG adducts in hepatocyte nuclei (brown, **bottom row**). p-, phosphorylated.

example, Western blotting confirmed greater expression in IP donor hepatocytes of JUN, phosphorylated MAPK8 (JNK), phosphorylated ERK-1/2, and phosphorylated p38 MAPK, which were observed at the mRNA level by RT-qPCR and were implicated by IPA in cell growth and

proliferation. Because cell-cycle checkpoint controls are often activated by DNA damage, which is a consequence of IR¹² and is regulated by ataxia telangiectasia mutated (Atm) pathways,²³ we examined this possibility. Immunostaining of IP donor liver showed presence of 8-oxo-2'-deoxyguanosine (8-oxo-dG) DNA adducts, which is highly characteristic of oxidative DNA damage (Figure 6B). Moreover, Atm protein was expressed less well in IP donor livers. Furthermore, we determined that 8-oxo-dG adducts were highly prevalent in IP donor hepatocytes. Taken together, these findings indicate that IP causes oxidative DNA damage, with multiple gene expression changes, eventually perturbing hepatocyte cycling and causing impairment in hepatocellular replication.

Discussion

The present results provide significant information about hepatic consequences of IP, particularly over the long term. In our models, IR and IP were effective in reproducing ischemia-related processes and events; changes in hepatic tissue 5 days after these manipulations included evidence for ischemia (GGT expression) and for oxidative stress (HMOX1 expression, antioxidant enzymes, lipid peroxidation, 8-oxo-dG adducts), as well as for protection from secondary CCl₄-induced injury. To our knowledge, the persistent nature of these hepatic alterations over many days had not previously been demonstrated, because other studies of IP had extended for hours, but not days. Moreover, in the present study hepatocytes from IR and IP donors reproduced major aspects of these changes, including antioxidant enzyme activities and protection from secondary insults, which also had not previously been established. However, IP was eventually deleterious for cells, as indicated by impaired proliferation capacity after cell isolation and transplantation, which was due to DNA damage and cell-cycle checkpoint restrictions.

The overall significance of these findings should extend to biological mechanisms related to IP. Insight into these basic mechanisms is still evolving, although the various processes and molecular pathways involved are quite complex.^{1,7} To focus on organ repair and regeneration, we examined some, but not all, aspects of cellular responses after IR or IP.

To a major extent, short-term outcomes after hepatic IR are determined by inflammation, typically induced by activated neutrophils, monocytes (Kupffer cells), natural killer T cells, and the like, which contribute reactive oxygen species and proinflammatory cytokines and chemokines (eg, TNF- α , IL-6) during early reperfusion.¹ Such IR-type changes produce considerable hepatic and endothelial injury after cell transplantation, with additional vascular mechanisms (eg, endothelin-1) contributing to pro-oxidant injury.^{18,19} The ability of hepatocytes to undergo DNA synthesis within the liver after exposure to CCl₄ indicated that IR does not abolish

their entry into late G1, S, or G2/M, phases that are characterized by Ki-67 expression.²⁴ Compensatory liver regeneration in some instances is accomplished by DNA synthesis and polyploidization, without necessarily involving cell division, although this may be difficult to establish in the intact liver without further assays.²⁵ In the present study, the close correlation between the degree of liver necrosis and the extent of Ki-67 expression with acute CCl₄ toxicity verified that the Ki-67 measure reports on the process of compensatory liver regeneration. We interpreted our finding of less liver necrosis and hepatic Ki-67 expression after IP to indicate hepatoprotection, as established by studies including biochemical assays of antioxidant defense mechanisms, and thereby lowered requirement for compensatory liver regeneration.

In hepatocytes, acquisition after IP of superior antioxidant defenses (including protection from secondary insults) should have improved cell survival, and thereby cell engraftment, if these mechanisms were highly relevant in this setting. Hepatocytes from IP donors engrafted better than hepatocytes from IR donors, indicating that IP had some benefits. However, IP donor hepatocytes did not engraft better than cells from healthy donors, indicating that benefits accruing from IP were insufficient for this application *in vivo*. The mechanisms underlying early clearance of transplanted hepatocytes (eg, immediate blood-mediated responses, inflammation, and vascular perturbations) seem to be shared by other cell types (eg, pancreatic islets²⁶), suggesting that IP could possibly confer some benefits to suboptimal organ donors. For example, this should be in agreement with the benefits of IP in protecting organs with pre-existing fatty changes,²⁷ although whether such donor organs can be of value for cell-therapy applications has not yet been examined. On the other hand, potential applications of IP to protect healthy transplanted livers are likely to be accompanied by the adverse effects of hepatocellular DNA damage and restriction of cell proliferation. Moreover, issues of DNA damage could be a serious problem after small-for-size liver transplantation, given the failure of liver regeneration in this setting.²⁸

Our cell transplantation model of liver repopulation additionally elicited information on whether hepatocytes exposed to IR and IP can actually divide and produce daughter cells, because in that situation transplanted cell numbers should have increased. In the retrorsine–PH liver repopulation model,²² native hepatocytes are damaged by DNA adducts of retrorsine and by oxidative DNA damage induced by PH, which culminate in proliferation advantages to transplanted healthy cells.^{18,19} This principle of selective proliferation advantage has been effective in other settings; for example, hepatic DNA damage by radiation followed by IR-induced hepatic DNA damage synergistically led to extensive liver repopulation with healthy transplanted hepatocytes.¹⁵ In natural disease settings, oxidative hepatic DNA damage (eg, by copper toxicosis in Wilson's disease models) also permitted proliferation of healthy transplanted

hepatocytes.¹⁷ By contrast, when transplanted cells themselves experienced oxidative DNA damage, such as after PH in donors or with radiation of cells before transplantation, their proliferation capacity was attenuated, despite proliferation advantages in the retrorsine–PH model.²⁹ It was therefore reasonable to conclude that decreased liver repopulation capacity of IP donor hepatocytes was related to persistent oxidative DNA damage in these cells. These types of perturbations due to IP were evidenced by 8-oxo-dG DNA adducts and greater expression of cell-cycle regulatory genes in hepatocytes before their transplantation into animals.

Previously, knowledge of changes in gene networks was limited to a very early time (30 minutes) after IR or IP, although perturbations were observed in genes involved in cellular growth and proliferation.³⁰ Among various cellular pathways identified in the present study, MAPK signaling was of particular interest, because many intracellular genes activated by oxidative stress, cytokines, and the like may coordinately regulate MAPK signaling and related genes, including genes for well-studied kinases with liver activities, such as p38 MAPK and MAPK8.^{30,31} Pathways involving these genes intersect with cell-cycle proteins regulated by the ERK-1/2 pathway. Western blotting confirmed that activated forms of these proteins were expressed more in IP donor hepatocytes. Thus, these and other related proteins likely play roles in hepatocyte proliferation, but in a negative manner and over prolonged periods.

For example, excessive activity of p38 MAPK after oxidative stress may induce cell-cycle checkpoint arrest in G1/S or G2/M via coordinate regulation of various genes, including *CDKN2A*, *CDKN2B*, *TP53*, and *RB1*, after the oxidative stress of IR.^{32,33} Among other regulators, *MAP2K1IP1* (which encodes for an interacting protein that binds specifically to MAP2K1 and MAPK2) was recently found to inhibit self-renewal, proliferation, and differentiation in stem cells,³⁴ although its roles in primary hepatocytes are unknown. Similarly, cell stress and inflammatory cytokines activate MAPKAPK5, which promotes cell senescence via p53 pathways.³⁵ Expression of MAP4K1, which may up-regulate phosphorylation of JUN or MYC, plays roles in stress responses and growth regulation in concert with MAPK8IP2, which is a scaffold protein that may modulate MAPK8 pathway activity.³⁶ We observed greater expression in IP donor hepatocytes of Mos, which phosphorylates ERK-1/2 to stabilize FOS, while inhibiting CDK4 activity to block G1/S.³⁷

Because transcription factors such as NFYAC4, MYC, MEF2C, ETS1/2, FOS, and MAPK8 serve pleiotropic roles in cell cycling and other processes,^{38,39} further molecular complexities seem inevitable. For example, oxidative stress arrests cell cycling by prolonging chromatin binding of phosphorylated ERK and of FOS activation, thereby inhibiting FOS to FRA1 switching on chromatin loci and thus leading to decreased CCND1 expression, thus contributing to G1/S arrest.^{40,41} Also, MAPKs (primarily

MAPK8) activate a JUN and FOS complex (AP-1) for regulating cell death pathways.⁴¹ We found greater expression in IP donor hepatocytes of several cyclins, including Cnd2, which normally advances G1/S by complexing with CDK4 and CDK6. However, simultaneous expression of cell-cycle suppressors such as CDKN2A, CDKN2B, p53, RB1, and SFN was likely more potent in interfering with G1/S.⁴² Furthermore, products of other genes (eg, stratifin) may have acted downstream of p53 pathways to additionally block G2/M and prevent proliferation of damaged cells escaping G1/S controls,⁴³ as should have been the case in IP donor hepatocytes with DNA damage.

Thus, our findings establish possible long-term fates of cells after IP, along with potential roles of intracellular signaling mechanisms in cell survival, differentiation, proliferation, and death. These mechanisms should be of general significance for better understanding of how exposure of organs to IR or IP eventually bears on the long-term health of their cells. Because residual cells in organs after such IR or IP could be at certain disadvantages compared with completely healthy cells, avoiding imbalances in healthy and damaged cell subsets could be important. This could help explain instances in which IP has little or no benefit, or even adverse effect, such as in liver transplantation,^{9,10} where IP-related cell damage and restrictions in cell proliferation could have contributed. On the other hand, suitable ways to mitigate DNA damage and recruitment of deleterious cell signaling pathways, as identified here, should benefit application of IP. Drug-based approaches should be of interest, because pharmacological targets capable of reproducing IP have already been considered (eg, phosphodiesterase inhibition⁴⁴).

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.07.002>.

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