• *BASIC RESEARCH* •

Identification of differentially expressed genes after partial rat liver ischemia/reperfusion by suppression subtractive hybridization

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Abstract

AIM: To identify potential diagnostic target genes in early reperfusion periods following warm liver ischemia before irreversible liver damage occurs.

METHODS: We used two strategies (SSH suppression subtractive hybridization and hybridization of cDNA arrays) to determine early changes in gene expression profiles in a rat model of partial WI/R, comparing postischemic and adjacent nonischemic liver lobes. Differential gene expression was verified (WI/R; 1 h/2 h) and analyzed in more detail after warm ischemia (1 h) in a reperfusion time kinetics (0, 1, 2 and 6 h) and compared to untreated livers by Northern blot hybridizations. Protein expression was examined on Western blots and by immunohistochemistry for four differentially expressed target genes (*Hsp70, Hsp27, Gadd45a and IL-1rI*).

RESULTS: Thirty-two individual WI/R target genes showing altered RNA levels after confirmation by Northern blot analyzes were identified. Among them, six functionally uncharacteristic expressed sequences and 26 known genes (12 induced in postischemic liver lobes, 14 with higher transcriptional expression in adjacent nonischemic liver lobes). Functional categories of the verified marker genes indicate on the one hand cellular stress and tissue damage but otherwise activation of protective cellular reactions (AP-1 transcription factors, apoptosis related genes, heat shock genes). In order to assign the transcriptional status to the biological relevant protein level we demonstrated that Hsp70, Hsp27, Gadd45a and IL-1rI were clearly up-regulated comparing postischemic and untreated rat livers, suggesting their involvement in the WI/R context.

CONCLUSION: This study unveils a WI/R response gene set that will help to explore molecular pathways involved in the tissue damage after WI/R. In addition, these genes especially *Hsp70* and *Gadd45a* might represent promising new candidates indicating WI/R liver damage.

INTRODUCTION

Prolonged warm ischemia followed by reperfusion is a main determinant of hepatic injury in patients undergoing hemorrhagic shock[1], late sepsis[2], trauma[3], liver resection or transplantation[4]. While cold storage that appears to cause injury mainly to sinusoidal endothelial cells[5], WI/R has damaging effects on both, hepatocytes and endothelial cells[6]. Liver damage initiated by transient warm ischemia and aggravated during reperfusion occurs in a biphasic pattern[4,7,8]. In the acute phase (<6 h post ischemia) activated Kupffer cells release reactive oxygen species (ROS) and proinflammatory cytokines. These mediators initiate a complex network of intercellular signalling between endothelial and parenchymal cells[4,9] resulting in platelet and neutrophil recruitment[4,8]. Sinusoidal microcirculation is disturbed due to endothelin-induced into cell contraction and endothelial cell swelling[10]. The subacute reperfusion phase (6-24 h) is characterized by an immense influx of neutrophils, which infiltrate into the liver parenchyma after selectin and immunoglobulin-mediated endothelial adhesion[4,8,11]. Activated neutrophils cause hepatocyte damage by superoxide, ROS[7] and protease[12] release and also via receptor/ligand mediated cell-cell contacts[8]. In addition, ROS can inactivate endogenous protease inhibitors facilitating protease-mediated hepatocyte injury[13]. Consequently irreversible tissue injuries including microcirculatory disturbance[14], sinusoidal cell loss[15] and hepatic cell death[16] may result from WI/R, finally leading to liver dysfunction or failure.

Although much work has been done on certain components, taking part in WI/R liver damage, the exact

interactions of mediators and signal transduction in the involved cells remain unclear. One possible way to resolve this complex network is to compare entire gene expression profiles of liver tissues shortly after WI/R before irreversible liver damage occurs and not affecting specimens in the acute reperfusion phase. Apart from general insights into the involved mechanisms, identification of new marker genes with differential screening methods an improve the diagnosis of early WI/R injury and may influence therapeutic strategies. In order to identify such genes, two different methods were used in the present study (SSH and screening of cDNA-arrays) to determine differentially expressed genes in an autologous system of partial rat liver WI/R (1 h/2 h).

Complementary DNA arrays provide a powerful tool to compare gene expression patterns, since many known genes or sequences can be examined in a single experiment[17]. In contrast, the polymerase chain reaction (PCR) based method SSH[18] is a useful technique to compare two entire poly(A)⁺RNA populations and allows the identification of previously unknown genes. Fragments of digested cDNA, are compared by one round of subtractive hybridization with high subtraction efficiency and normalized representation of abundant or rare, differentially expressed genes. In the subsequent suppression PCR, only differentially expressed sequences are amplified exponentially, while suppression of equally expressed sequences occurs[18].

In the present study, the SSH technique and the hybridization of 'Atlas Arrays' (Clontech) were chosen to compare the transcriptional pattern of postischemic liver lobes and adjacent nonischemic liver tissues after 1 h partial ischemia and 2 h reperfusion. After initial screening and validation on Northern blots, 32 differentially expressed genes were identified. Beside genes with known physiological function, six functionally uncharacteristic sequences were identified *via* SSH, providing a novel source for marker genes indicating WI/R liver damage. Expression profiles of all verified genes were established in detail, comparing healthy rat livers to WI/R-injured tissues in reperfusion time kinetics. Protein expression was investigated for four targets (Hsp70, Hsp27, Gadd45a and IL-1-RI) to correlate the transcriptional status to the protein level.

For the first time, SSH and Atlas array techniques were used in this standard *in vivo* model of WI/R to identify and verify differentially expressed genes after WI/R that represent potential candidates for the early detection of WI/R liver damage.

MATERIALS AND METHODS
Animals and model of WI/R
Rats were housed in two to three per cage with access to water and standard pellet food. The night before surgery, animals were fasted but received water ad libitum. After induction of anaesthesia (Ketamine 10 mg/kg IM and Phenobarbital 18 mg/kg IP), a median laparotomy was performed and the left liver lobe was mobilized. In all animals, warm ischemia of 1 h was induced in the left liver lobe by placing a hemoclip across the pedicle, followed by reperfusion periods of 0, 1, 2 or 6 h. Left and right liver lobes were extracted, snap frozen and stored at -80 ℃. Additionally healthy rat livers were explanted directly after laparotomy. During the surgical procedure, body temperature was monitored and maintained at 36.8-37.2 ℃ using heating pads.

Experiments were approved by the Committee of Animal Care, Regierungspräsidium Karlsruhe, Germany, and performed in accordance to German legislation on protection of animals.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels
To define the hepatocellular injury, AST and ALT serum levels were measured using routine tests (ADVIA 120, Bayer, Leverkusen).

Liver histology
Formalin-fixed and paraffin-embedded liver sections were stained with hematoxylin and eosin (HE) for conventional morphological evaluation.

RNA isolation
RNA was extracted from 40 mg of snap frozen specimens. Twenty micrometers of cryosections were homogenized using QIAshredder columns (Qiagen, Hilden, Germany) and total RNA was isolated with the Rneasy Mini kit (Qiagen). To enrich poly(A)⁺RNA, we used the Oligotex-Direct mRNA kit (Qiagen). RNA quantification was performed spectrophotometrically and RNA integrity was examined by agarose gel electrophoresis.

SSH experiments
SSH was performed as described[18] using reagents supplied with the PCR Select cDNA Subtraction kit (Clontech, Heidelberg, Germany). We used 2.5 μg poly(A)⁺RNA of the postischemic lobe from one rat liver after partial WI/R (1 h/2 h) and 2.5 μg poly(A)⁺RNA derived from the corresponding nonischemic liver lobe of the same animal for forward and reverse SSH experiments. PCR products from both SSH experiments were directly cloned into pCR2.1TOPO vector and TOP10F' cells (Invitrogen, Groningen, Netherlands).

First and second screening of SSH clones on reverse Dot and Southern blots
SSH clone inserts were amplified from LB-Kanamycin overnight cultures, using the nested adapter-1 and -2R primers (PCR Select cDNA kit, Table 1), flanking each candidate cDNA after subtraction, as described[18]. In the first screening, PCR products (200 ng/dot) were passed to two identical sets of nylon membranes (GeneScreen, Perkin Elmer Biosystems, Weiterstadt, Germany). In the Southern blot differential approach PCR products were size-fractionated on duplicate agarose gels, including a RT-PCR-amplified *β-Actin* fragment as positive control on each gel (Table 1), and transferred to nylon membranes (GeneScreen).

In the first differential screening, complex probes were synthesized from 0.5 μg postischemic and the corresponding nonischemic liver lobe poly (A)⁺RNA of the SSH starting material using the SMART PCR cDNA Synthesis kit properly

Table 1 PCR primers and annealing temperatures used for target amplification and labelling ([α-32P]-dCTP)

Description	Accession number	Primer sequence (5′ → 3′)	Size[1] (bp)	Annealing temperature (℃)
Adapter-1		TCGAGCGGCCGCCCGGGCAGGT		68
Adapter-2R		AGCGTGGTCGCGGCCGACGT		
β-Actin forward	V01217	2161-ATGTTTGAGACCTTCAACAC-2180	483	56
β-Actin reverse	V01217	2743-AACGTCACACTTCATGATGG-2724		
Fos forward	XM_234422	695-TGGTGAAGACCATGTCAGGCG-715	404	60
Fos reverse	XM_234422	1098-ATTGAGAAGAGGCAGGGTGAAGG-1076		
Jun forward	NM_021835	367-GGAAACGACCTTCTACGACGATG-389	519	60
Jun reverse	NM_021835	885-GGGTTGAAGTTGCTGAGGTTGG-864		
Jund forward	NM_138875	465-AGTACGCAGTTCCTCTACCCTAA-487	413	58
Jund reverse	NM_138875	877-ATGTCGATGGGCGACAGTGGA-857		
Hsp27 forward	M86389	157-GTTTCCCGATGAGTGGTCTCAGT-179	378	63
Hsp27 reverse	M86389	534-TCAGGGGACAGGGAAGAGGACAC-512		
Hsp60 forward	X54793	913-CCAGGGTTTGGGGACAACAGG-933	380	59
Hsp60 reverse	X54793	1293-TCTTGTAGCATTGAGAGCATCTG-1271		
Hsp70 forward	L16764	2129-GGCTAGAGACAGACTCTTGATGG-2151	276	58
Hsp70 reverse	L16764	2404-CTCAGTTTGTAGGGATGCAAGG-2383		
Gadd45a forward	L32591	51-GAAGATCGAAAGGATGGACACGG-73	307	63
Gadd45a reverse	L32591	357-GCTCTCAGCGGGGCTCTTGTC-337		
Ldlr forward	X13722	1337-CTGTGGGTTCCATAGGGTTTCTG-1359	561	60
Ldlr reverse	X13722	1897-GTTTGGAATCAACCCAATAGAGG-1875		

(Clontech). A purification step using the High Pure PCR Purification kit (Roche, Mannheim, Germany) was included before random [α-32P]-dCTP (Amersham Pharmacia Biotech, Freiburg, Germany) labeling with 300 ng of the respective cDNAs and HexaLabel DNA Labeling kit (MBI Fermentas, St. Leon-Rot, Germany). For the second screening, we performed complex probes with 400 μg total RNA of four pooled nonischemic and 400 μg total RNA of the four pooled, matched postischemic lobes after partial WI/R (1 h/2 h). Each lobe was represented in equal amounts. RNA-pools were subjected to desoxyribonuclease-I digestion (DNAseI Amp Grade, Invitrogen) before purification of poly(A)+RNA. Linear [α-32P]-dCTP labeling occurred during reverse transcription (Superscript-II, Invitrogen) using 0.5 μg poly(A)+RNA of each RNA-pool as template. Non incorporated nucleotides and primers were separated by NICK Columns (Amersham Pharmacia Biotech).

Equal amounts of [α-32P]-dCTP labeled probes, (1×10^6 counts/min/mL hybridization solution) were used for hybridization. Membranes were pre-hybridized for 2 h and hybridized for 18 h at 65 ℃ with heat denatured probes and washed under stringent conditions as described[19]. Filters were exposed with intensifying screens to BioMax MS-1 x-ray films (Kodak).

Northern blot analysis

For Northern blot synthesis 3 μg total RNA was transferred after agarose gel electrophoresis to N+-Hybond membranes (Amersham). Plasmid DNA of SSH clones was prepared from 3 mL LB-Kanamycin overnight cultures using Quantum Prep Plasmid Miniprep kit (Bio-Rad Laboratories, Munich, Germany) for probe generation. Plasmid DNAs (100 ng) were [α-32P]-dCTP labeled by PCR using Taq-polymerase, Mix-C (dATP, dGTP, dTTP, 0.2 mmol/L each), dCTP (0.006 mmol/L), [α-32P]-dCTP (0.8 μCi/μL), sense and antisense primers (Table 1, 0.5 μmol/L each).

Adapter-1 and adapter-2R primers were used to label insert DNAs of the chosen SSH clones. Non incorporated [α-32P]-dCTP was removed with High Pure PCR Purification kit columns (Roche). Hybridization procedures as above.

Sequencing analysis

PCR fragments were sequenced with an ABI Prism Big Dye Terminator Cycle Sequencing kit (P/N4303151, Perkin Elmer) and the nested-PCR-primer-1 (Table 1) which binds to adaptor-1 flanking the amplified SSH-fragments, or gene specific primers (Table 1). Sequencing reactions were analyzed on an ABI Prism 310 Genetic Analyzer (Perkin Elmer).

The obtained sequences were compared to DNA databases at the National Center for Biotechnology Information (NCBI) on the BLASTN server (http://www. ncbi.nlm.nih.gov/gorf/bl2.html).

Complementary DNA 'Atlas Array' analysis

Two 'Atlas Arrays' (Atlas Rat cDNA Expression Array #7738-1; Clontech) were hybridized with complex probes generated from 1 μg desoxyribonuclease-I digested poly (A)+RNA preparations as described in the kit manual. Rat RT-PCR-amplified fragments (Table 1) of the resulting candidate genes were cloned in pCR2.1 vector and TOP10' cells (Invitrogen). Target identity was confirmed after sequencing (see above).

Western blot analysis

Rat liver cryosections (40 mg) were homogenized in two-fold loading buffer according to the Laemmli system[20] and subjected to SDS polyacrylamide gel electrophoresis. Proteins were transferred on Immobilon-P membranes (Millipore, Eschborn, Germany), blocked in 50 g/L non-fat milk powder and incubated overnight (4 ℃) with the following primary antibodies: anti-Hsp70 (1:1 000, sc-24, Santa Cruz Biotechnologies, Heidelberg, Germany) anti-Hsp27 (1:2 000, SPA-810, Stressgen, Victoria, Canada), anti-

Gadd45a (1:1 000, sc-792, Santa Cruz), anti-IL-1RI (1:1 000, sc-689, Santa Cruz) or anti-actin-β (#69100, ICN Biomedicals, Eschwege, Germany). Subsequent to washing procedures, filters were incubated for 1 h (25 ℃) with horseradish peroxidase-coupled anti-rabbit-IgG or anti-mouse-IgG (W4011; Promega, Mannheim; 315-035-003, Dianova, Hamburg, Germany; 1:10 000 each). Signals were visualized by enhanced chemiluminescence (ECL detection kit, Amersham) and exposure to ECL hyperfilms (Amersham).

Hsp70 and Gadd45a immunohistochemistry

The LSAB rat kit (DakoCytomation, Hamburg, Germany) was used to stain paraffin embedded liver sections. Immunohistochemistry with cryoconserved tissues was performed with the universal Vectastain Elite ABC kit (Vector, Burlingame, USA). Both procedures were performed according to manufacturer's instructions. Anti-Hsp70 (1:1 000, sc-24, Santa Cruz) and anti-Gadd45a (1:200, sc-792, Santa Cruz) were utilized as primary antibodies.

Statistical analysis

AST and ALT levels as well as densitometric results of analyzed Northern blot hybridizations are expressed in mean±SE.

RESULTS

### Liver histology and dysfunction after partial warm ischemia/ reperfusion

In order to define the reperfusion time point at which liver dysfunction is initiated but morphologically not yet detectable, animals were subjected to 1 h warm ischemia and 2 or 6 h reperfusion (n = 5 per group). Serum aspartate aminotransferase or alanine aminotransferase (AST/ALT) levels were measured and compared to liver enzymes in sera of five untreated animals to assess general liver dysfunction. AST and ALT levels were increased in both experimental groups with higher levels after 6 h reperfusion as compared to healthy animals (Figure 1A). For Northern and Western blot experiments, an additional liver series after 1 h partial ischemia and 0, 1, 2 or 6 h of reperfusion was prepared. To monitor liver damage, serum AST and ALT concentrations were determined before and after WI/R. AST levels were in a normal range before WI/R and in untreated rat livers (AST: 27.8±4.8 u/L; ALT: 23.6±5.7 u/L) but increased in all postischemic specimens, indicating strongest organ damage 6 h post ischemia (AST: 660 u/L; ALT: 840 u/L) (Figure 1B).

To include WI/R relevant histopathological changes in the postischemic liver tissues HE stained sections were examined for all rat livers. In untreated rats, almost normal liver architecture was observed in histology (Figure 2A). A low number of infiltrated cells were noticed 2 and 6 h post ischemia. Comparing untreated specimens to rat liver lobes after 1 h warm ischemia and 2 h reperfusion, no irreversible liver damage could be detected morphologically (Figure 2B). Vacuolation of hepatocytes and a few single pyknotic or single necrotic cells were restricted to the reperfusion time point 6 h post ischemia representing reversible and irreversible liver destructions, respectively (Figure 2C). However, only mild signs of liver damage were observed, no large necrotic areas could be detected in any analyzed tissue section.
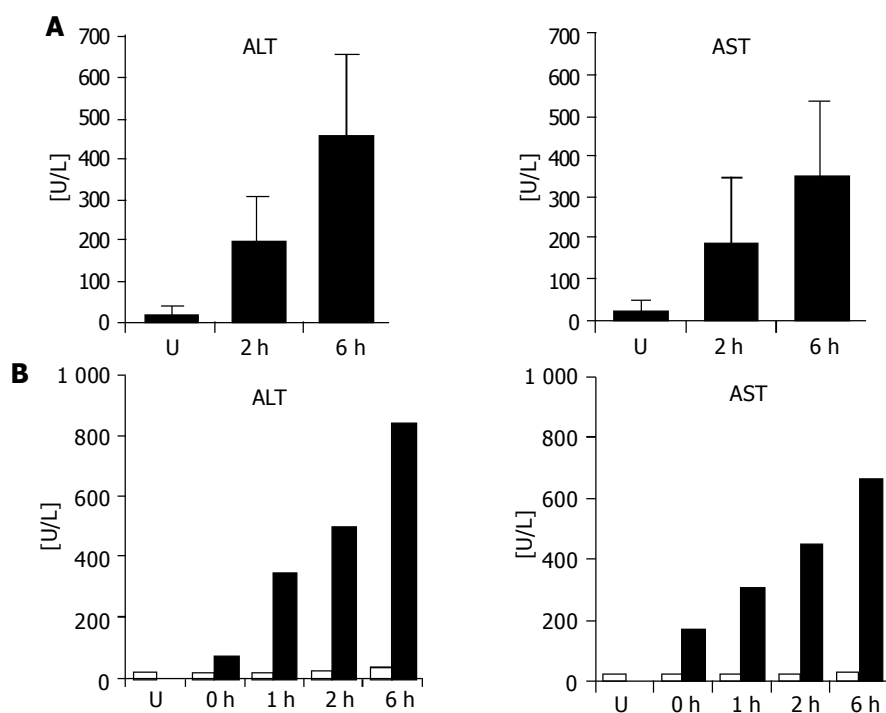
Figure 1 Serum AST/ALT levels after WI/R compared to untreated animals. A: AST/ ALT levels in untreated (U) animals and rats subjected to 1 h warm ischemia and 2 or 6 h reperfusion (2, 6 h) indicating strongest liver damage after 6 h reperfusion (n = 5 per experimental group); B: Serum AST/ALT levels in untreated animals (U; n = 3) and rat livers of the additionally prepared reperfusion time kinetics (1 h ischemia, 0, 1, 2, 6 h reperfusion; n = 1/time point). Liver enzymes were measured immediately after laparotomy (white bars) and after WI/R (black bars).
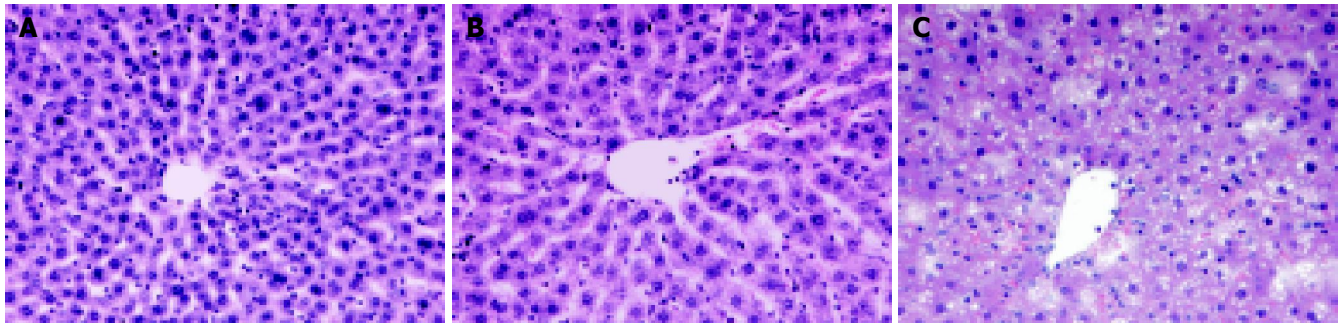
Figure 2 Liver histology after WI/R compared to untreated animals. HE stained sections of untreated rat livers and after WI/R. Almost normal liver architecture in untreated animals (A) and after 2 h reperfusion (B). Vacuolation and hepatocyte swelling after 6 h reperfusion (C).

For the identification of differentially expressed genes *via* SSH and cDNA array hybridizations, 1 h warm ischemia and 2 h reperfusion was chosen as experimental setting.

Identification of differentially expressed genes in postischemic vs nonischemic liver lobes after WI/R via SSH technology

To compare transcriptional profiles of postischemic (I-lobe) and the adjacent nonischemic liver tissue (C-lobe), two SSH experiments were performed with poly(A)$^+$RNA of one animal subjected to partial liver WI/R (1 h/2 h). A total number of 1 565 candidate clones were obtained, among them 809 clones from forward (I-lobe enriched transcripts) and 756 clones from reverse subtraction (C-lobe enriched transcripts). Differential dot blot screening of all 1 565 candidate clones was performed using complex probes generated from the same poly(A)$^+$RNA sources, that were used as starting material for SSH experiments.

A total number of 163 clones (10.4%) showed significantly different hybridization signal intensities between the two complex probes. Sequencing analysis revealed that these 163 clones were derived from 89 individual target genes. Of these, 28 genes up-regulated in the examined I-lobe and 61 genes up-regulated in the corresponding C-lobe were subjected to a second screening on reverse Southern blots with complex probes derived from pooled poly(A)$^+$RNA of four I-lobes and pooled poly(A)$^+$RNA of the four matched C-lobes after WI/R (1 h/2 h). *β-Actin* RT-PCR fragments were chosen as positive controls on each blot. Figure 3A shows representative examples of the differential screening approaches. To monitor transcriptional differences, hybridization volumes were normalized to the *β-Actin* signal of the individual blots, before we originated the ratios of pool-C *vs* pool-I hybridized signals. Figure 3C summarizes signal differences after densitometric measurements of the 89 analyzed genes. Gene selection for further analysis could be subdivided into seven genes with higher relative cDNA amounts in the pooled I-lobes (pool-C/pool-I $\leqslant$0.5), 20 clones showing higher transcriptional level in pool-C (pool-C/pool-I $\geqslant$2) and five noninformative candidates.

Identification of genes, differentially expressed in C- vs I-lobes after WI/R with 'Atlas Array' hybridizations

Two Clontech 'Atlas Arrays', consisting of spotted cDNAs corresponding to 588 functionally known genes, were hybridized with pooled cDNA of matched postischemic and nonischemic rat liver lobes. We started with 1 µg poly (A)$^+$RNA pools of the four rat livers used for the secondary SSH screening after WI/R (1 h/2 h). A comparison of signal intensities after normalization against *β/γ-Actin* and the gene coding for the ribosomal protein S29 revealed 17 genes up-regulated in the I-lobe pool and 13 genes up-regulated in the C-lobe pool. To compensate the low number of I-lobe enriched targets, resulting from the SSH screening, ten up-regulated and three down-regulated candidate genes (in the I-lobes) were selected for further investigation. Figure 3D shows the hybridization result of the two 'Atlas Arrays' after 4 h exposition.

Verification of differential transcriptional expression of SSH and 'Atlas Array' derived target genes

After the long run of screening procedures, we further analyzed 42 candidate genes obtained from SSH and 'Atlas Array' experiments (including three genes identified with both methods) by Northern blot hybridizations. Gene expression was analyzed with RNA of up to four paired C- and I-lobes, extracted after partial WI/R (1 h/2 h).

In total, 32 genes (76.2%) showed a reproducible differential expression pattern (for $\geqslant$3 matched pairs). Fourteen genes were up-regulated in the I-lobes while 18 genes showed higher expression in the corresponding C-lobes. Mean fold increase of the paired lobes ranged from 1.4 to 74.3. Figure 4 shows the hybridization results for all 32 differentially expressed genes for two postischemic and the two matched nonischemic liver lobes. Table 2 gives an overview of the differentially expressed genes including official gene name abbreviations and densitometric results of the analyzed Northern blots. The differentially expressed genes can be divided into seven groups, according to their physiological functions of their encoded proteins (Table 2). They include genes that are involved in cellular stress response, DNA damage and apoptosis, genes coding for transcription factors of the AP-1 family, genes coding for immune response proteins as well as acute phase proteins and proteins involved in cellular metabolism. The last category comprises expressed but functionally not characterized sequences.

Transcriptional expression profiling between untreated and postischemic rat livers in a reperfusion time kinetic

To establish more comprehensive gene expression profiles of the 32 identified targets after partial ischemia we analyzed
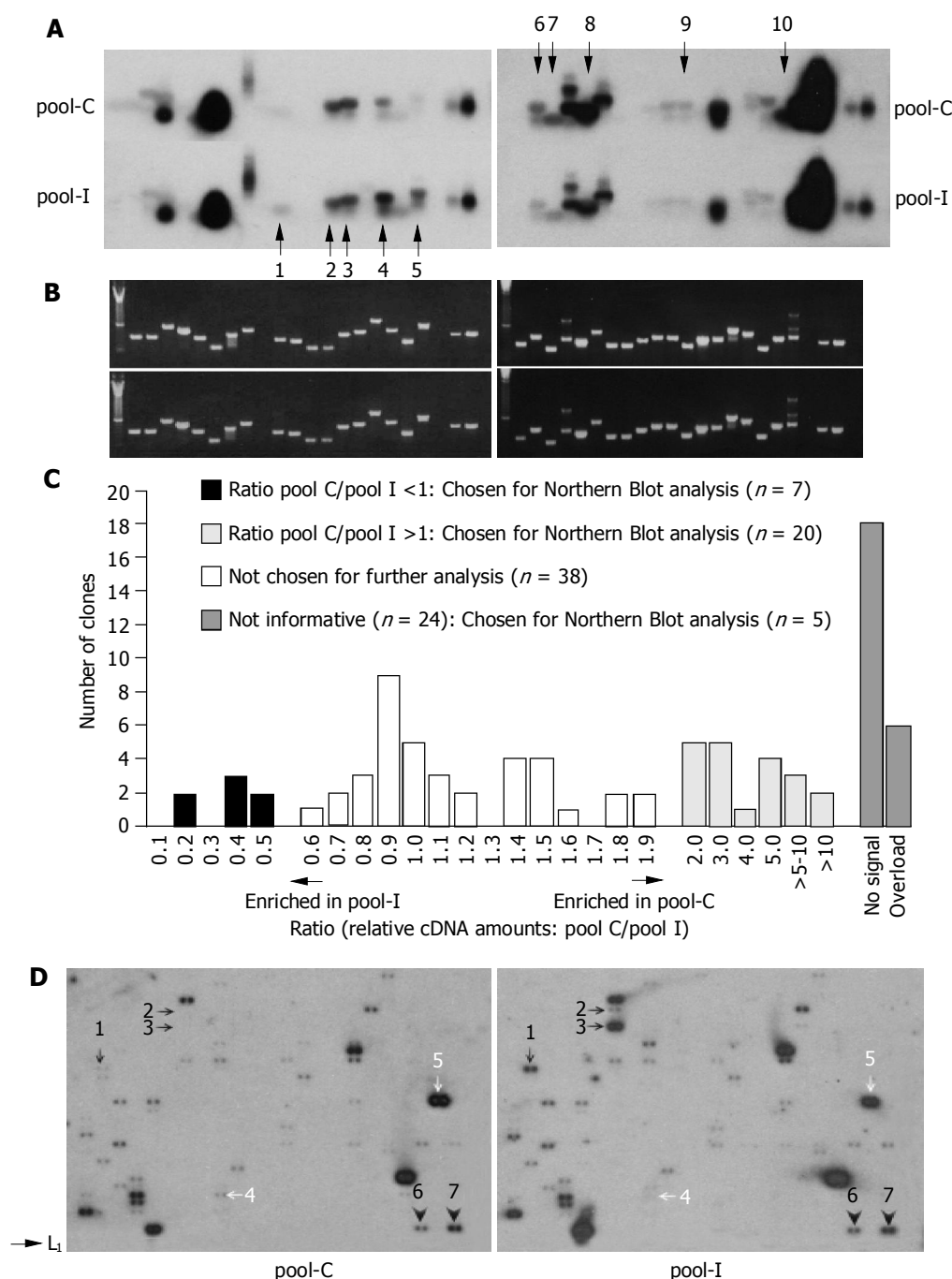
Figure 3 Second screening of SSH-clones and 'Atlas Arrays'. Two identical sets of Southern blots and two Atlas-Arrays were hybridized with complex probes of four pooled C-lobes or matched I-lobes after partial WI/R (1 h/2 h). A: Typical results of two corresponding Southern blots. The upper membranes were screened with the C-lobe probe (pool-C) and the lower membranes show the result after hybridization with the I-lobe probe (pool-I) pool-C/pool-I ratios of signal intensities after densitometry: 0.4 (1, 4), 1.1 (2, 3), 0.2 (5), 2.0 (6), 2.6 (7), 2.8 (8), 2.2 (9), 7.0 (10). β-Actin-positive controls ([1]50 ng/lane, [2]200 ng/lane); B: Ethidium bromide-stained agarose gels before blotting; C: Distribution of pool-C/pool-I ratios of all 89 analyzed genes; D: Exemplary hybridization results of the two cDNA arrays after 4 h exposition. Numbered black or white arrows indicate spots of up- or down-regulated genes in the I-lobe cDNA-pool, which were among others chosen for Northern blot hybridizations. *Jun* (1), *Hsp27* (2), *Hsp70* (3), *IL-1RI* (4) and *Fgb* (5). Arrow heads point to spotted housekeeping gene cDNAs; β-Actin (6), ribosomal protein S29 (7), 1 spotted genomic DNA to assess contaminations in the RNA preparations, used for probe synthesis.

RNA of untreated rat livers as well as RNA of the additionally prepared liver series after 1 h partial ischemia and 0, 1, 2 or 6 h of reperfusion on Northern blots.

Figure 5A shows the resulting Northern blot data for the 14 I-lobe up-regulated genes. Ten genes were identified that were clearly up-regulated in the matched postischemic lobes as compared to the untreated livers and the C-lobes of the experimental animals: *Hsp27, Hsp70, Hsp86, Hsp105,*

Hsp60, Hsp10, Jund, Fos, Gadd45a and *EST-Rn.2835.* All analyzed heat shock genes as well as *Jund, Gadd45a* and *EST-Rn.2835* showed highest transcriptional levels after 6 h reperfusion equal to the highest serum AST/ALT levels in the analyzed reperfusion time kinetics (compare Figure 5A to Figure 1B). Two genes, *Ldlr* and *EST-Rn.160092,* showed higher expression levels in the untreated livers as well as in the I-lobes after 2 h reperfusion, whereas in the matched
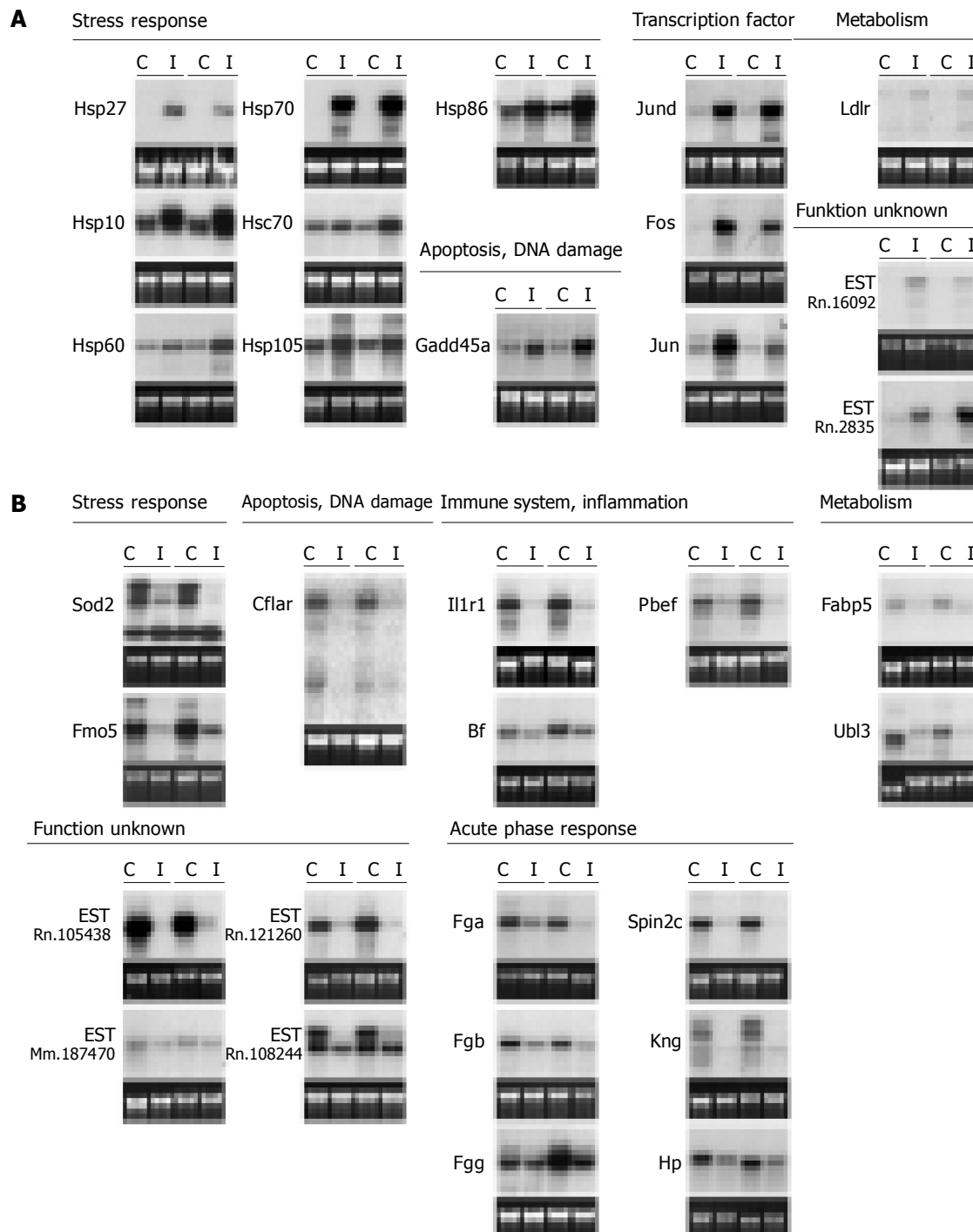
Figure 4 Northern blot data of verified target genes (SSH and 'Atlas Array') after partial WI/R (1 h/2 h). Exemplarily, liver lobes of two animals are shown. Matched liver lobes (C- and I-lobe) are underlined. Abbreviations of the analyzed genes are written on the left of every Northern blot. For full titles see Table 2. 28s rRNA bands of the ethidium bromide-stained gels are shown below the respective blots as loading controls. 14 genes with enriched RNA (A) and 18 down-regulated genes (B) in the I-lobes.

C-lobes RNA levels were lower. The reperfusion time kinetics indicates that these two genes are down-regulated with ongoing reperfusion time and liver damage; this effect was seen earlier in the C-lobes than in the I-lobes (Figure 5A).

Figure 5B demonstrates the transcription levels of 17 genes up-regulated in the C-lobes. Twelve genes have comparably low RNA levels in the analyzed I-lobes and the untreated rat livers but are clearly induced in the C-lobes, comprising of genes coding for plasma proteins including acute phase genes, *Pbef, IL-1RI, Sod2, Ubl3, Fabp5* as well as three ESTs. Except for the genes coding for *IL-1RI, Bf, Sod2* and the *EST-Rn.121260,* all genes with induced expression in the C-lobes showed highest transcriptional levels 6 h post ischemia. Figure 5B also presents some genes with delayed increased gene expression in the I-lobes compared to the corresponding C-lobes after 6 h reperfusion and untreated rat livers (*Pbef, Spin2c, Fgg, Sod2, Fabp5, EST-Rn.121260* and *EST-108244*).

Table 2 Summary of Northern blot expression data of rat liver lobes after partial WI/R (1h/2h)

Description	Up-regulated genes (C-lobes vs I-lobes; $n = 14$)						
	UniGene ID	Accession number	% Identity[1]	Method	Mean fold increase[2]	SE[3]	WI/R citation[4]
Stress response							
Heat shock 10 ku protein 1 (Hspe1, Hsp10)	Rn.106093	NM_012966	id	SSH	2.8 ($n = 4$)	1.4	
Heat shock protein, 27 ku (Hsp27)	Rn.3841	M86389	id	A	13.7 ($n = 4$)	1.6	[21, 27]
Heat shock protein, 60 ku (liver) (Hsp60)	Rn.102058	X54793	id	A	1.4 ($n = 4$)	1.4	
Heat shock 70 ku protein 1A (Hspa1a, Hsp70)	Rn.1950	L16764	id	SSH/A	64.0 ($n = 4$)	1.6	[22, 36-39]
Heat shock 70 ku protein 8 (Hspa8, Hsc70)	Rn.120392	M11942	id	SSH	1.5 ($n = 4$)	1.5	[37, 40]
Heat shock protein 1, alpha (Hspca, Hsp86)	Rn.3277	AJ297736	id	SSH	2.5 ($n = 4$)	1.1	[39]
Similar to heat shock protein 105 ku alpha (LOC288444, Hsp105)	Rn.37805	XM_213699	id	SSH	1.9 ($n = 4$)	1.1	
Cell cycle arrest, DNA damage, apoptosis							
Growth and DNA-damage-inducible transcript-1 (Gadd45a)	Rn.10250	L32591	id	A	3.7 ($n = 4$)	1.2	
Transcription factor							
Jun D proto-oncogene (Jund)	Rn.46225	NM_138875	id	A	4.6 ($n = 4$)	1.3	[9, 29]
c-fos mRNA (Fos)	Rn.103750	XM_234422	id	A	16.3 ($n = 4$)	1.9	[9, 28, 29, 36, 37, 41]
Avian sarcoma virus 17 oncogene homolog (Jun)	Rn.93714	NM_021835	id	A	2.5 ($n = 3$)	1.5	[9, 28, 29, 36, 41]
Cellular metabolism							
Low density lipoprotein receptor (Ldlr)	Rn.10483	X13722	id	A	9.1 ($n = 4$)	1.4	
Gene product function unknown							
ESTs	Rn.16092	BM391881	id	SSH	5.2 ($n = 4$)	1.2	
ESTs	Rn.2835	BG664888	id	SSH	3.6 ($n = 4$)	1.4	

Description	Down-regulated genes (C-lobes vs I-lobes; $n = 18$)						
	UniGene ID	Accession number	% Identity[1]	Method	Mean fold decrease	SE	WI/R citation
Stress response							
Superoxide dismutase 2, mitochondrial (Sod2)	Rn.10488	NM_017051	id	SSH	3.8 ($n = 4$)	1.3	[9, 42, 43, 44]
Flavin containing monooxygenase 5 (Fmo5)	Rn.7038	AF458413	id	SSH	3.3 ($n = 4$)	1.3	
Cell cycle arrest, DNA damage, apoptosis							
FADD-like apoptosis regulator (Cflar)	Rn.28010	AF244366	id	SSH	3.2 ($n = 3$)	1.0	
Immune system, inflammation							
B-factor, properdin (Bf)	Rn.109148	XM_215326	id	SSH	1.8 ($n = 4$)	1.2	[45]
Pre-B-cell colony-enhancing factor (Pbef)	Rn.38030	AB081730	id	SSH	5.3 ($n = 3$)	1.6	
Interleukin 1 receptor, type I (IL-1rI)	Rn.9758	M95578	id	SSH/A	11.5 ($n = 4$)	1.2	[35]
Acute phase response							
Haptoglobin (Hp)	Rn.10950	K01933	id	SSH	3.1 ($n = 4$)	1.2	[46, 47, 47-49]
Fibrinogen-α (Fga)	Rn.98846	X86561	id	SSH	2.3 ($n = 3$)	1.0	[38, 44, 48]
Fibrinogen-β (Fgb)	Rn.11416	U05675	id	SSH/A	2.7 ($n = 3$)	1.3	[38, 44, 48]
Fibrinogen-γ (Fgg)	Rn.1702	J00734	id	SSH	1.8 ($n = 4$)	1.2	[38, 44, 48]
T-kininogen (Kng)		M14370	id	SSH	5.0 ($n = 4$)	1.4	[50]
Serine protease inhibitor (Spin2c)	Rn.128	Aj225628	id	SSH	13.3 ($n = 4$)	1.4	
Cellular metabolism							
Ubiquitin-like 3 (Ubl3)	Mm.21846	AF044223	id	SSH	2.9 ($n = 4$)	1.4	
Fatty acid binding protein 5 (Fabp5)	Rn.5811	U13253	id	SSH	3.4 ($n = 4$)	1.1	
Gene product function unknown							
ESTs	Rn.121260	BF559707	98%	SSH	4.6 ($n = 3$)	1.3	
ESTs (LOC361579)	Rn.105438	BG447490	id	SSH	74.3 ($n = 4$)	1.2	
ESTs	Mm.187470	XM_129912	93%	SSH	1.7 ($n = 4$)	1.2	
Rattus norvegicus similar to TIM2 (LOC287222)	Rn.108244	AY255103	id	SSH	2.6 ($n = 3$)	1.1	

[1]Percentage of sequence identity of tagged cDNAs in comparison to the sequence of the listed Accession No. (id means ≥99% sequence identity); [2]mean signal differences of up to four rat livers after WI/R (1 h/2 h); number of analyzed liver lobe pairs (matched C- and I-lobe of one animal) are shown in parenthesis. Mean fold in- or decrease ≥2.0 are shown in bold letters; [3]standard error; [4]literature citations associated with liver WI/R; citations in bold letters with implied gene expression data.

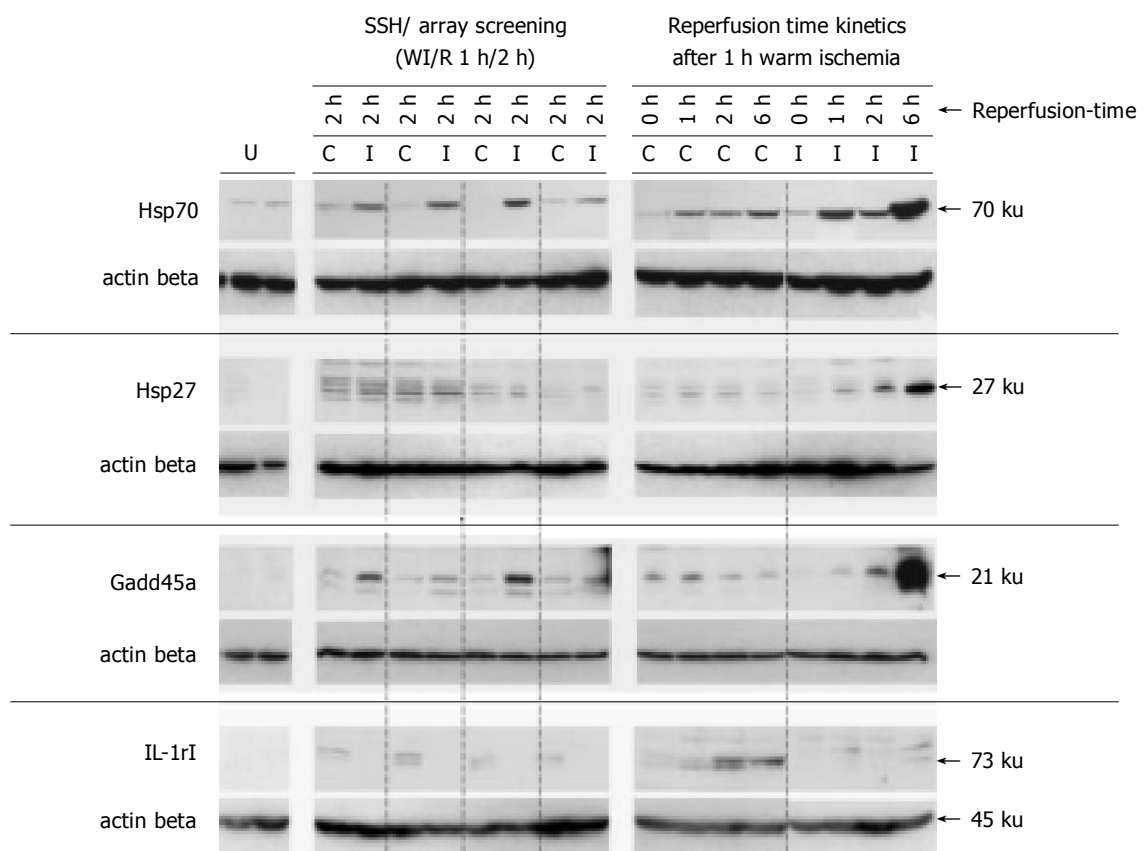### *Hsp70, Hsp27, IL-1RI and Gadd45a protein expression*

We analyzed the protein levels of four verified target genes (*Hsp70, Hsp27, Gadd45a and IL-1RI*) on Western blots after 1 h partial ischemia and 2 h reperfusion ($n = 3$), in the specimens of the representative reperfusion time kinetics (0, 1, 2 and 6 h reperfusion; $n = 1$) and untreated rat livers ($n = 2$) as shown in Figure 6.

Although increased transcript levels of the *Hsp27* gene were observed after 1 h reperfusion (Figure 5A), protein levels were elevated slightly in the I-lobes after 2 h and more pronounced after 6 h of reperfusion. Furthermore, Figure 6 shows increased IL-1RI amounts in the analyzed nonischemic lobes (1, 2 and 6 h reperfusion) compared to protein levels in the corresponding postischemic lobes and untreated livers.
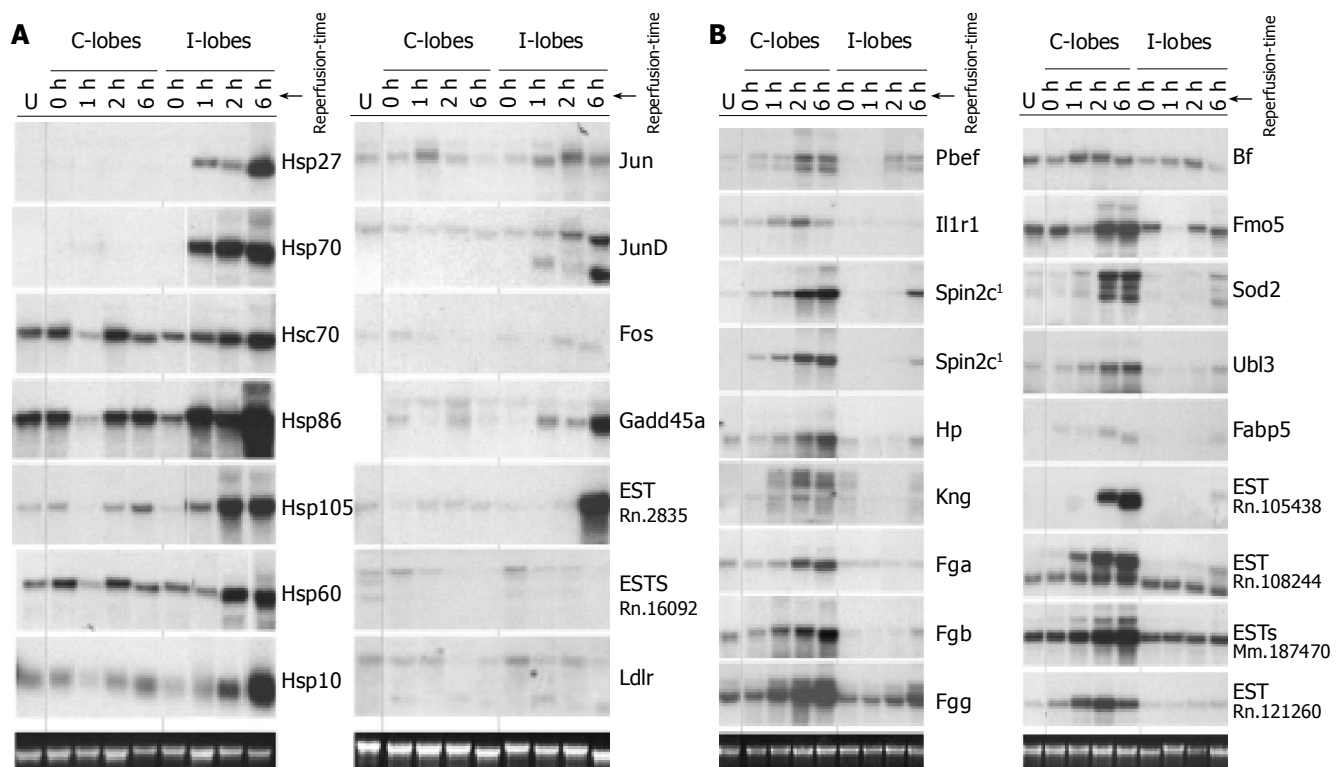
Figure 5 Expression profiling (Northern blot analysis) of 31 genes, untreated and postischemic rat livers after partial WI/R in a reperfusion time kinetics. A: Twelve I-lobe up-regulated genes and two down-regulated sequences after WI/R; B: Genes with enriched RNA in the C-lobes, untreated liver (U), two overlapping Spin2c probes used for independent Northern blot experiments (*1*).

Figure 6 Expression of Hsp70, Hsp27, Gadd45a, IL-1rI and β-Actin proteins in rat livers after partial WI/R (Western blot data). Two untreated rat livers (U); C-lobes and I-lobes after WI/R (C and I).
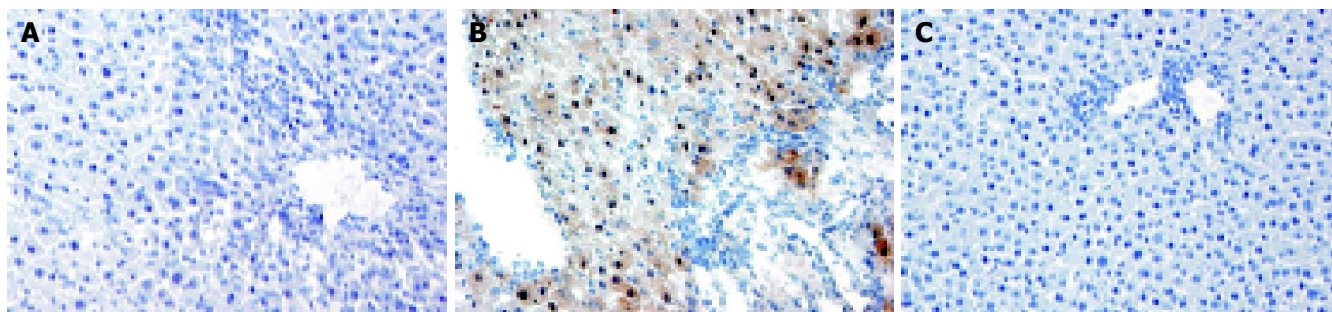
Figure 7 Gadd45a immunohistochemistry exemplarily after 1h/6h WI/R (cryosections). A: C-lobe after WI/R (1 h/6 h); B: I-lobe after WI/R (1 h/6 h); C: Negative control, lacking the primary antibody in the immunohistochemical staining procedure.

Increased Gadd45a protein could be detected as early as after 2 h of reperfusion but was more elevated after 6 h of reperfusion in the postischemic liver lobes as compared to untreated rat livers and respective C-lobes (Figure 6). Immunohistochemical Gadd45a staining of fresh frozen liver tissues demonstrated induced Gadd45a expression after 2 and 6 h of reperfusion, with cytoplasmic and nuclear staining in parenchymal cells (Figure 7). In untreated livers, in all analyzed C-lobes as well as in postischemic I-lobes up to 1 h of reperfusion, Gadd45a expression was absent or at basal levels (data not shown).

Hsp70 protein was clearly induced in the I-lobes after 1, 2 and 6 h of reperfusion (compared to the protein level of the adjacent C-lobes and untreated rat livers), as demonstrated by the Western blot experiments shown in Figure 6. Slightly up-regulated Hsp70 protein levels could also be noticed in the C-lobes with advanced reperfusion time compared to untreated, healthy rat livers. Looking at the immunohistochemical data, Hsp70 induction was observed 1 and 2 h after clamp removal in the analyzed I-lobes only in bile ducts, connective tissue and endothelial cells in portal fields (Figure 8). Additionally, positive staining was noticed in sinusoidal endothelial cells (SEC) and cells in the liver sinusoids. In the analyzed early reperfusion periods (WI/R 1 h/1 h or 1 h/2 h), no Hsp70 expression could be detected in hepatocytes, in contrast to the postischemic liver lobe after 6 h reperfusion. Here intense Hsp70 staining was mainly restricted to parenchymal cells in perivenular liver arreals (Figure 8). In comparison to histopathological changes after WI/R, parenchymal vacuolation correlated with the intense perivenular Hsp70 staining pattern in the I-lobe at this time point (Figure 8).

DISCUSSION

To date, no reliable parameters exist to allow early prediction of liver damage after hepatic resection or transplantation. Conversely, it would be of great clinical importance to have information about potential function of the liver remnant or graft. In this study we established gene expression profiles of 32 genes with differential expression in the acute phase after partial rat liver WI/R, in order to elucidate the genes and molecular pathways involved in the complex setting of WI/R mediated liver injury. For the first time, the two methods SSH and hybridization of 'Atlas Arrays' were used to obtain a comprehensive profile of differentially expressed genes in this standard WI/R animal model. To analyze, if

genes coding for functionally related proteins show similar expression patterns, steady state RNA levels were compared in ischemic/reperfused *vs* adjacent control liver lobes and healthy livers by Northern blot analysis. These gene patterns should reveal new makers for early WI/R liver injury.

In case of the SSH scenery, candidate genes were first screened with the raw material used for the SSH experiments and subsequently with pooled poly(A)$^+$RNA of four rat livers after 1 h/2 h WI/R, to reduce the number of candidate genes from 89 to 32. This second screening allows the comparison of SSH and 'Atlas Array' approaches, because the same pooled RNA source was used for both screening methods. Both procedures yielded a comparable set of differentially expressed genes. The slightly higher rate of differentially expressed candidates of 5.1% recovered from the 'Atlas Arrays' compared to 1.7% after SSH may result from the restriction of the spotted cDNAs on the arrays. The cDNA arrays used here include many gene families involved in signal transduction, cellular stress and inflammation pathways, known to play a role during WI/R, in contrast to the SSH-clones representing a potential of differentially expressed genes from a more unfiltered background. This fact could also explain why only three identical genes were identified with both methods (*Hsp70, IL-1rI and Fgb*). While the array technology allowed a faster identification of candidate genes, SSH yielded six functionally noncharacterized but *via* Northern hybridizations confirmed sequences, that were not included in any arrays, allowing for the detection of new marker genes in the WI/R context. After Northern blot analysis, differential expression could be confirmed for 76.9% (11/13) of 'Atlas Array' candidate genes and for 78.1% (25/32) of SSH candidate genes after WI/R (1 h/2 h), that included 26 genes or sequences differentially expressed in matched liver lobes of more than three animals with fold changes of relative transcript amounts ≥ 2.0 (Table 2). In addition to genes that were already described in experimental or clinical settings to be involved in WI/R liver injuries (Table 2), we verified transcriptional differential expression of 11 functionally known genes not described in this *in vivo* liver WI/R model before, but generally belonging to gene families that are known to be effected after WI/R. These genes were: *Hsp10, Hsp60, Hsp105* (coding for heat shock proteins), *Gadd45a* (apoptosis), *Ldlr* (metabolism) that were all induced in the clamped liver lobes and *Fmo5* (stress responsive gene), *Cflar* (apoptosis inhibitor), *Pbef* and *Spin2c* (immune system, acute phase gene), *Ubl3* and *Fabp5* (cellular
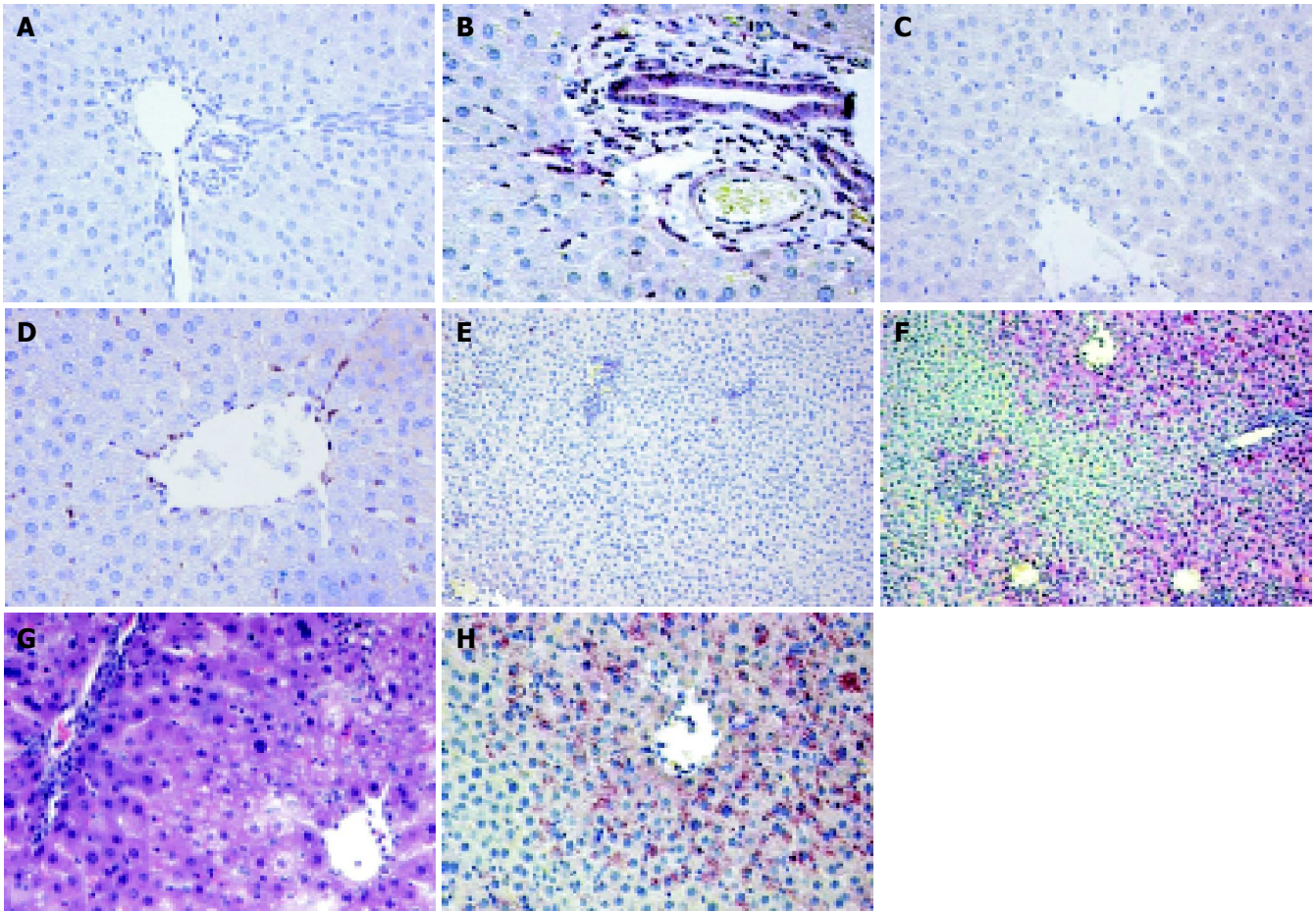
Figure 8 Hsp70 protein localization in rat livers after WI/R (paraffin-embedded sections). Hsp70 staining of paraffin-embedded liver tissues after partial WI/R. Up to 2 h of reperfusion Hsp70 was located in sinusoidal cells and cholangiocytes, connective cells, endothelial cells of portal areas only in the I-lobes (I-lobes after 1 h/2 h WI/R: B+D), while Hsp70 was not detectable in the C-lobes at these time points (C-lobes after 1 h/2 h WI/R: A+C). Parenchymal Hsp70 staining was only noticed after 6 h reperfusion (C-lobe: E; I-lobe: F+H).Comparison of perivenular vacuolization (HE staining: G) and Hsp70 protein expression in the affected hepatocytes after 1 h/6 h WI/R (H).

metabolism) with higher transcriptional levels in the analyzed C-lobes. Additionally, we showed for the first time transcriptional differences between postischemic and nonischemic lobes of the genes *Hsp27, Jund, Sod2 and IL-1rI* known to be involved in WI/R liver injury whose transcriptional status has so far remained unclear from literature data.

After the more detailed gene expression profiling including reperfusion time kinetics and livers of untreated animals (Figure 5), a more in-depth picture of gene expression was seen. First, up-regulated gene expression in one of the experimental groups (C- or I-lobes) *vs* healthy rat livers occured rather in early reperfusion phases than in the ischemic period (except *Spin2c* and *EST-Rn.121260*) that may be due to high energy nucleotide depletion in the ischemic period[4,9]. This finding also explains the delayed up-regulated gene expression in the I-lobes compared to the corresponding C-lobes after 6 h reperfusion and untreated rat livers for seven analyzed sequences. Secondly, genes coding for functionally related proteins show similar expression patterns in the analyzed tissue specimens that is now discussed in detail below.

Figure 5A summarizes the genes of main interest that are up-regulated in the postischemic lobes (I-lobes) compared to the matched C-lobes as well as to the normal livers. Eleven genes could be assigned to genes coding for heat shock proteins, one to apoptosis inductor and one belongs to the AP-1 transcription factor group.

Also known as molecular chaperones, the heat shock gene product family members are responsible to maintain correct protein folding; they are in part induced by denatured proteins produced during heat shock, ischemia or chemical noxes[21]. Interestingly, all important cytoplasmic, nuclear and mitochondrial chaperone systems were involved in our model of WI/R. Hsp70 the major inducible cellular chaperone and Hsp27, a member of the heterogeneous family of small heat shock proteins[21], showed the strongest up-regulation in our model. Hsp70 is accepted to be a sensitive marker indicating WI/R induced cellular stress during the early and subacute reperfusion phase. Hsp70 maintains correct protein folding[22,23] and has protective effects after chemical and ischemic preconditioning followed by liver WI/R[21,24]. It has been shown recently that Hsp70 is a potent inhibitor of apoptotic cell death, additionally explaining the protective effects after preconditioning inducing heat shock protein expression[23,25]. In our experiments, *Hsp70* and *Hsp27* gene transcripts were nearly absent in untreated rats and C-lobes after WI/R but were up-regulated in the I-lobes. Nearly all

published Hsp70 protein analyzes after WI/R do not include Hsp70 localization, but focus on ELISA or Western blot data. Our immunohistochemical results with Hsp70 restriction to ductal cells and SEC 1-2 h postischemia as well as intense parenchymal Hsp70 expression 6 h after reoxygenation of the clamped liver lobe, reflect the time course of this WI/R model[4]. This parenchymal Hsp70 protein staining 6 h after warm liver ischemia in correlation to hepatocyte vacuolation shows that Hsp70 is a sensitive marker for WI/R liver damage. Interestingly, the perivenular Hsp70 distribution could also be demonstrated in rat livers 48 h after heat shock exposure[26]. In the case of Hsp27, for the first time we demonstrated a significant protein up-regulation after partial liver WI/R. In experimental settings after kidney I/R it could be shown that Hsp27 is phosphorylated and activated after I/R; activated-p38 was involved in phosphorylation of Hsp27[27].

In the case of AP-1 transcription factors, which are involved in cellular regeneration or apoptosis[9,28], the immediate early genes *Fos, Jun* and *Jund* were up-regulated after WI/R. *Fos* and *Jun* gene expressions are known to be induced in the acute reperfusion phase after WI/R[28]. Our transcriptional data, concerning these two genes, are in agreement with the published ones. Furthermore, it could be shown that DNA binding activity of AP-1 dramatically increased in the acute reperfusion phase 1-3 h post-ischemia[29], predominantly of Jun and Jund hetero- and homodimers. AP-1 activity with Jund was mainly found to be up-regulated due to posttranslational modifications regulating rather anti-proliferative responses after WI/R[9,29]. This feature is underscored by the observation that levels of proliferating cell nuclear antigen (a proliferation marker) decrease in concordance with elevated Jund[28]. Now we close the circle and demonstrate transcriptional induction of *Jund* poly(A)$^+$RNA in the postischemic tissues after partial WI/R. Acting as a transcriptional complex, the up-regulation of these immediate early response genes can cooperatively induce the expression of inflammatory cytokines. This leads to neutrophil-mediated inflammation[9], thereby linking acute molecular events with early cellular damaging effects to the subacute inflammatory responses.

Gadd45a gene is regulated in correlation with the induction of apoptosis or as a consequence of stress response[30]. In our experiments, the *Gadd45a* gene was induced in the postischemic tissues ⩾1 h on RNA level and ⩾2 h on protein level. Gadd45a may play a role in p53 dependent and independent apoptosis[30] via activation of JNK and/or p38-MAPK signalling pathways[31]. Both are known to be part of signal transduction cascades in the acute reperfusion phase after liver WI/R[9]. Elevated apoptotic rates are recognized after WI/R although necrosis appears to be the principal mechanism of cell death after WI/R[16].

Looking at the different pathophysiological sequences after liver ischemia[4] as described in the introduction, reperfusion injury is initiated in the early or immediate reperfusion period (1-6 h) with activation of Kupffer cells and induction of microcirculatory disturbance. These changes lead to immense neutrophil inflammatory response, parenchymal apoptosis and more pronounced necrosis in

the later postischemic periods (>6 h)[16]. With the identification of genes already up-regulated 1-2 h post-liver clamping in the manipulated liver tissues (I-lobes), compared to the analyzed normal liver tissue, these marker genes indicate not only cellular stress but also tissue injury in the early reperfusion phase (1-2 h) with progressive aggravation. Considering our presented protein data, cellular stress is indicated by the chaperones Hsp27, Hsp70 and the apoptosis or growth arrest inducing protein Gadd45a, with clearly but slightly elevated protein levels in the early reperfusion period in the postischemic liver lobes and immense up-regulated protein synthesis at the later stage (6 h reperfusion).

In contrast to the discussed I-lobe induced genes, Figure 5B combines 18 sequences primarily identified as verified down-regulated after partial WI/R, comparing I-lobes and the corresponding C-lobes of our standard model (WI/R; 1 h/2 h), showing an interesting feature after expression profiling in contrast to the untreated specimens. These gene transcripts were enriched in the C-lobes that were under permanent blood flow throughout the whole of surgical procedure, with some genes indicating a delayed up-regulation in the post ischemic tissues (I-lobes) after 6 h reperfusion. This indicates that various mediators or direct effects of ROS, produced in the clamped liver lobe, systemically influence the adjacent perfused liver lobes during the ischemic period or subsequent to reperfusion. It is well known that TNFα and IL-1 and other mediators have effects not only on the local hepatic environment, but also on remote organs after WI/R[8,32,33], representing the main mechanism of multiorgan dysfunction after liver transplantation and resection[34]. Additionally, it could be shown that blocking IL-1R with its physiological antagonist (IL-1Ra) has beneficial effects after WI/R[35-50]. Our findings demonstrating the up-regulation of acute-phase response/inflammation genes in the adjacent C-lobes in contrast to the corresponding I-lobes after partial WI/R and to the untreated animals support this hypothesis, most notably, increased *IL-1RI* RNA and protein synthesis in the analyzed C-lobes (⩾1 h post ischemia). IL-1RI interacts with IL-1 and TNFα leading to inflammatory responses *via* NFκB activation[4,8,11]. Another source for mediators leading to altered gene expression in the C-lobes could be the splanchnic region due to portal hypertension as a result of incomplete decompression *via* the unclamped liver lobes in the case of partial WI/R, i.e., endotoxins, that are absorbed by Kupffer cells leading to the activation of these macrophages[32].

In conclusion, we present a new set of genes responsive to WI/R in early reperfusion periods before irreversible liver damage occurs. The fact that these genes (six noncharacterized sequences and 11 genes described for the first time in the liver WI/R context) include members of known involved pathways (heat shock activation, AP-1 involvement, apoptosis, and inflammatory response) confirms that the methods used in this study provide good tools to identify new WI/R indicating genes. It becomes clear that in these early reperfusion periods (1-2 h) destructive mechanisms mediated by up-regulation of proapoptotic genes are counteracted by the pronounced expression of protective acting genes (i.e., heat shock genes).

The question is which side will prevail.

As we analyzed IL-1RI, Gadd45a and Hsp27 exemplarily and for the first time in the liver WI/R context on the protein level, future studies should expand protein expression data of the identified marker genes. Differentially expressed ESTs need to be further characterized in experimental and clinical settings especially during liver transplantation and resection. The involvement of different functional compartments should stimulate the definition of a characteristic set of early WI/R markers to distinguish between reversible and irreversible injuries after liver surgery rather than to focus on single markers. Clearly, heat shock proteins as well as AP-1 and apoptosis-related genes and proteins should be part of this diagnostic spectrum.

ACKNOWLEDGEMENTS

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