Table 3-1. Representative miRNAs That Were Commonly Repressed in CH-B, CH-C, HCC-B, and HCC-C Compared with Normal Liver (Cluster)

miRNA	Parametric *P* Value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test *P* Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-219	7.3E-05	0.28	25/109	2.59E-04	Glypican-3, ERP5, PLK2, HIRA, HMG2	Regulatory T cell differentiation
					ACOX1	Fatty acid beta-oxidation
					NF-X1	MHC class II biosynthetic process
hsa-miR-320	9.8E-05	0.50	26/88	3.50E-06	Vimentin, ALP (*N*-acetyltransferase-like), SEC61 beta, G-protein alpha-i2, Filamin A	Protein kinase cascade
					Rac1, RhoG	Organelle organization and biogenesis
					Vinexin beta, Profilin I, Ca-ATPase3	Actin cytoskeleton organization and biogenesis
hsa-miR-154	2.7E-04	0.15	22/70	5.40E-06	OTR, NET1(TSPAN1), NAP1, Vimentin, PDIA3, cytochrome P-450 reductase	Regulation of apoptosis
					DLX2	Morphogenesis
					GUAC, ACAT1	Branched chain family amino acid catabolic process
hsa-miR-29c	1.8E-03	0.55	53/133	1.00E-06	FBX07, ASPP1, HSPA4, Cathepsin O, PDF, COL4A1, HSPA4, TIP30, CXADR	Cell-substrate adhesion
					NS1-BP, ALP (*N*-acetyltransferase-like), ACTR10, Beclin 1	Transcription, DNA-dependent
					SMAD6, LTBR(TNFRSF3), ENPP7	Apoptosis
hsa-miR-338	5.2E-03	0.46	30/101	3.60E-06	ID3, GATA-4, NFIA, FR-beta, CREST, HYOU1	Developmental process
					G3ST1, CAD,	Immune effector process
					FKBP12, LZIP, PDIA3, Schwannomin (NF2), CREST	Immune system process
hsa-miR-26a	6.3E-03	0.70	37/119	2.64E-05	*LIG4*, c-FLIP, GADD45 beta, DAPK1, PRDX4, LRP130	Response to stimulus
					Cyclin E, ZDHHC6, Tx1, ATG8 (GATE-16), WASP, C1s	DNA replication initiation
					COPG1	Ion transport
hsa-miR-126	8.1E-03	0.65	27/101	4.04E-03	ANP32B (april), HSPA4, RLI, LIV-1 (SLC39A6), PTP-MEG2, CD97, DHPR	Regulation of cellular protein metabolic process
					NFKBIA, NMI, MDH1, PDCD2	Response to stress
					SMAD6, ATP6AP2, ANP32B (april), NMI, HSPA4	Apoptosis
hsa-miR-325	8.7E-03	0.20	18/63	2.03E-04	TRADD, CREST, NEDD8, annexin IV, GPX2, PDF, TNFAIP1	Developmental process
					Glypican-3, ID1, PC-TP,	Multicellular organismal development
					SNRPB (Sm-B)	RNA splicing

*Ratio of HCC-B, HCC-C, CH-B, and CH-C to normal.
†The number of significant genes ($P < 0.05$) out of predicted target genes in which expression was evaluated in microarray.
‡Statistical assessment of presence of differentially expressed genes out of predicted target genes of miRNAs.
§Representative differentially expressed genes out of predicted target genes of miRNAs.
¶Representative pathway of differentially expressed genes out of predicted target genes of miRNAs.

the hypergeometrical distribution based on gene ontology terms. Because one gene is frequently involved in multiple pathways, all pathways corresponding to the genes with significance probability were listed.

Verification of Regulation of Candidate Target Genes by miRNAs. Anti-miRNA (Ambion) specific to 13 miRNAs (has-miR-17*, has-miR-20a, has-miR-23a, has-miR-26a, has-miR-27a, has-miR-29c, has-miR-30a, has-miR-92, has-miR-126, has-miR-139, has-miR-187, has-miR-200a, and has-miR-223) showing significant differences in expression were transfected into Huh7 cells using TransMessenger transfection reagent (QIAGEN, Valencia, CA), and loss of function of each miRNA was evaluated. Similarly, precursor miRNAs of five miRNAs (has-miR-23a, has-miR-26a, has-miR-27a, has-miR-92, and has-miR-200a) were also transfected into Huh7 cells, and gain of function of each miRNA was evaluated. The loss- and gain-of-function of miRNAs were evaluated via RTD-PCR. In addition, different gene expressions regulated by miRNAs were also evaluated via RTD-PCR.

HBV/HCV Infection Model Using Cultured Cells. The plasmid pHBV 1.2 coding the 1.2-fold length of the HBV genome was transfected into Huh7.5 cells using Fugene6 transfection reagent (Roche Applied Science, Indianapolis, IN). HBeAg production in culture medium was measured using Immunis HBeAg/Ab EIA (Institute of Immunology Co., Ltd., Tokyo, Japan).[13] The amount of HBV-DNA was measured via RTD-PCR (Supplementary Fig. 1A,B). JFH1-RNA was transfected into Huh7.5 cells using TransMessenger transfection reagent (QIAGEN) and the expression of the core protein was examined via immunofluorescence staining using anti-HCV core antibody (Affinity BioReagent, CO).[14,15] HCV-RNA amount was also measured via RTD-PCR (Supplementary Fig. 1A,B). JFH1/GND was used as a negative control. miRNA expression was quantitated by RTD-PCR 48 hours after transfection.

Results

Expression of miRNA in Liver Tissue. A panel of miRNA was successfully amplified from liver tissues via RTD-PCR. The representative amplification profile of miRNA as determined with RTD-PCR is shown in Fig. 1. To assess the reliability and reproducibility of this assay system, we first measured RNU6B in duplicate from all samples in different plates. The mean difference in Ct values of RNU6B expression within the same samples was 0.08 ± 0.05 (mean ± standard deviation), indicating the high reproducibility of this assay. All Ct values from each reaction were collected, and Ct variation obtained by each probe from all patients was calculated. Although RNU6B was frequently used as the internal control, the standard Ct variation was relatively high (Ct, 27 ± 1.94), suggesting that the variances in its value depend on the state of liver disease (N, CH and HCC). Therefore, we selected has-miR-328 as the internal control with the smallest standard deviation (Ct, 30 ± 0.60). The relative expression ratio of individual miRNA to has-miR-328 was calculated and applied to the following analysis using a BRB-array tool.

Hierarchical cluster analysis revealed that the expression profiles of the 188 miRNAs from each patient were roughly classified into normal liver, HBV-infected liver (CH-B+HCC-B; HBV group), and HCV-infected liver (CH-C+HCC-C; HCV group) (Fig. 2A). HCV viremia in two patients with CH-C was persistently cleared by interferon therapy before HCC development. The background liver of one of these patients was clustered in the normal group and those of others in the HCV group. Although these two patients were not clearly differentiated from others, some miRNAs such as miR-194, miR-211, and miR-340 that were down-regulated in the HCV group were significantly up-regulated in two patients (Fig. 3, cluster 2).

The present CH and HCC expression data were obtained from the same patient; however, each sample clustered irrespective of pairs in all but two patients. miRNA expression profiling was therefore more dependent on the disease condition than on the paired condition, as also confirmed by the Dunnett test.[12] We then attempted to classify the expression profiles into HBV and HCV groups using supervised learning methods (Table 2-1). HBV and HCV groups were significantly differentiated at an 87% accuracy ($P < 0.001$). The normal liver and CH (CH-B + CH-C) and CH and HCC (HCC-B + HCC-C) were also significantly differentiated at a 90% rate of accuracy. These results suggest that different stages of liver disease (normal, CH, and HCC) can be differentiated from each other based on the miRNA expression profile, as well as HBV and HCV infection.

To examine the relationship among five categories of groups, namely, N, CH-B, CH-C, HCC-B and HCC-C, we attempted to differentiate the five groups using a supervised learning algorithm (binary tree classification) used for classifying three or more groups. SVM was used as a prediction method. Expression profiles were first classified into groups N (normal) and non-N (non-normal) (CH-C, CH-B, HCC-C, and HCC-B) (node 1) ($P < 0.01$). The non-N group was then classified into HBV and HCV (node 2) ($P < 0.01$). The HBV group was further classified into CH-B and HCC-B (node 3) ($P < 0.01$), and the HCV group was further classified into CH-C and HCC-C (node 4) ($P < 0.01$) (Fig. 2B, Table 2-2). Thus, the findings support the notion that differences in miRNA expression between HBV and HCV are as distinct as those between CH and HCC.

Out of 20 miRNAs that differentiated node 1 classification (Table 2-2), 12 also differentiated node 3 or node 4 classification. The remaining eight miRNAs specifically differentiated node 1 classification. They were down-regulated in the HBV and HCV groups compared with the normal group (Fig. 3, cluster 1). Nineteen miRNAs differentiated node 2 classification (Table 2-2) and the hierarchical clustering using these miRNAs clearly differentiated the HBV and HCV groups (Fig. 3, cluster 2). There were 15 and 14 miRNAs that differentiated node 3 and 4 classifications, respectively (Table 2-2). Hierarchical clustering using these miRNAs revealed that these miRNAs differentiated CH-B and HCC-B as well as CH-C and HCC-C, respectively; 17 miRNAs were down-regulated in HCC, and six were up-regulated in HCC (Fig. 3, cluster 3).

Table 3-2. Differentially Expessed miRNA Between HCC-B, CH-B, and HCC-C, CH-C, and Their Representative Target Genes (Cluster 2)

miRNA	Parametric *P* Value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test *P* Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-190	1.2E-05	2.06	21/68	4.47E-02	Chk1, C2orf25, VRK2, USP16, STAF65(gamma)	Regulation of cell cycle
					AP1S2, RNASE4	Mitotic cell cycle
					PPP2R1B, ARHGAP15, UBPY	Negative regulation of apoptosis
hsa-miR-134	2.3E-04	5.74	11/58	3.40E-06	VKDGC, SH2B, MALS-1, DDB2	Multicellular organismal process
					BCRP1	Regulation of viral reproduction
					DDB2	Lipid biosynthetic process
hsa-miR-151	2.8E-04	1.82	12/62	6.41E-01	RGS2, UFO, AK2, USP7	G-protein signaling
					eIF4G2, USP7	Regulation of translation
					SLC22A7	Organic anion transport
hsa-miR-193	5.0E-04	1.67	23/95	9.30E-01	G-protein alpha-11, p130CAS, VAV-1, PDCD11	Cell motility
					Colipase, ACSA	Energy coupled proton transport
					DCOR	Intracellular signaling cascade
hsa-miR-133b	1.7E-03	2.42	20/97	3.69E-02	DDB2, Bcl-3, Cystatin B	Proteasomal protein catabolic process
					Rab-3, RAG1AP1, KCNH2, DCOR	Regulation of biological quality
					AL1B1	Carbohydrate metabolic process
hsa-miR-324-5p	2.9E-03	1.51	27/121	1.90E-06	SKAP55, VAV-1, DDB2, E2A, NIP1	Cellular developmental process
					MEMO (CGI-27), Rab-3	Cellular structure morphogenesis
					COPG1, GPX3, OAZ2	Glutathione metabolic process
hsa-miR-182*	3.1E-03	2.23	28/123	< 1e-07	Alpha-endosulfine, HCCR-2, Thioredoxin-like 2, TPT1, USP7	Translation initiation in response to stress
					DDB2, TPT1	Cellular developmental process
					JIP-1	JNK cascade
hsa-miR-105	4.6E-03	4.38	18/68	4.74E-05	Beta-2-microglobulin, HLA-B27	Antigen processing and presentation
					PIMT, IL-17RC	Immune response
					MHC class I, CDK9, ERG1, Desmocollin 3	
hsa-miR-211	5.3E-03	25.61	10/56	2.00E-04	PSMD5, SLC26A6	Proteasomal protein catabolic process
hsa-miR-20	5.7E-03	1.52	27/113	5.28E-03	Noelin, SC4MOL, Thioredoxin-like 2, CCL5, NALP3	Regulation of apoptosis
					Hic-5/ARA55, USP16, MAP4, Ferroportin 1	Positive regulation of cellular process
					TOP3A, PLRP1	Oxygen transport
hsa-miR-191	6.7E-03	1.39	25/79	7.55E-04	CDK9, GPS2, CLTA, LXR-alpha	Nucleic acid metabolic process
					ACSA	Acetyl-CoA biosynthetic process
					UGCGL1, SGPP1	Metal ion transport
hsa-miR-340	8.5E-03	1.48	17/81	3.73E-03	FKBP12, DCOR,	Calcium ion transport
					Gelsolin, VAV-1, ARF6	Actin cytoskeleton organization and biogenesis
					HXK3	Glucose catabolic process
hsa-miR-194	8.7E-03	1.67	13/74	5.90E-01	Cyclin B1, Serglycin	M phase of mitotic cell cycle
					PTE2	Acyl-CoA metabolic process
					SLC7A6	Carbohydrate utilization
hsa-miR-23a	1.9E-04	0.46	14/97	< 1e-07	*RGL2*, MANR, MEK1 (MAP2K1), Caspase-3, AZGP1	Protein kinase cascade
					FRK, Pyk2(FAK2), CSE1L	Cellular developmental process
					AZGP1	Defense response
hsa-miR-142-5p	4.9E-04	0.40	25/89	9.10E-06	Sirtuin4, PAI2, PSAT, RIL, CDC34, SPRY1	Metabotropic glutamate receptor
					E4BP4, DNAJC12, WWP1, PAIP1, PASK, rBAT	Regulation of gene expression
					VCAM1, CaMK I, WWP1, FHL3	Cell-matrix adhesion
hsa-miR-34c	5.1E-04	0.20	31/129	7.30E-06	Diacylglycerol kinase, zeta, PLC-delta 1, ATP2C1, PAI2	Manganese ion transport
					MLK3(MAP3K11), MEK1(MAP2K1), CDC25C, MRF-1, XPC	Protein kinase cascade
					GNT-IV	Inflammatory cell apoptosis

Table 3-2. Continued

miRNA	Parametric *P* Value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test *P* Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-124b	8.6E-04	0.32	25/120	7.10E-05	E2F5, Rad51, Jagged1	Muscle development
					MLK3(MAP3K11), RGS1	Intracellular signaling cascade
					COL16A1	MAPKKK cascade
hsa-let-7a	1.0E-03	0.45	28/136	9.35E-04	RAD51C, CoAA, hASH1, Cockayne syndrome B, Caspase-1, PP5	Response to DNA damage stimulus
					PLC-delta 1, MANR, ACADVL	Fibroblast proliferation
					HGF, NGF	Cellular developmental process
hsa-miR-27a	3.9E-03	0.59	18/108	1.19E-02	COL16A1, RIL, RhoGDI gamma, ANP32B (april)	Cytoskeleton organization and biogenesis
					VE-cadherin, NTH1, GATA-2, E4BP4	Response to external stimulus
					RAD51C	DNA recombination

*Ratio of HCC-B, CH-B, to HCC-C,CH-C.

†The number of significant genes ($p < 0.05$) out of predicted target genes in which expression was evaluated in microarray.

‡Statistical assesment of presence of differentially expressed genes out of predicted target genes of miRNAs.

§Representative differentially expressed genes out of predicted target genes of miRNAs.

¶Representative pathway of differentially expressed genes out of predicted target genes of miRNAs.

These results indicate that there were two types of miRNAs—one associated with HBV and HCV infection (cluster 2), the other associated with the stages of liver disease (clusters 1 and 2) that were irrelevant to the differences in HBV and HCV infection.

Differential miRNAs and Their Candidate Target Genes and Signaling Pathways. Differentially expressed miRNAs are shown in Table 3. In addition to the expression ratios of miRNAs in each group, the number of genes analyzed on the microarray predicted to be the target genes of miRNAs and that which actually showed significant ($P < 0.05$) differences in expression are also shown. Based on the frequencies and levels of expression of differential genes, the significance of regulation of these gene groups by miRNAs was evaluated using Hotelling T2 test (BRB ArrayTools) (Table 3). The representative candidate target genes and their signaling pathways by each miRNA were shown one by one (Table 3). The signaling pathways regulated by all differential miRNAs in each category of groups are shown in Table 4.

Eight miRNAs were down-regulated in the HBV and HCV groups compared with the normal group (Table 3-1; Fig. 3, cluster 1). These miRNAs were associated with an increased expression of genes related to cell adhesion, cell cycle, protein folding, and apoptosis (Tables 3-1, 4-1), and possibly with the common feature of CH irrespective of the differences in HBV and HCV infection.

Nineteen miRNAs clearly differentiated the HBV and HCV groups (Fig. 3, cluster 2, Table 3-2). Thirteen miRNAs exhibited a decreased expression in the HCV group, and six showed a decreased expression in the HBV group. miRNAs exhibiting a decreased expression in the HCV group regulate genes related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism. On the other hand, those exhibiting a decreased expression in the HBV group regulate genes related to cell death, DNA damage and recombination, and transcription signals. These findings reflected the differences in the gene expression profile between CH-B and CH-C described (Tables 3-2, 4-2).[10] Interestingly, although these miRNAs were HBV and HCV infection–specific, some of them were reported to be tumor-associated miRNAs, suggesting the possible involvement of infection-associated miRNAs in HCC development.

Twenty-three miRNAs clearly differentiated CH and HCC that were irrelevant to the differences in HBV and HCV infection. Seventeen miRNAs were down-regulated in HCC that up-regulated cancer-associated pathways such as cell cycle, adhesion, proteolysis, transcription, translation, and the Wnt signaling pathway (Tables 3-3, 4-3). Six miRNAs were up-regulated in HCC that down-regulated all inflammation-mediated signaling pathways, potentially reflecting impaired antitumor immune response.

Relationship Between Expressions of Infection-Associated miRNA in Liver and Cultured Cells Infected with HBV and HCV. To clarify whether the expression of infection-associated miRNA is regulated by HBV and HCV infection, we investigated the relationship between changes in miRNA in liver tissues and those in miRNA in Huh7.5 cells in which infectious HBV or HCV clones replicated. To evaluate the replication of each clones in Huh7.5 cells, we measured time-course changes in the amounts of HBV-DNA and HCV-RNA in Huh7.5 cells transfected with pHBV1.2 and JFH1-RNA, respectively, by RTD-PCR (Supplementary Fig. 1A). The expression of HBV proteins was examined by measuring the amount

Table 3-3. Differentially Expessed miRNA Between CH and HCC and Their Representative Target Genes (Cluster 3)

miRNA	Parametric p-value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test *P* Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-139	4.50E-06	0.42	19/106	2.70E-03	Cyclin B1, DHX15, MCM5, Histone H2A	Mitotic cell cycle
					RBCK1, SYHH	Protein catabolic process
					ILK, IGFBP7, SAFB, CTR9	Response to external stimulus
hsa-miR-30a-3p	2.50E-05	0.49	26/144	1.73E-02	GGH, Pirin, ZNF207, Annexin VII	Regulation of oxidoreductase activity
					ILK, LTA4H, ABC50, GNPAT	Cell-matrix adhesion
					DLC1	Morphogenesis
hsa-miR-130a	7.00E-05	0.50	22/108	1.07E-02	SPHM, PPP2R5D, RHEB2, SPHM	Mitotic cell cycle
					MLK3(MAP3K11), Otubain1, TIMP4	Protein modification process
					NRBP	Cell differentiation
hsa-miR-223	3.40E-04	0.39	14/90	6.52E-03	Ephrin-A1, Midkine, FDPS	Cell morphogenesis
					K(+) channel, subfamily J	Notch signaling pathway
hsa-miR-187	3.55E-04	0.12	16/66	6.76E-04	HFE2, Otubain1	Negative regulation of programmed cell death
					PRSS11, SUPT5H, RAG1AP1	Developmental process
					PLOD3	Mitochondrial ornithine transport
hsa-miR-200a	6.86E-04	0.18	20/141	2.15E-02	*CDC25B, KAP3, CDK2AP2,* CHKA	Cell communication
					POLD	DNA replication
					CPSF4	RNA splicing
hsa-miR-17-3p	8.42E-04	0.58	28/108	8.98E-04	MLK3(MAP3K11), Tip60, ACBD6, DOC-1R, DAX1, RBCK1	Protein kinase cascade
					WNT5A, 14-3-3 gamma, DHX15	BMP signaling pathway
					HFE2, MCM5	DNA recombination
hsa-miR-99a	1.17E-03	0.53	33/163	9.52E-03	Calpain small subunit, Thoredoxin-like 2, Survivin	Cytokinesis
					IBP2, DNA-PK, KAP3,	Intracellular signaling cascade
					NFE2L1, PARP-1, HDAC11	Regulatory T cell differentiation
hsa-miR-200b	1.57E-03	0.18	24/147	2.72E-02	HSP47, HMG2, NRBP	Regulation of cell cycle
					SNX17	Cell motility
					Ephrin-A1	Receptor protein signaling pathway
hsa-miR-125b	1.82E-03	0.55	26/114	1.03E-01	COL4A2, TIP30, HSP47, MSP58	Cell adhesion
					MLK3(MAP3K11), ERK2 (MAPK1), ERK1 (MAPK3), PLOD3	Nuclear translocation of MAPK
					Otubain1, SCN4A(SkM1)	Ubiquitin-dependent protein catabolic process
hsa-miR-30e	2.10E-03	0.65	24/151	4.30E-02	Cyclin B1, XTP3B, GAK, Annexin VII, MIC2, NRBP	Mitotic cell cycle
					MSS4	Protein localization
					S100A10	Calcium ion transport
hsa-miR-199a*	4.26E-03	0.35	11/71	7.16E-02	BUB3, Cyclin B1, LMNBR	Mitotic cell cycle
					PRAME	Cardiac muscle cell differentiation
hsa-miR-122a	6.31E-03	0.51	11/80	1.01E-03	JAB1, APEX, Clathrin heavy chain	Base-excision repair
					PARN	Translational initiation
					DDAH2	Regulation of cellular respiration
hsa-miR-199a	8.77E-03	0.35	18/94	3.56E-02	IL-13, MLK3(MAP3K11), CLK2, ACP33	Protein amino acid phosphorylation
					PAFAH beta, SPA1, CLCN4	Small GTPase mediated signal transduction
hsa-miR-326	9.00E-03	0.57	29/147	2.25E-01	Midkine, ENT1, IP3KA, PSMC5, ANCO-1	Regulation of programmed cell death
					Thy-1, MCM6, Tip60, VILIP3	Cell-matrix adhesion
					COMP, Cathepsin A	Blood vessel development
hsa-miR-92	9.60E-03	0.81	28/140	2.47E-02	*TUBGCP2,* Fibrillin 1, PIPKI gamma, KAP3	Rho protein signal transduction
					SNX15, BCAT2	LDL receptor and BCAA metabolism
					IGFBP7, FZD6, COPS6	Adenosine receptor signaling pathway
hsa-miR-221	3.40E-06	3.34	16/67	3.59E-01	Lck, Kallistatin, Neuromodulin, LFA-3, PA24A, AZGP1, MSH2	Immune response-activating signal transduction
					KYNU, PMCA3	DNA repair

Table 3-3. *Continued*

miRNA	Parametric p-value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test *P* Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-222	6.50E-06	2.23	18/85	1.59E-02	Thrombospondin 1, Lck, MSH2, ATF-2, CITED2, Kallistatin	Cell motility
					PGAR	Triacylglycerol metabolic process
					KYNU	DNA replication
hsa-miR-301	5.22E-05	1.96	14/71	1.16E-01	Beta-2-microglobulin, PPCKM, PRC, Fra-1, PPCKM, ACAT2	Antigen processing and presentation
					BMPR1B, ARMER, EHM2, RBBP8	Meiotic recombination
					Neuromodulin, LDLR	Cell motility
hsa-miR-21	7.67E-03	1.57	19/81	1.86E-04	Btk, Fra-1, MSH2, Collectrin, Adipophilin	Regulation of T cell proliferation
					RNASE4, AGXT2L1	Peptidyl-tyrosine phosphorylation
					SARDH	Natural killer cell activation during immune response
hsa-miR-183	2.46E-02	3.51	13/86	3.36E-01	Hdj-2, PEMT, Lck, MKP-5, Chondromodulin-I, ABCA8	Cell differentiation
					IL-16, MTRR, SerRS	Methionine biosynthetic process
hsa-miR-98	5.22E-02	1.32	24/130	2.95E-04	ACAA2, LTB4DH, ACADVL, DECR, S14 protein,	Fatty acid metabolic process
					Rapsyn, Kallistatin, ENPEP, Beta crystallin B1	Multicellular organismal process
					CYP4F8	Prostaglandin metabolic process

*Ratio of HCC to CH.
†The number of significant genes ($P<0.05$) out of predicted target genes in which expression was evaluated in microarray.
‡Statistical assesment of presence of differentially expressed genes out of predicted target genes of miRNAs.
§Representative differentially expressed genes out of predicted target genes of miRNAs.
¶Representative pathway of differentially expressed genes out of predicted target genes of miRNAs.

of HBeAg released in culture medium (Supplementary Fig. 1B). HCV protein expression was examined by evaluating the core protein expression after 48 hours by fluorescence immunostaining (Supplementary Fig. 1C). RNA was extracted from the Huh7.5 cells 48 hours after gene transfection, and miRNA expression pattern in the cells was compared with those in liver tissues. We found a strong correlation between differences in miRNA expression between liver tissues of the HBV and HCV groups, and those in miRNA expression between Huh7.5 cells transfected with HBV and HCV clones ($r = 0.73$, $P = 0.0006$) (Fig. 5). These results revealed that differences in the expression of infection-associated miRNA in the liver between the HBV and HCV groups are explained by changes in miRNA expression caused by HBV and HCV infections.

Verification of Regulation of Candidate Target Genes by miRNA. Anti-miRNAs (Ambion) specific to 13 miRNAs (has-miR-17*, has-miR-20a, has-miR-23a, has-miR-26a, has-miR-27a, has-miR-29c, has-miR-30a, has-miR-92, has-miR-126, has-miR-139, has-miR-187, has-miR-200a, and has-miR-223) showing significant differences in expression were transfected into Huh7 cells to examine loss of function of the miRNAs. Five miRNAs (has-miR-23a, has-miR-26a, has-miR-27a, has-miR-92, and has-miR-200a) showed a decreased expression by more than 50%. Precursor miRNAs of these miRNAs were also transfected into the cells to examine the gain of function of the miRNAs (Supplementary Fig. 2). It was confirmed that the expressions of target genes of the five miRNAs (LIG4 [by has-miR-26a]; RGL2 [by has-miR-23a]; Rad51C [by has-miR-27a]; KAP3, CDC25B, KAP3, CDK2AP2, POLD, and CPSF4 [by has-miR-200a]; and TUBGCP2, SNX15 and BCAT2 [by has-miR-92]) were increased by the suppression of the miRNAs induced by anti-miRNAs and were decreased by the overexpression of precursor miRNAs (Supplementary Fig. 3).

Discussion

miRNA plays an important role in various diseases such as infection and cancer.[1-3] In this study, we examined miRNA expression profiles in normal liver and HCC, including nontumor lesions infected with HBV or HCV. Although the expression profiles of miRNAs in HCC have been reported,[16-18] most of the studies were performed using a microarray system. Because we thought that miRNAs could not produce enough detection signals owing to their short length, we applied a highly sensitive and quantitative RTD-PCR method for miRNAs. Moreover, global gene expression in the same tissues was ana-

Table 4-1. Pathway Analysis of Targeted Genes by miRNAs that Were Commonly Repressed in CH-B, CH-C, HCC-B, and HCC-C Compared with Normal Liver (Cluster 1)

No.	Pathway Name	P Value
Down-regulated miRNA in CH-B,HCC-B,CH-C and HCC-C (possibly up-regulating target genes)		
1	Cell adhesion_Platelet-endothelium-leukocyte interactions	1.11E-02
2	Cell cycle_S phase	2.18E-02
3	Protein folding_Protein folding nucleus	2.43E-02
4	Cell cycle_G1-S	3.07E-02
5	Development_Cartilage development	3.89E-02
6	Protein folding_Folding in normal condition	3.89E-02
7	Proteolysis_Connective tissue degradation	3.99E-02
8	Proteolysis_Proteolysis in cell cycle and apoptosis	4.31E-02
9	Signal Transduction_BMP and GDF signaling	5.81E-02
10	Immune_Antigen presentation	6.05E-02

lyzed via cDNA microarray to examine whether the differentially expressed miRNAs could regulate their target genes. Because the absolute standard of miRNA is not available at present, and miRNA expression was compared within the samples and genes analyzed in this study, there might be possible errors when a larger number of samples and genes were analyzed.

Using these systems, we found that the expression profile in miRNAs was clearly different according to HBV and HCV infection for the first time. The differences were confirmed by the nonsupervised learning method, hierarchical clustering (Fig. 2A), and supervised learning methods based on SVM at an 87% accuracy ($P < 0.001$) (Table 2-1). As similarly described, the expression profile in miRNAs was significantly different according to the progression of liver disease (normal, CH, and HCC) in this study. The present CH and HCC expression data were derived from the same patient, and some microarray analyses suggested that the noncancerous liver tissue can predict the prognosis of HCC.[19,20] We examined whether the miRNA expression of paired samples was similar or independent using the Dunnett test[12] (Supplementary Data). Our data indicated that miRNA expression profiling was more dependent on the disease condition than on the paired condition, although the issue of paired samples should be taken into account carefully.

Binary tree prediction analysis and detailed assessment of hierarchical clustering revealed two types of differential miRNAs, one associated with HBV and HCV infection, the other associated with the stages of liver disease that were irrelevant to the differences in HBV and HCV infection. We found that differences in miRNA expression between liver tissues with HBV and HCV (HBV/HCV) were strongly correlated with those in miRNA between cultured cell models of HBV and HCV infection (HBV/HCV) ($r = 0.73$ $P = 0.0006$) (Fig. 5). Thus, there exist HBV- and HCV-infection–specific miRNAs that potentially regulate viral replication and host gene signaling pathways in hepatocytes.

Table 4-2. Pathway Analysis of Targeted Genes by Differentially Expressed miRNAs Between HBV-Related Liver Disease (CH-B,HCC-B) and HCV Related Liver Disease (CH-C,HCC-C Cluster 2)

No.	Pathway Name	P Value
Down-regulated miRNA in CH-C,HCC-C (possibly up-regulating target genes)		
1	Immune_Phagosome in antigen presentation	5.80E-04
2	Muscle contraction	1.05E-03
3	Immune_Antigen presentation	5.75E-03
4	Cell cycle_Meiosis	1.49E-02
5	Reproduction_Male sex differentiation	2.06E-02
6	Cell adhesion_Platelet aggregation	2.77E-02
7	Transport_Synaptic vesicle exocytosis	3.56E-02
8	Inflammation_Kallikrein-kinin system	3.73E-02
9	Inflammation_IgE signaling	4.10E-02
10	Development_Skeletal muscle development	5.02E-02
Down-regulated miRNA in CH-B,HCC-B (possibly up-regulating target genes)		
1	Signal Transduction_Cholecystokinin signaling	1.15E-04
2	Inflammation_NK cell cytotoxicity	5.29E-03
3	Signal transduction_CREM pathway	5.31E-03
4	Reproduction_GnRH signaling pathway	7.80E-03
5	DNA damage_DBS repair	1.02E-02
6	Cell cycle_G2-M	1.63E-02
7	Development_Neuromuscular junction	2.07E-02
8	Apoptosis_Apoptosis mediated by external signals	2.42E-02
9	Reproduction_FSH-beta signaling pathway	2.92E-02
10	Cell adhesion_Amyloid proteins	3.81E-02

Table 4-3. The Pathway Analysis of Targeted Genes by Differentially Expressed miRNAs Between CH and HCC (Cluster 3)

No.	Pathway Name	P Value
	Down-regulated miRNA in HCC (possibly up-regulating target genes)	
1	Cytoskeleton_Spindle microtubules	2.15E-03
2	Transcription_Chromatin modification	5.27E-03
3	Proteolysis_Ubiquitin-proteasomal proteolysis	6.43E-03
4	Cell adhesion_Cell-matrix interactions	7.30E-03
5	Cell cycle_Meiosis	7.83E-03
6	DNA damage_Checkpoint	1.69E-02
7	Reproduction_Progesterone signaling	1.94E-02
8	Apoptosis_Apoptotic mitochondria	3.14E-02
9	Translation_Regulation of initiation	4.22E-02
10	Signal transduction_WNT signaling	4.26E-02
	Up-regulated miRNA in HCC (possibly down-regulating target genes)	
1	Inflammation_IgE signaling	1.05E-02
2	Inflammation_Kallikrein-kinin system	2.46E-02
3	Inflammation_Innate inflammatory response	2.51E-02
4	Inflammation_Histamine signaling	4.25E-02
5	Inflammation_Neutrophil activation	4.55E-02
6	Chemotaxis	4.68E-02
7	Inflammation_IL-12,15,18 signaling	5.16E-02
8	Inflammation_NK cell cytotoxicity	7.25E-02
9	Cell cycle_G0-G1	7.53E-02
10	Inflammation_Complement system	7.72E-02

	HBV/HCV	
	Tissue	Huh7.5
hsa-miR-20	0.61	0.36
hsa-miR-23a	-1.12	-1.3
hsa-miR-27a	-0.75	-1.51
hsa-miR-34c	-2.29	N.D.
hsa-miR-105	2.13	N.D.
hsa-miR-124b	-1.63	-10.54
hsa-miR-133b	1.28	-3.64
hsa-miR-134	2.52	-0.63
hsa-miR-142-5p	-1.34	-4.39
hsa-miR-151	0.86	-0.29
hsa-miR-182*	1.16	0.37
hsa-miR-190	1.04	1.32
hsa-miR-191	0.48	1.16
hsa-miR-193	0.74	-0.03
hsa-miR-194	0.74	0.76
hsa-miR-211	4.68	5.26
hsa-miR-324-5p	0.59	1.16
hsa-miR-340	0.57	1.68
hsa-let-7a	-1.14	-4.51

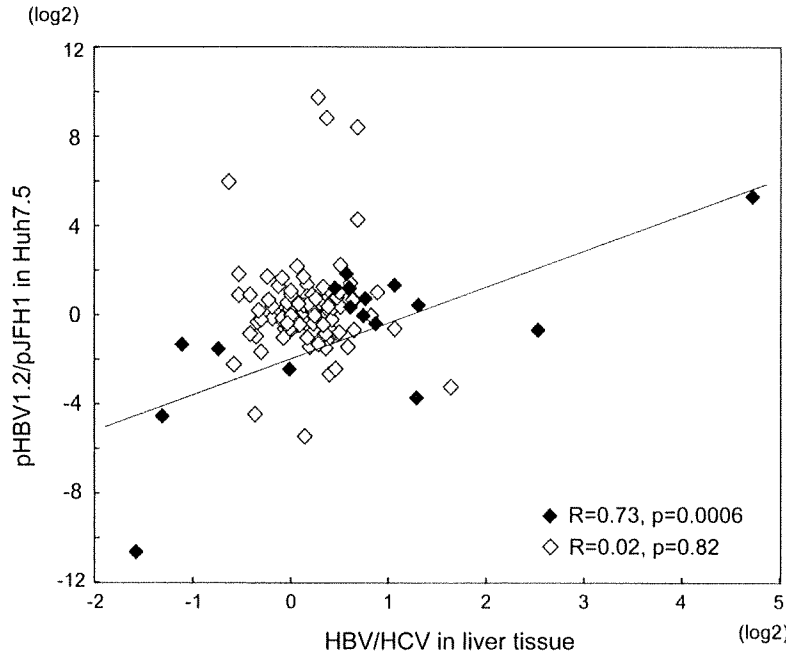

Fig. 5. Correlation between differences in miRNA expression between liver tissues infected with HBV and HCV and those in miRNA expression between cultured cell models of HBV and HCV infections. A total of 140 of 188 miRNAs were confirmed to be expressed in Huh7.5 cells. There was a significant correlation of infection-associated miRNA (closed lozenge) in vitro and in vivo ($r = 0.73$, $P = 0.0006$), but none for the other 121 miRNAs (open lozenge) ($r = 0.02$, $P = 0.82$).

The pathway analysis of targeted genes by miRNAs revealed that 13 miRNAs exhibiting a decreased expression in the HCV group regulate genes related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism. Six miRNAs showing a decreased expression in the HBV group regulate genes related to cell death, DNA damage and recombination, and transcription signals. These findings reflected differences in the gene expression profile between CH-B and CH-C as described.[10] Many of the miRNAs were down-regulated in the HCV group rather than in the HBV group. It has been reported that human endogenous miRNAs may be involved in defense mechanisms, mainly against RNA viruses.[21] On the other hand, it is suggested that endogenous miRNAs may be consumed and reduced by defense mechanisms, especially those against RNA viruses.

Although the expressions of these HBV- and HCV-infection–specific miRNAs were irrelevant to the differences in CH and HCC (Fig. 3, cluster 2), some of them have been reported to play pivotal roles in the occurrence of cancer. For example, has-let-7a regulates ras and c-myc genes,[22] and has-miR-34 is involved in the p53 tumor suppressor pathway.[23] These miRNAs were down-regulated in the HBV group, possibly participating in a more aggressive and malignant phenotype in HCC-B rather than in HCC-C. High expression of has-miR-191 was shown to be significantly associated with the worse survival in acute myeloid leukemia,[24] and has-miR-191 was overexpressed in the HBV group compared with the HCV group. On the other hand, has-miR-133b, which was reported to be down-regulated in squamous cell carcinoma,[25] was repressed in the HCV group compared with the HBV group. Some hematopoietic-specific miRNAs such as has-miR-142-5p were up-regulated in the HCV group. Therefore, these miRNAs were not only HBV and HCV infection–associated but also tumor-associated. These findings indicate different mechanisms of development of HCC infected with HBV and HCV (Fig. 6).

Following HCC development, common changes in miRNA expression between HCC-B and HCC-C appeared (Fig. 3, cluster 3). The 23 miRNAs mentioned above clearly differentiated CH and HCC that were irrelevant to the differences in HBV and HCV infections. Seventeen miRNAs were down-regulated in HCC, which up-regulated cancer-associated pathways. Six miRNAs were up-regulated in HCC that down-regulated all inflammation-mediated signaling pathways, potentially reflecting impaired antitumor immune response in HCC. These results suggest that common signaling pathways are involved in HCC development from CH, and that HBV- and HCV-specific miRNAs participate in generating HCC-specific miRNA expressions (Fig. 6). Therefore, these miRNAs might be good candidates for molecular targeting to prevent HCC occurrence, because they reg-

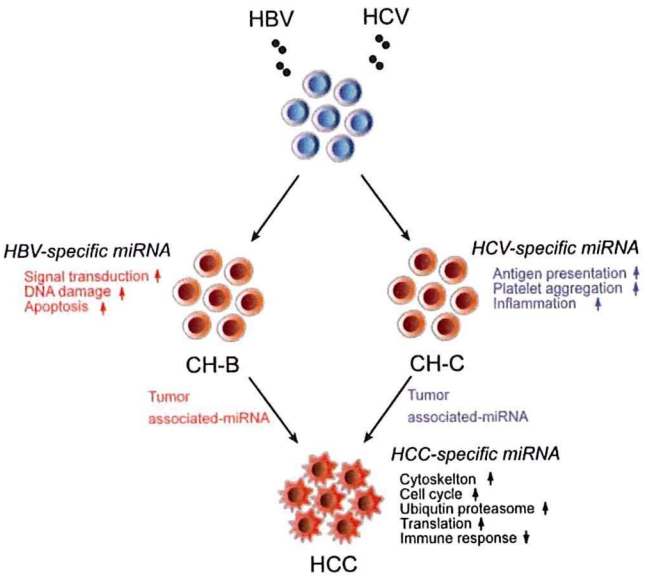

Fig. 6. Infection-associated and HCC-specific miRNAs and liver disease progression.

ulate a common signaling pathway underlying HCC-B and HCC-C development.

In conclusion, we showed that miRNAs are important mediators of HBV and HCV infections as well as liver disease progression. Further studies are needed to enable more detailed mechanistic analysis of the miRNAs identified here and to evaluate the usefulness of miRNAs as diagnostic/prognostic markers and potential therapeutic target molecules.

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Altered Hepatic Gene Expression Profiles Associated With Myocardial Ischemia

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Background—Acute coronary syndrome is sometimes accompanied by accelerated coagulability, lipid metabolism, and inflammatory responses, which are not attributable to the cardiac events alone. We hypothesized that the liver plays a pivotal role in the pathophysiology of acute coronary syndrome. We simultaneously analyzed the gene expression profiles of the liver and heart during acute myocardial ischemia in mice.

Methods and Results—Mice were divided into 3 treatment groups: sham operation, ischemia/reperfusion, and myocardial infarction. Mice with liver ischemia/reperfusion were included as additional controls. Marked changes in hepatic gene expression were observed after 24 hours, despite the lack of histological changes in the liver. Genes related to tissue remodeling, adhesion molecules, and morphogenesis were significantly upregulated in the livers of mice with myocardial ischemia/reperfusion or infarction but not in those with liver ischemia/reperfusion. Myocardial ischemia, but not changes in the hemodynamic state, was postulated to significantly alter hepatic gene expression. Moreover, detailed analysis of the signaling pathway suggested the presence of humoral factors that intervened between the heart and liver. To address these points, we used isolated primary hepatocytes and showed that osteopontin released from the heart actually altered the signaling pathways of primary hepatocytes to those observed in the livers of mice under myocardial ischemia. Moreover, osteopontin stimulated primary hepatocytes to secrete vascular endothelial growth factor-A, which is important for tissue remodeling.

Conclusions—Hepatic gene expression is potentially regulated by cardiac humoral factors under myocardial ischemia. These results provide new insights into the pathophysiology of acute coronary syndrome. **(*Circ Cardiovasc Genet.* 2010;3:68-77.)**

Key Words: coronary disease ■ genetics ■ liver ■ myocardial infarction

In addition to chest pain, acute coronary syndrome (ACS) is sometimes accompanied by systemic manifestations, such as proinflammatory responses, activation of the coagulation-fibrinolytic system, and lipid metabolism.[1–3] These are considered to be systemic reactions involving multiple organs, which exacerbate the cardiac events.

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C-reactive protein, coagulation factors, and protein C, the levels of which fluctuate in ACS, are liver-specific factors. Although these reports were based on a limited number of factors, the observations suggest a close relation between the liver and myocardial ischemia and imply that the liver plays a pivotal role in the pathophysiology of ACS.

cDNA microarray technology allows simultaneous analysis of the expression levels of thousands of genes. Genome-based expression profiling provides useful information on the molecular pathogenesis of various diseases as well as disease progression and prognosis.[4–7] Previous microarray studies have examined the molecular dynamics of the myocardium induced by myocardial ischemia.[8,9] However, global gene expression analyses applied to the liver affected by myocardial ischemia have not been reported.

In this study, we examined the responses of hepatic gene expression to myocardial ischemia. Given the systemic inflammation that characterizes ACSs, we postulated that regulation of hepatic genes occurs by inflammatory mediators and not by alterations in hemodynamics or hepatic perfusion. Therefore, we used whole-genome transcriptional profiling to identify hepatic genes selectively regulated in myocardial ischemia.

Methods

This study was approved by institutional and governmental animal research committees and was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). C57BL/6J mice (n=46; body weight, 24.1±1.4 g; 8 to 10

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Table 1. Biochemical Assessment

		6 h				24 h			
	Before Operation	Sham	I/R	Infarction	Liver I/R	Sham	I/R	Infarction	Liver I/R
No.	5	5	5	5	5	6	5	5	5
CPK, U/L	944±98	5031±646	11597±1272*	19830±1154*	8673±1379	1702±181	1913±184	2939±515†	1595±349
AST, U/L	94±4	674±41	899±21*	1858±59*	414±43	200±19	277±14	661±28*	163±22
ALT, U/L	58±4	119±9	115±6	153±7	143±18	46±3	64±6	107±11*	42±3
LDH, U/L	652±32	2684±206	3432±80†	5264±111*	2478±446	681±72	867±37	2095±164*	862±209

Values are presented as mean±SE. CPK indicates creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; and LDH, lactate dehydrogenase.

*$P<0.01$ compared with sham.

†$P<0.05$ compared with sham.

weeks of age; Charles River Laboratories, Yokohama, Japan) were divided into the following treatment groups: sham operation (n=11), ischemia/reperfusion (I/R; n=10), myocardial infarction (MI; n=10), liver I/R (n=10), and sham operation plus hydralazine (n=5). Hepatic gene expression was evaluated among these groups, and the results were further investigated in primary mouse hepatocytes.

Additional Methods

An expanded Methods section containing details of animal surgery, hydralazine group, liver I/R group, blood sampling and analysis, histopathological analysis, blood pressure and heart rate measurements, microarray experiments, processing of cDNA microarray data, extraction of significantly upregulated cardiac and hepatic genes, pathway analysis, ELISA for secreted osteopontin and vascular endothelial growth factor (*VEGF*), primary hepatocyte experiments, and quantitative real-time detection polymerase chain reaction (RTD-PCR) is available in the online-only Data Supplement.

All microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database with the series accession number GSE14843.

Data Analysis

The data are presented as the mean±SEM for each group of mice and were analyzed by ANOVA with Bonferroni post hoc test for multiple comparisons. Statistical analyses of blood sampling, blood pressure, and heart rate were performed with the Steel (heterogeneity of variance) multicomparison test. Significance was set at $P<0.05$. Statistical analyses were performed with SAS statistical software (SAS Institute Japan, Tokyo, Japan).

Results

Establishment of Cardiac I/R or MI in Mice

Cardiac I/R or MI was successfully induced in normal C57BL/6J mice. The levels of cardiac enzymes, such as creatine kinase, aspartate aminotransferase, and lactate dehydrogenase, increased significantly after 6 hours in the I/R group and showed markedly greater increases in the infarction group compared with the sham group (Table 1). In addition, the normalization of these enzyme levels was reduced after 24 hours in the infarction group.

Histologically, azan or hematoxylin/eosin staining showed wall thinning, coagulation necrosis, and transmural fibrosis in the risk area in the infarction group but not in the I/R group (data not shown). As shown in Table 2, no significant differences were found in heart rate or blood pressure after 24 hours compared with the preoperative values in the sham and I/R groups, whereas a decrease in blood pressure was found in the infarction group.

Histological Assessment of the Liver After Cardiac I/R or MI

The I/R and infarction groups showed a minimal, but transient, increase in alanine aminotransferase ALT. Although alanine aminotransferase may be released from the myocardium[10] rather than from the liver, to exclude the effect of the transient change in hepatic venous pressure associated with cardiogenic shock, we examined histological changes in the liver after myocardial I/R or infarction. No histological abnormalities were observed in the shocked liver, as indicated by the lack of hepatocyte necrosis in acinar zone 3 in the sham, I/R, and infarction groups (Figure 1a, 1c, 1e, and 1g; hematoxylin/eosin staining). In addition, no signs of liver congestion were observed, as indicated by the lack of dilatation of the terminal hepatic venules and adjacent sinusoids in the sham, I/R, or infarction group (Figure 1b, 1d, 1f, and 1h; silver staining).

On transmission electron microscopy, no ischemic changes, such as swelling or loss of cristae in the mitochondria, a mixed irregular pattern or swelling of the rough endoplasmic reticulum, or dilatation or indistinct appearance of the sinusoids, were observed in the sham, I/R, or infarction group (Figure 2A through 2C). Based on these results, histological analysis did not demonstrate the presence of shock or congestive liver in the I/R or infarction group.

Changes in the Hepatic Gene Expression Profile After Cardiac I/R or MI

Although no histological changes were observed in the liver after cardiac I/R or MI, significant changes in gene expression were noted. Hierarchical clustering analysis, which is a nonsupervised learning method that includes 23 281 nonfil-

Table 2. HR, sBP, and mBP

		24 h		
	Before Operation	Sham	I/R	Infarction
HR, bpm	575±27	553±30	553±27	568±16
sBP, mm Hg	105±2	102±2	95±1	80±4*
mBP, mm Hg	78±3	74±2	63±2	56±4†

Values are presented as mean±SE. HR indicates heart rate; sBP, systolic blood pressure; and mBP, mean blood pressure.

*$P<0.01$ compared with sham.

†$P<0.05$ compared with sham.

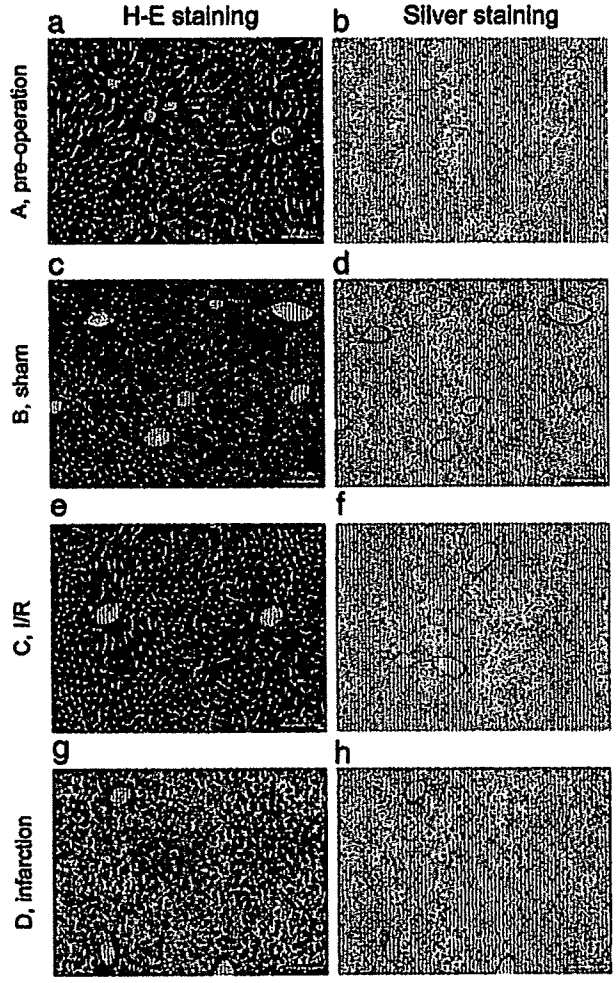

Figure 1. Histological comparison of hematoxylin/eosin staining and silver staining of the liver after 24 hours. Preoperation (A), sham (B), I/R (C), and infarction (D). Scale bars represent 100 μm. Hematoxylin/eosin staining (a, c, e, and g); silver staining (b, d, f, and h). No indication of shock or congestive liver was observed in any group (magnification, ×200).

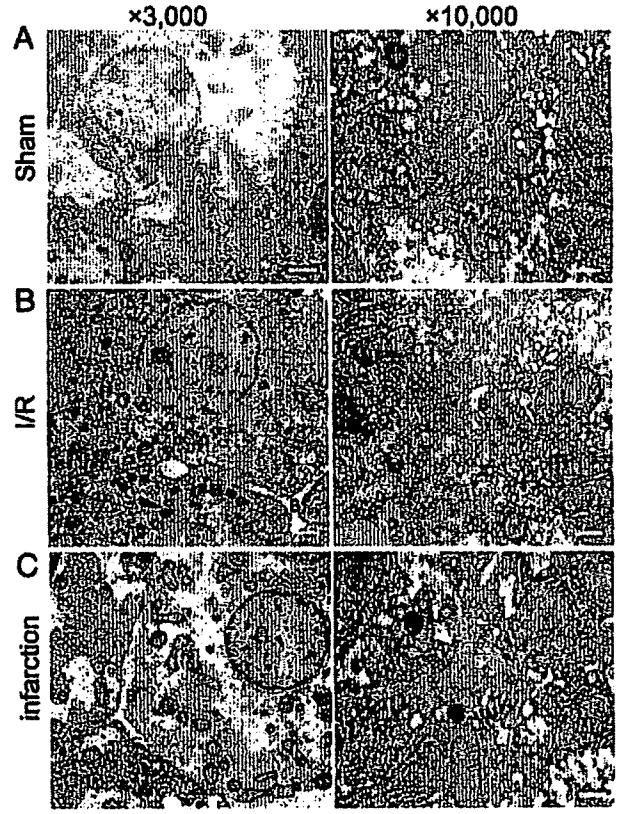

Figure 2. Representative electron microphotographs of the liver after 24 hours. Sham (A), I/R (B), and infarction (C). Scale bars represent 2 μm on the left (magnification, ×3000) and 500 nm on the right (magnification, ×10 000). No indication of shocked liver was observed in any group. M indicates mitochondria; rER, rough endoplasmic reticulum; B, bile canaliculi; N, nucleus.

tered genes, produced clusters for the I/R or infarction group and the sham-operated group (data not shown). Because nonfiltered genes may include those that are unchanged in all samples, which generated "noise" that prevented efficient gene clustering, we filtered out these genes with different stringency and performed hierarchical clustering. Hierarchical clustering with 9165 (log-ratio variations >40th percentile) or 5156 (log-ratio variations >50th percentile) filtered genes clearly demonstrated clusters for the I/R or infarction group after 24 hours, for the I/R or infarction group after 6 hours, and for the sham group after 6 and 24 hours (supplemental Figure I). Hierarchical clustering with 773 (log-ratio variations >80th percentile) or 96 (log-ratio variations >90th percentile) filtered genes showed more detailed and clearer clusters for the I/R group after 24 hours, for the infarction group after 24 hours, for the I/R or infarction group after 6 hours, and for the sham group after 6 and 24 hours (Figure 3). Thus, by filtering out "noise" genes, more detailed and clearer clustering could be obtained, thus addressing the reliability of the analysis.[11] The increased robustness (R-index) and decreased discrepancy (D-index) of clustering with filtering conditions supported this finding (supplemental Figure I; expanded Methods and Results).

Class prediction analysis, a supervised learning method based on the compound covariate predictor, was performed with various clinical parameters, including provocation (I/R or infarction), 6 hours (I/R or infarction after 6 hours), 24 hours (I/R or infarction after 24 hours), and time (sham or 6 hours, sham or 24 hours, and 6 or 24 hours). The results

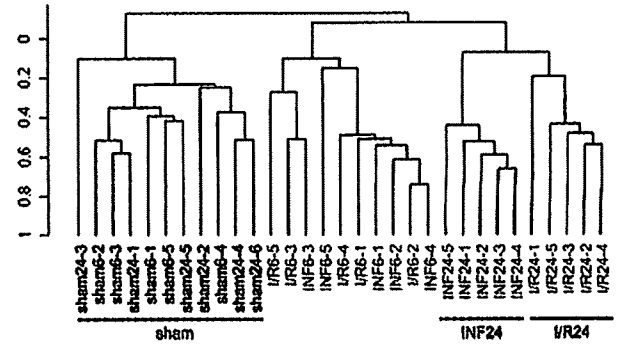

Figure 3. Hierarchical clustering analysis with 96 filtered genes (genes with log-ratio variation in the 90th percentile and data missing >5% were excluded). The resulting dendrogram shows clear clusters for the I/R group after 24 hours, the infarction group after 24 hours, and the sham group after 6 and 24 hours.

Table 3. Class Prediction Analysis (Supervised Learning Methods)

Classifier Category	Clinical Group	Total No. of Classes	No. of Cases Misclassified	Classifier *P*	Mean Percent of Correct Classification	No. of Genes in the Classifiers ($P<0.002$)
Provocation	I/R	10	2	0.02	80	85
	INF	10	2			
6 h	I/R	5	2	0.48	50	23
	INF	5	3			
24 h	I/R	5	0	0.015	100	218
	INF	5	0			
Time	Sham	11	0	0.001	90	644
	6 h	10	2			
	Sham	11	0	<0.0005	95	3380
	24 h	10	1			
	6 h	10	1	0.004	85	747
	24 h	10	2			

INF indicates infarction.

indicated that provocation, 24 hours, and time significantly classified these models (Table 3).

Both nonsupervised and supervised learning methods indicated differences in hepatic gene expression profiling among sham, 6 hours, and 24 hours after heart provocation, and different heart provocation (I/R or infarction) may generate differences in hepatic gene expression, especially 24 hours after provocation.

Identification of Genes Differentially Expressed Between I/R and Infarction

Because the filtering process may result in loss of important genes, for identification of differentially expressed genes among different groups, we used a class comparison analysis tool (http://linus.nci.nih.gov/BRB-ArrayTools.html). Class comparison analysis ($P<0.0005$) among the 5 groups (ie, sham, I/R-6, I/R-24, infarction-6, and infarction-24) was performed, and genes that were differentially expressed among the 5 groups were extracted. On 1-way hierarchical clustering analysis of the extracted genes and heat map, 6 gene clusters were assigned on the basis of the gene expression patterns (Figure 4). Of the 6 groups, group 2 showed significant upregulation for I/R and infarction after 24 hours compared with the other groups. Group 3 showed upregulation for I/R, but not for infarction, after 24 hours. Group 4 showed downregulation for I/R and infarction after 24 hours compared with the other groups. Group 5 showed downregulation for infarction after 24 hours compared with the other groups. Representative genes (>3-fold difference in *t* value) and frequent pathways observed in each group (based on the MetaCore database) are listed in supplemental Tables I through IV.

Interestingly, in group 2, genes related to tissue remodeling, adhesion molecules, and morphogenesis were significantly upregulated. This may be related to the induction of tissue repair factors, such as antigenic factor and myocardiogenic factors, associated with I/R or infarction. In addition, genes involved in the cell cycle and apoptosis and neuron-related genes, such as retinoblastoma 1, angiopoietin-like 4, apoptotic peptidase-activating factor 1, transformation-related protein 53 (*p53*), and Eph receptor B1, were preferentially expressed. The expression of group 2 genes was significantly correlated with serum creatine kinase levels, suggesting that these genes reflect the severity of cardiac damage. Especially, ($R=0.856$, $P<e^{-07}$) and apoptotic peptidase-activating factor 1 ($R=0.856$, $P<e^{-07}$) were highly correlated with creatine kinase (supplemental Table I).

In group 3, in addition to the genes described earlier, chemokine and hormone gene pathways involved in interleukin (IL)-8 and androgen or estrogen receptor signaling were upregulated, suggesting that more tissue repair and bioreactive signaling pathways were activated. This may reflect the presence of a living myocyte I/R condition. In group 4, genes involved in lipid catabolism, immune response, proteolysis, and oxidative stress, such as apolipoprotein A-II, CD7 antigen, and reduced nicotinamide-adenine dinucleotide phosphate oxidase 1, were downregulated in the infarction and I/R groups after 24 hours. In group 5, genes involved in muscle and neurite morphogenesis, such as myosin (heavy polypeptide 11, smooth muscle) and ephrin A5, were significantly downregulated in the infarction group after 24 hours.

Effects of Hemodynamic State on Hepatic Gene Expression Profile

To exclude the possibility that changes in hemodynamic state induced alterations in hepatic gene expression, we examined the livers of mice subjected to liver I/R. For liver I/R, gentle occlusion of the hepatic artery and portal vein was applied so that the extent of liver injury was comparable with those in the myocardial I/R and infarction models (Table 1).

We analyzed the gene expression profile of the liver I/R group by using the same extracted genes as shown in Figure 4. The gene expression patterns induced in the myocardial I/R and infarction groups are clearly different from those in the liver I/R group (Figure 5), except for the group 3 gene cluster in myocardial I/R. It should be noted that the group 3 gene cluster was upregulated in the myocardial I/R group at 24

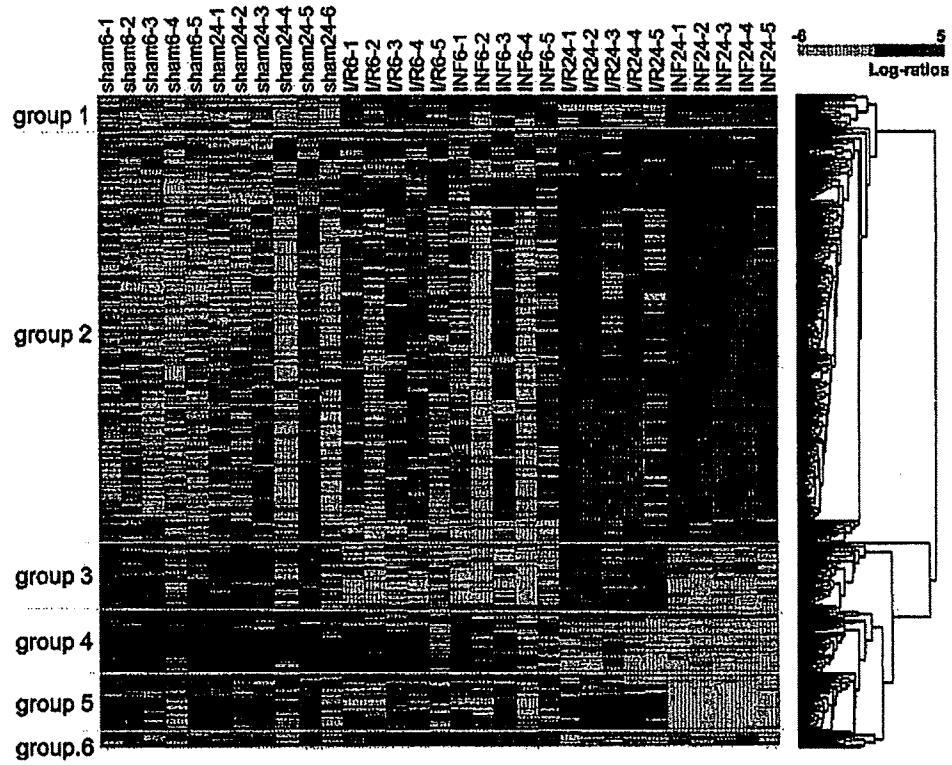

Figure 4. One-way hierarchical clustering and a heat map of 1166 genes that were extracted by class comparison analysis ($P<0.0005$). Each column corresponds to a sample, and each row represents a gene. The gene cluster data are graphically presented as colored images: red indicates upregulated genes, and green indicates downregulated genes. The genes with the most similar patterns of expression are adjacent to one another. Detailed definitions of each group are given in the text. Representative genes and frequently observed pathways are listed in supplemental Tables I through IV.

hours after provocation, whereas it was upregulated from 6 hours after provocation in the liver I/R group. Therefore, the delayed changes in hepatic gene expression in the myocardial I/R and infarction models may be due to different mechanisms resulting from liver I/R.

The assessment of liver weight revealed no differences between the myocardial I/R and infarction groups (supplemental Table V). This result supports our histological findings and indicates an absence of liver congestion in the myocardial I/R and infarction groups.

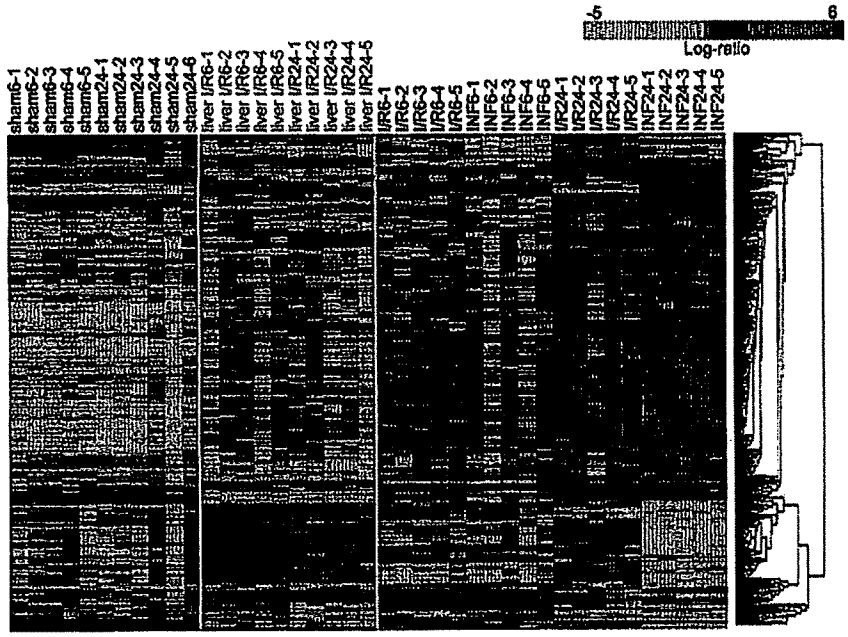

Figure 5. One-way hierarchical clustering and a heat map of the liver I/R group and others with the same extracted genes as shown in Figure 4. Each column corresponds to a sample, and each row represents a gene. The gene cluster data are graphically presented as colored images: red indicates upregulated genes, and green indicates downregulated genes. The genes with the most similar patterns of expression are adjacent to one another. Gene expression patterns induced in the liver I/R group clearly differed from those in the myocardial I/R and infarction groups.

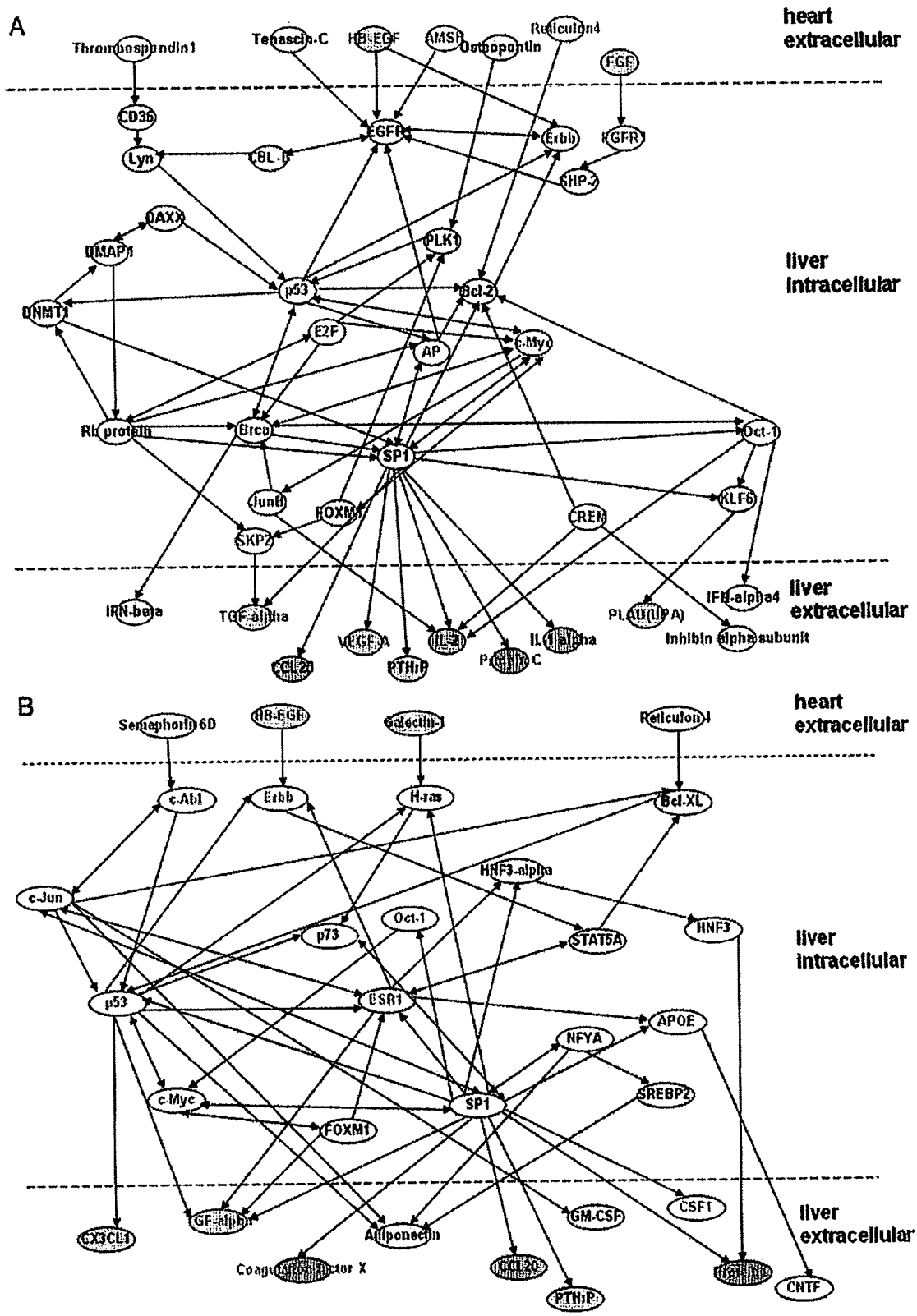

Figure 6. A, Postulated gene network of differentially expressed genes in infarction. B, Postulated gene network of differentially expressed genes in I/R. Detailed definitions of heart-extracellular, liver-intracellular, and liver-extracellular are given in the Methods. Yellow ovals indicate genes related to angiogenesis; green ovals, genes related to coagulation-fibrinolysis; blue ovals, genes related to inflammation; and red characters, genes upregulated in microarray analysis of primary hepatocytes treated with osteopontin. The network diagrams consist of representative genes. All abbreviations are defined in supplemental Tables VI through XI.

Detailed Gene Network Analysis Between the Liver and Heart in Myocardial Ischemia

Several factors can affect the liver, including humoral factors released from the ischemic myocardium, the hemodynamic state, or the autonomic nervous system. We focused on the possibility that humoral factors released from the heart may affect the liver. Cardiac gene expression profiles induced by myocardial ischemia were investigated to identify cardiac genes affecting the liver. To obtain a detailed and comprehensive gene network for the liver and heart, individual data from the liver after 24 hours were integrated with pooled data from the risk area and nonrisk area of the heart. Initially, we divided the heart and liver genes into 3 groups: heart-extracellular, liver-intracellular, and liver-extracellular. To find the network among these induced genes, published results for the interactions of individual genes were integrated with these results by using MetaCore software (GeneGo, St. Joseph, Mich). Direct interactions between individual genes were sought. Genes were excluded according to the following criteria: (1) heart-extracellular, no output signal into liver-intracellular; (2) liver-intracellular, no bidirectional signals; and (3) liver-extracellular, no input signal from liver-intracellular. As expected, the network of these differentially expressed genes involved complex interactions of individual genes; however, representative signaling pathways for MI or I/R injury were identified (Figure 6).

During MI, fibroblast growth factor, osteopontin, and heparin-binding epidermal growth factor-like growth factor (*HB-EGF*) were upregulated in the heart and may have been systemically secreted. Endothelial growth factor receptor and fibroblast growth factor receptor-1 may play important roles in receiving these signals in the liver. Transcription factors such as *p53*, myelocytomatosis oncogene, *trans*-acting transcription factor 1, and octamer-binding transcription factor 1 are important molecules in the regulation of these signaling pathways. Protein C, VEGF-A, and urokinase were expected to be systemically secreted from the liver (supplemental Tables VI through VIII). After infarction, genes involved in inflammation, the coagulation-fibrinolytic system, and angiogenesis showed preferential expression. After I/R, heparin-binding epidermal growth factor was upregulated in the heart and was expected to be systemically secreted. V-*erb*-a erythroblastic leukemia viral oncogene homolog 4 (avian) may play an important role in receiving these signals in the liver. Transcription factors such as *trans*-acting transcription factor 1, *p53*, estrogen receptor-1α, and signal transducer and activator of transcription 5A are potentially important molecules for regulation of these signaling pathways. Protein C, coagulation factor X, ciliary neurotrophic factor, and colony-stimulating factor-1 (macrophage) (*CSF-1*) were expected to be systemically secreted from the liver. In I/R, angiogenesis-related genes were preferentially upregulated (supplemental Tables IX through XI). On comparison of the expression profiles of the heart and liver, genes expressed at significantly higher levels in the heart than in the liver were designated as He, and those expressed at significantly higher levels in the liver than in the heart were designated as Li. Genes expressed in both the heart and liver were described as He/Li (supplemental Tables VIII and XI). In this analysis, most of the factors that were expected to be secreted from the liver induced by I/R and infarction were liver-specific. Most of these genes were not significantly upregulated in the liver I/R groups.

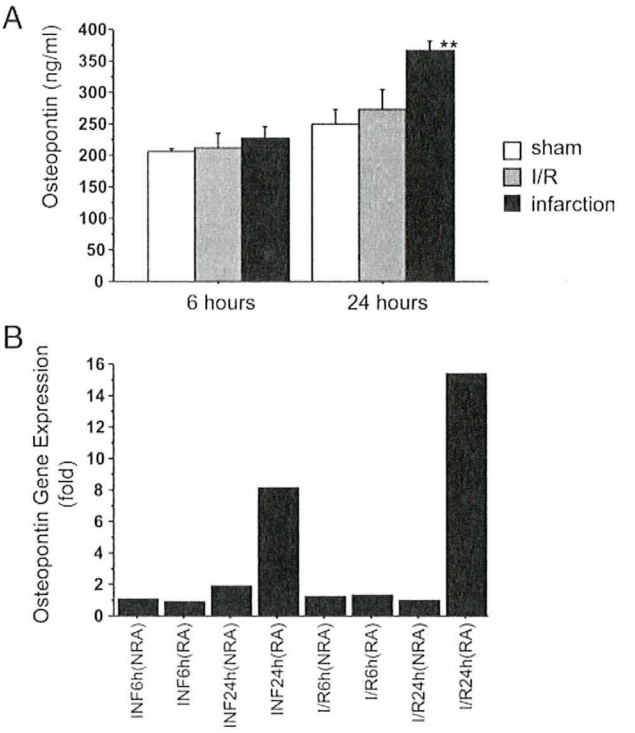

Figure 7. A, The time course of the serum osteopontin concentrations in sham, I/R, and infarction groups. The assessment of serum osteopontin by ELISA in the sham, I/R, and infarction groups after 6 and 24 hours. The serum concentrations of osteopontin after 24 hours were 365.7±14.6 ng/mL, 273.0±30.6 ng/mL, and 249.2±23.4 ng/mL in infarction, I/R, and sham groups, respectively. Error bars represent the SEM. **$P<0.01$ compared with sham. B, The changes in osteopontin gene expression in the infarcted and reperfused heart. INF indicates infarction; NRA, nonrisk area; RA, risk area.

Serum Osteopontin Concentrations in Mice

Of the infarction-induced, cardiac-secreted factors that were expected to stimulate multiple liver genes, we quantified the serum levels of osteopontin by ELISA. Serum osteopontin concentration was significantly increased in the infarction group compared with the sham group ($P=0.0012$) after 24 hours (Figure 7A). In addition, the changes in osteopontin gene expression in the infarcted and reperfused heart are shown in Figure 7B.

Signaling Pathway in Primary Hepatocytes Treated With Osteopontin

To determine whether ischemia-induced, cardiac-secreted factors affected hepatic gene expression, we investigated the effects of osteopontin on primary mouse hepatocytes (supplemental Materials and Methods); 979 genes were upregulated and 734 genes were downregulated ($P<0.05$ and fold change >2.0 determined by class comparison analysis) by osteopontin in primary hepatocytes (GSE14843). The most frequent pathway processes observed among upregulated genes as determined with the use of MetaCore software are

shown in supplemental Table XII. Osteopontin upregulated signaling pathways of protein C, angiogenesis, cell adhesion, etc, which were observed in groups 2 and 3 gene clusters in the mouse liver under conditions of myocardial ischemia (Figure 4; supplemental Tables I and II). The role of osteopontin in the postulated gene network connecting the liver and heart in myocardial ischemia is shown in Figure 6. Interestingly, many of the genes included in the postulated gene network were actually activated by osteopontin ($P<0.05$ or fold change >2.0 by class comparison analysis) in primary hepatocytes. Unexpectedly, osteopontin activated *HB-EGF*, thrombospondin 1, and fibroblast growth factor, which were released from the ischemic heart (Figure 6; supplemental Table VI) in primary hepatocytes. These results indicated that these proteins were released from the liver and from the heart under conditions of myocardial ischemia through osteopontin, and an autocrine signaling pathway may exist in the liver.

Among the candidate hepatic-secreted factors under conditions of myocardial ischemia (Figure 6A; supplemental Table VIII), we quantified the levels of *VEGF-A* in the supernatants of primary hepatocytes treated with osteopontin. The concentration of *VEGF-A* measured by ELISA was significantly increased in the supernatants of primary hepatocytes treated with osteopontin (n=6) compared with the mock group (n=7; $P=0.0042$; supplemental Figure II). Thus, important factors for tissue remodeling could be released from the liver through humoral factors, such as osteopontin, that are released from the heart under conditions of myocardial ischemia.

Quantitative RTD-PCR

We performed a quantitative RTD-PCR with TaqMan probes. In the I/R group, protein C, coagulation factor X, *CNTF*, and *CSF-1* were upregulated in the liver. In the infarction group, protein C, urokinase, and *VEGF-A* were upregulated in the liver (supplemental Figure IIIA). In the hepatocytes treated with osteopontin, protein C, coagulation factor X, ciliary neurotrophic factor, *CSF-1*, urokinase, and *VEGF-A* were upregulated compared with the mock group (supplemental Figure IIIB). These results were consistent with those of cDNA microarray analyses performed in this study.

Discussion

The liver is an essential organ that synthesizes many bioactive proteins, including acute-phase inflammatory proteins (eg, C-reactive protein and IL-6) and coagulation factors. Therefore, it has been speculated that the liver may be involved in systemic reactions that modify the pathophysiology of ACS, although this possibility has not been addressed in detail.

In this study, we examined the gene expression profiles of the livers of mice affected by myocardial I/R or infarction. Marked changes in hepatic gene expression were observed after 24 hours, despite the lack of histological changes in the liver. These changes were essentially restored to normal after 3 to 7 days (data not shown). These findings may not be due to hemodynamic changes during myocardial I/R or infarction. Instead, inflammatory mediators or humoral factors released from the affected heart may be responsible for the observed alterations in hepatic gene expression. This was further confirmed by investigation of signaling pathways in primary hepatocytes induced by osteopontin, a candidate humoral factor released from the ischemic myocardium in vitro.

To exclude the possibility that these changes in gene expression were due to systemic hypotension during I/R or infarction, we performed an additional experiment involving liver I/R to examine whether a pattern of gene expression similar to that in the myocardial I/R and infarction groups could be observed in the liver. Hepatic gene expression in the liver I/R group was completely different from those in the myocardial I/R and infarction groups, with the exception of a small gene cluster (group 3). Although the group 3 gene cluster was upregulated in both the liver I/R and myocardial I/R groups at 24 hours after provocation, peak expression was delayed in the myocardial I/R group compared with the liver I/R group. A recent report of extended observations of cytokine expression in murine hepatic I/R injury indicated that the levels of expression of tumor necrosis factor-α, IL-1β, and IL-6 peaked within 4 hours and returned to baseline at 24 hours.[12] In contrast, in the myocardial I/R and infarction models, these cytokines peaked $\approx$24 to 48 hours and decreased at 7 days.[13] These findings were consistent with those of this study (data not shown). Therefore, the delayed peak of hepatic gene expression observed in this study may be correlated with the extent of inflammation in the myocardium after destruction of myocytes, rather than changes in the hemodynamic state of the liver. The lack of histological changes in the liver in the myocardial I/R and infarction models supported these suggestions, although the influence of hemodynamic state on hepatic gene expression should be carefully considered.

Interestingly, genes related to tissue remodeling, adhesion molecules, and morphogenesis were significantly upregulated in the livers of mice that were subjected to I/R or infarction. This may be related to the induction of tissue repair factors such as angiogenic or myocardiogenic factors in the heart undergoing I/R or infarction. In support of this notion, in addition to the genes upregulated during infarction, chemokines and hormonal factors, including IL-8, androgen, and estrogen receptor genes, were upregulated during I/R. These findings may reflect the presence of living myocytes and the greater release of tissue repair and bioreactive factors during I/R than during infarction.

A recent study that included a sequential analysis of ischemic mouse heart with quantitative RT-PCR demonstrated expression of IL-1β, IL-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1, and granulocyte-CSF at 6 and 24 hours.[13] These results were essentially consistent with those of our microarray analysis of pooled RNA extracted from heart specimens (data not shown).

In this study, the hepatic RNA samples were not pooled but were used to analyze the hepatic gene expression profiles individually. This strategy was successful, in that our microarray results were consistent with those produced from pooled or nonpooled liver specimens. Moreover, it facilitated the statistical evaluation of differentially expressed genes

among the various groups and revealed dynamic changes in hepatic gene expression through clustering analysis.

We analyzed the network connecting the heart-extracellular genes and liver-intracellular genes induced after I/R injury or infarction by using expression data from pooled heart samples and averaged the expression data for individual liver samples. The results suggested that factors secreted from the heart altered gene expression in the liver. By detailed analysis of signaling pathways, we identified 9 candidate genes (eg, *Osteopontin*, *HB-EGF*, *Reticulon 4*) that were upregulated in the heart and were expected to be systemically secreted and to regulate gene expression in the liver (Figure 6). Moreover, we identified the factors that were expected to be secreted from the liver induced by these signaling pathways, such as protein C, coagulation factor X, *CNTF*, *CSF-1*, and angiogenesis-related genes. These factors were expected to be systemically secreted from the liver and to modulate the pathophysiology and outcome of ACS. It has been reported that protein C prevents myocardial I/R injury,[14] *VEGF* enhances capillary density and improves cardiac function,[15] and urokinase is essential for cardiac functional recovery after acute myocardial infarction.[16]

Of the factors that were expected to be secreted from the heart, we confirmed that infarction increased the serum osteopontin concentration after 24 hours. Osteopontin is essential for the development of myocytes, tissue repair, and angiogenesis, and its downstream products, eg, polo-like kinase, were upregulated in the liver. To confirm these findings, we examined the signaling pathways in primary hepatocytes treated with osteopontin. Osteopontin activated signaling pathways of protein C, angiogenesis, and cell adhesion (supplemental Table XII) by inducing the expression of protein C, urokinase, *VEGF-A*, *CSF-1*, factor X, and ciliary neurotrophic factor (*CNTF*) in primary hepatocytes, which was confirmed by RTD-PCR or ELISA (supplemental Figures II and IIIB). Moreover, many other genes involved in the postulated gene network associating the liver and heart (Figure 6A and 6B) were actually activated in primary hepatocytes treated with osteopontin, confirming this signaling pathway. These results suggest that humoral factors play important roles in signal transduction from the ischemic myocardium to the liver.

Although our results addressed humoral factors from the heart that may affect hepatic gene expression, the effects of other factors, such as autonomic nerves, should also be considered. Because the liver has rich sympathetic and parasympathetic innervation,[17–19] it is possible that sympathetic hyperactivity affects hepatic gene expression. Although hydralazine has been reported to activate sympathetic nerves,[20,21] we observed no differences in gene expression in the hydralazine-treated group compared with the sham-operated group (data not shown). Therefore, autonomic nerves seemed to have little effect on hepatic gene expression determined in this study.

In conclusion, we reported new insights into the pathophysiology of ACS, which may facilitate identification of the mechanisms by which an acute coronary event causes systemic reactions. Further studies are needed to determine whether early therapeutic targeting of the liver during an acute coronary event has any beneficial effect on the clinical outcome in these patients.

Study Limitations

Although we confirmed that the serum osteopontin concentration was increased during myocardial ischemia, other proteins that could potentially be secreted from the heart and liver were not assayed. Further studies are needed to determine whether these proteins, including osteopontin, actually affect hepatic gene expression as observed in this study.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Acute coronary syndrome (ACS) is accompanied by systemic changes in inflammation, coagulation, and metabolism, which may affect the outcome and prognosis of ACS. These systemic reactions are not explained by cardiac events alone. Several lines of evidence suggest that patients with fatty liver disease have a high risk of developing cardiovascular diseases, and it is possible to speculate that the liver is involved in a systemic reaction that modifies the pathogenesis of ACS. However, the relation between liver and myocardial ischemia in the acute ischemic phase has not been elucidated so far. In this investigation, we simultaneously analyzed the gene expression profiles of the liver and heart during acute myocardial ischemia in mice and observed the presence of humoral factors that intervened between the heart and liver. These humoral factors were released from the heart and influenced the liver to secrete important tissue remodeling factors. One of these humoral factors, osteopontin, a widely expressed glycoprotein, was increased in the ischemic heart and altered the gene expression of hepatocytes to produce important tissue remodeling factors (such as vascular endothelial growth factor-A). Our observations suggest that hepatic gene expression is potentially regulated by humoral factors of cardiac origin provoked by myocardial ischemia, and we provide direct evidence that the liver is involved in a systemic reaction that accompanies ACS. Our findings provide potential new insights into the pathophysiology of ACS.

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F Literature Search
G Funds Collection

Knowledge of *Vibrio vulnificus* infection among Japanese patients with liver diseases: A prospective multicenter study

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Summary

Background: *Vibrio vulnificus* (*V. vulnificus*) is a seafood-borne infectious pathogen that can be lethal to humans. The infection has been correlated with pre-existing liver disease, particularly liver cirrhosis. Awareness of *V. vulnificus* infection among Japanese citizens is low, despite the increasing number of patients with hepatocellular carcinoma (HCC). The present study was conducted to assess the level of knowledge of patients with liver disease regarding *V. vulnificus* infection.

Material/Methods: Questionnaires were sent to patients with chronic liver disease who had been treated by liver specialists at 14 medical institutes.

Results: Of 1,336 patients, 304 (22.8%) had liver cirrhosis, and 732 (54.8%) had comorbidities of this disease. Only 14.5% (194/1,336) of patients had knowledge of *V. vulnificus* infection. Of 304 patients with liver cirrhosis, 17.4% (53/304) of the patients had knowledge of *V. vulnificus* infection. Of 60 patients with liver cirrhosis and diabetes mellitus, 11 (18.3%) patients had knowledge of *V. vulnificus* infections. Even when the patients with high risk factors such as liver cirrhosis and diabetes mellitus had knowledge of *V. vulnificus* infections, most ate raw seafood without regard to season.

Conclusions: Patients with chronic liver diseases and their physicians need to be better educated about *V. vulnificus* infection and its prevention.

key words: ***Vibrio vulnificus*** • **liver diseases** • **hepatitis C virus (HCV)** • **hepatocellular carcinoma (HCC)**

Abbreviations: ***V. vulnificus*** – *Vibrio vulnificus*; **HCV** – Hepatitis C virus; **HBV** – Hepatitis B virus; **HCC** – Hepatocellular carcinoma; **PBC** – primary biliary cirrhosis; **AIH** – autoimmune hepatitis; **ICD** – International Classification of Diseases

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