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ORIGINAL ARTICLE

Basic Study

Changes in gene expression in liver tissue from patients with fulminant hepatitis E

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Abstract

AIM: To study host gene expression and number

of immune cells in liver tissues from patients with fulminant hepatitis E (FH-E).

METHODS: Microarray-based expression profiling was done using Illumina Human WG-6_v3_BeadChip arrays on post-mortem liver tissue from 5 patients with FH-E, and compared with similar tissue from 6 patients with fulminant hepatitis B (FH-B; disease controls) and normal liver tissue from 6 persons. Differential expression was defined as ⩾ 2.0-fold change with Benjamini-Hochberg false discovery rate below 0.05 using t-test in liver tissue from FH-B and FH-E, than healthy liver tissue. For some genes that showed differential expression in FH-E, microarray data were validated using quantitative reverse transcription PCR. Differentially expressed gene lists were then subjected to "Gene Ontology" analysis for biological processes, and pathway analysis using BioCarta database on the DAVID server. In addition, tissue sections were stained for $CD4^+$, $CD8^+$ and $CD56^+$ cells using indirect immunohistochemistry; cells staining positive for each of these markers were counted and compared between groups.

RESULTS: Compared to normal livers, those from patients with FH-E and FH-B showed differential expression of 3377 entities (up-regulated 1703, downregulated 1674) and 2572 entities (up 1164, down 1408), respectively. This included 2142 (up 896, down 1246) entities that were common between the two sets; most of these belonged to metabolic, hemostatic and complement pathways, which are active in normal livers. Gene expression data from livers of patients with FH-E but not those of FH-B showed activation of several immune response pathways, particularly those involving cytotoxic T cells. The fold-change values of mRNA for selected genes in livers from FH-E than in normal liver tissue determined using quantitative reverse transcription PCR showed excellent concordance with microarray analysis. At immunohistochemistry, $CD8^+$ T cells showed an increase in liver biopsies from both FH-E [median 53.4 per arbitrary unit area (range 31.2-99.9)] and FH-B [median 49.3 (19.3-51.0); P = 0.005] compared to control liver tissue [median 6.9 (3.1-14.9)].

CONCLUSION: FH-E patients show $CD8^+$ T cell infiltration and increased gene expression of cytotoxic T cell pathways in liver, suggesting a possible pathogenetic role for these cells.

Key words: Cytotoxic T cells; Gene expression; Hepatitis E; Hepatitis E virus; Immune response; Liver biopsy; Microarray; Natural killer cells; Pathogenesis

Core tip: Data on pathogenesis of hepatitis E virus (HEV) infection, which is a common cause of acute hepatitis in several developing countries, are quite limited. This manuscript reports our data on microarray-based gene expression analysis and immunohistochemistry in liver tissue from patients with HEV infection, as compared to liver tissue from patients with hepatitis B virus infection and normal liver tissue. These data advance the current knowledge about the pathogenesis of HEV infection.

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INTRODUCTION

Hepatitis E virus (HEV), a member of genus *Hepevirus* in family *Hepeviridae*, consists of 32-34 nm diameter, icosahedral, non-enveloped virions[1]. It has a 7.2-kb single-stranded, positive-sense RNA genome with three open reading frames (ORFs) that code for a viral non-structural polyprotein (ORF1), the major capsid protein (ORF2) and a phosphoprotein with possible regulatory functions (ORF3)[1]. The virus has at least four genotypes, named 1 to 4[2]; however, these all belong to a single serotype.

HEV infection is common in developing countries of Asia and Africa, where it causes an acute disease, known as hepatitis E, either as water-borne outbreaks or as sporadic cases[3,4], caused by infection through the fecal-oral route. The disease occurs predominantly among young adults and resembles acute hepatitis caused by other hepatotropic viruses. It is usually self-limiting, with overall case fatality rate below 0.5%[3]. However, some patients, in particular pregnant women, develop severe liver injury that progresses to fulminant hepatic failure (FH), which is often fatal. In these areas, the virus belongs most often to genotype 1 and sometimes to genotype 2[3,4].

In areas with lower disease endemicity, such as Europe, North America, Japan, *etc.*, occasional cases with locally-acquired HEV infection have been reported[5]. These are mainly elderly men, often with other co-existing diseases, who are believed to acquire infection with genotype 3 or 4 HEV through ingestion of undercooked meat of HEV-infected animals[6]. In immunosuppressed persons, the infection may become persistent[6].

To develop therapeutic measures against hepatitis E, it is important to understand the pathogenesis of liver injury in this disease. In infection with other hepatotropic viruses, such as the hepatitis A, B and C viruses, tissue injury is mediated not by the infectious agent but is related to the host immune response[7-11]. We have shown that HEV infection is associated with activation of specific cellular immune responses in the

peripheral blood[12-16]. However, little data are available on the liver tissue from hepatitis E. Therefore, the mechanism of liver injury in this disease remains unclear.

We therefore studied gene expression profile in liver biopsies from patients with FH due to acute hepatitis E (FH-E), in comparison with those from healthy adults and patients with FH due to acute hepatitis B (FH-B; a disease control group).

MATERIALS AND METHODS

Patients and specimens

Post-mortem needle liver biopsies were obtained from patients dying of FH-E (n = 5) or of FH-B (n = 6). The biopsies were obtained within 30 min after death, and separate pieces were collected in RNA*Later* (Qiagen) and stored at -80 ℃ for gene expression analysis, and in formalin for histology and immunohistochemistry. In addition, a blood specimen was collected from each subject, for biochemical tests and serological markers of viral hepatitis.

Patients dying more than 2 wk after the onset of disease, and those with major sepsis or clinical evidence of pre-existing liver disease were excluded. Diagnosis of acute hepatitis E was based on detection of IgM anti-HEV (Genelabs, Singapore) in the absence of hepatitis B surface antigen (HBsAg), IgM anti-HBc antibody, anti-hepatitis C virus (HCV) antibody and IgM anti-hepatitis A virus (HAV) antibody (all from BioMerieux, Marcy l'Etoile, France), and that of acute hepatitis B on detection of HBsAg and IgM anti-HBc, in the absence of IgM anti-HEV, anti-HCV and IgM anti-HAV.

In addition, normal liver tissue was obtained from six persons undergoing partial hepatic resection for focal diseases, such as hydatid liver disease, gallbladder cancer without biliary obstruction.

The study was reviewed and approved by Sanjay Gandhi Postgraduate Institute of Medical Sciences institutional review board. Written, informed consent was obtained from all patients or their families, as appropriate.

RNA isolation

Total RNA was isolated from tissue biopsies using RNeasy Protect minikit (Qiagen, Carlsbad, CA). RNA concentration was measured using NanoDrop 1000 spectrophotometer (Nanodrop, Wilmington, DE). RNA integrity was assessed using Bioanalyzer 2100 (Agilent, Santa Clara, CA). Specimens with A260/A280 and A260/A230 ratios > 1.9, and RIN > 8.0 were processed further.

Microarray analysis

Gene expression profiling was done using Illumina Human WG-6_v3_BeadChip arrays (Illumina, San Diego, CA), each containing more than 46000 entities (gene probes or probesets) derived from NCBI, RefSeq and UniGene databases. Poly(A)-RNA was reverse transcribed to complementary DNA (cDNA), using an oligo(dT)-primer that contained a phage T7 RNA polymerase promoter sequence at its 5′-end, followed by conversion to double-stranded cDNA. The double-stranded cDNA was transcribed *in vitro* to yield large quantities of biotin-labelled anti-sense RNA, which was then hybridised to the bead arrays at 55 ℃ for 16-18 h, and scanned using an Illumina iScan reader.

Bioinformatic analysis

The array intensity data were initially analysed using Illumina Genome Studio Gene Expression Module (v1.1.1) (Illumina, Cambridge, United Kingdom) for visualisation and normalisation. After quantile normalisation and background correction using medians within the BeadStudio software, the data were exported for further analysis in GeneSpring GX software 11.5 (Agilent).

Differential expression between various subject groups was analyzed using Illumina Custom Algorithm. The normalized data were first subjected to quality check using principal component analysis. This was followed by analysis to determine genes showing differential expression (fold-change $\geqslant$ 2.0 and Benjamini-Hochberg false discovery rate < 0.05 using *t*-test) in liver tissue from patients with FH-B and FH-E, as compared to healthy liver tissue. Such gene lists were then subjected to "Gene Ontology" (GO) analysis for biological processes, and pathway analysis using BioCarta database on the DAVID server (http://david.abcc.ncifcrf.gov). Separate analyses were done for genes that were differentially expressed in both FH-E and FH-B, and those differentially expressed in only one of these.

Validation of microarray results

For some genes that showed differential expression in FH-E, microarray data were validated using quantitative reverse transcription PCR (qRT-PCR). In brief, cDNA was prepared using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) from 2 μg of RNA. This was followed by real-time PCR in 20-μL reactions comprising of 50 ng cDNA, primers (Appendix 1) and SYBR Green (Applied Biosystems).

The validation assay included liver tissue for all the subjects used for microarray analysis (a biopsy piece other than that used for microarray analysis) and four additional healthy liver tissues. All assays included RNA from peripheral blood mononuclear cells of a healthy person as a calibrator. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S rRNA was used as housekeeping genes. Relative fold-change was determined for each specimen and gene from cycle threshold (Ct) values as follows, and compared to fold-change in expression in microarray data:

Table 1 Characteristics of study subjects in the three groups

Characteristic	Healthy controls (n = 6)	Fulminant hepatitis B (n = 6)	Fulminant hepatitis E (n = 5)
Age (yr)	52 (30-58)	24 (16-60)	28 (18-32)
Gender (Male:Female)	4:2	4:2	1:4
Duration of illness before death (d)	-	9 (6-14)	10 (7-13)
Maximum total serum bilirubin (mg/dL)	0.7 (0.4-1.4)	21 (13.1-41.4)	25 (5.4-32.0)
Serum ALT[a] (IU/L)	30 (21-58)	1939 (524-5795)	770 (240-1302)
Serum AST (IU/L)	28 (18-35)	859 (250-2345)	458 (332-1000)
Alkaline phosphatase (IU/L)	-	257 (131-427)	306 (164-561)
Serum albumin (g/dL)	-	2.8 (2.0-3.3)	2.8 (2.1-3.2)

Data are shown as median (range), except for gender distribution; patients with fulminant hepatitis B and fulminant hepatitis E were comparable in all variables except serum ALT levels ([a]P < 0.05, Mann-Whitney U test). ALT: Alanine aminotransferase.

$\Delta\Delta Ct = [(\Delta Ct_{\text{gene of interest}} - \Delta Ct_{GAPDH})_{\text{patient}} - [(\Delta Ct_{\text{gene of interest}} - \Delta Ct_{GAPDH})_{\text{calibrator sample}}]$ and Fold change = $2^{-\Delta\Delta Ct}$.

Immunohistochemistry on liver biopsies

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin, and examined to confirm the presence of acute inflammation in patients with FH, and absence of disease in controls.

In addition, immunohistochemistry (IHC) was performed for specific T-cell subsets ($CD4^+$ and $CD8^+$) and NK cells ($CD56^+$). In brief, formalin-fixed, paraffin-embedded 3-μm liver sections on silanized glass slides (Dako) were fixed at 60 ℃ overnight. The slides were deparaffinised in xylene and rehydrated in graded alcohol. Antigen retrieval was done in 10 mmol/L EDTA (pH 9.0) at 98 ℃ for 30 min. After washing with distilled water and Tris-buffered saline (TBS; pH 7.4), endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 30 min in dark. Sections were then incubated with monoclonal primary antibodies [prediluted mouse anti-human CD8 (clone C8/144B), CD4 (clone 4B12) or CD56 (clone 123C3) (Dako, Denmark)] for 1 h at room temperature, followed by peroxidase enzyme-labelled secondary antibody (Dako) for 30 min. Diaminobenzidine was used as chromogen to detect the bound antibodies. Slides were counterstained with Mayer's hematoxylin for 1 min, cleared and mounted with DPX (Sigma-Aldrich). Stained cells were counted in five fields, averaged and expressed as number in an area measuring 73000 μm^2.

Statistical analysis

Inter-group comparisons were done using t-test with Benjamini-Hochberg correction in case of gene expression data and Mann-Whitney U test for other quantitative data. Relationship of fold-change in the microarray and real-time PCR data was assessed using Pearson's correlation coefficient. The statistical methods of this study were reviewed by Dr. Rakesh Aggarwal from Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India.

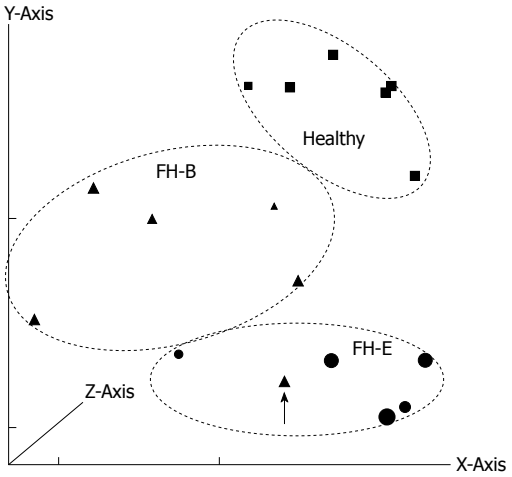

Figure 1 Principal component analysis of gene expression data from various specimens included in the study. The components along X, Y and Z axes were 62.54%, 21.17%, and 8.88%, respectively. Patients with fulminant hepatitis E (FH-E) are represented using circles, those with fulminant hepatitis B (FH-B) using triangles and healthy controls using square symbols. The size of markers varies according to their placement on the Z axis. Patients with FH-E and FH-B clustered separately, except for one patient with FH-B who was an outlier (arrow).

RESULTS

Demographic features and biochemical findings in the three subject groups are shown in Table 1. Alanine aminotransferase levels were higher in FH-B than in FH-E.

Principal component analysis

On principal component analysis of gene expression data (Figure 1), all specimens except one (with FH-B) showed clustering with other specimens in the same group.

Microarray gene expression

A total of 3377 entities, each representing a discrete gene, showed differential expression of ⩾ 2-fold with P-value of < 0.05 in liver tissue from patients with FH-E than that from healthy persons (Figure 2); this included 1703 entities with over-expression and 1674 showing reduced expression in FH-E. In liver tissue from FH-B, 2572 entities showed differential expression than in healthy livers (up-regulation 1164, down-regulation 1408).

Of the entities differentially expressed in FH-E or FH-B compared to normal liver, 2142 were common (up-regulation in both 896, down-regulation in both 1246) (Figure 2, Appendix 2); none of these entities showed discordance in the direction of differential expression between FH-E and FH-B. In contrast, 1235

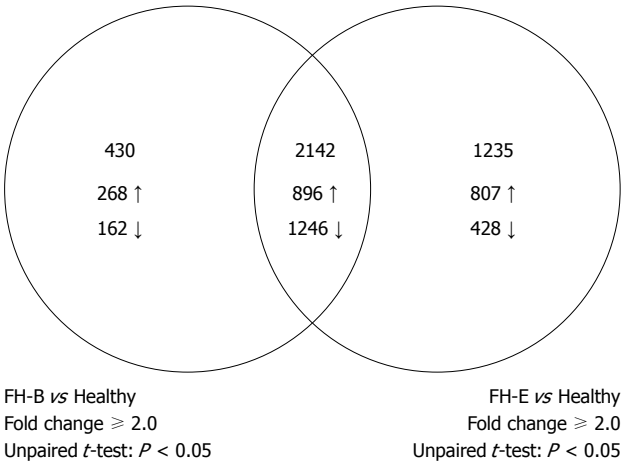

Figure 2 Venn-diagram showing comparison of number of entities differentially expressed in liver tissue from fulminant hepatitis B or E, as compared to healthy liver tissue. The intersection of two circles shows entities that were differentially expressed in both fulminant hepatitis E and fulminant hepatitis B.

entities (Appendix 3) were differentially expressed in FH-E but not in FH-B, and 430 entities (Appendix 4) were differentially expressed in FH-B but not in FH-E.

Gene ontology analysis

Genes differentially expressed in FH-E and FH-B livers: Table 2 lists the biological processes that showed differential regulation on GO analysis of the 2142 entities differentially expressed in both FH-E and FH-B. These included several metabolic processes in which the liver plays an active part, such as general body metabolism, or those whose proteins are mainly synthesized in the liver, such as coagulation pathways and complement system. Further, both the disease conditions showed differential regulation of innate immune responses and humoral immune responses mediated by B cells.

Genes differentially expressed in FH-E but not in FH-B: On GO analysis, the 1235 entities that showed differential expression in FH-E but not in FH-B were related mainly to the immune system pathways (Table 3); for each of these immune pathways, several genes showed upregulation.

Genes differentially expressed in FH-B but not in FH-E: GO analysis of these entities did not reveal significant changes in any specific pathway.

Pathway analysis

Genes differentially expressed in both FH-E and FH-B: Pathway analysis for the 2142 entities differentially expressed in both FH-E and FH-B using the BioCarta database showed downregulation of several pathways related to metabolic processes, hemostasis, and complement system (Table 4). Appendix 5 shows individual genes involved in each of these pathways.

Genes differentially expressed in FH-E but not in FH-B: BioCarta pathways whose genes were significantly over-represented among genes differentially expressed in liver tissue from FH-E compared to normal liver but not in FH-B were mostly related to cellular immune mechanisms (Table 4), *e.g.,* T cytotoxic cell surface molecules, CTL mediated immune response against target cells, T helper cell surface molecules, co-stimulatory signal during T-cell activation, T cell receptor signalling pathway, Lck and Fyn tyrosine kinases in initiation of TCR activation, IL-7 signal transduction, T cell receptor and CD3 complex, IL 17 signalling pathway. Several individual genes involved in these pathways were upregulated in patients with FH-E (Appendix 6).

Genes differentially expressed in FH-B but not in FH-E: Pathway analysis of these 430 entities did not reveal significant change in any pathway.

Validation using qRT-PCR

The fold-change values of mRNA for selected genes (Appendix 1) in livers from FH-E than in normal liver tissue determined using qRT-PCR showed excellent concordance with those at microarray (Figure 3), using either of the two housekeeping genes.

Immunohistochemistry

IHC showed a few $CD4^+$ positive T-cells and occasional NK cells in all the biopsies. In FH-B as well as FH-E, $CD8^+$ T-cells were found in both the liver parenchyma and the portal areas (Figure 4). The median number of $CD8^+$ T cells was greater in liver tissues from both FH-E [median 53.4 (range 31.2-99.9)]; $P = 0.005$, 2-sided Mann-Whitney *U* test) and FH-B [49.3 (19.3-51.0); $P = 0.005$] than in controls 6.9 (3.1-14.9); however, there was no significant difference between FH-E and FH-B.

DISCUSSION

Host tissue injury during a viral infection may be caused either by virus-induced cell death, or by immune-mediated killing of infected cells. Understanding the underlying mechanism may not only help understand the pathogenesis of a particular viral disease but also provide a lead to development of strategies for countering the host injury. In infection with hepatitis A, B or C virus, liver injury is mediated primarily by the host immune response[7-11]. However, the mechanisms of liver injury in HEV infection remain unclear.

Initial attempts at delineation of mechanism of liver injury caused by HEV were based on observations during experimental HEV infection in non-human primates. In these studies, liver injury appeared after viremia and fecal viral excretion had started declining

Table 2 Gene Ontology analysis (biological processes) for gene entities differentially expressed in liver tissue from fulminant hepatitis E as compared to normal liver tissue, as well as in fulminant hepatitis B as compared to normal liver tissue

Gene ontology accession	Gene Ontology term	*P* value
GO:0055114	Oxidation-reduction process	2.68E-40
GO:0006082	Organic acid metabolic process	4.75E-39
GO:0043436	Oxoacid metabolic process	2.17E-38
GO:0019752	Carboxylic acid metabolic process	4.09E-38
GO:0006629	Lipid metabolic process	3.94E-28
GO:0044281	Small molecule metabolic process	9.06E-27
GO:0032787	Monocarboxylic acid metabolic process	3.56E-22
GO:0016054	Organic acid catabolic process	4.22E-19
GO:0046395	Carboxylic acid catabolic process	4.22E-19
GO:0044255	Cellular lipid metabolic process	1.35E-17
GO:0044282	Small molecule catabolic process	5.16E-17
GO:0044712	Single-organism catabolic process	5.16E-17
GO:0006631	Fatty acid metabolic process	2.71E-16
GO:0006520	Cellular amino acid metabolic process	4.43E-16
GO:0044711	Single-organism biosynthetic process	8.25E-14
GO:1901605	Alpha-amino acid metabolic process	1.07E-13
GO:0044283	Small molecule biosynthetic process	1.60E-13
GO:1901564	Organonitrogen compound metabolic process	2.46E-11
GO:0009063	Cellular amino acid catabolic process	4.46E-11
GO:0008610	Lipid biosynthetic process	8.46E-11
GO:0072376	Protein activation cascade	3.87E-10
GO:0008202	Steroid metabolic process	4.10E-10
GO:0016053	Organic acid biosynthetic process	6.26E-10
GO:0046394	Carboxylic acid biosynthetic process	6.26E-10
GO:1901566	Organonitrogen compound biosynthetic process	1.94E-08
GO:0008152	Metabolic process	4.34E-08
GO:1901606	Alpha-amino acid catabolic process	4.58E-08
GO:0072329	Monocarboxylic acid catabolic process	1.40E-07
GO:0007596	Blood coagulation	1.98E-07
GO:0050817	Coagulation	1.98E-07
GO:0007599	Hemostasis	2.83E-07
GO:1901615	Organic hydroxy compound metabolic process	4.14E-07
GO:0042060	Wound healing	4.86E-07
GO:0006956	Complement activation	6.77E-07
GO:0009062	Fatty acid catabolic process	1.71E-06
GO:0006066	Alcohol metabolic process	1.82E-06
GO:0008652	Cellular amino acid biosynthetic process	2.42E-06
GO:0050878	Regulation of body fluid levels	5.46E-06
GO:0051186	Cofactor metabolic process	5.50E-06
GO:0006694	Steroid biosynthetic process	8.32E-06
GO:0044242	Cellular lipid catabolic process	1.09E-05
GO:0005996	Monosaccharide metabolic process	0.000012
GO:0051346	Negative regulation of hydrolase activity	1.28E-05
GO:0005975	Carbohydrate metabolic process	1.35E-05
GO:0010876	Lipid localization	5.30E-05
GO:0044710	Single-organism metabolic process	5.82E-05
GO:0006732	Coenzyme metabolic process	6.57E-05
GO:0010951	Negative regulation of endopeptidase activity	7.22E-05
GO:0019318	Hexose metabolic process	7.95E-05
GO:0006869	Lipid transport	8.02E-05
GO:0065008	Regulation of biological quality	8.96E-05
GO:0010466	Negative regulation of peptidase activity	0.0001
GO:0006006	Glucose metabolic process	0.0001
GO:0042558	Pteridine-containing compound metabolic process	0.0002
GO:0043648	Dicarboxylic acid metabolic process	0.0002
GO:0006958	Complement activation, classical pathway	0.0004
GO:0006091	Generation of precursor metabolites and energy	0.0004
GO:0044262	Cellular carbohydrate metabolic process	0.0004
GO:0006575	Cellular modified amino acid metabolic process	0.0005
GO:0016051	Carbohydrate biosynthetic process	0.0005
GO:0072330	Monocarboxylic acid biosynthetic process	0.0006
GO:0002455[1]	Humoral immune response mediated by circulating immunoglobulin	0.0010
GO:0044723	Single-organism carbohydrate metabolic process	0.0010
GO:0019320	Hexose catabolic process	0.0011
GO:0016052	Carbohydrate catabolic process	0.0014
GO:0044724	Single-organism carbohydrate catabolic process	0.0014
GO:0006820	Anion transport	0.0016
GO:0006007	Glucose catabolic process	0.0017
GO:0046365	Monosaccharide catabolic process	0.0018
GO:0046835	Carbohydrate phosphorylation	0.0020
GO:1901617	Organic hydroxy compound biosynthetic process	0.0020
GO:0002253[1]	Activation of immune response	0.0021
GO:0016125	Sterol metabolic process	0.0022
GO:0006096	Glycolysis	0.0023
GO:0019395	Fatty acid oxidation	0.0037
GO:0034440	Lipid oxidation	0.0037
GO:0046165	Alcohol biosynthetic process	0.0038
GO:0006959[1]	Humoral immune response	0.0042
GO:0009611	Response to wounding	0.0044
GO:0006957	Complement activation, alternative pathway	0.0044
GO:0006760	Folic acid-containing compound metabolic process	0.0044
GO:0045087[1]	Innate immune response	0.0053
GO:0052548	Regulation of endopeptidase activity	0.0077
GO:0046364	Monosaccharide biosynthetic process	0.0077
GO:0006790	Sulfur compound metabolic process	0.0078
GO:0051179	Localization	0.0079
GO:0052547	Regulation of peptidase activity	0.0083
GO:0046942	Carboxylic acid transport	0.0083
GO:0015849	Organic acid transport	0.0095
GO:0016042	Lipid catabolic process	0.0113
GO:0015711	Organic anion transport	0.0113
GO:0051234	Establishment of localization	0.0113
GO:0006810	Transport	0.0119
GO:0044092	Negative regulation of molecular function	0.0126
GO:0019319	Hexose biosynthetic process	0.0131
GO:0072378	Blood coagulation, fibrin clot formation	0.0132
GO:0006558	L-phenylalanine metabolic process	0.0146
GO:0006559	L-phenylalanine catabolic process	0.0146
GO:0000038	Very long-chain fatty acid metabolic process	0.0146
GO:0051289	Protein homotetramerization	0.0146
GO:0002252[1]	Immune effector process	0.0153
GO:0008203	Cholesterol metabolic process	0.0153
GO:0009074	Aromatic amino acid family catabolic process	0.0212
GO:0048806	Genitalia development	0.0212
GO:0016064[1]	Immunoglobulin mediated immune response	0.0214
GO:0050819	Negative regulation of coagulation	0.0214

GO:0043086	Negative regulation of catalytic activity	0.0224
GO:0034754	Cellular hormone metabolic process	0.0243
GO:0019724[1]	B cell mediated immunity	0.0263
GO:0051262	Protein tetramerization	0.0279
GO:0050778[1]	Positive regulation of immune response	0.0292
GO:0050776[1]	Regulation of immune response	0.0341
GO:0006633	Fatty acid biosynthetic process	0.0372
GO:0003333	Amino acid transmembrane transport	0.0389
GO:0016126	Sterol biosynthetic process	0.0416
GO:0006811	Ion transport	0.0420
GO:0006094	Gluconeogenesis	0.0474
GO:0001676	Long-chain fatty acid metabolic process	0.0474

[1]These pathways are related to immune responses.

and coincided with the appearance of anti-HEV antibodies[17-19]. These findings suggested that host immune response was responsible for the hepatocyte killing. This was followed by attempts to determine whether HEV was capable of producing a cytopathic effect in *in vitro* models. However, these efforts were largely unsuccessful due to failure of this virus to grow well in cell culture. Recent successful culture[20], though in low titers, of some genotypes 3 and 4 HEV isolates has revealed absence of cytotoxicity[21,22], providing some support to the hypothesis that liver injury in HEV infection is unlikely to be virus-mediated.

Human data on cellular immune responses during HEV infection are limited to studies on peripheral blood mononuclear cells (PBMCs). In these studies, we and others have shown proliferation of HEV-specific $CD4^+$ and $CD8^+$ cells, and increased production of various cytokines by PBMCs during acute and convalescent phases of hepatitis E[14,16]. In a study that looked at patients with different disease severities, HEV-specific T-cell responses in PBMCs were weaker in patients with FH-E than in those with uncomplicated acute hepatitis E[12]. However, observations made on circulating immune cells may not faithfully represent those in the immune cells infiltrating the liver, because of preferential localization of certain cells, including virus-specific T cell subsets, in the inflamed tissue[23,24]. Thus, there is a need to study changes in the liver tissue from patients with hepatitis E.

In the current study, we found changes in expression of genes involved in a variety of immune responses, including the innate, humoral and cellular immune pathways in liver tissue from patients with FH-E. Of these, changes in innate and humoral immune responses were also observed in FH-B, indicating that HEV infection may share some of the pathways causing liver cell injury with HBV infection. In contrast, activation of cellular immune pathways was found only in FH-E, and not in FH-B, indicating a specific role for cellular immune response in inducing liver injury during HEV infection.

In particular, we found several T-cell surface molecules such as CD2, CD3 (γ, δ and ε chains) and CD8-alpha to be up-regulated in liver tissue from patients with FH-E. Furthermore, several T-cell signalling molecules, such as Fyn, phospholipase C gamma-1, NFκB1, protein kinase C and ras-related C3 botulinum

Table 3 Results of Gene Ontology analysis (biological processes) for gene entities differentially expressed in liver tissue from fulminant hepatitis E as compared to normal liver, but not in that from fulminant hepatitis B as compared to normal liver

Gene Ontology term	*P* value
Immune system process[1]	1.67E-11
Immune response[1]	4.81E-09
Regulation of immune system process[1]	5.06E-08
Positive regulation of immune system process[1]	2.72E-06
Defense response	1.04E-05
Response to wounding	1.90E-05
Signaling	7.78E-05
Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains[1]	2.40E-04
Adaptive immune response[1]	2.40E-04
Response to stimulus	3.53E-04
Regulation of cell activation	4.65E-04
Inflammatory response[1]	6.89E-04
Cell adhesion	0.0010
Biological adhesion	0.0010
Positive regulation of immune response[1]	0.0015
Cell activation	0.0018
Signal transduction	0.0019
Regulation of leukocyte activation[1]	0.0020
Activation of immune response[1]	0.0021
Oxidation reduction	0.0021
B cell mediated immunity[1]	0.0023
Leukocyte mediated immunity[1]	0.0026
Positive regulation of response to stimulus	0.0027
Regulation of locomotion	0.0028
Regulation of immune response[1]	0.0030
Phospholipid efflux	0.0032
Lymphocyte mediated immunity[1]	0.0032
Regulation of cell migration[1]	0.0048
Regulation of cell motility[1]	0.0048
Response to stress	0.0050
Regulation of lymphocyte activation[1]	0.0056
Regulation of cellular component movement	0.0064
Signal transmission	0.0067
Immunoglobulin mediated immune response[1]	0.0067
Signaling process	0.0067
Small molecule metabolic process	0.0083
Cholesterol efflux	0.0124
Immune effector process[1]	0.0154
Negative regulation of apoptosis	0.0161
Positive regulation of cell activation	0.0176
Positive regulation of leukocyte activation[1]	0.0176
Regulation of response to stimulus	0.0177
Negative regulation of cell death	0.0179
Negative regulation of programmed cell death	0.0179
Regulation of lipid metabolic process	0.0180
Signaling pathway	0.0206
Acute inflammatory response[1]	0.0241
Phospholipid transport	0.0257
Regulation of fatty acid metabolic process	0.0273
Alcohol metabolic process	0.0282
Cholesterol transport	0.0310
Sterol transport	0.0472

[1]These pathways are related to immune responses.

Table 4 Pathways whose genes were found to be over-represented among entities differentially expressed in both fulminant hepatitis E and fulminant hepatitis B, and in fulminant hepatitis E but not in fulminant hepatitis B (derived using BioCarta through DAVID resource)

BioCarta pathway	Gene count	*P* value
In both FH-B *vs* normal, and FH-E *vs* normal		
Complement pathway	10	0.0002
Intrinsic prothrombin activation pathway	10	0.0002
Nuclear receptors in lipid metabolism and toxicity	13	0.0022
Alternative complement pathway	6	0.0069
Extrinsic prothrombin activation pathway	6	0.0113
Lectin induced complement pathway	6	0.0172
Classical complement pathway	6	0.0172
Acute myocardial infarction	6	0.0248
Vitamin C in the brain	4	0.0385
CBL mediated ligand-induced downregulation of epidermal growth factor receptors	5	0.0543
Catabolic pathways for methionine, isoleucine, threonine and valine	3	0.0955
In FH-E *vs* normal but not in FH-B *vs* normal		
T cytotoxic cell surface molecules	7	0.0005
T helper cell surface molecules	6	0.0043
Cytotoxic T lymphocyte mediated immune response against target cells	7	0.0022
Co-stimulatory signal during T-cell activation	7	0.0085
Role of epidermal growth factor receptor transactivation by G protein-coupled receptors in cardiac hypertrophy	6	0.0092
T cell receptor signaling pathway	10	0.0049
Lck and Fyn tyrosine kinases in initiation of T cell receptor activation	5	0.0187
Interleukin-7 signal transduction	5	0.0446
Mitochondrial carnitinepalmitoyltransferase system	3	0.0529
T cell receptor and CD3 complex	3	0.0529
Interleukin-17 signaling pathway	5	0.0562

toxin substrate (rac) were also overexpressed in these livers. These findings may indicate a role for activated $CD8^+$ T cells in the causation of liver injury in HEV infection. An over-expression of CTLA4, which is expressed only on activated T cells, also supports this hypothesis. An alternative explanation for the overexpression of CD2 in liver tissue could be the presence of this marker on the NK cells. Importantly, the increased expression of CD2 in the liver tissue was also associated with over-expression of perforin, the main cytolytic protein contained in the $CD8^+$ T cells and NK cells. The above findings, taken together, indicate that immune-mediated cytotoxicity of $CD8^+$ or NK cells against virus-infected cells may play a role in producing liver damage in HEV infection.

Some other findings in our study may also point to alterations in $CD8^+$ cells. In viral infections, the number of effector CD8 T cells contracts over time with the formation of a population of protective memory cells, which is maintained by IL-7 and IL-15. Our finding of activation of IL-7-mediated signalling in patients with hepatitis E possibly reflects this phenomenon. In addition, the genes for signal transducer and activator of transcription 5 (STAT5), a key molecule involved in the survival of effector and memory CD8 cells[25], and for BCL2, a key survival molecule that is upregulated by STAT5, were overexpressed in FH-E.

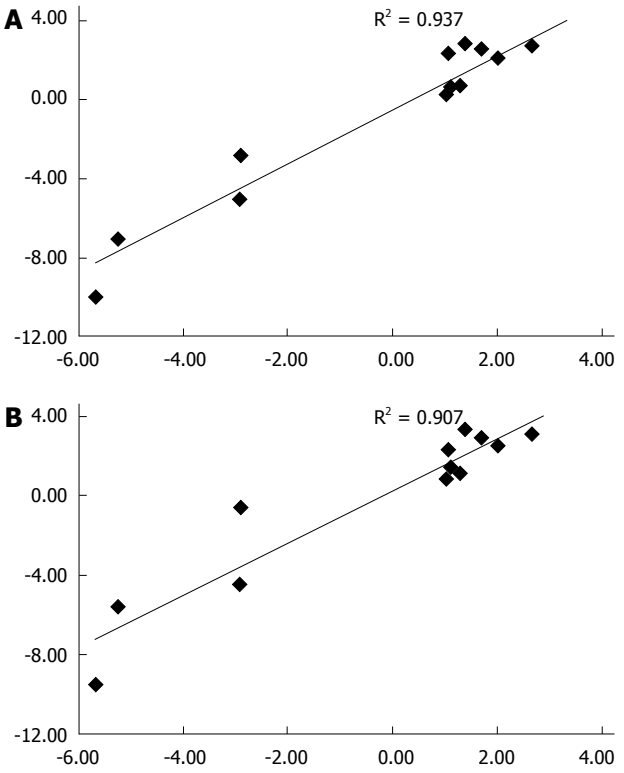

Figure 3 Relationship of fold-change in RNA expression of selected genes in patients with fulminant hepatitis E and fulminant hepatitis B on real-time polymerase chain reaction with fold-change in RNA expression of the same genes on microarray analysis. A and B show the data when reverse transcription polymerase chain reaction results were normalized using the GAPDH gene and 18S rRNA gene as housekeeping gene, respectively.

The differential expression in livers from patients with FH-E of several genes which were unaffected in FH-B could have been because of changes in any of the several cell types present in the liver, including hepatocytes, Kupffer cells, cholangiocytes, endothelial cells, stellate cells and a variety of immune cells. However, the demonstration of predominant infiltration with $CD8^+$ T cells in the FH-E livers in the current study as well as in two previous reports[26,27], and the fact that the genes that showed differential expression are not expressed much in other cell types, indicate the $CD8^+$ T cells were the most likely source. It may be pertinent to note that since the immune cells constitute only a minority of all the cells in the liver, the absolute gene expression changes within the intrahepatic immune cells must be more marked than is indicated by the overall gene expression data. It may thus be interesting in future to undertake studies on such immune cells after recovering these using techniques such as laser dissection microscopy.

It may be pertinent to compare our results with those from a recent study of gene expression in serial

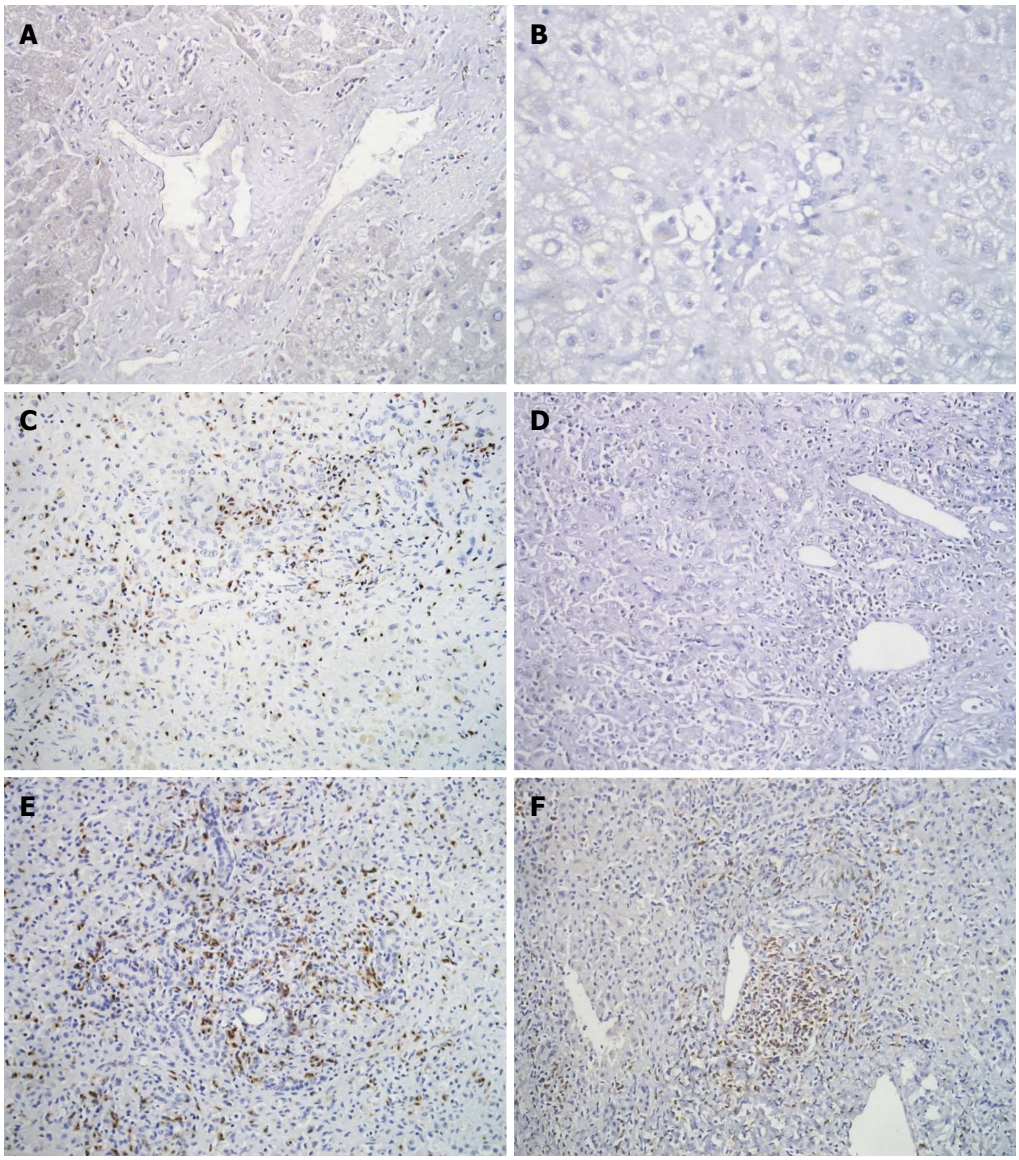

Figure 4 Immunohistochemistry for CD8$^+$ cells in liver biopsies shows occasional cytotoxic T-cells in portal tracts of controls (A), and moderate infiltration in HBV (C) and HEV (E); immunostaining for CD4 shows absence of helper T-cells in portal areas of control (B), an occasional cell in HBV (D) and some small aggregates of helper T-cells in HEV (F). A and B: Diaminobenzidine × 400; C-F: Diaminobenzidine × 200. HBV: Hepatitis B virus; HEV: Hepatitis E virus.

liver tissues from chimpanzees with experimental HEV infection[28]. In this study, differential expression was limited to a few genes that belonged predominantly to the innate immune response pathways, and was weaker than that observed in chimpanzees with HCV infection. Further, the number of upregulated genes peaked sooner after the onset of viremia in HEV infection than with HCV infection. We did find altered expression of some genes involved in innate immune responses; however, these genes were different from those showing significant changes during HEV infection in the chimpanzee study. In this context, it is important to note that comparison of our data with those from the experimentally-infected chimpanzees may not be valid. Our patients had severe liver disease, whereas experimental HEV infection in primates is milder[19,29]. Further, time-kinetics of different types of immune responses vary, with innate immune responses being prominent during the initial phase after viral infection; thus, in human HEV infection, such responses may occur before an infected person becomes symptomatic and hence not picked up. Also, the experimental animals had been inoculated by the intravenous route, whereas humans acquire infection through the oral route.

Though we studied patients with FH-B primarily as disease controls, it may be interesting to compare liver in this condition with healthy livers. Livers from patients with FH-B showed infiltration with CD8$^+$ cells, but no differential expression of genes belonging to cellular immune response pathways. Previous gene expression data in this disease are available from only two Italian patients[30,31]. These patients too showed prominent CD8$^+$ T cell infiltration with little change in expression of genes associated with T cell activity and a prominent upregulation of B cell response, similar to

our observations.

Interestingly, though liver tissues from both FH-E and FH-B in our study showed prominent $CD8^+$ T cell infiltration, only FH-E was associated with a cytotoxic T cell transcriptional signature *e.g.,* increased expression of perforin. This suggests that the infiltrating $CD8^+$ T cells in the two diseases behave quite differently. This may hold the key to pathogenesis of liver injury in HEV infection.

We also found reduced expression of several genes associated with metabolic, hemostatic and complement pathways in liver tissue from FH-E as well as FH-B. Liver is a metabolically active organ which produces several body proteins. Since FH, irrespective of its cause, is characterized by marked destruction of hepatocytes, reduction in the expression of these proteins was thus expected in both FH-E and FH-B, as a consequence of the massive liver injury.

Our data are limited by a small sample size, the use of post-mortem tissue, and of patients with severe liver injury at only one time-point. These limitations are related to the fact that liver biopsy, being invasive, is ethically unacceptable in patients with acute liver disease, forcing us to use tissue collected immediately post-mortem. Though our findings may not be entirely applicable to acute uncomplicated hepatitis E, these should be seen in light of the current absence of any human data on immune events in the liver in acute hepatitis E. Further, we compared our data to those from a control group of post-mortem biopsies from FH-B, which would have been susceptible to similar artefacts as those in FH-E.

In conclusion, liver tissue from our patients with FH-E showed infiltration with $CD8^+$ T cells and overexpression of genes involved in T cell immune responses, especially those related to T cell activation, cytotoxicity and IL-7 signalling. The latter changes were not observed in FH-B, and hence were specific to hepatitis E. These data suggest that the severe liver damage in FH-E is mediated by the host T-cell immune response. Further work on this aspect should help us better understand the pathogenesis of liver injury in hepatitis E.

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COMMENTS

Background

Infection with hepatitis E virus is the most common cause of acute hepatitis in the world. The mechanism of liver injury in this disease and its pathogenesis are yet fully understood. A better understanding of these may allow attempts at therapeutic intervention in patients with this disease.

Research frontiers

Microarray techniques allow study of expression of several genes in tissues from patients with a particular disease and its comparison with those from healthy controls and persons with similar diseases. These techniques have been applied for the study of pathogenesis of several diseases.

Innovations and breakthroughs

The authors used microarray techniques to determine the expression of various genes in the liver tissues from patients dying of hepatitis E and compared this to that in healthy liver tissue (control tissue) and liver tissues from patients dying of a disease with similar morphologic changes but caused by infection with another virus (hepatitis B; disease controls). The data showed several differences in gene expression between these groups. In particular, livers of patients with fulminant hepatitis E, but not those of hepatitis B, showed activation of several immune response pathways, particularly those involving cytotoxic T cells. This difference was observed even though tissues from both hepatitis B and E showed infiltration with cytotoxic T cells.

Applications

All data suggest that immune cells may play a role in the pathogenesis of hepatitis E, though further work is required in this regard.

Peer-review

Minor improvements, such as addition of statistical methods to the manuscript.

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