Expression and distribution of immunoglobulin G in the normal liver, hepatocarcinoma and postpartial hepatectomy liver

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The liver has the extraordinary properties of regeneration and immune tolerance; however, the mechanisms governing these abilities are poorly understood. To address these questions, we examined the possible expression of immunoglobulins in the human and rat liver and the relationship of IgG expression to hepatocyte proliferation, metastasis, apoptosis and immune tolerance. Immunohistochemistry, *in situ* hybridization, laser-guided microdissection and reverse transcription-PCR were performed to examine the expression of IgG in normal human and rat liver, severe combined immunodeficient mouse (SCID) liver and human liver cancers and corresponding cell lines. Small interfering RNA (siRNA) was transfected into cultured hepatocarcinoma cells to downregulate the expression of IgG heavy chain genes. Cell proliferation and apoptosis were assayed with flow cytometry. Cell metastasis was assayed with a Transwell cell assay. Partial hepatectomy (70%) was performed in rats to examine the relationship between hepatocyte IgG and hepatocyte proliferation. IgG, together with essential enzymes for its synthesis, were expressed in the cytoplasm of hepatocytes of normal adult human and hepatoma patients and rat livers, SCID mouse liver and BRL-3A, L-02 and HepG-2 cell lines. Downregulation of IgG inhibited cell proliferation and metastasis and promoted apoptosis. Postsurgery livers expressed significantly more IgG than the livers before surgery and decreased to the original levels when hepatocytes stopped regeneration. IgA and IgM but not IgD and IgE were also positive in hepatocytes. Our findings demonstrate that normal and malignant hepatocytes are capable of synthesizing immunoglobulin, which has important roles in hepatocyte proliferation, apoptosis and cancer growth with profound clinical implications.

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The liver is a complex organ performing many functions, including synthesis of plasma proteins and hormones, breakdown of toxic and waste substances and clearing of antibody–antigen complexes. During fetal development, the liver is also a source of hematopoietic stem cells.¹ Liver has two unique characteristics, one is its strong proliferation capability and another is its immune-tolerance ability.^{2–4} A large number of studies have addressed the mechanisms governing these two properties.^{4,5}

IgG is present in both the fetal and adult livers and is known to be locally produced by B lymphocytes or transported from the blood in the form of immune complexes.^{6–8} In addition, in both adult humans and rats, high concentrations of IgG have been found in hepatic and gall bladder bile.^{9–12} Most of the IgG present in bile is thought to

be generated by B lymphocytes in the liver.^{13,14} However, the precise origin of IgG in the bile has not been well established and its role is not well appreciated.

Given the potent proliferative potential and immune tolerance of the liver and previous findings that IgG can also be synthesized and released by non-lymphocytic cancer cells and normal cells in some immune-privileged organs,^{15–19} we hypothesized that the hepatocytes may also synthesize IgG that would have an impact on liver regeneration and repair.

MATERIALS AND METHODS

Ethics Statement

All animals used in this study were cared appropriately and approved by the Institutional Animal Care Instructions of the Ethics Committee for Research of the Shantou University

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Medical College. Consents of using surgical and autopsy human tissue samples were obtained from the patients and/or their families.

Preparation of Human and Animal Liver Samples and Cell Lines

Fresh human liver tissues were obtained from hepatectomy of 12 patients with hepatocarcinomas from the Second Affiliated Hospital, Shantou University Medical College. Both cancerous and adjacent normal portion of the dissected liver were obtained. Twenty cases of formalin-fixed, paraffin-embedded normal human liver specimens were obtained from autopsy of the Forensic Medicine Department of Shantou University Medical College.

Thirty-two Sprague–Dawley rats (250–300 g weight), 8-week old, and 5 Balb/c rats were obtained from the Laboratory Animal Center of Shantou University Medical College (Shantou, China). Eight C.B-17 SCID mice, 8–10-week old, were obtained from Beijing Vital River Laboratories. Animals were kept under standard conditions with 12-h light/dark cycles.

Rats and SCID mice were anesthetized with intraperitoneal injection of 10% chloral hydrate (4.5 ml/kg body weight). Livers and spleens were immediately dissected and then fixed with 4% paraformaldehyde for 24 h. A portion of the fresh livers and spleens was frozen in liquid nitrogen for RNA extraction.

Rat hepatocyte cell line (BRL-3A), human normal liver cell line (L-02) and human liver cancer cell line (HepG-2) were purchased from Cell Bank (Shanghai Institute for Biological Sciences, Shanghai, China). BRL-3A cell line was cultured in DMEM (GIBCO) containing 5% FCS at 37 °C in a humidified atmosphere with 5% CO₂. L-02 and HepG-2 cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% FAS at 37 °C in a humidified atmosphere with 5% CO₂.

Immunohistochemistry

Immunohistochemistry was performed by a standard protocol.²⁰ Briefly, paraffin tissue sections were dewaxed, rehydrated through graded ethanol washes and incubated with 3% hydrogen peroxide for 30 min, and antigen retrieval was performed by heating the sections at 95 °C in 0.01 mol/l citrate buffer (pH6.0) for 15 min. Slides were then washed with PBS for 15 min and treated with 10% normal goat serum (Invitrogen) (diluted with PBS) for 30 min and incubated with the primary antibody at 4 °C overnight. The reaction products were detected with 3-amino-9-ethyl-carbozole (AEC) substrate-chromogen kit after incubating with the secondary antibody of Dako REAL EnVision Detection Kit (Dako, Carpinteria, CA) for 30 min and washing with 0.1 M PBS at room temperature. Staining with AEC resulted in red signals. Human and rat spleen sections were used as positive controls for IgG and CD19. Rat fetal liver sections were used as positive controls for AFP and

albumin. Rat fetal bone marrow section served as a positive control for C-kit. Sections incubated with PBS instead of primary antibody were used as negative controls.

The following antibodies were used as primary antibodies: Antibodies to human proteins/peptides: Polyclonal rabbit anti human IgG (γ chain specific, 1:4000; Dako), monoclonal mouse anti-human ALB (1:200; Santa Cruz, CA, USA), monoclonal mouse anti-human CD19 (marker for human B cell, 1:200; Zhongshan Golden Bridge, Beijing, China), monoclonal mouse anti-human AFP (1:200; Santa Cruz), monoclonal mouse anti-human RAG1 (1:200; Santa Cruz), monoclonal mouse anti-human RAG2 (1:200; Santa Cruz), monoclonal mouse anti-human AID (1:200; Santa Cruz), polyclonal rabbit anti-human IgA (1:200; Zhongshan Golden Bridge), polyclonal rabbit anti-human IgM (1:200; Zhongshan Golden Bridge), polyclonal rabbit anti-human IgD (1:200; Thermo, Fremont, USA), polyclonal rabbit anti-human IgE (1:200; Thermo), and monoclonal mouse anti-human Ki-67 (1:200; GeneTex, Taiwan).

Antibodies to rat proteins/peptides: Polyclonal rabbit anti-rat whole molecular IgG (1:200; Abcam, Hong Kong, China), rat IgG constant region (IgG Fc, 1:200; Bethyl Laboratories, Texas, USA), rat Ig kappa light chain (Ig κ , 1:200; Bethyl Laboratories), rat alpha fetal protein (AFP, marker for immature hepatocytes; 1:200; DakoCytomation, Glostrup, Denmark), rat albumin (marker for hepatocytes; 1:200; DakoCytomation), rat CD45RA (marker for leukocyte common antigen; 1:200; Bioss, Beijing, China), and rat C-kit (marker for hematopoietic stem cell; 1:200; DakoCytomation).

Stain-Decolorize-Stain (SDS) Method for Human and Rat Hepatocytes

The stain-decolorize-stain (SDS) method was performed to show the expression patterns of IgG and albumin according to a recently established procedure.²¹ Briefly, immunohistochemistry was performed on human and rat liver slides with IgG antibodies. After visualizing and taking photos, these slides were decolorized with 80% alcohol for half an hour at room temperature and then heated in a microwave oven for 10 min to remove the bound antibodies and the labeling color. Then these slides were incubated with Ki-67 antibodies. After visualizing with AEC kit, photos were taken at the same fields as for the first immunostaining. Controls were performed to ensure no cross-reaction between the two primary antibodies or between the primary antibody to the first antigen and the secondary antibody to the second antigen and *vice versa* as described for the technology.²¹

Immunofluorescence

BRL-3A, L-02 and HepG-2 Cells were cultured on cover slips and fixed in 4% paraformaldehyde for 10 min. After rinsing in 0.01 M PBS for three times, the slides were incubated overnight with the primary antibody, which was diluted in 0.01 M PBS containing 0.2% Triton X-100, 10% normal

donkey serum and 0.5% BSA. Rabbit anti-rat IgG (whole molecular), rabbit anti-rat albumin, rabbit anti-human IgG Fc, mouse anti-human Ig κ , Ig λ , RAG1, RAG2 and AID antibodies were used as the primary antibody; 0.01 M PBS was used as a negative control. After incubating with donkey anti-rabbit IgG antibody labeled with FITC for 1 h at room temperature, the cover slides were mounted with Vectashield mounting medium with 1.5 μ g/ml DAPI (Vector, Burlingame, CA). The photos were taken with a Confocal Laser Scanning microscopy FV1000 (Olympus, Japan).

Antibody Preabsorption Test

To assure the specificity of immunostaining, antibody preabsorption tests were carried out using various concentrations of corresponding antigens to preabsorb the primary antibodies before immunostaining as described previously.²² Rabbit anti-human IgG antibody (1:4000 diluted as working solution, Dako) and different concentration of human IgG (as an antigen from human serum, Sigma, St Louis, MO, USA) were mixed. Then, the mixed solution was used as the primary antibody. The proportions of molar concentration between the antibodies and their antigens were 1: 1, 1: 5 and 1:10.

In Situ Hybridization

With rat, mouse and human IGHG1 RNA probes, ISH was performed following a procedure described previously.¹⁶ Deparaffinized and dehydrated tissue sections were fixed in 4% paraformaldehyde for 10 min after antigen retrieval and then hybridized for 18–20 h at 45 °C with rat, mouse or human IGHG1 cRNA probes. After hybridization, sections were washed in 2 $\times$ SSC plus 50% formamide at 50 °C for 15 min and 2 $\times$ SSC twice at 37 °C for 15 min. The sections then were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics, Rotkreuz, Switzerland) for 1 h. 5-Bromo-4-chloro-3-indolyl phosphate (Promega, Madison, WI, USA) were used for colorization. Human and rat spleen tissues incubated with anti-sense probe were used as positive controls and with sense probe as negative controls.

LMD and RT-PCR of Hepatocytes

Frozen rat and normal human liver samples were sectioned at 10 μ m and mounted on slides, and slides containing frozen sections were quickly fixed in 70% ethanol for 5 min, stained with hematoxylin for 1 min and then quickly rinsed in diethyl pyrocarbonate-treated water. Slides were dried in a fume hood for 5 min and then were microdissected with a laser-guided microdissection machine (Leica Microdissection System, Leica, Solms, Germany). The total RNA were isolated from microdissected hepatocytes with NP-40. Reverse transcription was carried out with the Superscript III First-Strand Synthesis System for RT-PCR Kit (Invitrogen).

Total RNA of cultured cells was extracted with Trizol reagent (Invitrogen). Reverse transcription was carried out

using the SuperScript III Reverse Transcriptase (Invitrogen). To exclude the contamination of genomic DNA, we treated all RNA samples with RQ1 RNase-free DNase (Promega). We used the LA Taq Polymerase (TaKaRa Bio, Inc., Siga, Japan) for the amplification of IGHG1, Ig κ , Ig λ , RAG1, RAG2 and AID. Raji cells were used as positive controls for IGHG1. Water, instead of primers, was used as a negative control. The primers used for PCR are listed in Supplementary Tables S2 and S3.

SDS-PAGE and Western Blotting

To analyze cytosolic proteins, cultured cells were harvested and washed twice with ice-cold PBS and lysed in cell lysis buffer (Cell Signaling Technology, Inc., Beverly, MA, USA) for 15 min on ice. The protein concentration of the cell lysate was calculated with a BCA kit (Pierce Biotechnology, USA). Lysate proteins (40 μ g/well) were separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred onto a polyvinylidene difluoride membrane and incubated with primary antibodies, including polyclonal rabbit anti-human IgG (γ chain specific, 1:4000; Dako) and monoclonal mouse-anti human β -actin (1:1000; Zhongshan Golden Bridge, Beijing, China) at 4 °C overnight. All the membranes were washed with TBST for 15 min at room temperature before incubation with secondary antibodies. Goat anti-rabbit IgG-HRP (1:5000; Dako) and goat anti-mouse IgG-HRP (1:5000; Dako) were used as secondary antibodies. Immunoreactive results were observed with Odyssey Infrared imager (Li-Cor Biosciences, NE).

siRNA Design

The siRNA insert sequence designed to silence expression of IgG heavy chains is as follows: siRNA (GenePharma, Shanghai, China): 5'-CCAAGGACACCCUCAUGAUTT-3' (sense), 5'-AUCAUGAGGGUGUCCUUGGTT-3' (anti-sense) and negative control (scrambled siRNA): 5'-UUCUCCGAA CGUGUCACGUTT-3' (sense), 5'-ACGUGACACGUUCGGA GAATT-3' (anti-sense).

Transfection of siRNA into Cultured Cells to Inhibit IgG Synthesis

The transfection of siRNA was performed according to a published protocol.²³ Briefly, L-02 and HepG-2 cell lines were cultured in six-well plates at 10 $\times$ 10⁴ cells/well, incubated overnight at 37 °C in RPMI 1640 with 10% fetal bovine serum. Twenty-four hours later, the supernatants were discarded and replaced by RPMI 1640 without fetal bovine serum. siRNA that silences the expression of IgG and scrambled siRNA with X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) were separately added into the cell culture. Six hours later, the supernatants were discarded again and replaced by RPMI 1640 with 10% fetal bovine serum. The cells were cultured and harvested at different time points to examine cell proliferation, apoptosis and metastasis.

Analysis of Cell Proliferation

A MTS cell proliferation assay was carried out in a 96-well plate according to the manufacturer's instructions (CellTiter 96 AQ One Solution Cell Proliferation Assay Kit, Promega). After being treated with siRNA or scrambled siRNA for 3 days, L-02 and HepG-2 cells were transported into 96-well plates and 20 μ l MTS was added to each well, and after incubation at 37 °C in a 5% CO₂ incubator for 3 h, the OD value at 490 nm was read. A time course experiment was performed to monitor cell viability. All experiments were performed in triplicate and repeated three times to assure reproducibility of the results.

Analysis of Cell Apoptosis

A cell cycle and apoptosis analysis was carried out according to the manufacturer's instructions (Cell Cycle and Apoptosis Analysis Kit, Beyotime, Jiangshu, China). After being treated with siRNA or scrambled siRNA for 3 days, L-02 and HepG-2 cells were collected and incubated with Annexin V-FITC at 4 °C for 30 min in the dark. After adding 300 μ l PBS to each sample, the cells were incubated with propidium iodide (PI), and cell apoptosis was detected with flow cytometry (Becton Dickinson, San Diego, CA).

Analysis of Cell Metastasis

Cell migration assays were performed in a 24-well plate with a protocol described previously.²⁴ Briefly, after being treated with siRNA or scrambled siRNA for 3 days, L-02 and HepG-2 cells were collected and counted. About 5×10^4 cells were added to each chamber (Corning, USA). After 24 h, the membrane was stained with H&E, and six random fields per membrane were photographed with a light microscope. The migrating cells were counted, and the average numbers were calculated.

Animals and Surgery

Twenty-eight adult male Sprague-Dawley rats (250–300 g) were randomly assigned to four groups (seven animals in each group, five rats for PH, one for sham operation and one for normal control). All animal were fed in standard laboratory chow *ad libitum* with free access to water. After an overnight fast, rats were anesthetized with sodium pento-

barbital (30 mg/kg, intraperitoneally), and the large left lateral lobe and median lobes were ligated and removed according to the method of Higgins and Anderson.²⁵ At the indicated time points (0, 1, 3, 5, 7 or 10 days after surgery), the animals were euthanized, and the livers were collected and examined.

Image Analysis

The percentage of IgG γ - or Ki-67-positive hepatocytes was measured in the rat livers with or without 70% hepatectomy with an image analyzing software (Image J 2x2.147, NIH Bethesda, MD). We counted positive cells by randomly choosing 10 areas in $\times 20$ view of every case. At least 500 cells were counted in each case.

Statistical Analysis

The differences in the IgG γ expression before and after siRNA treatment were analyzed with the Student's *t*-test. Difference in the ratio of positive hepatocytes of IgG and Ki-67 expression between the livers before and after partial hepatectomy was tested using the paired *t*-test. The correlation between IgG and Ki-67 expression was calculated with the Pearson's correlation. Two-sided *P*-values of <5% were considered statistically significant. The calculations were carried out with SPSS 12.0.

RESULTS

Expression of IgG and its mRNA in the Normal Human Liver and Hepatocarcinoma

IHC on serial sections of the normal human livers were positive for IgG in the cytoplasm of most human normal hepatocytes. The IgG-expressing cells were also positive for the hepatocyte marker albumin. The B lymphocyte marker CD19 was expressed at low levels in the human liver; only one or two cells were positive for CD19 in about 700 cells (Supplementary Figure S1(A)). Most of B lymphocytes were distributed in the portal area (Supplementary Figure S1(B)). Positive signals for CD19, IgG and AFP were detected in the lymphocytes of the human spleen and liver cancer cells (Figure 1a).

To verify the specificity of anti-human and anti-rat IgG antibodies, the antibodies were preabsorbed with IgG Fab,

Figure 1 IgG expression in the hepatocytes of normal adult human and liver cancer. (a) IgG expression in the normal hepatocytes of adult human livers and liver cancer by immunohistochemistry. (A–C) are serial sections of normal adult human liver. Positive immunoreactivity for IgG (A) and albumin (B) were seen in the same hepatocytes. IgG and albumin had identical distributions, but preabsorption tests confirmed that the two antibodies do not cross-react. More evidence is shown in Supplementary Figure S2 and Supplementary Figure S6. CD19 (C) was scattered positive in B lymphocytes. (D) Human spleen had lymphocytes positive for CD19, used as a positive control. (E) Human hepatoma was positive for AFP and IgG (F). (Bar = 20 μ m). (b) Antibody preabsorption test. (A–C) are the serial sections of normal human liver incubated with anti-human IgG antibody, which was preincubated with human IgG before immunostaining. The ratio of antibody to antigen was 1:0, 1:1 and 1:10 as shown in A–C, respectively. (c) The expression of IgG mRNA in normal human hepatocytes with ISH. (A–C) are serial sections of adult human liver. (A) Positive signals for IgG were seen in hepatocytes by immunohistochemistry. (B) Positive mRNA signals were seen in the same hepatocytes by *in situ* hybridization. (C) No positive signal was seen with the sense probe. (d) IgG mRNA transcript amplification with laser-microdissected (LMD) RT-PCR of adult human normal hepatocytes. (A) Normal hepatocytes of the adult human liver section before LMD. (B) Same section after LMD. (C) mRNA amplification from the LMD-captured hepatocytes. Transcripts of IGHG1, RAG1, RAG2 and AID were detected in captured hepatocytes. Normal human spleen was a positive control. Water was a negative control. β -Actin was an internal control.

IgG Fc and whole IgG before immunostaining was performed. We found that increasing concentrations of pure human or rat IgG whole molecular but not its fragments were able to completely abolish IgG immunoreactivity, thereby confirming that the antibodies are against IgG (Figure 1b and Supplementary Figure S2).

IgG mRNA-positive signals by ISH were specifically localized to the cytoplasm of IgG-expressing hepatocytes of the human livers (Figure 1c(A and B)). To avoid possible contamination by other cell types, laser-guided microdissection was used to capture hepatocytes. We observed amplifications of IGHG1, RAG1, RAG2 and AID gene transcripts in laser-captured human hepatocytes with RT-PCR. No positive signal for CD19 or CD68 was detected, which excluded contamination by B lymphocytes and macrophages (Figure 1dC).

Expression of IgG and its mRNA in Normal Rat Liver Tissue and Cell Lines

IHC on serial sections of normal rat livers were positive for IgG in the cytoplasm of most hepatocytes. The IgG-expressing cells were also positive for the hepatocyte marker albumin in rat liver (Figure 2a(A and B)). The hematopoietic stem cell marker C-kit was sporadically detected. The B lymphocyte marker CD19 was detected only in a few scattered B lymphocytes within the interstitial tissue of rat liver. The leukocyte marker CD45RA was detected only in a few leukocytes in the interstitial tissue of rat liver. The immature hepatocyte marker AFP was negative in the adult rat livers (Figure 2a(C–E)). Positive signals for IgG and CD45RA were detected in the rat spleen lymphocytes. The albumin and AFP-positive signals were detected in the fetal rat livers, and C-kit-positive cells were observed in the fetal rat bone marrow (Figure 2a(G–K)).

IgG mRNA-positive signals were specifically localized to the cytoplasm of IgG-expressing hepatocytes in the rat livers with ISH (Figure 2b(A and B)).

LMD was carried out to capture pure rat hepatocytes (Figure 2c(A and B)). Positive signals for IGHG1, CK18 and albumin were abundantly detected, whereas signals for CD19, CD34 and CD68 were negative in the captured rat hepatocytes with RT-PCR, which excluded the contamination of B lymphocytes, hematopoietic progenitor cells and macrophages (Figure 2c).

IgG and albumin were detected in rat hepatocyte cell line BRL-3A with IF (Figure 2d(A and B)). IgG mRNA was also detected in BRL-3A cell line with ISH (Figure 2d(C and D)). IGHG1 and CK18, but not CD19, were amplified from BRL-3A cells with RT-PCR (Figure 2e).

Expression of IgG, Related Enzymes and their mRNAs in L-02 and HepG-2 Cell Lines

Ig κ , Ig λ , Ig γ , RAG1, RAG2 and AID proteins were all positive by immunofluorescence staining with corresponding antibodies in the normal human hepatocyte cell line L-02 and

the human hepatoma cell line HepG-2 (Figure 3a). IgG mRNA were detected in L-02 and HepG-2 cells with ISH (Figure 3b). IGHG1, RAG1, RAG2 and AID but not CD19 were amplified in L-02 (Figure 3c) and HepG-2 cell lines (Figure 3d) with RT-PCR.

Expression of IgG and its mRNA in SCID Mouse Hepatocytes

IHC on serial sections of SCID mouse livers showed co-localization of both IgG γ and Ig κ with albumin protein in the same hepatocytes (Supplementary Figure S3(1A–C)). The spleens of SCID mice were found to contain no IgG (Supplementary Figure S3(1E)), demonstrating that the SCID mice were truly B cell deficient. IgG was detected in the lymphocytes of the spleen of BALB/c mice as a positive control.

When ISH and IHC were performed on serial sections of SCID mouse liver tissue, IGHG1 mRNA signals co-localized in cells positive for IgG Fc and albumin (Supplementary Figure S3(2A–C)), which further confirmed that IgG is produced by hepatocytes in SCID mice. The spleens of SCID mice were negative for ISH with IGHG1 anti-sense probes (Supplementary Figure S3(2E)), which further verified that those animals were B lymphocyte deficient.

SCID mouse livers were positive for IGHG1 mRNA by RT-PCR (Supplementary Figure S3(3)). The lack of CD19 and IGHG1 mRNA amplification from the SCID mouse spleen tissue again established that the SCID mice do not have B lymphocytes.

Expression of IgA and IgM in Normal Human Hepatocytes

With the same set of techniques and specific antibodies (Supplementary Table S1), we detected IgA and IgM in adult human hepatocytes, but IgD and IgE were negative (Supplementary Figure S4(1)). The expression of IgM in liver tissue was also confirmed with ISH at the mRNA level (Supplementary Figure S4(2)).

Downregulation of IgG Expression with siRNA

siRNA of IgG heavy chain was transiently transfected into L-02 and HepG-2 cells, and the transfect efficiency of L-02 (Figure 4aB) and HepG-2 cells (Figure 4aD) was 95.8% and 93.3%, respectively. The expression of IgG heavy chain in the IgG siRNA-treated cells was much lower than that of the scrambled siRNA-treated L-02 (Figure 4bA) and HepG-2 cells (Figure 4bB) measured with western blotting. In L-02 cells, IgG expression relative to β -actin was 0.442 when treated with scrambled siRNA, but that decreased to 0.176 when treated with IgG siRNA (Figure 4cA). In HepG-2 cells, when treated with IgG siRNA, IgG expression relative to β -actin was 0.045, which is significantly lower than that in cells treated with scrambled siRNA (0.409).

Figure 2 IgG expression in the normal hepatocytes of adult rats and the BRL-3A cell line. (a) IgG expression in normal hepatocytes of adult rat livers with IHC. (A–E) are serial sections. Positive signals for IgG (A) and albumin (B) were seen in the same hepatocytes. AFP (C), CD45RA (D) and C-kit (E) were negative. Liver (F) and spleen (L) were negative when primary antibodies were replaced by PBS. Rat spleen lymphocytes were positive for IgG (G) or CD45RA (J) antibody. Albumin (H) and AFP (I) were positive in the rat fetal hepatocytes. C-kit was positive in the rat bone marrow (K). (Bar = 20 μ m). (b) Expression of IgG mRNA in the normal hepatocytes of adult rat livers with ISH. In the serial sections of adult rat liver (A–C) and spleen (D–F), the expression of IgG was seen in the hepatocytes (A) and spleen (D) with IHC. Positive mRNA signals using IGHG1 anti-sense probe hybridization were seen in normal hepatocytes (B) and spleen (E). The specificity of ISH was established by the absence of signal with a sense probe (C and F). (Bar = 30 μ m). (c) IgG mRNA transcript amplification with laser-guided microdissection (LMD) RT-PCR in the adult rat hepatocytes. (A) Liver section before LMD. (B) The same liver section after LMD. (C) mRNA amplification from the LMD-captured hepatocytes. Transcripts of IGHG1, CK18 and albumin were detected. β -Actin was an internal control. (Bar = 25 μ m). (d) IgG protein and IGHG1 mRNA expression with IF and ISH in the BRL-3A cell line. IgG (A) and albumin (B) signals were detected. (C) Positive signals of IGHG1 mRNA were observed with a rat IGHG1 anti-sense probe. (D) The negative control was a sense probe. (Bar = 10 μ m). (e) IgG mRNA expression in BRL-3A cells with RT-PCR. IGHG1 and CK18 were amplified from BRL-3A cells. Normal rat spleen was a positive control. Water was a negative control. β -Actin was an internal control.

Downregulation of IgG Inhibited the Proliferation of L-02 and HepG-2 Cells

MTS assay showed that the proliferation of L-02 (Figure 5aA) and HepG-2 cells (Figure 5aB) was started to be inhibited by specific IgG siRNA compared with untreated or scrambled

siRNA on the second day. On the third day, the proliferation rate of L-02 and HepG-2 was almost half that of untreated or scrambled siRNA-treated cells. However, the inhibitory effects started to reverse on the fourth day, and the three treatment groups showed the same proliferation rate on the sixth day. The

Figure 3 IgG and relevant enzymes for its synthesis are expressed in L-02, HepG-2 cell lines at the protein and mRNA level. (a) The expression of Igκ, Igλ, Igγ, RAG1, RAG2 and AID in the cytoplasm of the cultured L-02 and HepG-2 cells by immunofluorescence. (b) The expression of IgG mRNA in L-02 and HepG-2 cells by *in situ* hybridization. Positive signals were detected with an IGHG1 anti-sense probe in L-02 (A) and HepG-2 (C). No positive signal was seen with the IGHG1 sense probe (B and D). (c) Expression of IGHG1, RAG1, RAG2 and AID mRNA in the normal human liver cell line L-02. The transcriptions of IGHG1, RAG1, RAG2 and AID were amplified by RT-PCR. Raji cells were a positive control. Water was a negative control. GAPDH was an internal control. (d) Expression of IGHG1, RAG1, RAG2 and AID mRNA in the human hepatoma cell line HepG-2. The results and controls were the same as for L-02 cells.

inhibitory effect in HepG-2 cells lasted longer, reversing on the fifth day and at the same proliferation rate on the sixth day.

Downregulation of IgG-Promoted Apoptosis of L-02 and HepG-2 Cells in Transwell Assay

An annex-V assay combined with flow cytometry 72 h after transfection with IgG siRNA or scrambled siRNA showed that apoptosis of L-02 (Figure 5bA) and HepG-2 cells (Figure 5bB) was increased by the introduction of IgG siRNA, compared with parental or scrambled siRNA. The apoptosis rate of L-02 cells treated with scrambled siRNA was 1.35%. However, the apoptosis rate increased to 2.68% when treated with IgG siRNA. In HepG-2 cells, the apoptosis rate was 20% when treated with IgG siRNA, which is significantly higher than that treated with scrambled siRNA (3.95%).

Downregulation of IgG Inhibited Metastasis of L-02 and HepG-2 Cells

Transwell assays of L-02 and HepG-2 cells showed that migrating cells decreased by approximately 60% in L-02 cells (Figure 5cA) and by about 70% in HepG-2 cells (Figure 5cB) when transfected with IgG siRNA in comparison with the scrambled siRNA or untreated control.

IgG Expression was Significantly Increased in the Regenerated Liver and Strongly Correlates with Ki-67 Expression after Partial Hepatectomy (PH)

We counted IgG- and Ki-67-positive hepatocytes after immunohistochemistry and calculated their positive ratio. We found that, before surgery, positive signals for IgG (Figure 6aG) and Ki-67 (Figure 6aA) were only weakly pre-

Figure 4 IgG was significantly downregulated at 72 h after transfection with siRNA. (a) Transfection efficiency of siRNA in L-02 and HepG-2 cell lines was assessed with IF and FACS analysis. Transfection efficiency of L-02 cells (A and B) and HepG-2 cells (C and D) were assayed 6 h after transfection. (b) IgG downregulation efficiency of siRNA in L-02 (A) and HepG-2 (B) cell lines was assessed by western blotting. 'IgG siRNA' represents cells transfected with siRNA, which downregulated the expression of IgG. β-Actin was an internal control. (c) Histogram of IgG expression in L-02 and HepG-2 cells with various treatments based on the western blotting results. IgG was significantly ($P < 0.05$) downregulated after treatment with siRNA.

sent in a small number of hepatocytes. The average positive ratio of IgG was 3.39% and the Ki-67 positive ratio was 0.8%. However, after 70% hepatectomy, the expression of Ki-67 (Figure 6a(B–F)) and IgG (Figure 6a(H–L)) in hepatocytes of rats increased substantially. One day after surgery, the Ki-67- and IgG-expressing hepatocytes increased in both number and staining intensity (Figure 6a(B and H)). The average positive ratio of IgG was 12.79% and that of Ki-67 was 13.04%, both of these were significantly higher than those before surgery ($P < 0.05$) (Figure 6bA). This trend lasted until 7 days after surgery, and during this period, about 9.3% hepatocytes expressed IgG and about 11.7% hepatocytes expressed Ki-67. Seven days after surgery, the Ki-67- and IgG-expressing hepatocytes began to decrease in number and staining intensity (Figure 6a(E and K)). Some hepatocytes expressed Ki-67 and IgG strongly 10 days after surgery, and

the remaining hepatocytes fall back to the presurgery level (Figure 6a(F and L), Figure 6bA)). IgG and Ki-67 expression pattern were analyzed and found that they are closely related (Figure 6bB)). This pattern of distribution indicates that IgG is likely associated with cell proliferation.

DISCUSSION

This study shows that both IgG and its mRNA were present in the adult human and rat hepatocytes by immunohistochemistry, immunofluorescence, *in situ* hybridization and LMD-assisted RT-PCR. IgG was also detected in the human liver cell line L-02, the rat hepatocyte cell line BRL-3A and the human hepatoma cell line HepG-2. Detection of IgG protein and gene transcripts in the livers of SCID mice, which lack B lymphocytes, further suggests that the IgG found in hepatocytes was not absorbed from circulation but locally

synthesized. The expression of essential genes necessary for the synthesis of immunoglobulin, including RAG1, RAG2 and AID in hepatocytes, further supports local synthesis of IgG. The significant reduction of IgG synthesis when the cultured liver cells were transfected with siRNA to inhibit specific IgG gene expression provided additional evidence that these cells can synthesize IgG. Previous studies have shown that the neonatal Fc receptor (FcRn), which is known to transport IgG across epithelial layers, is functionally expressed on the apical surface of hepatocytes.¹⁴ Therefore, it is possible that the IgG found in hepatocytes may have two origins; ie, the blood, via receptor internalization, and local production by hepatocytes themselves.

When IgG expression was inhibited with siRNA, cell proliferation and migration were reduced and apoptosis was increased. In addition, following partial hepatectomy, IgG expression was markedly increased alongside Ki-67 in the rapidly regenerating liver and decreased when the liver stopped regeneration. Therefore, a role for locally produced IgG in liver cell growth and regeneration may be indicated. Previously, it has been reported that tumor-derived IgG was a growth factor for cancer cells. Blockage of tumor-derived IgG with either anti-sense DNA or anti-human IgG antibody were found to increase programmed cell death and inhibited growth of cancer cells *in vitro*. It was also found that administration of anti-human IgG antibody suppressed the growth of an IgG-secreting carcinoma cell line in immunodeficient nude mice.^{15,23} Li *et al* found that cancerous immunoglobulin promoted cell proliferation and inhibited antibody-dependent cell-mediated cytotoxicity.²⁶ It is possible that hepatocyte-produced IgG acts as a growth factor to participate in signaling pathways, leading to cell proliferation and apoptosis in normal hepatocytes and hepatocarcinomas. Therefore, hepatocyte-produced Ig may also affect liver transplant, regeneration and repair.

In our study, we also found that IgA and IgM, but not IgD and IgE, were produced by hepatocytes. It has been reported that bile contains high concentrations of IgG and IgA, but the origin of the biliary Ig has not been fully clarified.^{10–12} One potential source is that biliary luminal Ig is produced by local immunoglobulin-secreting B lymphocytes within the liver.¹³ Another potential source of biliary IgG is the circulation, with FcRn mediating the transport of IgG from the blood into the bile.¹⁴ Based on our findings, it is possible that part of the biliary IgG is produced by hepatocytes and subsequently secreted into the bile.

It has been reported that biliary IgG possesses specific antibacterial activity in the absence of specific immunization,

suggesting ongoing production of IgG against a variety of potential gastrointestinal pathogenic events.^{27,28} In addition, IgA is the most abundant immunoglobulin in mucosal immunity^{29,30} and it has been reported that IgA levels in the intestinal mucosa in acute liver necrosis patients were decreased significantly, but the mechanisms are not well understood.³¹ Based on these studies and our new findings, we hypothesize that the liver may provide immunoprotection for the biliary tract and the intestines through secreting IgG and IgA into the bile and subsequently into the gut. IgG was shown to be secreted by cultured cancer cells.¹⁵ Niu *et al* demonstrated that tumors can secrete IgG into circulation with a SCID mouse model.²³ They inoculated colorectal cancer cells into SCID mice, and IgG was detected in the sera with increasing IgG levels as the tumor grew. Therefore we speculate that IgG can be synthesized and secreted into extracellular space. However, direct evidence for this theory has not been obtained in this study. The significance of IgM, IgD and IgE production by hepatocytes is not clear.

In recent years, there has been increasing recognition of the liver as a lymphoid organ.^{32–34} Not only intrahepatic hematopoietic cells, but also non-hematopoietic cells, including stellate cells, sinusoidal endothelial cells and hepatocytes, appear to have roles in the induction of tolerance against both harmless antigens and the induction of immune response against deleterious pathogens.^{34,35} Hepatocytes promote the induction of immune tolerance owing to their capacity to induce clonal deletion of naive CD8+ cells when these T cells are primed by antigens presented by hepatocytes.³⁵ Our finding that hepatocytes have the ability to produce Ig adds an extra dimension to the concept of the liver as a lymphoid organ. It should be noted that one of the antibodies to IgG used in this study was polyclonal, and therefore its cross-reactivity to other antigens, although unlikely, cannot be completely ruled out.

Taken together, our data show that hepatocytes are capable of synthesizing immunoglobulin and that there is a significant upregulation of this synthesis during liver regeneration. Downregulation of IgG had marked effects on liver cell proliferation, apoptosis and migration. This is the first time that IgG is found to be produced by hepatocytes in the normal human and rat livers. We also proposed a new mechanism of the liver to participate in gut mucosal immunity and a possible explanation for liver immune tolerance. The potential pathological and clinical significance of local synthesis of IgG by hepatocytes calls for further investigation.

Figure 5 IgG promotes cell proliferation and metastasis and inhibits apoptosis. (a) Proliferation of L-02 (A) and HepG-2 (B) cells transfected with either IgG siRNA (blue) or scrambled siRNA (NC) (red) was examined with methylthiazol tetrazolium. (b) Apoptosis of L-02 (A) and HepG-2 (B) cells transfected with either IgG siRNA or scrambled siRNA (NC) was analyzed with flow cytometry. (c) Metastasis of L-02 (A) and HepG-2 (B) cells transfected with either IgG siRNA or scrambled siRNA (NC) was analyzed with a Transwell assay. Bottom panel shows representative photographs; top panel shows quantitative evaluation.

Figure 6 IgG was significantly upregulated and positively correlates with the expression of Ki-67 following partial hepatectomy. (a) IgG and Ki-67 expression before and after partial hepatectomy (PH) in rats with IHC. Sections (A and G) were livers before PH. Sections (B and H), (C and I), (D and J), (E and K) and (F and L) were livers collected 1, 3, 5, 7 and 10 days after PH, respectively. Sections (G–L) were IgG immunoreactivity, and Sections (A–F) were Ki-67 immunoreactivity. (b) Histogram and graph of IgG and Ki-67 expression at different time points following partial hepatectomy.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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