

# Constitutive Expression and Regulation of Rat Complement Factor H in Primary Cultures of Hepatocytes, Kupffer Cells, and Two Hepatoma Cell Lines

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**SUMMARY:** The 155-kd soluble complement regulator factor H (FH), which consists of 20 short consensus repeats, increases the affinity of complement factor I (FI) for C3b by about 15 times. In addition to its cofactor activity, it prevents factor B from binding to C3b and promotes the dissociation of the C3bBb complex. The primary site of synthesis of FH, as well as of FI, is the liver, but the cell types responsible for the hepatic synthesis of both factors have not yet been clearly identified. In contrast to FI-mRNA, which was detectable only in hepatocytes (HC), FH-specific mRNA was identified in both HC and Kupffer cells (KC). As calculated for equal amounts of mRNA isolated from both cell types, FH-specific mRNA was found to be nearly 10-fold higher in KC than in HC, leading to the conclusion that KC are an abundant source of FH. Of the investigated proinflammatory cytokines IL-6, TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ , only IFN- $\gamma$  up-regulated FH-specific mRNA up to 6-fold in both primary HC and KC. This was also demonstrable on the protein level. However, FH-specific mRNA was not inducible in the rat hepatoma cell line H4IIE, which did not express FH-specific mRNA and could not be up-regulated in FAO cells that constitutively expressed FH-specific mRNA. This demonstrates that transformed cell lines do not reflect FH regulation in isolated primary HC. In addition to IFN- $\gamma$ , the endotoxin lipopolysaccharide (LPS) up-regulated FH-specific mRNA nearly 10-fold in KC after stimulation at concentrations of 10 or 1 ng/ml. In contrast, concentrations of up to 2  $\mu$ g LPS/ml did not show any effect on HC. Our data suggest that LPS does not regulate the expression of FH in HC. (*Lab Invest* 2002, 82:183–192).

The regulation of complement activation at the level of C3 and C4 is mediated by several receptors and soluble regulatory proteins. These include complement receptor 1 (CD35), complement receptor 2 (CD21), decay-accelerating factor (CD55), membrane cofactor protein (CD46), factor I (FI), and factor H (FH). These proteins, with the exception of FI, form the “regulators of complement activation” family (Hourcade et al, 1989). Their genes are closely linked on chromosome 1 in man and mouse. This illustrates that this region on the long arm of chromosome 1 in humans is analogous to that region in mice (Klickstein et al, 1985). The serine protease FI could be located to chromosome 4 in man (Goldberger et al, 1987) and to chromosome 3 in mouse (Minta et al, 1996).

The potentially deleterious complement system is strictly regulated to prevent damage in the absence of pathologic situations (Hill et al, 1992; Mulligan et al, 1992; Piddlesden et al, 1994; Smith et al, 1993). On

some host cell surfaces, membrane-bound complement regulatory factors are expressed only at low levels. Thus, reduced protection against complement attack may be compensated by the soluble regulator FH, which has a critical role in complement inactivation in the fluid phase (Meri and Pangburn, 1990; Pangburn and Müller-Eberhardt, 1978) and acts in concert with FI. FH is a single-chain protein of 155 kd. It consists of 20 short consensus repeats (SCR) similar to other members of the regulators of complement activation family that are also, in part or completely, composed of such structural units of 60 amino acids (aa), each of which are highly conserved. The concentration of FH in human plasma is 300 to 600  $\mu$ g/ml, ie, about 10-fold the serum concentration of FI. The affinity of human FI for its substrate C3b is 15 times higher in the presence of FH than in the absence of any cofactor (DiScipio, 1992). FH further promotes the dissociation of the C3bBb complex (ie, it has decay-accelerating activity) (Weiler et al, 1976; Whaley and Ruddy, 1976) and prevents factor B from binding to C3b (DiScipio, 1981; Kazatchkine et al, 1979). FH consists of 1216 aa in mouse (Kristensen and Tack, 1986) and 1231 aa in man (Ripoche et al, 1988a). The 42-kd FH-like protein/reconectin (FHL-1) is encoded by the same mRNA by which the FH-protein is en-

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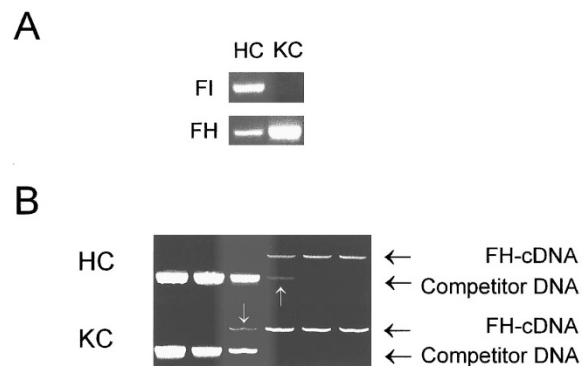
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coded. As a truncated splicing form of the FH-specific mRNA, it comprises the SCR 1-7 (Schwaeble *et al*, 1987, 1991) and is present in human serum at concentrations of 20 to 50  $\mu\text{g/ml}$ . As does FH, FHL-1 acts as a cofactor of FI for C3b/C4b cleaving (Misasi *et al*, 1989) and also promotes the dissociation of the C3bBb complex (Kühn *et al*, 1995) in addition to having some unique biologic functions (Hellwege *et al*, 1997; Johnsson *et al*, 1998). The primary site of synthesis of FH is the liver (Zipfel and Skerka, 1994). Extrahepatic cell types such as human umbilical vein endothelial cells (HUVEC) (Brooimans *et al*, 1989, 1990; Ripoche *et al*, 1988b), peripheral blood monocytes (Whaley, 1980), cells of the monocyte-macrophage series (De Ceulaer *et al*, 1980), primary skin fibroblasts (Katz and Strunk, 1988), fibroblast-like L cells (Munoz-Canoves *et al*, 1989; Vik, 1999), primary myoblasts and rhabdomyosarcoma cell lines (Legoedec *et al*, 1995), glioma cell lines (Gasque *et al*, 1992), and glomerular mesangial cells (van den Dobbelsteen *et al*, 1994) have also been reported to express FH. These cell types may function as local sources of FH and thereby reduce tissue damage caused by local complement activation. As a model for the investigation of the hepatic synthesis and regulation of FH, various hepatoma cell lines of mouse (+/+ Li) (Vik, 1996) and man [Hep3b (Luo and Vik, 1999; Schwaeble *et al*, 1991), HepG2 (Lappin *et al*, 1992; Schwaeble *et al*, 1991), and HepG3, HepG4, H4 (Schwaeble *et al*, 1991)] have been used in the past. In the present study, we show the first approach to investigate the constitutive expression of FH in primary, cultured liver cells, ie, in hepatocytes (HC) and in Kupffer cells (KC) of rat as well as in two rat hepatoma-derived cell lines (FAO and H4IIE). We also used the proinflammatory cytokines IL-6, TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$ , and LPS to investigate their potential influence on the expression of FH, both on the mRNA and on the protein level in primary HC and KC as well as in the two hepatoma cell lines.

## Results

### *Constitutive Expression of FH in HC and KC*

First, the constitutive expression of FH in HC and KC was investigated. Conventional semiquantitative PCR with equal amounts of mRNA used for reverse transcription resulted in an amplificate that was much more intense when it had been derived from KC than from HC. This demonstrates that the FH-specific mRNA content is much higher in KC than in HC (Fig. 1A). In contrast, the FI-specific amplificate could only be generated from HC-derived cDNA (Fig. 1A) but not from KC-derived cDNA. This confirms a differential constitutive hepatic expression of the two proteins because KC express much more FH-specific mRNA than HC, and HC but not KC seem to be the only source of FI in the liver. A faint band sometimes visible in KC-derived amplificates of FI (data not shown) was most probably a result of the contamination of KC with sinusoidal endothelial cells. HC preparations were



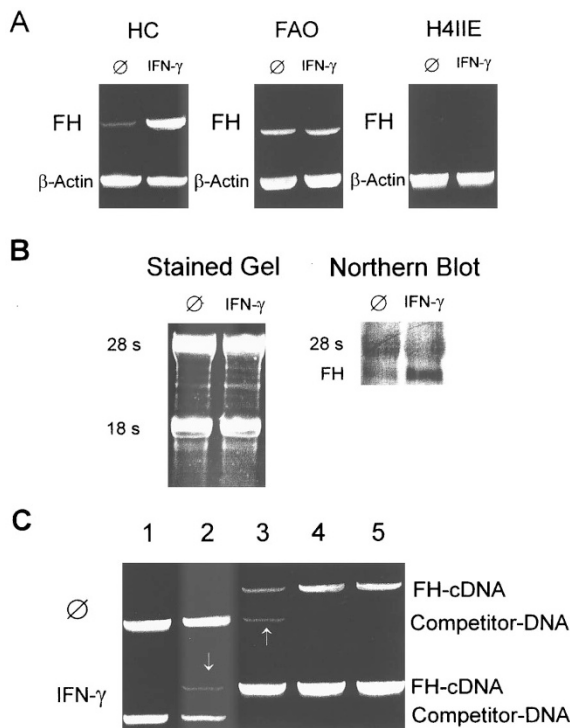
**Figure 1.**

Expression of specific mRNA for complement factors I (FI) and H (FH) in isolated hepatocytes (HC) and Kupffer cells (KC) as detected by RT-PCR assays. A, Demonstration of specific mRNA for FI and FH in isolated HC and KC by RT-PCR assays. B, Quantification of FH-specific constitutive mRNA expression in HC and KC by quantitative-competitive PCR. Equal amounts of HC-derived and KC-derived cDNA were amplified together with 10-fold dilutions of an external  $\lambda$ -standard consisting of 500 nucleotides. The white arrows indicate the points of near equimolarity of the HC- or KC-derived cDNA (upper bands) and the  $\lambda$ -standard DNA (lower bands). The expression of FH-specific mRNA in HC and KC differs by a factor of close to 10.

almost pure, whereas KC preparations contained up to 4% contaminating cells, of which sinusoidal endothelial cells were predominant. To quantify the difference in FH-specific mRNA of HC and KC, equal amounts of total mRNA were isolated from both cell types and used for a quantitative-competitive PCR, which was performed by coamplifying 10-fold dilutions of the competitor. The aim of this assay was only to determine the degree by which FH-specific mRNA differs in HC and KC or, in the following experiments, to which degree it was up-regulated in both cell types, and not to compare absolute molarities of standard  $\lambda$ -DNA and FH-cDNA of HC and KC. For this reason only the lanes with equal intensities of the amplificates had to be compared. As shown in Fig. 1B, the equilibrium between FH-cDNA and competitor DNA showed a shift by a factor of close to 10, demonstrating that the FH-specific cDNA is nearly 10-fold higher in KC than in HC. For control purposes amplificates of FH and FI were sequenced using the dideoxy chain termination method and were identified to be part of the corresponding cDNA nucleotide sequences (not shown).

### *Interferon- $\gamma$ -Mediated Up-Regulation of FH-Specific mRNA in Primary Cultures of HC But Not in the Hepatoma Cell Lines FAO and H4IIE*

Because IFN- $\gamma$  had been found to be an effective up-regulator of FH protein secretion in several primary and malignantly transformed cell lines (Brooimans *et al*, 1989; Luo and Vik, 1999; Ripoche *et al*, 1988b; Schwaeble *et al*, 1991; Vik, 1996), its potential effects were investigated in primary rat HC and the rat hepatoma cell lines H4IIE and FAO. Using conventional RT-PCR, it was demonstrated that only primary HC up-regulated FH-specific mRNA after exposure to IFN- $\gamma$  (Fig. 2A). The faint signal in unstimulated HC



**Figure 2.**

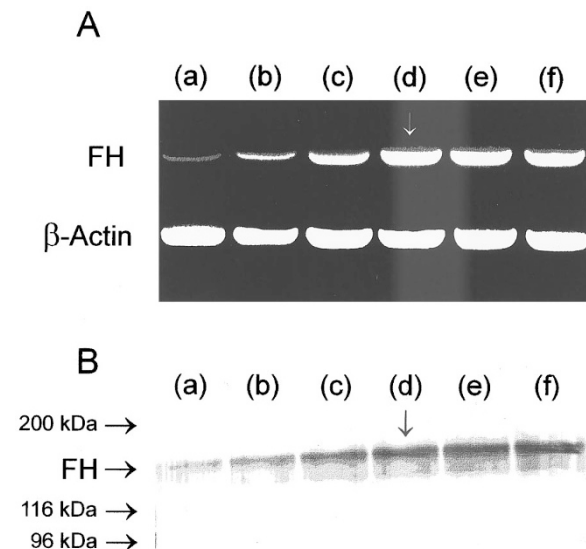
Up-regulation of FH-specific mRNA in HC, FAO, and H4IIE cells after their stimulation with IFN- $\gamma$ . **A**, Comparison of IFN- $\gamma$ -mediated up-regulation in HC, FAO, and H4IIE cells. After incubation of the cells with IFN- $\gamma$  (100 U/ml), mRNA was extracted and FH-specific mRNA was determined by RT-PCR assays. Only HC respond to IFN- $\gamma$  with an increase in FH-specific mRNA. The constitutive expression of FH is low in HC, higher in FAO, and absent in H4IIE cells. As a control  $\beta$  actin-specific cDNA was coamplified. **B**, IFN- $\gamma$ -dependent up-regulation of the expression of FH-specific mRNA in HC as shown by Northern blot analysis. *Left*, Gel electrophoresis with staining of extracted mRNA as a control for loading equal amounts of mRNA of both IFN- $\gamma$ -treated and untreated cells. *Right*, Northern blot analysis of FH-specific mRNA extracted from HC without stimulation and after stimulation of the cells with IFN- $\gamma$  (100 U/ml). **C**, Competitive PCR for the quantification of FH-specific mRNA from IFN- $\gamma$ -treated and untreated HC. The arrows indicate the points of near equimolarity. The difference between IFN- $\gamma$ -treated and untreated cells is about 7-fold.

increased clearly after stimulating these cells with 100 U/ml of IFN- $\gamma$ , whereas the signal for  $\beta$ -actin did not change. In contrast, the strong FH signal of FAO hepatoma cells did not increase after a stimulation with IFN- $\gamma$  (Fig. 2A). In H4IIE hepatoma cells that did not show a constitutive FH-specific mRNA expression, stimulation with IFN- $\gamma$  did not lead to the induction of FH-specific mRNA (Fig. 2A). These results clearly show that both FAO and H4IIE hepatoma cell lines do not represent the physiologic behavior of primary HC and suggest once more that transformed cells should not be used as models of primary cells. Because of the observed differences between HC and the hepatoma cell types, all further investigations were performed with primary HC. As shown in Figure 2B, the data obtained by conventional PCR were confirmed by Northern blot analyses. Cells were again cultured for 48 hours, with IFN- $\gamma$  for the last 24 hours before harvest. Figure 2B (left) in which the RNA was stained with Radiant Red RNA Stain shows equal intensities of RNA bands, ie, mainly of the rRNA bands

of 28S (4.7 kB) and 18S (1.9 kB) in both lanes. In contrast, the FH-specific Northern blot signals in the range of 4.3 kB (Fig. 2B, right) were clearly increased after stimulation with IFN- $\gamma$ . Densitometric analyses revealed an FH-specific signal increase of between 5- and 8-fold. In addition to the Northern blot analyses, which are at best semiquantitative, the up-regulation of FH-specific mRNA in HC was quantified by competitive PCR. As shown in Figure 2C, the equilibrium between competitor DNA and FH-cDNA of unstimulated cells is reached in lane 3, whereas it shifts to lane 2 (ie, by a 10-fold dilution of the competitor) after stimulation with IFN- $\gamma$ . Because the equilibrium was not reached completely, the up-regulation must be between 5- and 10-fold as the intensity of the FH-specific amplicate is only half as intensive as the amplicate of the competitor. The data of a 6- to 7-fold up-regulation of FH-specific mRNA in primary HC are in good accord with the densitometric analyses of the Northern blot signals (Fig. 2B), which revealed increases between 5- and 8-fold (not shown). As a control mRNA specific for the IFN- $\gamma$  receptor was identified in HC by RT-PCR analyses (not shown).

#### **Dose-Response Relation of the Up-Regulation of FH-specific mRNA and Secreted FH Protein by Stimulation with IFN- $\gamma$**

IFN- $\gamma$  induced an increase in FH-specific mRNA (Fig. 3A) and in secreted FH protein (Fig. 3B) in a dose-dependent manner. Using doses of 0, 10, 20, 50, 100,



**Figure 3.**

Dose-dependent increase in FH-specific mRNA (A) and secreted FH protein (B) after treatment with IFN- $\gamma$ . **A**, HC were cultured for 48 hours, with the last 24 hours stimulated with increasing doses of IFN- $\gamma$  (a, 0 U/ml; b, 10 U/ml; c, 20 U/ml; d, 50 U/ml; e, 100 U/ml; f, 200 U/ml). A plateau of HC-specific mRNA is reached by treatment of the cells with 50 U/ml IFN- $\gamma$  (white arrow/lane d). **B**, Immunoblot analysis of culture supernatants from stimulated and unstimulated HC. Proteins were concentrated by precipitation, and 30  $\mu$ g each was applied to the gel. HC had been cultured for 96 hours, with the last 72 hours incubated with increasing doses of IFN- $\gamma$  (a, 0 U/ml; b, 10 U/ml; c, 20 U/ml; d, 50 U/ml; e, 100 U/ml; f, 200 U/ml). The arrow indicates the signal, obtained with 50 U/ml IFN- $\gamma$  with which a plateau level was reached.

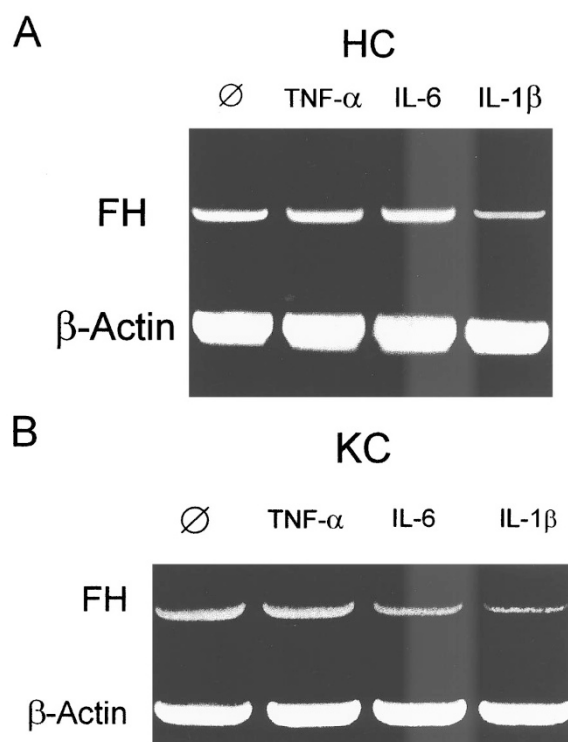
and 200 U/ml and investigating the FH-specific mRNA by conventional RT-PCR analyses, it was shown that the weak basal signal (Fig. 3A, *lane a*) rose with 10 and 20 U/ml IFN- $\gamma$  (*lanes b and c*) and reached a plateau at a dose of 50 U/ml (*lane d*). Thus, the dose of 100 U/ml used for the stimulation assays is sufficient to result in a clear increase in the signals of FH expression without being supraphysiologic. The data obtained by RT-PCR were supported by immunoblot analyses using the novel anti-rat FH mAb 4-7D for which FCS-free supernatants were harvested from cultures of HC after a cultivation period of 96 hours, with the last 72 hours in the presence of IFN- $\gamma$ . As shown in Figure 3B, the FH-specific immunoblot signal in the range of 150 kd increased from the basal level without stimulation (*lane a*) after stimulation of the cells with 10 and 20 U/ml and reached a plateau at 50 U/ml (*lane d*). There was no further increase with higher concentrations. The basal FH-specific signal (Fig. 3B, *lane a*), in comparison to the signal obtained after stimulation of the cells with 100 U/ml IFN- $\gamma$  (Fig. 3B, *lane e*), was increased by about 4- to 5-fold according to densitometric analyses (not shown). Thus, the IFN- $\gamma$ -mediated up-regulation of FH-specific mRNA of 6- to 7-fold is also reflected in an increase in FH protein released from HC.

#### ***IL-1 $\beta$ Down-Regulates the Expression of Rat FH Whereas IL-6 and TNF- $\alpha$ Have No Effect***

Cytokines that had not shown an up-regulating effect on the expression of FH were investigated more systematically. Double the concentrations of cytokines recommended by the suppliers were used for the stimulation of primary HC, FAO, and H4IIE hepatoma cells. IL-1 $\beta$  was used at a dose of 200 U/ml, TNF- $\alpha$  and IL-6 at doses of 400 U/ml. As demonstrated by conventional RT-PCR analyses (Fig. 4), IL-6 and TNF- $\alpha$  did not exhibit any influence on the FH-mRNA expression in primary HC and KC. However, IL-1 $\beta$  reproducibly down-regulated the FH-specific mRNA expression in HC and KC at doses of 100 U/ml (Fig. 4). In H4IIE hepatoma cells that did not express FH-specific mRNA constitutively, none of these cytokines induced FH-specific mRNA (not shown). In FAO hepatoma cells in which the constitutive expression of FH-specific mRNA was higher than in primary cells, none of the cytokines showed any effect, ie, the down-regulating effect of IL-1 $\beta$  on primary HC was not demonstrable in FAO cells (not shown).

#### ***LPS and IFN- $\gamma$ Up-Regulate FH-Specific mRNA and Protein Secretion in KC***

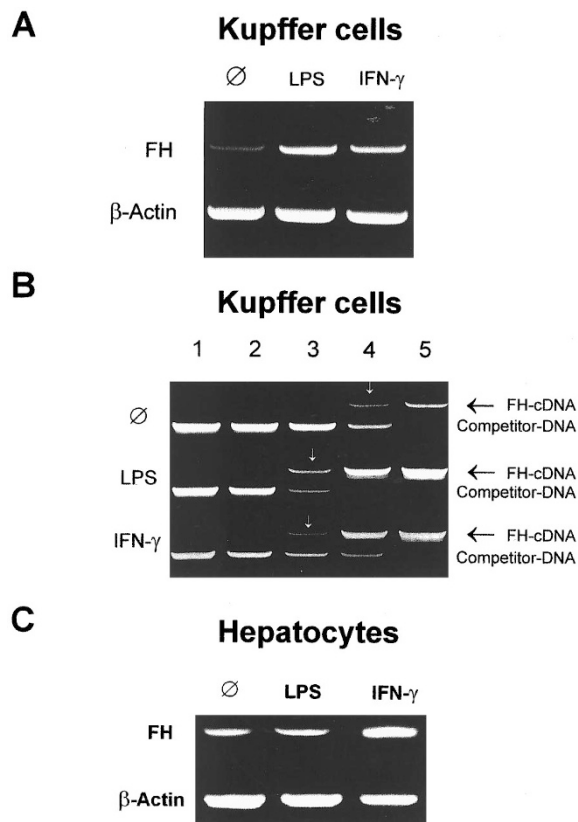
The effects of LPS on primary KC and HC were also investigated. The LPS concentration of 1  $\mu$ g/ml, which in former experiments (Minta, 1988) resulted in a nearly 3-fold up-regulation of FH synthesis in premonocytic U-937 cells, is known to be too high for a specific receptor-mediated signal transduction. Therefore concentrations of 10 or 1 ng/ml were used in this study. They resulted in reproducible effects on



**Figure 4.**

RT-PCR analysis of equal amounts of cDNA derived from HC (A) cultured for 48 hours and KC (B) cultured for 72 hours without or with stimulation for the last 24 hours using TNF- $\alpha$  (400 U/ml), IL-6 (400 U/ml), or IL-1 $\beta$  (200 U/ml). No differences in the FH-specific mRNA expression were detected in HC and KC that were stimulated with TNF- $\alpha$  or IL-6 at higher concentrations. The FH-specific amplicate obtained after treatment of HC and KC with IL-1 $\beta$  was decreased (already at a dose of 100 U/ml). Rat cDNA coding for  $\beta$ -actin was coamplified and used as a control.

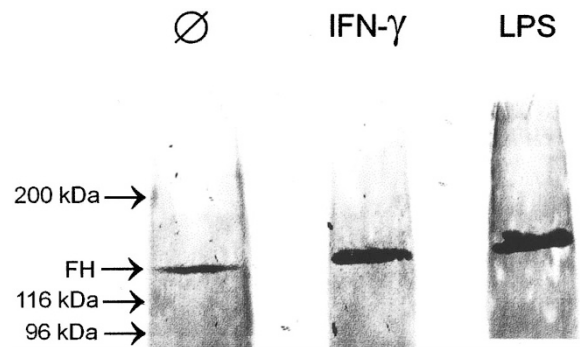
the up-regulation of FH-specific mRNA and on protein secretion. The LPS-mediated up-regulation of FH-specific mRNA at 1 ng/ml was more intensive than that mediated by IFN- $\gamma$  (100 U/ml) as shown by RT-PCR analyses (Fig. 5A). No additional increase was seen when 10 ng/ml were used for the stimulation. The activation of KC (by IFN- $\gamma$  as well as by LPS) was, in addition, demonstrated by the up-regulation of the cytokines IL-6 and IL-1 $\beta$  on the mRNA level in these cells (not shown). There was no difference in the up-regulation of either cytokine whether 1 ng/ml LPS or 10 ng/ml LPS was used. Thus, LPS at a concentration of 1 ng/ml is sufficient for its maximal effect on KC (not shown). The quantifications of the LPS- and the IFN- $\gamma$ -mediated increases in FH-specific mRNA were performed by quantitative-competitive RT-PCR. As shown in Figure 5B, the equilibrium between FH-specific cDNA (*upper lanes*) and competitor DNA (*lower lanes*), which in unstimulated KC was in *lane 4*, shifted after LPS treatment (1 ng/ml) to *lane 3*, ie, by a factor of close to 10. Nearly the same result was obtained when KC were treated with IFN- $\gamma$ . But in this case the FH-specific amplicate was less pronounced when compared with the amplicate after LPS treatment, which reached a complete equilibrium with the amplicate of the competitor (Fig. 5B). This illustrates, in good accord with the data obtained by conventional



**Figure 5.**

RT-PCR analyses of FH-specific mRNA in HC and KC after their stimulation with LPS or IFN- $\gamma$ . A, RT-PCR analysis of mRNA derived from KC cultured for 72 hours without or with stimulation of the cells by LPS (1 ng/ml) or, as a positive control, by IFN- $\gamma$  (100 U/ml) for the last 24 hours. As a control cDNA coding for  $\beta$ -actin was coamplified. B, Quantification of the up-regulation of FH-specific mRNA in KC after stimulation of the cells with LPS (1 ng/ml) or IFN- $\gamma$  (100 U/ml) by competitive PCR. Equal amounts of FH-specific cDNA were coamplified with 10-fold dilutions of a  $\lambda$ -standard DNA. The white arrows denote the points of near equimolarity and indicate that the FH-specific mRNA expression between unstimulated KC and LPS-stimulated KC differs by a factor of close to 10 and between unstimulated KC and IFN- $\gamma$ -stimulated KC by a factor of close to 6. C, RT-PCR analysis of equal amounts of mRNA derived from HC that had been cultured for 48 hours without or with stimulation using LPS (1  $\mu$ g/ml) or with IFN- $\gamma$  at a dose of 100 U/ml as a positive control. As a control  $\beta$ -actin-specific cDNA was coamplified.

PCR, that LPS (although used at a concentration as low as 1 ng/ml) is more effective than IFN- $\gamma$  used at a dose of 100 U/ml. The LPS-dependent up-regulation of FH, however, could not be demonstrated in HC (Fig. 5C). Up to a concentration of 2  $\mu$ g/ml the endotoxin was ineffective, whereas IFN- $\gamma$  clearly up-regulated FH-mRNA. This supports the hypothesis that LPS does not have a direct effect on HC. The effects of the LPS-mediated up-regulation of FH-specific mRNA in KC were supported by the "quantification" of the FH protein in the supernatants of KC. As shown in Figure 6, the immunoblot analyses of proteins precipitated from supernatants of KC (30  $\mu$ g each) that had been stimulated with IFN- $\gamma$  or LPS showed an enhanced signal. KC as well as HC displayed an increased secretion of FH after stimulation with IFN- $\gamma$ . In addition, LPS at low concentrations (1 ng/ml) also induced an increase of FH in KC but not in HC.



**Figure 6.**

Immunoblot analysis of secreted FH protein from the culture supernatants of KC treated with IFN- $\gamma$  or LPS. Proteins in the culture supernatants were concentrated by precipitation, and 30  $\mu$ g each were applied to the gel. KC had been cultured for 96 hours, with the last 72 hours without an additional stimulus or with IFN- $\gamma$  (100 U/ml) or LPS (1 ng/ml).

## Discussion

The liver is the prime source of complement proteins in the blood. It has been claimed that complement FH also is mainly produced by this organ, although experimental evidence using primary cells of the liver has not yet been provided. Many hepatoma cells of human or murine origin such as Hep3b (Luo and Vik, 1999; Schwaeble et al, 1991), HepG2 (Lappin et al, 1992; Schwaeble et al, 1991), HepG4 (Schwaeble et al, 1991), H4 (Schwaeble et al, 1991), HUH7 (Friese et al, 1999), or +/+ Li (Vik, 1996) have been investigated with respect to the expression of FH. Most of the analyzed hepatoma cell lines (HepG2, HepG3, H4) have been found not to express the FH protein constitutively, whereas the human line HUH7 (Friese et al, 1999) and the murine line +/+ Li (Vik, 1996) have been found to produce FH in the absence of stimulation. The data concerning the induction of FH in these hepatoma cell lines are also controversial. For the hepatoma cell lines HepG2, HepG3, HepG4, and H4, it has been described that FH-specific mRNA expression cannot be induced by IFN- $\gamma$  (Lappin et al, 1992; Schwaeble et al, 1991). In contrast to these findings, Luo and Vik (1999) reported that Hep3b cells that did not express FH constitutively could be stimulated to express FH protein upon treatment with IFN- $\gamma$ . The only human hepatoma cell line that constitutively expressed FH (HUH 7) exhibited an IFN- $\gamma$ -dependent up-regulation of this molecule (Friese et al, 1999) similar to the murine +/+ Li cell line (Vik, 1996). This inconsistent profile of the investigated cell lines prompted us to investigate the constitutive expression and regulation of FH in cultures of primary rat HC and KC. In this study evidence was obtained that hepatoma-derived cell lines do not represent the physiologic behavior of nonmalignant HC, with regard to the expression and regulation of complement FH in liver cells. H4IIE cells from rat, like most of the human hepatoma cell lines investigated in former studies, do not constitutively express FH-specific mRNA. In addition, FH could not be induced in this cell line by the cytokines IL-6, TNF- $\alpha$ , IL-1 $\beta$ , or IFN- $\gamma$ . The other

hepatoma cell line, FAO, exhibits a higher constitutive expression of FH-specific mRNA than primary HC. However, this basal expression could not be augmented by the four cytokines used here.

These data are in contrast to a former study of our group in which the regulation of complement FI was investigated (Schlaf et al, 2001). In this study all regulatory effects mediated by IL-6 on HC were also observed with the hepatoma cell lines H4IIE and FAO. A comparison of both studies shows that hepatoma-derived cells, or malignantly transformed cells, need not necessarily display the physiologic behavior of a wild-type cell as concerns the expression of a given protein. Therefore conclusions drawn from results obtained with hepatoma cells should not be extended to wild-type cells without further analyses. For instance, HepG2 cells, FAO, and H4IIE cells express the C5a receptor (CD88) constitutively, but primary HC do not unless they are stimulated with proinflammatory cytokines such as IL-6 or IL-1 $\beta$  (Schieferdecker et al, 1997, 2000; Schlaf et al, 1999).

We investigated the basal expression and regulation of FH in primary HC and KC isolated from rat liver. An unexpected, high level of FH-specific mRNA was found in KC. As calculated for equal amounts of mRNA isolated from both cell types, the conclusion must be drawn that in normal rat liver KC are an abundant source of FH because the yield of FH-specific mRNA is nearly 10-fold higher in KC than in HC as calculated from the results of the quantitative-competitive RT-PCR. However, it is still difficult to estimate whether the whole KC population in the liver synthesizes more FH than the whole HC population because HC amount to 65% of the liver cells and KC to only about 7%. The data obtained by quantitative-competitive PCR suggest that the lower number of KC might be compensated for by their higher level of FH expression. Further analyses will have to measure quantitatively FH protein in culture supernatants of both cell types of rat liver by ELISA. We are presently developing such a test in our laboratory that will enable us to determine the amount of FH released per cell with and without stimulation and to determine the proportion of FH released by each cell type.

Although the constitutive expression of FH-specific mRNA is—as calculated above—much higher in KC than in HC, it can still be up-regulated by IFN- $\gamma$ . Although it had not been shown before in these cell types, the IFN- $\gamma$ -mediated up-regulation that was observed here for HC and KC was not unexpected because this phenomenon had formerly been observed in HUVEC (Brooimans et al, 1989; Dauchel et al, 1990; Lappin et al, 1992; Ripoche et al, 1988b; Schlaf et al, 2001), in primary myoblasts and in two rhabdomyosarcoma cell lines (CRL 1558 and HTB 153) (Legoedec et al, 1995), in primary fibroblasts (Friese et al, 1999; Katz and Strunk, 1988; Lappin et al, 1992; Schwaeble et al, 1991), in fibroblast-like L cells (Munoz-Canoves et al, 1989), in monocytes (Lappin et al, 1992), in the murine liver cell line +/+Li (Vik, 1996), and in the human Hep3b hepatoma cell line (Luo and Vik, 1999) and may therefore be a common feature. In the present study, the proinflam-

matory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$  did not up-regulate FH. This is in accord with the study of Williams and Vik (1997), who characterized the 5' flanking region of the human complement FH gene. Using the luciferase reporter gene in promoter assays, they found that only IFN- $\gamma$  was able to increase the mRNA level of FH in Hep3b hepatoma and U118-MG astrogloma cells. The down-regulation of FH observed after treatment of HC and KC with IL-1 $\beta$  was previously described for HUVEC (Brooimans et al, 1989; Ripoche et al, 1988b). Therefore, like the IFN- $\gamma$ -mediated up-regulation, it also seems to be a general mechanism for the regulation of the FH-specific expression.

Of special interest are the differences between HC and KC in the LPS-mediated up-regulation of FH. Our data on the LPS-mediated up-regulation of FH in KC are supported by Minta (1988) who described an LPS-mediated, 3-fold up-regulation of secreted FH protein in the premonocytic cell line U937, which expresses FH mainly in a membrane-bound form (Malhotra and Sim, 1985; Minta, 1988). Minta (1988) used LPS at a concentration of 1  $\mu$ g/ml, which is known to be far too high to represent a specific, receptor-mediated signal, eg, via LPS-binding CD14 molecules (LPS receptor) and TOLL-like receptors (Kopp and Medzhitov, 1999). A concentration as high as 1  $\mu$ g/ml may lead to unspecific effects (Wright, 2000). For this reason a possible LPS-mediated effect was investigated with much lower LPS concentrations (10 and 1 ng/ml). As analyzed by quantitative-competitive RT-PCR, FH-specific mRNA in KC was up-regulated up to 8-fold at 1 ng/ml LPS. IFN- $\gamma$ , which has been shown to be the main up-regulator of FH in HC, increased FH-specific mRNA in KC up to 4-fold. These data suggest that, especially after stimulation with endotoxins, KC are an abundant source of FH. In contrast to KC, no LPS-mediated effect on HC either at low concentrations (10 or 1 ng/ml) or at concentrations up to 2  $\mu$ g/ml was observed, strengthening the hypothesis that LPS may not have direct effects on HC. The results of this investigation are in accord with other recent studies in which the LPS-mediated induction of the receptor for the anaphylatoxin C5a could not be induced directly by LPS. Most probably, the endotoxin exerted its effects via proinflammatory cytokines that are released by KC upon stimulation with LPS at concentrations as low as 0.1 ng/ml (HL Schieferdecker, C Mäck, and G Schlaf, unpublished data). First results using FACS analyses and immunocytochemistry with mAbs against the rat CD14 molecule demonstrated that rat HC, in contrast to KC, did not carry the glycosyl phosphatidyl inositol (GPI)-anchored membrane-bound form of this protein but express only the soluble molecule (sCD14). But generally, an involvement of CD14 in the LPS-mediated signal transduction of KC is controversial (R. Landmann, Basel/Switzerland, personal communication, 2001) because there seem to exist ways of LPS-mediated signal transduction beyond the CD14/LBP (LPS-binding protein)-mediated mechanism.

Taken together our data show that the activation of the complement pathways may be regulated by proinflammatory cytokines and by LPS. IFN- $\gamma$ , the most prominent function of which is to activate macrophages, adjusts the balance between activation and inhibition of the complement system by the up-regulation of the inhibitory FH in primary HC and KC of rat. Thus, under inflammatory conditions, it may contribute to the homeostasis of the complement system.

## Materials and Methods

### *Animals, Cytokines, Cells, and Cell Lines*

Male Wistar rats used for the isolation of HC, weighing 200–250 gm (Winkelmann, Borchon, Germany), were kept for the isolation of HC on a 12-hour day/night rhythm with free access to water and a standard rat diet (Ssniff, Soest, Germany).

Recombinant human IL-6, which is known to function in rodents (Taga and Kishimoto, 1997), was from Boehringer Mannheim (Mannheim, Germany); recombinant rat (rr) IL-1 $\beta$  was from Strathmann (Hannover, Germany); and rrIFN- $\gamma$  and rrTNF- $\alpha$  were from R&D Systems (Wiesbaden, Germany). LPS-endotoxin from *Escherichia coli* 026:E6 was from Sigma (Deisenhofen, Germany).

HC were isolated without using collagenase according to the method of Meredith (1998). The liver was perfused at 37° C in a noncirculating manner via the portal vein with Ca<sup>2+</sup>-free Krebs-Henseleit buffer containing 15 mM glucose, 2 mM lactate, 0.2 M pyruvate, and 2 mM EDTA. The flow rate was 10 ml/minute. The liver was excised after 45 minutes, its capsule was opened, and the cells were suspended in Krebs-Henseleit buffer containing 1 mM CaCl<sub>2</sub> and filtered through nylon gauze (mesh diameter 60  $\mu$ m). Debris was removed by two washing steps at 50 $\times$ g. Viable HC were purified using a Percoll gradient of 58%. Their homogeneity was assessed by their typical light microscopic appearance and by FACS analyses using an anti-dipeptidyl-peptidase (CD26) mAb (Biozol, Eching, Germany), which does not bind to liver cell types other than HC. Purity was found to be 98% to 100%. KC were prepared by a combined collagenase/pro-nase perfusion and were purified by Nycodenz density gradient centrifugation and subsequent centrifugal elutriation using a Beckmann JE-6 elutriation rotor in a J-21 Beckmann centrifuge (Beckmann, München, Germany) according to the method of Eyhorn et al (1988). The rat hepatoma-derived cell lines H4IIE and FAO were from the cell pool of the Institute of Biochemistry and Molecular Cell Biology (Georg-August University, Göttingen, Germany). KC were cultured in a medium consisting of 80% RPMI, 20% medium 199 [medium 80/20] with 10% FCS supplemented for the first 24 hours. HC were cultured in medium 80/20 supplemented with 10<sup>-4</sup> M dexamethasone and 10<sup>-5</sup> M insulin for the whole time and with 4% newborn calf serum added for the first 24 hours. For the investigation of FH synthesis after the first 24 hours, HC and KC were cultured in medium 80/20 for an additional 72

hours on collagen-coated (HC) or uncoated dishes (KC).

### *Northern Blot Analysis*

Total cellular RNA was extracted from HC using the RNeasy total kit (Qiagen, Hilden, Germany). RNA was denatured with formamide and formaldehyde, and electrophoresis was performed on gels containing formaldehyde. After capillary transfer overnight onto Hybond-N nylon membranes (Amersham-Pharmacia, Freiburg, Germany), the separated mRNA transcripts were probed with a digoxigenin (dig)-labeled cDNA probe generated with the rat FH-specific primers FH sense (5'-GTGACGTGGTGAATATGATTGCA-3') and FH antisense (5'-TTCAGACCACTGTCCTCTACACA-3'), resulting in an amplicon of 1003 nucleotides. The dig probe had been generated using a DNA-dig labeling and detection kit (Boehringer Mannheim), which works with random primer hexamers. Prehybridization was performed in Ultra-Hyb solution (Ambion, Austin, Texas) for 45 minutes at 42° C followed by hybridization with the dig-labeled probe overnight in the same solution at 42° C. After several washes, the nylon membranes were treated according to the instructions in the kit. They were first blocked with a special blocking solution and then incubated with an anti-digoxigenin antibody for 30 minutes. After two washes the color reaction was developed for at least 6 hours according to the supplier's instructions.

### *Reverse PCR Amplification to Generate the Northern Blot Hybridization Probe for FH and FH-Specific Signals*

Messenger RNA from all cells was prepared with the RNeasy total kit (Qiagen). The subsequent transcription into cDNA was performed using the Super Script Preamplification system (Gibco-BRL, Eggenstein, Germany). PCR was carried out with the primer FH sense (5'-GTGACGTGGTGAATATGATTGCA-3') and FH antisense (5'-TTCAGACCACTGTCCTCTACACA-3'), resulting in an amplicon of 1003 nucleotides. This amplicon did not represent the nucleotides of the first seven SCR, ie, it represented only the mRNA of FH but not of FHL-1/reconectin. PCR was performed using *Taq*-polymerase (Red-*Taq*; Sigma) with denaturing at 94° C for 1 minute, annealing at 56° C for 1 minute, and extension at 72° C for 1 or 2 minutes in 32 cycles (conventional semiquantitative PCR) or in 35 cycles (quantitative-competitive PCR and generation of the hybridization probe for Northern blots). For control purposes amplified cDNA was sequenced using the dideoxy chain termination method and was identified to be part of rat FH cDNA. As a control for RT-PCR, a primer pair was used to amplify rat  $\beta$ -actin cDNA. The sense primer was (5'-GATATCGCTGCGCTCGTCGTC-3') and the antisense primer was (5'-CCTCGGGGCATCGGAACC-3'), resulting in an amplicon of 749 nucleotides. Complement FI was amplified using the sense primer (5'-GTCTTCTGCCAGCCRTGGCAGAG-3') [R=A+G] and the antisense primer (5'-GTRATGCAGTCCACCTCACCATT-3') [R=A+G]

(accession number Y18965), resulting in an amplicon of 707 nucleotides. As markers for the LPS-dependent activation of KC, rat IL-6-specific cDNA (accession number E02522) was amplified using the primer pair IL-6 sense (5'-CTGACCAC-AGTGAGGAATGT-3') and IL-6 antisense (5'-TGGAAATGAGAAAAGAGTTG-3'), resulting in an amplicon of 499 nucleotides. For the same reason, IL-1 $\beta$ -specific cDNA (accession number E01884) was amplified using the primer pair IL-1 $\beta$  sense (5'-GGGCGGTTCAAGGCATAA-3') and IL-1 $\beta$  antisense (5'-CAGCAGGAGGCATTTTGT-3'), resulting in an amplicon of 484 nucleotides. The rat IFN- $\gamma$  receptor was identified by RT-PCR using the degenerated primers sense (5'-TCRGTGCCTRYACCRACKAATGTT-3') and antisense (5'-CAAGGACTTRGGTAAYATTATGCT-3') [Y=C+T, R=A+G, K=G+T] because only the receptors of mouse (Munroe and Maniatis, 1989) and man (Aguet et al, 1988) have been cloned.

### Quantitative-Competitive PCR

For quantitative-competitive PCR, a constant amount of cDNA from HC or KC, without or after stimulation, was coamplified with a  $\lambda$ -DNA standard of 500 nucleotides (TaKaRa Biomedicals, Tokyo, Japan) flanked by chimeric segments. Coamplification using combined primers was performed in 5- or 10-fold dilution steps of the standard under the conditions outlined above. After amplification PCR products were separated in 1.5% agarose gels. Bands were visualized by ethidium bromide staining and amplicates of standard  $\lambda$ -DNA and FH were checked for equal staining intensity.

### Generation of mAbs and Immunoblot Analysis

mAbs were generated against FH purified from rat serum according to the method established by Daha and van Es (1982). Four immunizations with 100  $\mu$ g protein each in TiterMax adjuvant (Sigma) were performed, and anti-FH titers were monitored in an ELISA assay with immobilized rat FH detected by 2-fold dilutions of 1:50 prediluted antiserum from immunized mice. The fusion of spleen cells with Ag8.653 myeloma cells was performed according to standard protocols using cells from the animal with the highest serum anti-rat FH titer. Screening of the undiluted supernatants from hybridoma cultures was, in a first step, performed by an ELISA with the immobilized FH protein as the antigen. Supernatants that scored positive were screened in an immunoblot assay against SDS gel-separated and then blotted rat FH protein or serum samples. For this 4  $\mu$ l of rat serum per lane were boiled in SDS sample buffer for 5 minutes and then separated using an SDS minigel system (Biometra, Göttingen, Germany). The separated samples were transferred onto a nitrocellulose sheet under a current of 150 mA for 2.5 hours using a semi-dry transfer chamber (Multiphor-Novablot, Pharmacia-Amersham, Freiburg, Germany). The nitrocellulose sheet was blocked with 2% BSA (30 minutes), and each lane was excised and incubated with undiluted supernatants of the selected hybridomas for 3 hours.

The secondary antibody goat anti-mouse IgG (absorbed against rat, human, bovine, and horse serum proteins) (Dianova, Hamburg, Germany) was used at a dilution of 1:4000 for 2 hours. The color reaction was performed with diaminobenzidine (Sigma) and H<sub>2</sub>O<sub>2</sub>. Hybridomas of which the supernatants recognized the FH band in the range of 150 kd were cloned and expanded.

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