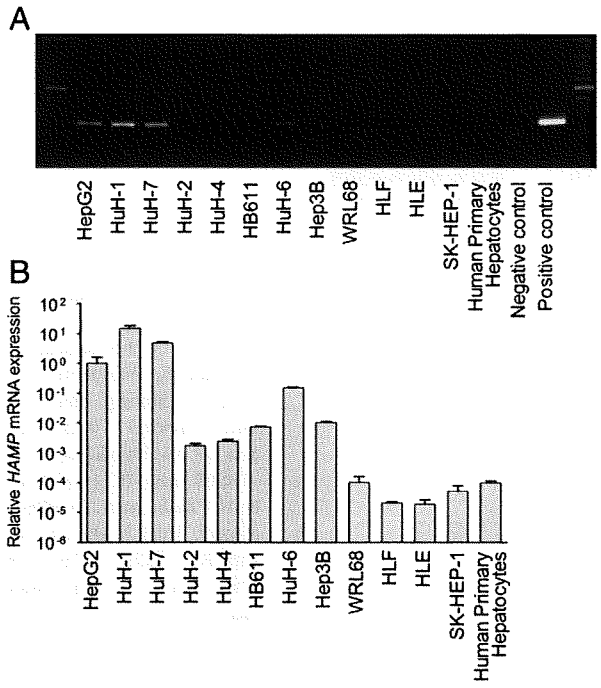


**Figure 3.** Expressions of *HAMP* mRNA in hepatoma-derived cell lines determined by qRT-PCR. (A) Qualitative RT-PCR showed that the expressions of *HAMP* mRNA were quite different among hepatoma-derived cell lines. (B) The expression levels of *HAMP* mRNA were standardized by 18*S* rRNA. Relative *HAMP* mRNA expression levels are shown as fold expression over the average of *HAMP* mRNA of HepG2 cells. HepG2, HuH-7, and HuH-1 cells highly express *HAMP* mRNA, while other cell lines exhibits only slight or moderate *HAMP* mRNA expressions.

human holo-Tf [24],] we believe ours is the first study showing upregulation of hepcidin at the peptide level by human holo-Tf in human cells. The physiological function of this effect is, however, not apparent since only hepcidin-25 is known to be involved in iron metabolism.

### 3.5 Determination of the changes of hepcidin expression in responses to various stimulations of HepG2 cells

The HepG2 cell line is one of the most frequently used hepatoma-derived lines for research and secretes mainly hepcidin-25, the only isoform reported to interact with ferroportin, into the culture medium.

Hepcidin expression has been reported to be regulated by inflammatory cytokines such as IL-6 and IL-1β; hence, HepG2 cells were stimulated with these cytokines. As shown in Fig. 5A, IL-6 significantly upregulates hepcidin-25, in agreement with earlier reports. A slight increase of hepcidin-22 was observed with IL-1β stimulation, but no obvious upregulation was seen in hepcidin-25. Iron overload has been reported to upregulate hepcidin expression *in vivo*, but addition of holo-Tf and FAC in the medium suppressed the expression of hepcidin-25. These results conflict with those of some *in vivo* investigations, but other transcriptional studies showed similar data to ours. Reasons for these discordances are still unknown.

Of interest, FAC increased hepcidin-22 expression by an unknown mechanism. DFO suppressed hepcidin-25 expression as expected change, but hepcidin-22 expression was increased. To investigate the effects of bacterial infections, LPS was added to media, and it significantly increased both hepcidin-22 and -25. Hypoxia is also reported to decrease hepcidin expressions [25], while in our studies $CoCl_2$ increased expression of both hepcidin-22 and -25. The furin inhibitor decreased hepcidin-25, but surprisingly hepcidin-22 was increased.

We then determined whether the inclusion of FBS influenced expressions of hepcidin types. Expressions of hepcidin-22 and -25 increased as higher concentrations of FBS were provided in the culture media, an effect that may have been due to the presence of cytokines in the FBS. HepG2 cells were then studied with various stimulants in FBS-free media. As shown in Fig. 5B, hepcidin expression levels were all lower than those determined in FBS-containing medium, and were almost at the limit of measurement by our method, but IL-6 upregulated hepcidin-25 expression. Holo-Tf and FAC depressed both hepcidin-22 and -25, as did the furin inhibitor. The finding that inclusion of FBS significantly influenced the expression of hepcidin deserves consideration from *in vitro* research using cultured cells.

## 4 Discussion

First developed for assaying prohepcidin [17], studies have used ELISA for measuring hepcidin in serum and urine. Lack of information about the physiological properties and importance of prohepcidin in clinical samples makes interpretation of these studies difficult. The main active isoform of hepcidin is believed to be hepcidin-25, but little information is available about how much translated prohepcidin in hepatocytes is released intact. In fact, Valore and Ganz have pointed out recently that most hepcidin released from the cells is the mature 25-residue form produced by furin [13]. Most recently, Ganz *et al.* developed a novel ELISA system for human serum hepcidin and it is expected that this method will be a powerful tool for clinical investigations, but it is unclear whether this method can be applied for *in vitro* research [23].

Methods utilizing MS-based modalities such as SELDI-TOF-MS have been widely used for measuring hepcidin in serum and urine samples [19]. However, the reliability of SELDI-TOF-MS for quantifying multiple molecules such as hepcidin isoforms is still unclear.

We recently developed a method utilizing LC/ESI-MS/MS for quantification of hepcidin [22]. We aimed to improve and extend this method to apply it for measurement of

 

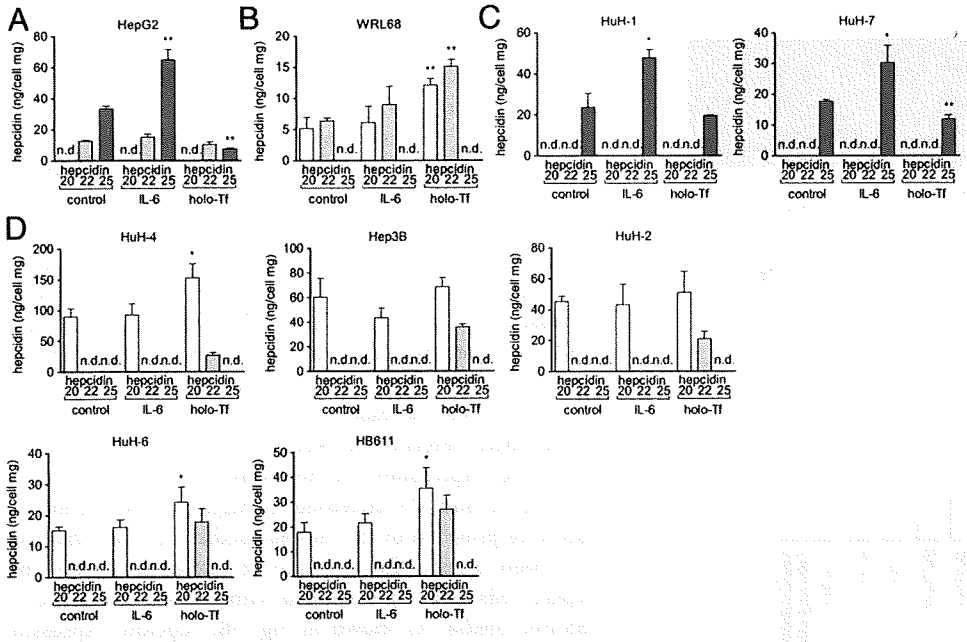


**Figure 4.** Quantification of hepcidin isoforms in the culture media of hepatoma-derived cell lines. The patterns of the expression of hepcidin isoforms were different among cell lines, and divided into four groups. (A) HepG2 cells, (B) WRL68 cells, (C) HuH-1 and HuH-7 cells, and (D) HB611, Hep3B, HuH-2, HuH-4, and HuH-6 cells. $^{*}p<0.05$, $^{**}p<0.01$, n.d.: not detected.

hepcidin secreted in culture media by hepatoma-derived cell lines. Our present assay, using MS with trichloroacetic acid precipitation, succeeds in this. Moreover, the new method can simultaneously detect and distinguish hepcidin-20, -22, and -25. The linear relationship between the peak area and hepcidin concentration provides simultaneous quantification of hepcidin-20, -22, and -25 isoforms. To our knowledge, this is the first report for simultaneous and quantitative measurement of hepcidin isoforms, applicable to evaluating hepcidin levels and their response to various stimulations for research using cultured cells. We believe that this method can be applied to clinical as well as research studies, thereby providing new information about hepcidin isoforms levels in serum. Determination of hepcidin isoforms may also be a biomarker for differential diagnosis and evaluation of disease activity in clinical studies, although further investigation is needed.

One advantage of our method is that it does not depend upon an antibody against hepcidin. Specificity of antibodies used for quantification of hepcidin requires validation to exclude the possibility that they recognize two or three isoforms of hepcidin simultaneously. Our method can also measure many samples in a relatively short time, so that it is useful for clinical samples and samples from *in vitro* research. However, it does require internal standards of hepcidin isoforms and mass spectrometers but still may be of interest in diverse laboratories.

We found differences in expression of *HAMP* mRNA among cell lines derived from hepatocytes. This finding indicates that such differences must be considered in using these cell lines for research in hepcidin expression.

HLE, HLF, and SK-HEP-1 cells exhibited low *HAMP* mRNA expression in qRT-PCR and did not secrete detectable hepcidin. They may have lost some physiological functions common to hepatocytes.

There were unexpected differences of secretion and response to various stimulations of hepcidin isoforms among cell lines. The cell lines that secreted detectable hepcidin in our study can be divided into at least four groups, suggesting that hepatocytes in the liver *in vivo* might possess different characteristics from each other. We believe this is the first report of the variety of hepcidin isoforms' expression patterns in hepatoma-derived cell lines. Possibly, one subset of hepatocytes is involved in only iron metabolism, while another line is involved in both iron metabolism and the antimicrobial system.

Care should be taken in evaluating hepcidin expression from transcriptional levels because we did not find any obvious correlation between *HAMP* mRNA expression and hepcidin secretion (Figs. 3 and 4). Moreover, different cell lines exhibit different patterns of hepcidin isoforms' secretion. Our data indicate that HuH-7 cells and Hep3B cells each express an mRNA of the *HAMP* gene as determined by RT-PCR. However, HuH-7 cells secrete only hepcidin-25 into the culture medium, and Hep3B cells secrete hepcidin-20 but no detectable hepcidin-25. These observations indicate a risk of misinterpretation if only transcriptional studies are performed for investigation of hepcidin expression, especially for *in vitro* research.

In this study, we subjected HepG2 cells to various stimulations, and observed changes of hepcidin-22 and -25 levels in culture media. The changes of hepcidin-22 and -25 were not parallel; therefore, again the determination of only *HAMP* mRNA might lead to misinterpretation, so simultaneous determination of hepcidin isoforms is strongly recommended. We observed changes of hepcidin-25 that are

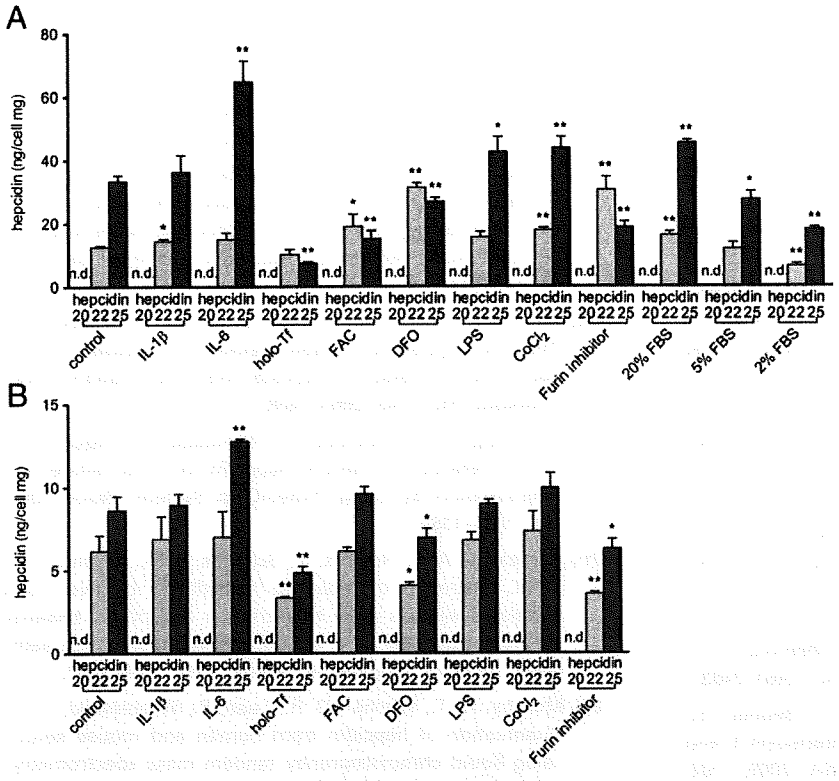


**Figure 5.** (A) Changes of hepcidin isoforms' expressions induced by various stimulations in the HepG2 cells. IL-6 (20 ng/mL), IL-1β pg/mL, holo-Tf (30 μM), FAC (100 μM), DFO (100 μM), $CoCl_2$ (50 μM), LPS (1 μg/mL), and furin inhibitor (50 μM) were added to the culture media of HepG2 cells as indicated. In addition, the effect of the concentrations of FBS on the expressions of hepcidin isoforms was determined. (B) HepG2 cells were incubated with serum-free medium UltraCulture. Hepcidin expression levels were all lower than those observed in FBS-containing medium. IL-6, IL-1β, holo-Tf, FAC, DFO, $CoCl_2$, LPS and furin inhibitor were also added to observe their effects on hepcidin isoforms' expressions. $^*p<0.05$, $^{**}p<0.01$, n.d.: not detected.

consistent with data previously reported elsewhere so that our method for quantification of hepcidin isoforms would be useful for investigating responses of hepatocytes to various stimulations. Observed changes that remain unexplained indicate a need for further investigation of the responses of hepatocytes to various stimulations in their expression of hepcidin isoforms.

We realize that varying concentrations of FBS might lead to different results even in the presence of identical stimulations. For example, the furin inhibitor decreased hepcidin-25 while hepcidin-22 was increased (Fig. 5A) in the presence of FBS. This suggests that the pathway for producing hepcidin-22 was activated when the pathway for producing hepcidin-25 was inhibited by furin inhibitor, thereby maintaining the total concentration of hepcidin although skewing the balance between isoforms. However, the precise mechanism of the effect is not known. Both hepcidin-22 and -25 were suppressed when cells were treated with furin inhibitor in FBS-free conditions (Fig. 5B), and this is contrary to the result observed in the presence of FBS. We speculated that the absence of FBS may stress the cells, increasing the sensitivity to furin inhibitor. We recognize that furin is a proprotein convertase acting on hepcidin expression at the posttranslational level [13], so that its inhibition should not be selectively affected by FBS. It is also possible, however, that unknown factors in FBS might upregulate hepcidin-22, since its concentration in FBS-free conditions could not be increased in our study. It may be advisable, therefore, to provide precisely controlled concentrations of FBS in further studies of expression of hepcidin isoforms *in vitro*, since FBS may already contain stimulants of hepcidin expression.

In conclusion, we have devised a method for simultaneous quantification of hepcidin-20, -22, and -25 in culture media by hepatoma-derived cell lines. Using this method, we determined the expression patterns of hepcidin isoforms and their responses to various stimulations in cultured cells, and we found that there are substantial differences among cell lines. We also found no obvious correlation between *HAMP* mRNA expressions and hepcidin isoforms' secretion. Levels of prohepcidin in the culture medium were too low to be detected by ELISA, indicating the necessity of directly measuring hepcidin instead of estimating it from prohepcidin measured by ELISA, especially *in vitro* studies. We believe that our method can contribute to *in vitro* research on the regulation of hepcidin expression, needed because the regulation of hepcidin expression is complex and difficult to investigate precisely *in vivo*.

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