Factor VIII expression and regulation in health and disease
Hollestelle, M.J.

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Chapter 3

Factor VIII expression in liver disease

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ABSTRACT

Liver disease is associated with markedly elevated plasma factor VIII (FVIII) levels, whereas the synthesis of many other coagulation factors and proteins is reduced. In order to define the mechanism of FVIII increase, we have determined the expression levels of FVIII, both at mRNA and protein level, in patients with liver disease who underwent partial liver resection. In addition, the expression of von Willebrand factor (VWF) and low density lipoprotein receptor-related protein (LRP), proteins known for their ability to modulate FVIII plasma levels, were examined. Tissue samples for RNA extraction were obtained from 4 patients with cirrhosis, 9 patients with liver failure without cirrhosis and 6 patients with liver metastasis of a colon or rectum carcinoma (control group). In patients with liver cirrhosis hepatic FVIII and LRP mRNA levels were significantly lower than controls (p < 0.010), while VWF mRNA was significantly higher (p < 0.050). Immunohistochemical analysis revealed that cellular VWF protein distribution was also increased in cirrhotic livers compared to liver tissue from patients with non-cirrhotic liver disease. In cirrhotic tissue enlarged portal veins appeared to overgrow FVIII producing sinusoidal endothelial cells. Similarly, the number of LRP-producing cells appeared to be lower in cirrhotic tissue than in controls. The plasma concentration of both FVIII and VWF was significantly higher in patients with cirrhosis than control subjects (p = 0.038 and 0.010 respectively). These results demonstrate that elevated plasma FVIII levels in liver cirrhosis are associated with increased hepatic biosynthesis of VWF and decreased expression of LRP, rather than increased FVIII synthesis.

INTRODUCTION

In contrast to other coagulation factors and proteins synthesized by the liver, the plasma concentration of factor VIII (FVIII) is frequently elevated in liver disease (reviewed in (1,2)). The pathogenic mechanism for the rise of FVIII is poorly understood but may result from a number of cellular and humoral abnormalities associated with liver disease. FVIII is expressed in a variety of tissues, including the liver, kidney, spleen, lungs and brain (3,4). A more detailed and quantitative study in mice revealed the highest levels of FVIII mRNA in both the liver and kidney (4). Reverse transcription PCR (RT-PCR) analysis of purified liver cells showed that FVIII gene expression was primarily confined to the sinusoidal endothelial cells (4,5). It is conceivable that in liver disease protein synthesis by sinusoidal endothelial cells and hepatocytes are affected to a different extent. This may explain the differences between the plasma concentration of the coagulation factors produced only by hepatocytes and the plasma concentration of FVIII.

In addition, the role of von Willebrand factor (VWF) in controlling FVIII levels should be considered. VWF is found at high levels in plasma in liver disease, particularly in acute liver failure (6) and at even higher levels in patients with liver cirrhosis (7,8). VWF is the natural carrier protein of FVIII. This interaction protects FVIII against proteolytic inactivation and premature clearance (reviewed in (9)). Very low levels of VWF and VWF
mRNA are seen in liver vascular endothelial cells (10); no, or extremely low VWF mRNA levels were detected in the sinusoidal endothelium (3-5). On the other hand, histochemical analysis of liver tissue revealed that in hepatic disease VWF expression is increased (11,12). Therefore, it is possible that elevation of VWF expression in liver disease may affect the plasma level of FVIII as well.

A factor that may also serve a role in regulating FVIII levels in plasma is the multifunctional endocytic receptor, low density lipoprotein receptor-related protein (LRP). To date, it has been well established that FVIII comprises multiple binding sites for LRP (13,14) and it has been proposed that LRP serves a role in controlling cellular uptake and subsequent degradation of FVIII (14,15). LRP is particularly expressed in the liver, notably Kupffer cells and hepatocytes (16). Consequently, in liver disease LRP expression might be decreased. This may impair the clearance of FVIII from the circulation, which, in turn, will result in increased plasma FVIII concentration.

In the present study, we measured FVIII gene expression by quantitative RT-PCR in hepatic tissue from patients with liver disease with different etiologies and related FVIII mRNA levels to VWF and LRP mRNA levels of these tissues. In addition, mRNA levels of factor V (FV) and antithrombin, typical markers of hepatocyte function, were quantified by RT-PCR. Transcript levels were related to the plasma concentrations of their respective translation products (FVIII, VWF, antithrombin, FV). Immunostaining of liver specimens (FVIII, VWF, LRP) was also performed to complement gene expression data.

**PATIENTS AND METHODS**

*Patients*

This study included 19 patients with liver disease (median age 60 years, range 27-76 years) who underwent partial liver resection. The diagnosis was based on standard clinical, biochemical and radiological data and confirmed by histopathological examination, including hematoxylin and eosin staining. The laboratory data of the patients on admission are shown in Table 1. The study was approved by the Protocol Review Board of the Academic Medical Center. Informed consent was obtained of all patients included in this study. The patients studied were divided in three groups: Group I consisted of patients with liver cirrhosis due to a proximal bile duct tumor (2) or viral hepatitis (2). These patients were all graded as Child A according to the Child-Pugh classification (17). Group II comprised patients with liver disease, who had no concomitant liver cirrhosis. Six patients of this group had a proximal bile duct tumor, 1 patient had biliary stone disease, 1 patient had echinococcal infection and 1 patient suffered from viral hepatitis. Group III consisted of patients, with liver metastases of a colon or rectum carcinoma and served as control group (18).
### Table 1. Clinical & laboratory demographics of patients studied

<table>
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<th>Group</th>
<th>Etiology</th>
<th>Sex</th>
<th>Age</th>
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<th>VWF (U/100 ml)</th>
<th>FV (U/100 ml)</th>
<th>AT (U/l)</th>
<th>ASAT (U/l)</th>
<th>ALAT (U/l)</th>
<th>CRP (mg/l)</th>
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| Normal range | 50-150 | 50-150 | 70-130 | 80-120 | < 40 | < 45 | < 7 |

FVIII, VWF, FV, AT = antithrombin, ASAT = aspartate-aminotransferase and ALAT = alanine-aminotransferase. *) Patients with liver cirrhosis; n.d. = not determined; underlined data indicates elevated levels, double underlined data indicates decreased levels.

**Plasma preparation**

Blood was collected (4.5 ml in 0.5 ml 3.1% sodium citrate) at the start of surgery during anaesthesia and before patients received interfering medication. Samples were centrifuged at 2500 g for 15 min at 4°C and plasma was stored at -70°C until tested. Protein levels were also measured on 9 patients the day before operation. No differences were observed compared with the values measured from samples collected at the start of surgery.
Assays

FV- and FVIII procoagulant activity was measured using one-stage clotting assays. FV antigen was measured by an ELISA using sheep anti-human FV IgG as coating antibody and peroxidase-conjugated sheep anti-human FV (both from Kordia, Leiden, The Netherlands) as the detecting antibody. FVIII- and VWF antigen were measured by ELISA according to previously published methods (19,20). Antithrombin activity was measured by a chromogenic assay according to the manufacturer's instructions (Chromogenix, Milano, Italy). Pooled plasma from 40 healthy individuals was used as the reference standard.

Liver tissue preparation and RNA extraction

Liver biopsies (5 x 5 mm) were obtained at the start of surgery and immediately frozen in liquid nitrogen. All liver biopsies were divided in two parts; one part was used for immunohistochemical analysis and the other part for total RNA isolation. Total RNA was isolated from the frozen tissues by acid guanidine thiocyanate-phenol-chloroform extraction as previously described (21) and then treated with RNase-free DNase (Promega Corp., Madison, WI) to remove contaminating genomic DNA. Extracted RNA was quantified by measuring absorption at 260 nm.

Real-time quantitative PCR

The concentration of various mRNAs (i.e. FVIII, FV, VWF, LRP, antithrombin, glucose-6-phosphate dehydrogenase (G6PD)) was determined using a quantitative real-time RT-PCR method with specific primer combinations (22-27). Total tissue RNA (1 μg) was reverse transcribed. Real-time PCR of the cDNA was performed using a LightCycler rapid thermal cycler (Roche Diagnostics Ltd, Lewes, UK) according to the manufacturer's instructions. Serial diluted solutions of the cDNA mixture were amplified using specific primers (Table 2). Amplified PCR product was measured using SYBR green. Serial dilutions of a cDNA mixture, prepared by reverse transcribing a commercial liver RNA sample (Clonetech, Palo Alto, CA), were used to prepare a standard curve. An undiluted sample of the standard was set as 100%. RNA levels were corrected for input differences and reverse transcription efficiencies by dividing all measured values by the corresponding mRNA levels of G6PD, a housekeeping gene. G6PD was chosen because initial studies demonstrated relative little variation (11%) between various samples compared to other genes considered as housekeeping gene. All RNA samples were analyzed three to four times. The mean values are reported. Based on eight to nine measurements of a given sample, the variation coefficient of the assay of respectively FVIII-, VWF-, FV-, antithrombin- and LRP mRNA was 10, 13, 7, 22 and 8%.
### Table 2. Sequences of PCR primers used

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<th>Gene</th>
<th>Primer sequences</th>
<th>Position</th>
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<td>5'-CCTTGGAAATCTCGCCAATA-3'</td>
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<td>155</td>
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<td>3' FVIII</td>
<td>5'-TCGTAGTTGGGTCTCTCT-3'</td>
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<tr>
<td>5' FV</td>
<td>5'-ATCAGAGCCAGTACAGAGA-3'</td>
<td>1357</td>
<td>236</td>
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<tr>
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<td>5'-AACACTGGGCATTTTC-3'</td>
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<tr>
<td>5' VWF</td>
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**Immunohistochemistry**

Acetone-fixed, frozen liver sections were blocked for endogenous peroxidase activity by incubating the slides for 10 minutes with 0.3% H$_2$O$_2$ and 0.1% NaN$_3$ in phosphate buffered saline (PBS). The slides were then incubated with rabbit anti-human VWF IgG (Dako, Glostrup, Denmark), sheep anti-human FVIII (Kordia) or goat anti-human LRP (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 hour. Subsequently, the slides were washed with PBS and treated with either peroxidase labeled horse anti-rabbit IgG (Sanquin, Amsterdam), peroxidase labeled donkey anti-sheep IgG (Kordia), or peroxidase labeled rabbit anti-goat IgG (Santa Cruz Biotechnology) for 30 minutes. After washing the slides with PBS, the peroxidase label was visualized by incubation with 0.03% H$_2$O$_2$ and 0.6 mg/ml diaminobenzidine in PBS for 10 minutes. The slides were then washed with distilled water and counterstained with hematoxylin (Vector Laboratories, Inc, Burlingame, CA) for 30 seconds. The slides were again washed with distilled water, dehydrated with ethanol and xylene and mounted with permanent mounting medium (Vector Laboratories) and examined by light microscopy. Immunohistochemistry was performed on liver sections of 4 patients without cirrhosis and 3 patients with liver cirrhosis.

**Statistical analysis**

All data was analysed using the Mann-Whitney U-test. The Spearman correlation coefficient was calculated to determine the relationship between two parameters.
RESULTS

Plasma levels of coagulation factors and other plasma proteins in liver disease

The plasma concentration of FVIII antigen was elevated in 13 of the 19 patients studied (Fig.1; Table 1). Also VWF antigen levels were frequently increased in these patients (15 of 19 patients). There was a positive correlation between VWF and FVIII antigen in all patients with liver disease \( (R = 0.66; p = 0.002) \). The majority of patients displayed normal FV antigen levels (13 of 19 patients, Fig.1; Table 1). In 5 patients FV levels were below the normal range. An elevated FV level was detected in one patient with a bile duct tumor \( (159 \text{ U/100 ml}) \). Antithrombin activity levels were decreased in 8 patients studied and normal in the other 11 patients with liver disease.

![Figure 1](image-url)

**Figure 1.** Plasma concentration of FVIII-, VWF- and FV antigen and antithrombin activity in liver disease. Group I: patients with liver cirrhosis (closed circles, patients with a bile duct tumor or a gallstone; closed triangles, patients with a liver infection), group II: miscellaneous (open circles, patients with a bile duct tumor or a gallstone; open triangles, patients with a liver infection), group III: liver metastasis (squares). Horizontal bars show median values. Grey area = normal range. *\( p = 0.038 \), **\( p = 0.010 \).
In patients with liver cirrhosis (group I, n = 4), FVIII and VWF plasma levels were significantly elevated, compared to the control patients (group III, n = 6, $p_{FVIII} = 0.038$ and $p_{VWF} = 0.010$, Fig.1). On the other hand, no significant differences in FV and antithrombin levels were seen between patients with liver cirrhosis and other patients studied. Levels of aspartate aminotransferase (ASAT, median: 116 U/l) and alanine aminotransferase (ALAT, median: 144 U/l) were significantly higher in patients with liver cirrhosis compared to control patients studied ($p_{ASAT/ALAT} = 0.016$; Table 1), indicative of hepatocellular injury. The ASAT and ALAT levels in the patients with hepatic disease associated with other etiologies (group II) were also significantly increased, but to a lesser extent (median$_{ASAT}$: 57 U/l, median$_{ASAT}$: 58 U/l, $p_{ASAT/ALAT} = 0.045$).

C-reactive protein (CRP) was slightly elevated in most patients (median: 12 mg/ml; normal < 7 mg/ml), except in the patient with echinococcus infection, who had an extremely high level (329 mg/l). No correlation was observed between CRP and plasma FVIII or VWF levels or the plasma concentration of the other proteins studied.

*mRNA levels of FVIII, VWF, LRP, antithrombin and FV in liver biopsies*

Significantly lower FVIII mRNA levels were found in patients with liver cirrhosis compared to the control patients in group III ($p = 0.010$; Fig.2). Thus, it appears that increased plasma levels of FVIII were not associated with increased transcriptional activity of FVIII gene, rather an inverse relationship was noted (though not significant). VWF mRNA levels,
on the other hand, were significantly increased in cirrhotic patients compared to the control patients \( (p = 0.038) \) and the patients with hepatic disease associated with other etiologies, who did not display liver cirrhosis \( \text{group II, } p = 0.006; \) Fig.2). This suggests that the observed elevated VWF plasma levels are caused, at least in part, by increased transcriptional activity of VWF in the liver. LRP mRNA levels in patients with liver cirrhosis were significantly lower compared to the control patients \( (p = 0.010, \) Fig.2), in contrast to the FVIII antigen levels in these patients, which were markedly elevated. In addition, a significant decrease in LRP mRNA levels was also seen for the patients in group II \( (p = 0.036) \) compared to the control patients.

**Figure 3. Immunostaining analysis of VWF antigen in human liver sections.** In a patient without cirrhosis a normal regular pattern was seen of hepatocytes and sinusoidal endothelial cells \( \text{(A)} \) with a large vessel \( \text{(large arrow, hematoxylin/eosin staining)} \). In a patient with cirrhosis \( \text{(B)} \) an increase in bile ducts \( \text{(small arrow)} \) was seen in combination with fibrosis \( \text{(white arrow)} \). A parenchymal nodule \( \text{(open arrow)} \) and large leukocyte infiltration was also observed \( \text{(arrow heads)} \). VWF antigen \( \text{(C)} \) was mainly localized in endothelial cells of larger vessels \( \text{(large arrow)} \) in a patient without cirrhosis, whereas in a patient with cirrhosis VWF antigen \( \text{(D)} \) was also detected in the neovascularature \( \text{(small arrow heads)} \) and to a lesser extent in the surrounding sinusoidal endothelial cells. \( \text{(Original magnification 100x)} \).

Significantly lower levels of FV mRNA were demonstrated in patients with liver cirrhosis compared to the control patients \( (p = 0.038) \). No significant difference in FV mRNA was seen when comparing patients with liver cirrhosis to the patients studied in group II. Significantly lower mRNA levels of antithrombin were found in patients with liver
cirrhosis compared to the control patients ($p = 0.010$, Fig.2). Furthermore, the patients in group II containing the non-cirrhotic liver patients with biliary obstruction, echinococcal infection or viral hepatitis, also showed significantly lower antithrombin levels, confirming the slightly affected hepatocyte function of these patients ($p = 0.050$).

Figure 4. Immunostaining analysis of FVIII antigen in human liver sections. FVIII antigen was mainly localized in sinusoidal endothelial cells (small arrows), both in hepatic tissue of patient without cirrhosis (A,B) and in a patient with cirrhosis (C,D). No FVIII was seen in endothelial cells of enlarged portal veins of the cirrhotic liver (open arrow; C,D). (Original magnification 100x (A,C) and 400x (B,D)).

Figure 5. Immunostaining analysis of LRP antigen in human liver sections. In a patient without liver cirrhosis LRP antigen is localized in hepatocytes and Kupffer cells (A,B). In a patient with liver cirrhosis enlarged portal veins were observed, showing less expression of LRP. LRP antigen was also seen in some cells of a leukocyte infiltration, probably monocytes and in bile duct cells (C,D). (Original magnification 100x (A,C) and 400x (B,D)).
Histology and immunohistochemistry of VWF, FVIII and LRP in the liver

Immunohistochemical analysis of VWF antigen in liver sections of patients with or without liver cirrhosis revealed two different patterns. In patients without cirrhosis, VWF antigen was only present in endothelial cells of larger vessels (Fig.3C). On the other hand, in patients with liver cirrhosis as visualized by hematoxylin/eosin staining of parallel sections of the same tissue (Fig.3B), positive VWF staining was also observed in the neovasculature of the enlarged portal areas and, to a lesser extent, in the surrounding sinusoidal endothelial cells (Fig.3D). Clearly, more VWF staining is seen in liver tissue of patients with liver cirrhosis when compared to patients without cirrhosis.

FVIII protein was only localized to the sinusoidal endothelial cells, both in patients with (Fig.4C,D), or without (Fig.4A,B) liver cirrhosis. No difference in FVIII distribution was observed. However, consistent with data at the mRNA level, less sinusoidal endothelial cells were seen in cirrhotic liver tissue, particularly in areas of enlarged portal veins.

In patients without liver cirrhosis LRP was clearly detected in hepatocytes and Kupffer cells (Fig.5A,B). On the other hand, in patients with liver cirrhosis expanding portal areas seemed to disturb the organization of hepatocytes expressing LRP (Fig.5C,D). Furthermore large leukocyte infiltrations were present in the portal areas with some LRP positive cells, probably monocytes (16).

DISCUSSION

Contrary to expectations, the increase in plasma concentration of FVIII in patients with liver failure does not appear to be associated with an increased expression of hepatic FVIII mRNA. In patients with liver cirrhosis, FVIII mRNA expression levels were low when compared to the control patients (p = 0.010), while plasma FVIII levels were clearly elevated (p = 0.038, Fig.1). No differences in cellular distribution of FVIII protein was observed between the patients studied (Fig.4). However, we noted that in cirrhotic tissue larger vessels seemed to overgrow sinusoidal, FVIII producing, endothelial cells. This may explain why in these tissues the expression level of FVIII mRNA was lower than in non-cirrhotic tissue. This pattern clearly differs from the expression profiles of hepatic FV and antithrombin mRNA where the circulating plasma protein levels of these typical hepatic marker proteins tended to correlate with their mRNA levels (data not shown).

In order to obtain insight into the apparently disparate cellular expression of FVIII and plasma FVIII concentrations, we also examined the expression of VWF, both at the mRNA and protein level. It is well documented that VWF is a significant factor in the regulation of plasma FVIII concentrations. VWF binds to FVIII and protects it against proteolytic attack and premature clearance (reviewed in (9,28,29)). In many pathophysiological conditions any change in plasma VWF levels is frequently coupled with a concordant change in the FVIII level (9,30) and/or half-life (31). Similarly, the present study shows a close correlation between plasma levels of FVIII and VWF. Less well understood is the cellular origin of the increased amount of VWF in the circulation and its role in
regulating FVIII at the cellular level. Previously, we and others have shown that the tissue distribution of FVIII and VWF mRNA synthesis in mice does not correlate. FVIII is primarily synthesized in the liver, notably sinusoidal endothelial cells (see above) (4,5), whereas sites of VWF synthesis has been identified throughout the vascular endothelium, particularly in the lung and brain, but not or to a minor extent, in liver tissue (3,4,10). The present study shows that, hepatic VWF synthesis is enhanced in liver disease. Therefore, it seems reasonable to assume that in liver disease hepatic synthesis of VWF does serve a role in controlling plasma FVIII levels. In all patients studied VWF mRNA was detectable in excised liver specimens. However, VWF mRNA concentration was highest in cirrhotic patients (Fig.2). Immuno-histochemical staining of liver tissue corroborates this observation. In cirrhotic tissue VWF antigen was detected in the neovascularure of enlarged portal areas and to a lower extent in the surrounding sinusoidal endothelial cells (Fig.3B). In contrast, in tissue from patients without cirrhosis VWF staining was only seen in larger vessels (Fig.3A). In tissue from these patients no newly formed vessels were observed. It has been observed that, in cirrhotic livers, transformation of sinusoids to capillaries and sinusoidal endothelial cells to vascular endothelial cells and concomitant increase in VWF expression may occur (11,12). This raises the possibility that in cirrhotic tissue, unlike healthy tissue, FVIII and VWF are co-expressed in sinusoidal endothelial(-derived) cells. Thus, our data suggests that in liver disease hepatic synthesis of VWF contributes to the regulation of the plasma FVIII level. Whether extra-hepatic VWF synthesis is also enhanced cannot be inferred from our study. The contribution is probably less significant because CRP levels were either not increased or only to a minor extent. As an acute phase protein, enhanced VWF expression is frequently associated with elevated CRP levels (7,32). This was apparently not the case in the patients with liver disease studied here.

Accumulated evidence suggests that LRP, a multi-ligand receptor that has been shown to play a role in the cellular uptake and degradation of FVIII, also serves a role in controlling plasma levels of FVIII (14). In mice that are deficient in hepatic LRP, FVIII levels are elevated and its half-life is increased (33). In addition, it has been shown that in mice injected with radio-labelleld FVIII, radioactivity was detected in the liver but not the kidney, suggesting that the liver serves an important role in the catabolism of FVIII (14). Similarly, administration of receptor-associated protein (RAP), which blocks the action of LRP, induced a sustained rise of FVIII levels (14,15). The present study demonstrates that the hepatic expression of LRP is decreased in liver disease, particularly in liver cirrhosis. This provides confirmation of the recent observations that liver-specific down-regulation of LRP expression affects plasma FVIII levels as well (33). Indeed, we noted a negative association between plasma FVIII levels and hepatic LRP mRNA in patients with liver cirrhosis compared to the control patients (not shown). Also immunohistochemical analysis suggested that the cellular distribution of LRP expression is disturbed in cirrhotic tissue (Fig.5).

In conclusion, the present study demonstrates that in patients with liver cirrhosis, elevated FVIII levels in plasma are not due to increased transcriptional activity of FVIII in
the liver. Instead, our study suggests that increased plasma FVIII concentrations are more likely caused by increased hepatic VWF biosynthesis. In addition, decreased levels of LRP expression may also contribute to the increased plasma FVIII levels. It should also be emphasised that other tissues/organs, such as kidney, spleen, lung and brain, express FVIII and thus may also play a role in controlling the plasma FVIII concentration (3,4). Their contribution to the circulating levels of FVIII could not be evaluated in this study. It should also be noted that we have assumed throughout that besides changes in gene expression, changes in other cellular events, such as protein folding and post-transcriptional modification or changes in mRNA stability, could be associated with liver disease and may dissociate the usual correspondence between mRNA and protein.

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