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Sequential Functions of CPEB1 and CPEB4 Regulate Pathologic Expression of Vascular Endothelial Growth Factor and Angiogenesis in Chronic Liver Disease

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BACKGROUND & AIMS: Vascular endothelial growth factor (VEGF) regulates angiogenesis, yet therapeutic strategies to disrupt VEGF signaling can interfere with physiologic angiogenesis. In a search for ways to inhibit pathologic production or activities of VEGF without affecting its normal production or functions, we investigated the post-transcriptional regulation of VEGF by the cytoplasmic polyadenylation element-binding proteins CPEB1 and CPEB4 during development of portal hypertension and liver disease. **METHODS:** We obtained transjugular liver biopsies from patients with hepatitis C virus–associated cirrhosis or liver tissues removed during transplantation; healthy human liver tissue was obtained from a commercial source (control). We also performed experiments with male Sprague-Dawley rats and CPEB-deficient mice (C57BL6 or mixed C57BL6/129 background) and their wild-type littermates. Secondary biliary cirrhosis was induced in rats by bile duct ligation, and portal hypertension was induced by partial portal vein ligation. Liver and mesenteric tissues were collected and analyzed in angiogenesis, reverse transcription polymerase chain reaction, polyA tail, 3′ rapid amplification of complementary DNA ends, Southern blot, immunoblot, histologic, immunohistochemical, immunofluorescence, and confocal microscopy assays. CPEB was knocked down with small interfering RNAs in H5V endothelial cells, and translation of luciferase reporters constructs was assessed. **RESULTS:** Activation of CPEB1 promoted alternative nuclear processing within noncoding 3′-untranslated regions of VEGF and CPEB4 messenger RNAs in H5V cells, resulting in deletion of translation repressor elements. The subsequent overexpression of CPEB4 promoted cytoplasmic polyadenylation of VEGF messenger RNA, increasing its translation; the high levels of VEGF produced by these cells led to their formation of tubular structures in Matrigel assays. We observed increased levels of CPEB1 and CPEB4 in cirrhotic liver tissues from patients, compared with control tissue, as well as in livers and mesenteries of rats and mice with cirrhosis or/and portal hypertension. Mice with knockdown of CPEB1 or CPEB4 did not overexpress VEGF or have signs of mesenteric neovascularization, and developed less-severe forms of portal hypertension after portal vein ligation. **CONCLUSIONS:** We identified a mechanism of VEGF overexpression in liver and mesentery that promotes pathologic, but not physiologic, angiogenesis, via sequential and nonredundant functions of CPEB1 and CPEB4. Regulation of CPEB4 by CPEB1 and the CPEB4 autoamplification loop induces pathologic angiogenesis. Strategies to block the activities of CPEBs might be developed to treat chronic liver and other angiogenesis-dependent diseases.

Keywords: Portal Hypertension; CPEB; Cytoplasmic Polyadenylation; Alternative RNA Processing.

BASIC AND TRANSLATIONAL LIVER

Chronic liver diseases, including liver cirrhosis, are major causes of death worldwide, but their therapeutic options still remain severely limited.[1] Pathologic angiogenesis triggered by vascular endothelial growth factor (VEGF) overproduction is central for liver disease progression and for development and maintenance of portal hypertension (PH),[2–5] the most devastating complication that develops in cirrhotic patients.[6] Hence, therapeutic targeting of angiogenesis has been proposed as a promising strategy.[2–5] However, the clinical benefit of antiangiogenic drugs is restricted because of significant adverse effects, including collapse of normal vasculature, vascular leakage,

***Authors share co-first authorship; §Authors share co-senior authorship.**

Abbreviations used in this paper: **ARE, AU-rich element; AurKA, Aurora kinase A; CPE, cytoplasmic polyadenylation element; CPEB, cytoplasmic polyadenylation element-binding protein; iKO, inducible knockout mice; mRNA, messenger RNA; PAS, polyadenylation site; PH, portal hypertension; PPVL, partial portal vein ligation; RACE, rapid amplification of complementary DNA ends; shRNA, short hairpin RNA; UTR, untranslated regions; VEGF, vascular endothelial growth factor.**

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and bleeding, as exemplified in patients with hepatocellular carcinoma and cirrhosis.[7] Most of these limitations arise from the fact that current anti-VEGF approaches are not selective for pathologic VEGF production, but instead inhibit also physiologic VEGF required for vascular homeostasis of healthy vessels and formation of vascular supply in many physiologic settings.[8,9] Deciphering mechanisms that regulate VEGF expression is therefore critical to achieve specific inhibition of pathologic generation of VEGF without affecting its physiologic production.

Although the prevailing paradigm of VEGF regulation focuses on its transcriptional activation,[9] independent research lines have revealed an equally important post-transcriptional mechanism mediated by RNA-binding proteins and microRNAs, operating on cis-regulatory elements within VEGF messenger RNA (mRNA) untranslated regions (UTRs).[10] For example, AU-rich elements, microRNA-target sites and a specific element recruiting the GAIT complex are negative regulators that have been implicated in VEGF mRNA silencing when VEGF synthesis is not needed, by repressing translation and/or reducing transcript half-life.[11–14] But, upon angiogenic stimuli and to support the high VEGF levels that drive pathologic angiogenesis, this translationally silent VEGF mRNA needs to be readenylated to achieve full activation. The mechanism mediating this VEGF transcript reactivation upon pathologic angiogenic stimulation remains unclear.

A possible explanation arises from the presence of cytoplasmic polyadenylation elements (CPEs) within non-coding VEGF 3′UTR.[15] CPEs are bound by CPE-binding proteins (CPEBs), a family of 4 members that recognize the same cis-acting element, but are regulated by different signal transduction pathways and have specific cellular functions.[16–22] Both CPEB1 and CPEB4 promote cytoplasmic-polyadenylation and translational activation of CPE-containing mRNAs when phosphorylated (activated), respectively, by the serine/threonine kinase Aurora kinase-A (AurKA) and by a yet unidentified kinase.[23,24] In addition to this cytoplasmic function, we have recently found that CPEB1 also shuttles to the nucleus, where it binds to CPE-containing pre-mRNAs and directs the use of alternative polyadenylation sites (PAS) and subsequent shortening of 3′UTRs, thereby modulating their translation efficiency in the cytoplasm.[25] Because we have also found that CPEB4 is required for xenografted tumor growth and neovascularization in pancreatic ductal adenocarcinoma and glioblastoma,[26] we propose that CPEBs could regulate cirrhosis and PH-associated pathologic VEGF expression and angiogenesis. Our studies identify a new molecular mechanism where sequential and coordinated functions of CPEB1 and CPEB4 regulate post-transcriptionally the "pathologically augmented" VEGF levels, but not the basal levels required for normal physiologic angiogenesis, suggesting that CPEBs could be clinically attractive therapeutic targets for pathologic angiogenesis in chronic liver disease and potentially other angiogenesis-dependent diseases.

Materials and Methods

Patients and Animals

Studies were approved by Research Ethic Committees of Hospital Clinic (Protocol #2011/6723), and Barcelona University and Scientific Park. Human samples of hepatitis C virus–related cirrhotic liver were obtained from transjugular liver biopsies or from explanted organs during transplantation, after informed consent signed by each patient. Human normal liver tissue was commercially obtained (AMS Biotechnology, Abingdon, UK). Male Sprague-Dawley rats and CPEB-deficient mice on a C57BL6 or mixed C57BL6/129 background were used along with corresponding age-matched wild-type mice. Generation of tamoxifen-inducible CPEB1 and CPEB4 knockout (iKO) mice, and induction of secondary biliary cirrhosis (bile duct ligation) and PH (partial portal vein ligation [PPVL]) are described in Supplementary Methods.

Molecular and Functional Analysis

Details of angiogenesis assays, translation of luciferase reporters in H5V cells, CPEB knockdown cells, luciferase assay, polyA tail assay, 3′ rapid amplification of complementary DNA ends (RACE), reverse transcription polymerase chain reaction, Southern blotting, immunoblotting, histologic analysis, immunohistochemistry, immunofluorescence, and confocal microscopy are provided in Supplementary Methods.

Statistical Analysis

Data are shown as mean ± SEM, mean ± SD, or mean ± range. Kolmogorov-Smirnov test was used to determine whether data were normally distributed ($P > .05$) or not ($P < .05$). Normally distributed results were compared with parametric statistical procedures (Student's t test and 2-way analysis of variance followed by Bonferroni's test for multiple comparisons. Non-normally distributed results were compared with nonparametric tests (Kruskal-Wallis one-way analysis of variance and Mann-Whitney U test). Significance was accepted at $P < .05$.

Results

CPEB1 and CPEB4 Are Required for Vascular Endothelial Growth Factor Expression and Angiogenesis

Because angiogenesis inhibition in CPEB4-depleted pancreatic adenocarcinoma-xenografted tumors pointed to a defect in secreted factor rather than to endothelial cell defect,[26] we performed in silico search for potential CPE-regulated mRNAs encoding proangiogenic secreted factors. We found that VEGF 3′UTR contains various CPE elements, making it susceptible to being regulated by CPEBs. Interestingly, we also identified several putative PAS in VEGF 3′UTR, being the more 5′ (PAS1) flanked by 2 CPEs (Figure 1*A*), suggesting that CPEB could mediate alternative 3′ UTR processing leading to shorter VEGF mRNA isoforms by alternative polyadenylation.

Alternative polyadenylation usually occurs by activation of "weaker" proximal PAS located 5′ of "stronger" distal PAS, the latter resulting in mRNAs containing the longer form of