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The gene expression of adrenomedullin, calcitonin-receptor-like receptor and receptor activity modifying proteins (RAMPs) in CCl_4-induced rat liver cirrhosis

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Abstract

This study was undertaken to determine AM expression in carbon tetrachloride (CCl_4)-induced liver cirrhosis developed with peritoneal ascites. Sprague–Dawley rats received subcutaneous injections of CCl_4 twice weekly in olive oil (1:1, 0.3 ml per kg body weight) for 6 or 12 weeks until ascites developed, or saline in olive oil as control. At 6 weeks, fibrosis developed and at 12 weeks cirrhosis developed with ascites formation. At both 6 and 12 weeks, increases in plasma renin and AM were evident, as was the gene expression of AM. At 12 weeks after CCl_4 injection, the gene expression of calcitonin-like-receptor (CRLR) and receptor activity modifying proteins (RAMP1, RAMP2 and RAMP3) were all elevated when compared to the control. The results suggest that liver cirrhosis increases mRNA expressions of AM, CRLR and RAMP1, RAMP2 and RAMP3 and that the increase in AM gene expression precedes the development of cirrhosis. The increase in AM synthesis as reflected by an increase in AM gene expression, together with a lack of increase in AM peptide at both 6 and 12 weeks may suggest an elevation of AM release. Given the potent vasodilatory action of AM, the increase in the synthesis and release of AM in the cirrhotic liver may also contribute to peripheral vasodilatation in liver cirrhosis.

Keywords: Adrenomedullin; Calcitonin receptor; RAMP; Liver cirrhosis

1. Introduction

Liver cirrhosis is associated with marked hemodynamic changes that are characterized by arterial vasodilation, increased cardiac output, and the development of portal hypertension in humans [1,2]. These are common features in patients with liver cirrhosis and especially those with ascites formation. Arterial vasodilation may contribute to the formation of ascites in liver cirrhosis and this vasodilation causes a marked decrease in systemic vascular resistance [3]. Recent evidence has suggested that arterial vasodilation in cirrhosis may be related to increasing circulating levels of AM [1,4,5] and other vasodilators such as CGRP [6], glucagons [3] and substance P [7]. In addition, elevated AM has been associated with activation of the renin–angiotensin system and functional renal impairment in cirrhosis [5].

The plasma AM level is increased in any disease condition that is associated with fluid retention and volume overload [8,9]. An elevated AM may be due to overproduction and/or reduced metabolic clearance [1]. Although AM is known to inhibit fibrosis in the heart [11,12], there is so far no report on the changes of AM gene expression in the cirrhotic liver. Moreover, the contribution of AM to the pathophysiology of liver cirrhosis is still unknown. There is no study on the adrenomedullin gene expression in the

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Table 1
PCR primers for rat AM, CRLR, RAMP1–3, HIF-1 α and inflammatory cytokines

Target gene	Forward primers	Reverse primers	Expected PCR band (bp)
AM	5′-ttcagcagggtatcggagc-3′	5′-ccgactgttcaatgctgcc-3′	614 bp
CRLR	5′-ccaaacagacttgggagtcactagg-3′	5′-gctgtcttctctttctcatgcgtgc-3′	323 bp
RAMP1	5′-cactcactgcaccaaactcgtg-3′	5′-cagtcatgagcagtgtgaccgtaa-3′	196 bp
RAMP2	5′-aggtattacagcaacctgcggt-3′	5′-acatcctctgggggatcggaga-3′	163 bp
RAMP3	5′-acctgtcggagttcatcgtg-3′	5′-acttcatccgggggggtcttc-3′	180 bp
IL-6	5′-cttccagccagttgccttct-3′	5′-gagagcattggaagttgggg-3′	496 bp
TNF-α	5′-cgtcgtagcaaaccaccaagc-3′	5′-accagggcttgagctcagctc-3′	296 bp
IL-1β	5′-ccttcttttccttcatctttg-3′	5′-accgcttttccatcttcttct-3′	372 bp
HIF-1α	5′-tgcttggtgctgatttgtga-3′	5′-ggtcagatgatcagagtcca-3′	209 bp
GADPH	5′-ccttcattgacctcaactacatggt-3	5′-tcattgtcataccaggaaatgagct-3′	833 bp

Abbreviations: AM, Adrenomedullin; CRLR, Calcitonin-receptor-like receptor; RAMP, Receptor activity modifying protein; IL-6, Interleukin-6; TNF-α, Tumor necrosis factor; IL-1β, Interleukin 1β; HIF-1α, Hypoxia-inducible factor 1; GADPH, Glyceraldehyde-3-phosphate dehydrogenase.

liver with fibrosis, a stage that precedes the manifestation of cirrhosis [10]. We hypothesize that the increase in AM gene expression in the liver precedes cirrhosis and is related to fibrosis formation. We further hypothesize that this may be the result of increase in NO and cytokines, and hypoxia, and is concomitant with an increase in gene expression of the receptor components of AM.

Previous studies reported that the vasodilator effect of AM is mediated through the G-protein coupled calcitonin-receptor-like receptor (CRLR), and receptor activity modifying protein 2 (RAMP2) or 3 (RAMP3) [13,14]. In this study, a rat model of liver cirrhosis with ascites development was established by subcutaneous injection of carbon tetrachloride (CCl_4). Our data indicate that in hepatic cirrhosis the gene expression of AM, CRLR (calcitonin-receptor-like receptor) and RAMPs (receptor activity modifying proteins) are all increased.

2. Materials and methods

2.1. Induction of hepatic cirrhosis

The experimental protocol was approved by the Committee on the Use of Animals in Teaching and Research of the Faculty of Medicine, the University of Hong Kong. Six-week-old male Sprague Dawley (SD) rats weighing 120–150 g were used. Two groups of 10 rats received subcutaneous injection of CCl_4 dissolved in olive oil (1:1, v:v) at a volume of 0.3 ml per kg body weight for 6 or 12 weeks while a corresponding group of 8 control rats received saline in olive oil. Meanwhile, all rats received sodium pentobarbital (0.3 g/L) [15] in drinking water 2 weeks prior to CCl_4 or saline injection until the end of the experiment in order to increase cytochrome P_{450} activity and therefore increase liver sensitivity to CCl_4 and CCl_4-induced damage. After 6 or 12 weeks, the rats were sacrificed by decapitation, and whole blood was collected into an ice-cooled 10 ml centrifuge tube containing EDTA (1 mg/ml blood) and aprotinin (500 KIU/ml blood) for determination of AM and NO_2^-/NO_3^- levels, and renin activity as well as for liver function tests. The liver was quickly excised, frozen on dry ice and stored at −70 °C until use for AM assay and total RNA extraction. Part of the hepatic tissue was cut into small cubes and fixed overnight in 10% buffered formalin for further processing for light microscopy (hematoxylin and eosin staining) and immunohistochemical staining for AM. Whole blood was centrifuged at 3800 r.p.m. for 20 min at 4 °C. The plasma was stored at −70 °C until the time of assay.

2.2. Histological characterization of liver

After fixing overnight in buffered formalin, the hepatic tissue was dehydrated by passage through graded ethanol series, cleared in xylene and embedded in paraffin blocks. The blocks were cut into 5-μm thick sections, and stained with hematoxylin and eosin according to standard procedures [16]. For the 12 week study, only the rats exhibiting histological signs of cirrhosis accompanied by accumulation of body fluid within the peritoneal cavity as signs of ascites formation were included in the data analysis.

2.3. Immunohistochemical staining for AM

This has been detailed in [17]. The paraffined sections were treated with 10% methanol and 3% hydrogen peroxide for 30 min, and then incubated with 1:1500 of AM antiserum (Phoenix, Belmont, CA) at 4 °C overnight. After washing, the sections were treated with a second antibody coupled to an avidin–biotin peroxidase. The avidin–biotin peroxidase complex in the sections was visualized by adding diaminobenzidine for 5–8 min. The sections were counterstained with hematoxylin and dehydrated in an alcohol series.

2.4. Measurement of plasma NO_3^- and NO_2^-

In this method, nitrate reductase was used to reduce nitrate to nitrite in the sample. Plasma samples were thawed immediately before assay and deproteinized by adding 2 vol. of ethanol and standing on ice for 30 min. After centrifugation at 1500 r.p.m. for 10 min at 4 °C, the deproteinized supernatant was assayed for total nitrate and nitrite using a colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI). The total nitrite after reduction (which represented total NO_3^-, NO_2^- in the sample) was determined by reaction with the Griess reagent. The absorbance of a purple azo dye was measured at 540 nm by a visible spectrophotometer. The plasma NO_3^- and NO_2^- levels were expressed as μmol/L or μM.