Elevated expression of caveolin-1 at protein and mRNA level in human cirrhotic liver: relation with nitric oxide

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Introduction

Caveolae are small invaginations of the cell surface and are thought to play a role in important physiological functions, such as cell surface signaling, endocytosis, and intracellular cholesterol transport.1 Unique membrane proteins, designated caveolin-1, -2, and -3, have been shown to be the major constituents of caveolae.2–4 Animal studies have revealed that caveolin-1 and -2 are present abundantly in vascular endothelial cells, adipocytes, smooth muscle cells, and fibroblasts.5 Caveolin-1, the first discovered marker protein for caveolae and the most extensively characterized, is implicated in regional signal transduction pathways.1 While most of the data on eNOS and caveolin-1 have been obtained using rat liver,9,10 there is relatively little information on the situation in intact human liver. We previously studied the immunohistochemical and immunoelectron microscopic localization of eNOS and caveolin-1 in normal human and cirrhotic liver tissues, and found increased immunoreactivity of caveolin-1 on SEC in cirrhotic liver.11 In the present study, we examined the expression of eNOS, caveolin-1, and calmodulin proteins by Western blotting, and we investigated the mRNA expression of eNOS and caveolin-1 by in situ hybridization.

Background. Caveolin, the principal structural protein of caveolae, binds with endothelial nitric oxide synthase (eNOS) leading to enzyme inhibition. This study examined the expression of caveolin and eNOS at the protein and mRNA levels in patients with hepatocellular carcinoma and hepatitis C-related cirrhosis, and in control noncirrhotic liver specimens obtained from patients with metastatic liver carcinoma.

Methods. Anti-eNOS, anti-caveolin-1, and anti-calmodulin antibodies were used for Western blotting. For in situ hybridization (ISH), human eNOS and caveolin-1 peptide nucleic acid probes were used with a catalyzed signal amplification system.

Results. Western blotting showed marked overexpression of caveolin-1 protein in cirrhotic liver, while caveolin-1 was almost undetectable in control liver tissue. Endothelial NOS was expressed at a slightly higher level in cirrhotic liver than in control liver tissue. Calmodulin was expressed abundantly in control liver tissue and at a low level in cirrhotic liver tissue. By ISH, eNOS mRNA was localized on portal vein and hepatic lining cells, and caveolin-1 mRNA was almost undetectable in normal liver tissue. In cirrhotic liver tissue, caveolin-1 mRNA was overexpressed on hepatic sinusoidal lining cells, while eNOS mRNA expression was similar to that in normal liver.

Conclusions. Enhanced caveolin-1 expression may be associated with a significant reduction in NO catalytic activity in cirrhosis.

Key words: caveolin-1, endothelial nitric oxide synthase, in situ hybridization, sinusoidal endothelial cell

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situ hybridization (ISH) in cirrhotic and noncirrhotic human liver tissues.

Patients, materials, and methods

Patients and materials

As controls, wedge biopsy specimens from non-cirrhotic portions of the liver were obtained from five patients (four men and 1 woman; aged from 52 to 68 years, with a mean age of 63.5 years) who underwent hepatectomy for metastatic liver carcinoma (four with colon carcinoma and one with gastric carcinoma). Cirrhotic liver specimens were obtained from macroscopically cirrhotic portions surgically resected from five patients (all men; aged from 58 to 67 years, with a mean age of 62.8 years) who had hepatocellular carcinoma combined with hepatitis C-related cirrhosis. The study was approved by the local ethics committee.

Western blotting

Western blotting was conducted using fresh control and cirrhotic liver tissues. Briefly, liver tissues were homogenized in 10 volumes of homogenization buffer (20 mM Tris-HCl [pH 7.5], 5 mM MgCl2, 0.1 mM phenyl methane sulfonil fluoride [PMSF], 20 mM pepstatin A, and 20 mM leupeptin), using a polytron homogenizer at setting 7 for 90 s. The homogenates were centrifuged at 100,000 g for 45 min. The membranes were washed three times, resuspended in 10 volumes of homogenization buffer, homogenized using a Teflon/glass homogenizer, and centrifuged. The membrane proteins thus obtained were used for immunoblotting. Proteins (20 mg/ml) were separated on sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE; 7.5% gel for eNOS, 12.5% gel for calmodulin, and 15% for caveolin-1) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The blots were blocked with 5% (w/v) dried milk in phosphate-buffered saline (PBS) for 30 min, incubated with anti-eNOS (×500), anti-caveolin-1 (×1000), or anti-calmodulin (×500) antibodies in 0.1% Tween 29 in PBS, and then processed by the Vectastain ABC system (Vector Laboratories, Burlingame, CA, USA). The immunoreactive bands were developed with diamino-benzidine solution containing 0.01% H2O2 at 60°C for 30 min. The sections were dewaxed in xyylene (twice for 15 min each), followed by a graded ethanol series, rehydrated in RNase-free distilled water, and incubated for 30 min in Target Retrieval Buffer (Dako, Glostrup, Denmark) preheated and maintained at 95°C. The slides were cooled at room temperature for 20 min and then digested with 20 µg/ml proteinase K (Dako) at room temperature for 30 min. The slides were rinsed in distilled water and rapidly air-dried. The air-dried sections were covered with approximately 15 ml of hybridization solution containing 10% (w/v) dextran sulfate, 10 mM NaCl, 30% (v/v) formamide, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) Ficoll, 5 mM Na2 ethylene diamine tetraacetic acid (EDTA), 50 mM Tris-HCl, pH 7.5, and 10 µg/ml PNA probe. ENOS antisense (fluorescein isothiocyanate [FITC]-GGTCTCTGATAGC CTTCA), eNOS sense (FITC-CGAAGAGCACCCT GAAAGT), caveolin-1 antisense (FITC-GGCTGATG CACTGAATCT), and caveolin-1 sense (FITC-CTACGCTACTTGA)4 probes were used. The slides were evenly covered with the hybridization solution and incubated in a moist chamber at 43°C for 90 min. Following hybridization, the coverslips were removed, and the slides were transferred to prewarmed Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris; pH 7.5, 1.0%) in a water bath at 49°C and washed for 30 min with gentle shaking (PNA Hybridization Kit; Dako, Tokyo, Japan). A nonisotopic, colorimetric signal amplification system (GenPoint kit, Dako) was used to visualize specific hybridization signals. Sections were incubated with an FITC-horseradish peroxidase reagent for 15 min, washed three times with Tris-buffered saline with Tween (TBST) (150 mM NaCl, 10 mM Tris; pH 7.5, 1.1% v/v Tween 20), and then immersed in a solution containing H2O2 and biotinyl tyramide for 15 min, followed by three washes with TBST. This catalyzed signal amplification step enhanced the deposition of biotin at the site of probe hybridization. The sections were then incubated in streptavidin-horseradish peroxidase reagent for 15 min and washed three times in TBST. Colorimetric signals were localized after incubation in diaminobenzidine solution containing 0.01% H2O2, and counterstaining with hematoxylin was carried out for light microscopic examination.

Results

We investigated the protein expression of eNOS, calmodulin, and caveolin-1 by Western blotting in normal and cirrhotic liver tissues. Samples containing 20 mg of membrane protein were subjected to electrophoresis on SDS/PAGE gel (eNOS, 7.5%; calmodulin, 12.5%; caveolin-1, 15%) and analyzed by blotting. Caveolin-1