Carcinoembryonic antigen-related cell-cell adhesion molecule C-CAM is greatly increased in serum and urine of rats with liver diseases

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Abstract C-CAM (rat cell CAM/human CD66a) is ubiquitous and multifunctional. It is involved in intercellular adhesion, signal transduction and cell growth inhibition. Structurally, it is related to the carcinoembryonic antigen. In the present study serum, bile and urine of rats with liver diseases were analyzed for the presence of cell CAM. After bile duct ligation and during galactosamine (GalN) hepatitis we found that large amounts of liver membrane-bound C-CAM are secreted or shed into blood. The serum level of another liver membrane-bound protein, LI-cadherin, is not increased. It was shown that C-CAM is also present in bile fluid, and for the first time that C-CAM is present in the urine of rats with liver diseases. A particularly high concentration was measured in the urine of rats suffering from GalN hepatitis.

Key words: Cell adhesion; Carcinoembryonic antigen family; Liver disease

1. Introduction

Rat C-CAM (cell-cell adhesion molecule), also known as cell CAM, is a ubiquitous, highly glycosylated, transmembrane protein with an apparent M_r of 110 000 [1–6]. C-CAM has been shown to be involved in different physiological functions (for review see [7]). Isolated rat hepatocytes or C-CAM transfected eukaryotic cells show strong cell-cell adhesion. This adhesion can be inhibited by specific antibodies indicating an intercellular adhesion function for C-CAM. For other carcinoembryonic antigen-related molecules, the same adhesive activity could be demonstrated [8,9]. C-CAM also acts as a bacterial and viral receptor [10–13], activates neutrophilic granulocytes [14], might be involved in bile salt export [15,16], serves as a substrate for different kinases [17–19] and suppresses the growth of prostate, colonic or breast cancer [20–22]. The involvement of C-CAM in different cellular functions suggests that loss of membrane-bound C-CAM by shedding or limited proteolysis may represent a critical response of the whole organism. In general only a few proteins are secreted or shed into body fluids. Albumin, immunoglobulins, transport proteins and coagulation factors represent the major protein fraction in blood. In individuals suffering from tumor growth, additional proteins are detectable in serum. Therefore some of these proteins, e.g. the carcinoembryonic antigen (CEA) [23,24], have been used as tumor marker molecules. In the present study we used specific antibodies to detect rat C-CAM in serum, bile and urine. From previous studies it is known that human C-CAM is present in serum or bile [25,26], but a more detailed study was needed to analyze whether it is useful for monitoring distinct liver diseases. As a model system we selected rats with liver diseases. Bile duct ligation was performed and D-galactosamine (GalN)-hepatitis was induced by 2-amino-2-deoxy-D-galactose [27]. As a tumor model we induced the Morris hepatoma 7777 [28]. We showed that C-CAM is shed into serum and bile, and that its serum level increases during liver disease progression. For the first time we report the presence of C-CAM in urine.

2. Materials and methods

2.1. Materials

Unless otherwise stated, chemicals and reagents were obtained from Merck, Serva, Sigma or Life Technologies, Germany. The different antisera and monoclonal antibodies have been characterized previously [6,29,30]. CHO wild type cells and Morris hepatoma (MH) 7777 cells were obtained from the American Tissue Culture Collection (ATCC); CHO/C-CAM transfectants have been described previously [31]. Wistar and Buffalo rats were inbred German stocks originally derived from Charles River Wiga.

2.2. SDS-PAGE, immunoblot and immunoprecipitation analysis

SDS-PAGE and electroblotting were done according to standard procedures. Membrane filters were blocked in 5% non-fat dry milk powder dissolved in PBS (150 mM NaCl, 0.3 mM KCl, 0.8 mM Na_2HPO_4, 0.1 mM KH_2PO_4, pH 7.2)/0.1% Tween-20. Membranes were incubated with the primary antibody and a peroxidase-coupled goat anti-mouse IgG (Dianova, Hamburg, Germany), each for 1.5 h at room temperature; antibody dilution was 0.5 μg/ml in PBS/0.1% Tween-20. Unbound antibodies were removed by three 15-min washes in PBS/0.1% Tween-20, and detection was carried out using an enhanced chemiluminescence detection kit (Amersham Buchler, Braunschweig, Germany) and X-ray exposure. For immunoprecipitation, 20 μl anti-C-CAM antiserum or anti-LI-cadherin antiserum was coupled to protein A-Sepharose (Pharmacia, Uppsala, Sweden). Preclearance of the antigen preparations with protein A-Sepharose avoided unspecific binding of proteins to the immunocomplex. C-CAM was precipitated from body fluids or membranes at 4°C overnight in PBS/1% Triton X-100/1 mM PMSF. Immunocomplexes were washed three times with 500 mM NaCl, 10 mM Tris-HCl pH 7.8, 1 mM EDTA, 1% Triton X-100, 1% BSA, 1 mM PMSF and twice with PBS. For semi-quantitative purposes Western blot analysis of the supernatants from all precipitation experiments was done. Staining of C-CAM and LI-cadherin was no longer possible in the supernatants after precipitation. Immunopurified antigens were applied to SDS-PAGE under reducing conditions. C-CAM and LI-cadherin were detected with the monoclonal antibodies Be 9.2 and 47.2, respectively, to avoid unspecific reactions during chemiluminescence detection. The antigen detection limit was 10 fmol. The coefficients of variation in amounts of detected antigens were 5–15% in different experiments.

2.3. Preparation of serum, urine and liver membrane protein fractions

Blood was taken from rats, and serum prepared according to standard procedures. 0.5 mg membrane proteins, 0.3 ml 1:3 diluted serum was precleared by incubation with protein A-Sepharose, urine was collected and directly used for immunoprecipitation. Bile was collected after opening the peritoneum, by aspiration directly from the

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main primary bile duct. Bile was diluted 1:3 with PBS prior to SDS-PAGE or immunoprecipitation. Liver and MH 7777 membrane fractions were prepared after homogenization by differential centrifugation as described [1,29,32]. Membranes were resuspended in PBS/1% Triton X-100 and completely solubilized for 2 h at 4°C. The supernatant after centrifugation at $100\,000 \times g$ contained the extracted membrane preparation.

2.4. Induction of diseases

The main primary bile duct was ligated after opening the peritoneum under anesthesia. The peritoneum was closed, and blood, liver or urine were taken 2 days later. Hepatitis was induced by intraperitoneal injection of 2-amino-2-deoxy-D-galactose (0 h and 24 h, 400 mg/kg body weight) as described [27,33]. Injection of galactosamine (GalN) was repeated 24 h later. After 48 h blood, liver and urine were taken. Normal healthy rats were used as a control for GalN hepatitis- and MH 7777-bearing rats. As a control for rats with bile duct ligation, the peritoneum of healthy rats was opened and closed without further manipulation. MH 7777 cells were inoculated into both hind legs of Buffalo rats as described previously [28].

3. Results

3.1. Characterization of C-CAM in serum, bile and urine

Western blot analysis did not detect C-CAM in the serum of normal rats. In contrast, the anti-C-CAM antiserum precipitated a 100-kDa immunoreactive protein band. No specific immunoreactivity was found against IgG from preimmune serum in the same M_r range, indicating that this 100-kDa protein is identical with C-CAM (Fig. 1). All other detectable proteins were found to bind unspecifically to the protein A-Sepharose complex, since they were not detected after pretreatment of the serum with protein A-Sepharose prior to precipitation. Using the monoclonal antibody Be 9.2 for Western blot detection C-CAM appeared as a distinct protein band (Fig. 2a). Since unspecifically binding proteins were not accumulated, this strategy was also used for semi-quantitative immunoprecipitation experiments. To check whether C-CAM is shed by proteolytic attack, we compared the difference in M_r of liver and serum C-CAM (Fig. 2b). Serum C-CAM has a slightly lower M_r than liver plasma membrane-bound C-CAM. Antibodies specific for the cytoplasmic tail of C-CAM did not precipitate C-CAM from serum, indicating shedding or limited proteolysis of C-CAM (Fig. 2c). We also

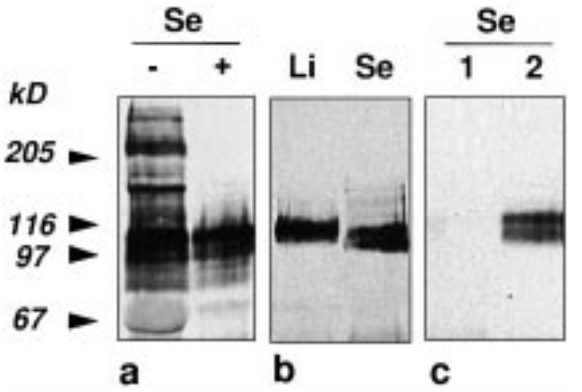

Fig. 2. Structural properties of serum-C-CAM. a: Immunoblot detection of serum proteins (Se) with polyclonal anti-C-CAM antibodies after precipitation. Precipitation was done before (−) and after (+) preclearance with protein A-Sepharose. b: Comparison of the M_r of liver C-CAM (Li) and serum C-CAM (Se) after precipitation and Western blot detection with the mAb Be 9.2. c: Evidence for loss of the cytoplasmic tail of serum C-CAM (Se) obtained by immunoprecipitation with an antiserum specific for the cytoplasmic tail of C-CAM followed by detection with the mAb Be 9.2; 1: Precipitation from serum, 2: precipitation from liver.

analyzed bile and urine for the presence of C-CAM. C-CAM was detectable in the bile of normal rats by Western blotting. Whereas transfected CHO cells express the long and the short C-CAM isoforms, bile C-CAM shows several distinct protein bands with slightly lower M_r ranging from 85 000 to 100 000. Specificity was shown with the monoclonal antibodies 38.9 and Be 9.2 (Fig. 3a). In contrast, specific antibodies did not precipitate C-CAM from urine (Fig. 3b).

3.2. Serum level of C-CAM in rats suffering from liver diseases

C-CAM was then analyzed in serum from rats with bile duct ligation, and in rats with induced hepatitis or hepatoma (MH 7777). The bile duct was tied up and serum was taken 4 days later. Hepatitis was induced chemically by galactosamine injection. Immunoprecipitation of C-CAM (Fig. 4) demonstrated that bile duct ligation caused a twofold increase of C-CAM, but not of LI-cadherin, which was measured as a

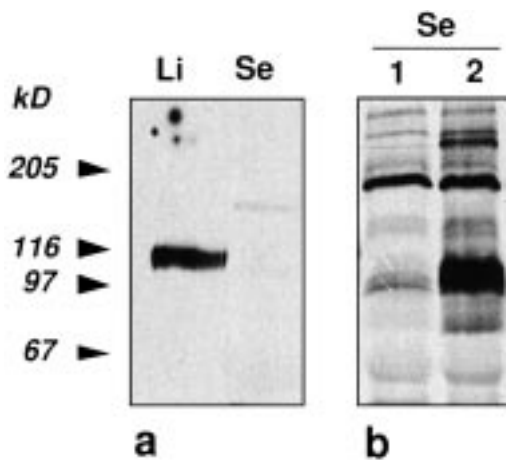

Fig. 1. Detection of C-CAM in rat serum. Immunoblot of liver membrane protein fraction (Li) and serum proteins (Se) (50 μg each) with polyclonal anti-C-CAM antibodies without (a) or after (b) C-CAM immunoprecipitation. 1: Pre-immune serum and 2: anti-C-CAM serum were used for precipitation.

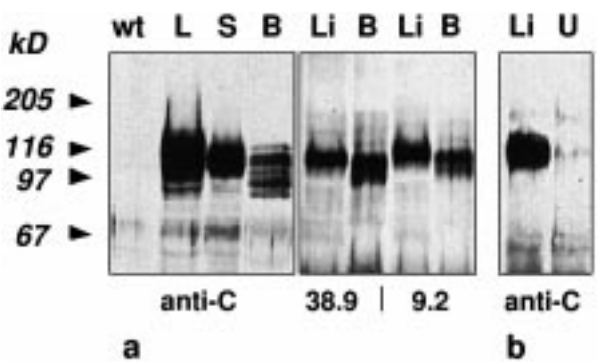

Fig. 3. C-CAM in bile liquid and urine. a, left: Western blot of proteins from bile (B) and for comparison from transfected CHO cells with a polyclonal antiserum (anti-C); (wt) wild type CHO cells, (L) long and (S) short isoform expressing CHO transfectants. a, right: C-CAM from bile (B) and for comparison from liver (Li) the mAbs 38.9 and Be 9.2 were used after precipitation with the anti-C-CAM antiserum. b: Western blot with a polyclonal antiserum (anti-C) of proteins from urine (U) and for control from liver (Li) after precipitation.

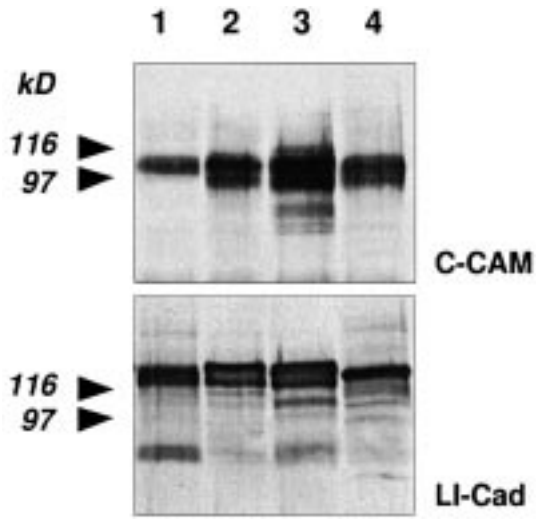

Fig. 4. Detection of C-CAM and LI-cadherin in serum of rats with induced liver diseases. (1) Healthy rats, (2) rats with bile duct ligation, (3) rats with induced GalN hepatitis and (4) rats with a growing Morris hepatoma 7777. C-CAM and LI-cadherin were precipitated with polyclonal antisera and detected with mAb Be 9.2 and mAb 47.2, respectively.

control in the same sera. Tumor growth (MH 7777) also results in high concentrations of C-CAM in serum. GalN hepatitis leads to the highest increase of C-CAM (3–4-fold) with no effect on the serum level of LI-cadherin. To evaluate whether the increased concentration of C-CAM is due to a decrease in membrane-bound C-CAM, we prepared membrane fractions of liver. Western blot analysis demonstrated that in rats with bile duct ligation and GalN hepatitis part of the C-CAM in serum results from shedding. The concentration of liver membrane-bound C-CAM is decreased to at least a third of normal, while the concentration of LI-cadherin in plasma membranes of damaged rat livers is unchanged. The

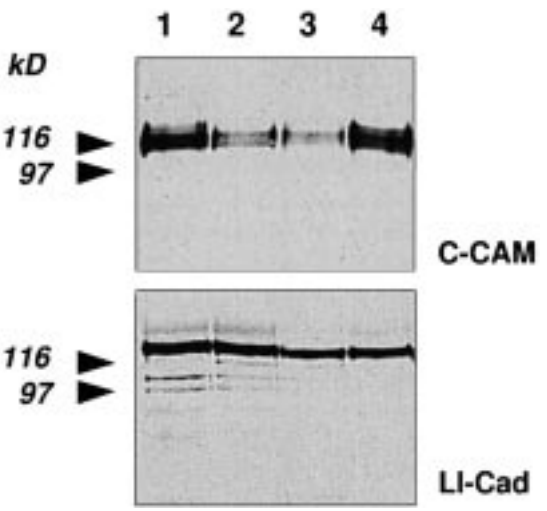

Fig. 5. Detection of C-CAM and LI-cadherin in liver membrane protein fractions of rats with induced liver diseases. (1) Healthy rats, (2) rats with bile duct ligation, (3) rats with induced GalN hepatitis and (4) rats with a growing Morris hepatoma 7777. C-CAM and LI-cadherin were precipitated with polyclonal antisera and detected with mAb Be 9.2 and mAb 47.2, respectively.

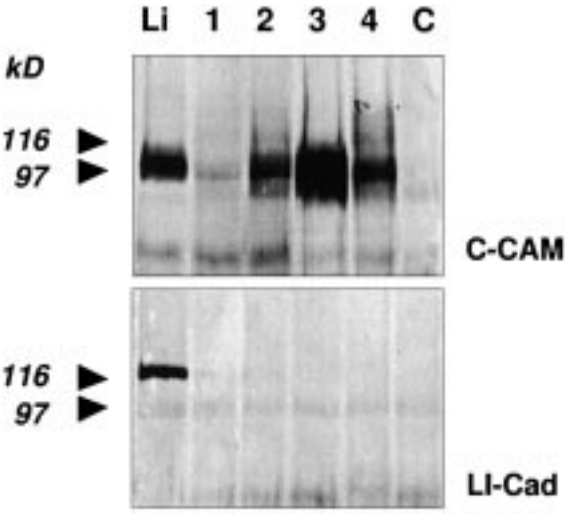

Fig. 6. Detection of C-CAM and LI-cadherin in urine of rats with induced liver diseases. (1) Healthy rats, (2) rats with bile duct ligation, (3) rats with induced GalN hepatitis and (4) rats with a growing Morris hepatoma 7777. As a control C-CAM was precipitated from liver (Li). Pre-immune serum was used for precipitation from the urine of rats with GalN hepatitis as a second control (C). C-CAM and LI-cadherin were precipitated with polyclonal antisera and detected with mAb Be 9.2 and mAb 47.2, respectively.

C-CAM concentration of liver plasma membrane fractions of rats bearing MH 7777 was not affected (Fig. 5).

3.3. C-CAM in urine of rats suffering from liver diseases

Urine of normal rats does not contain proteins ≥ 68 kDa. Surprisingly, the urine of diseased rats contains high concentrations of C-CAM (Fig. 6). The level of urine C-CAM is similar in rats with bile duct ligation and in rats bearing Morris hepatoma, and is highest in rats suffering from GalN hepatitis. GalN hepatitis leads to a fourfold increase in comparison with normal rats. In contrast, the albumin concentration in urine was unchanged (data not shown) and LI-cadherin was not detectable.

4. Discussion

Rat C-CAM is related to the CEA family (for review see [34,35]) showing greatest similarity to mouse and human biliary glycoproteins (*bgp*, BGP/human CD66a). The aim of this study was to determine whether the serum C-CAM level depends on inflammation and whether C-CAM could be used as a marker protein for these inflammations. So far, there are no data available for shedding of rat C-CAM. In view of the functions of C-CAM, such as growth inhibition activity, its loss would influence cell function in general.

We demonstrated by Western blotting that C-CAM is shed into serum and bile. Since membrane domain localization may be important for the degree of shedding by limited proteolysis, we investigated for comparison another liver plasma membrane-bound adhesion molecule, LI-cadherin [30], which is known to be associated with the basolateral membrane domain of hepatocytes. It was shown that the serum concentration of C-CAM but not of LI-cadherin is increased during bile duct ligation and GalN hepatitis. This is consistent with experiments on human BGP [25,26,36]. In the rat model we could show that large quantities of serum C-CAM arise

from the liver cell membrane itself. In contrast to LI-cadherin the serum level of C-CAM is increased in cases of hepatoma. This may reflect the reduction of C-CAM expression in hepatoma plasma membrane, compared with liver plasma membrane [1,37]. C-CAM expression in liver plasma membranes of hepatoma-bearing rats is not reduced, indicating shedding from hepatoma plasma membranes. The serum LI-cadherin level is generally not influenced by the induced liver diseases. The most surprising result of this study is that C-CAM is detectable in urine of rats with liver diseases, especially in rats suffering from hepatitis. This finding is not a result of disordered kidney function because neither LI-cadherin nor albumin, the major protein component in serum, is detectable in urine. The mechanism of C-CAM secretion into urine remains to be clarified.

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