Research Article

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Keratin 23 is a stress-inducible marker of mouse and human ductular reaction in liver disease☆

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Background & Aims: Keratins (K) constitute the epithelial intermediate filaments. Among them, K7/K19 are widely used markers of the regenerative liver response termed ductular reaction (DR) that consists of activated biliary epithelial cells (BECs) and hepatic progenitor cells (HPCs) and correlates with liver disease severity. In the present study we aimed to characterize K23 in the liver.

Methods: We analyzed the expression and localization of K23 in the digestive system under basal conditions as well as in various human and mouse liver diseases/stress models. Cell culture studies were used to study factors regulating K23 expression.

Results: In untreated mice, K23 was restricted to biliary epithelia. It was (together with K7/K19) markedly upregulated in three different DR/cholestatic injury models, i.e., multidrug resistance protein 2 (*Mdr2*) knockouts, animals treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine or subjected to bile duct ligation. *K23* levels correlated with the DR marker Fn14 and immunofluorescence staining showed a distinct co-localization with K7/K19. In chronic human liver disease, *K23* expression increased in patients with a more prominent inflammation/fibrosis. A dramatic upregulation (>200 times) was observed in patients with acute liver failure (ALF) and end-stage primary biliary cholangitis (PBC). Patients with alcoholic liver cirrhosis displayed increased K23 serum levels. In primary hepatocytes as well as hepatobiliary cell lines, treatment with TNF-related weak inducer of apoptosis (TWEAK), and the type I acute phase inducer interleukin (IL)-1β but not the type II inducer IL-6 elevated K23 expression.

Conclusions: K23 represents a specific, stress-inducible DR marker, whose levels correlate with liver disease severity. K23 may represent a useful non-invasive DR marker.

Lay summary: Ductular reaction represents a basic response to liver injury and correlates with liver disease severity. Our study identifies K23 as a novel ductular reaction marker in mice and humans.

Introduction

Keratins (K) are the intermediate filaments of epithelial cells and their appendages that are further subdivided into type I (including K9-K20) and type II (including K1-K8) proteins [1,2]. Both types assemble in an equimolar ratio to form obligate heteropolymers that are expressed in a tissue-specific manner. Consequently, each cell type displays a characteristic type I-type II keratin repertoire [1,2]. For example, K5/K14 and K1/K10 are produced in suprabasal and basal skin layers, respectively whereas K8/K18 constitute the major keratins of simple and glandular epithelia [1,2]. K7/K19/K20/K23 are the secondary simple epithelial keratins that are less abundant and have a more restricted expression pattern [1–3].

The tissue-specific expression of keratins that remains stable during dedifferentiation makes keratins widely used diagnostic tools in both neoplastic and non-neoplastic diseases [3–5]. Due to their abundance and release into the blood during stress situations, keratin fragments constitute attractive non-invasive disease markers [5,6]. Among them, antibodies M30 and M65 that recognize K18 epitopes, have attracted the most attention and are used to indicate the extent of hepatocellular damage and to predict disease outcome [5–7]. On the other hand, K7/K19 are absent from adult hepatocytes but are found in hepatic progenitor cells (HPCs), intermediate (i.e., not fully differentiated) hepatocytes and biliary epithelial cells (BECs) [7,8]. Because of that,

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K7/K19 constitute one of the most widely used ductular reaction (DRs) markers.

DR represents a basic regenerative response of the liver to injury [9,10]. It becomes even more important in patients with chronic liver diseases. In this situation, the normal liver regeneration that depends on division of mature hepatocytes becomes impaired [9,10]. Compared to humans, DR is typically less prominent in murine liver injuries where it is seen primarily in cholestatic injury models. DR takes place at the interface of the portal and parenchymal compartment and consists of activated BECs and HPCs. Within DR, HPCs are bipotential and give rise to both BECs and (intermediate) hepatocytes [11]. Given that the extent of DR reflects the severity of underlying liver disease [9,10], multiple studies showed that expression of K7/K19 increases with the severity of liver disease and represent a useful prognostic marker [12–17]. This might be of particular importance in severe, fulminant liver disorders such as alcoholic hepatitis where increased *K7* mRNA expression constituted an independent predictor of 90-day mortality [14]. Despite the obvious importance of DR, its extent can currently be evaluated only *in situ* since there are no established non-invasive DR markers. In that respect, CYFRA 21-1 assay detects K19 fragments in serum, however, its usefulness as a DR marker was not systematically analyzed [6].

To further explore the potential usefulness of keratins as diagnostic tools in liver disease, we analyzed the expression of K23 in the digestive system. K23 represents a largely unexplored, highly inducible keratin that was first detected in pancreatic cancer cell lines [18]. Since then, K23 was shown to be overexpressed in hepatocellular carcinoma and to constitute one of the most upregulated genes in steatohepatitis when compared to simple steatosis [19]. In our study, we demonstrate that K23 constitutes a specific DR marker that compared to both K7 and K19 is more restricted to the hepatobiliary system. K23 levels increase during the development of liver fibrosis and K23 expression becomes upregulated by the established DR inducer TNF-related weak inducer of apoptosis (TWEAK) or the type I acute phase inducer interleukin (IL)-1β. Moreover, K23 is found in the serum and is markedly elevated in patients with alcoholic liver cirrhosis when compared to liver-healthy subjects.

Materials and methods

Human liver specimen

Following 57 previously characterized samples from patients who underwent a liver biopsy at the University of Ulm and at the Bondy Liver Unit between the years 2006–2010 were analyzed [16]: alcoholic liver disease (ALD) (15 cases), chronic hepatitis B (HBV, 9 cases), HCV (13 cases), NASH (12 cases), 8 control samples. Tissues were cut in half and used either for histological staining or frozen for RNA isolation/biochemical analysis (see below). We also studied liver explants from 7 subjects with acute liver failure of indeterminate etiology (ALF) and 7 patients with primary biliary cholangitis (PBC Stage IV). For the confirmation cohort, a total of 150 non-tumor samples have been collected in two French University hospitals (Bordeaux and Créteil) between the years 1996 and 2014 from patients undergoing liver surgery due to hepatocellular carcinoma (Supplementary Table 1). The tissues were frozen immediately after surgery. The diagnosis of HBV/HCV was confirmed by a positive HBV DNA and HCV RNA (Cobas TaqMan/Amplicor; Roche, Basel, Switzerland) tests respectively, and all patients with viral hepatitis were treatment-naive. The diagnosis of ALD/NASH/ALF was based on laboratory parameters, histological findings, personal interview and an exclusion of other liver disease etiologies including viral infection, hereditary hemochromatosis, and autoimmune liver disease. ALD patients reported a daily alcohol consumption of >80 g in males and >60 g in females for at least 10 years. NASH patients had characteristic histological findings without significant alcohol consumption. The controls were patients with histologically normal livers who underwent a liver biopsy because of mildly elevated transaminase levels of unknown etiology (levels <twice the upper limit of normal) or liver surgery due to presence of liver metastases. The grading of inflammation and staging of fibrosis was done on 4 μm-thick liver sections using Desmet (HBV, HCV, controls) and Kleiner scoring criteria (ALD, NASH). To determine the impact of both parameters on K23 expression, samples were allocated to groups with minimal/mild/moderate/advanced fibrosis stage/inflammatory grade. HCV patients were subgrouped into individuals with "mild" (6 males/2 females) and "active" disease (3 males/2 females). A written informed consent to participate in the study was obtained from all patients and the analysis was in compliance with the 1975 Declaration of Helsinki as reflected in an approval by the Human Subjects Committees of the participating centers. To analyze publicly available data on K23 levels in liver disease, we searched the GEO database.

Animal experiments

Ten weeks old mice were fed with 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-supplemented diet (ssniff, Soest, Germany) for 4 weeks or subjected to bile duct ligation (BDL) for 21 days. For BDL, three months old mice were anaesthetized with isoflurane. Midline laparotomy was performed and common bile duct (CBD) was ligated with non-absorbable surgical silk (Perma-hand, Ethicon, Somerville, NJ). For partial hepatectomy (PH), 2 months old C57BL/6 mice were anaesthetized with rompun/ketamine and after a small subxiphoid incision, a two third hepatectomy was performed and the specimens were collected at indicated time points. As another model with pronounced DR, 20 weeks old multidrug resistance protein 2 (*Mdr2*) knockout (KO) males (FVB/N background) and their non-transgenic littermates were studied. Untreated, age- and sex-matched mice were used as controls. Mice were sacrificed via CO_2 inhalation followed by blood collection. Dissected livers were weighed, and pieces were put in 10% formaldehyde (histological staining), snap frozen in liquid nitrogen (biochemical analyzes) or submerged in RNAlater stabilization reagent (Ambion, Life Technologies GmbH, Darmstadt, Germany). CBDs, intestinal specimens and gallbladders (GBs) were taken for RNA and protein analysis. Serum parameters were measured in the clinical chemistry department of University Hospital Ulm and Aachen. Animal experiments were approved by the Institutional Animal Care Committees of the participating centres.

Cell culture experiments

The hepatocellular HuH7 (JCRB) and bile duct TFK-1 (DMSZ no: ACC-344) and MzCha1 [20] carcinoma cell lines were cultured in DMEM (HuH7, MzCha1) or alphaMEM (TFK-1; GIBCO-Invitrogen, Paisley, UK) medium supplemented with 10% fetal bovine serum (FBS-gold-PAA, Pasching, Austria), 1% penicillin-streptomycin (GIBCO-Invitrogen), 1% L-glutamine (GIBCO-Invitrogen), 2x MEM amino acids (both essential and non-essential) (TFK-1, MzCha1; GIBCO-Invitrogen) and 1 mM sodium pyruvate (TFK-1, MzCha1; GIBCO-Invitrogen). Cells were grown at 37 °C and 5% CO_2. Primary hepatocytes were isolated from 2–3 months old mice as described previously [21] and kept in hepatocyte culture medium (GIBCO-Invitrogen, Hepatozym-SFM 17705) supplemented with 10% FBS, 1% penicillin-streptomycin and L-glutamine (GIBCO-Invitrogen). To study the regulation of K23 expression, cells were treated for 6 h with IL-6 (40 ng/ml), IL-1β (20 ng/ml); both purchased from Sigma-Aldrich, Steinheim, Germany) or recombinant TWEAK (20 ng/ml; R&D Systems, Minneapolis, MN).

Reverse transcription and qRT-PCR analysis

Total RNA was isolated via RNeasy fibrous tissue, RNAeasy mini kits (Qiagen, Hilden, Germany) or Maxwell Tissue LEV Total RNA Purification Kit (Promega, Madison, WI). RNA isolation from CBDs and GBs was performed with a kit from Ambion (Life technologies; #AM1931). The RNA samples were translated to cDNAs with Superscript II reverse transcriptase kit and oligo-dT primers (Invitrogen, Darmstadt, Germany), quantitec cDNA translation kit (Qiagen) or MultiScribe reverse transcriptase and random hexamers (Applied Biosystems, Grand Island, NY). The relative expression of genes of interest was determined using specific primers (Supplementary Table 2). Human RPLPO (18S ribosomal RNA) and mouse L7 (ribosomal RNA) were used as internal control. For the confirmation cohort, qRT-PCR was performed using pre-designed TaqMan probes (Hs00210096_m1 for KRT23) and the BioMark HD reader (Fluidigm, South San Francisco, USA). Expression data (Ct values) were acquired using the Fluidigm RT-PCR Analysis software (4.1.3) and the $2^{-\Delta\Delta CT}$ method with R18S as calibrator.