Research Article

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Hepatitis C virus and alcohol: Same mitotic targets but different signaling pathways

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Background & Aims: Chromosomal aberrations are frequently observed in hepatitis C virus (HCV)- and alcohol-related hepatocellular carcinomas (HCCs). The mechanisms by which chromosomal aberrations occur during hepatocarcinogenesis are still unknown. However, these aberrations are considered to be the result of deregulation of some mitotic proteins, including the alteration of Cyclin B1 and Aurora kinase A expression, and the phosphorylation of gamma-tubulin. Our study aims at investigating changes in expression of the above mentioned proteins and related intracellular pathways, in *in vitro* and *in vivo* models of both HCV- and alcohol- dependent HCCs.

Methods: In this study, the molecular defects and the mechanisms involved in deregulation of the mitotic machinery were analyzed in human hepatoma cells, expressing HCV proteins treated or not with ethanol, and in liver tissues from control subjects ($n = 10$) and patients with HCV- ($n = 10$) or alcohol-related ($n = 10$) HCCs.

Results: Expression of Cyclin B1, Aurora kinase A, and tyrosine-phosphorylated gamma-tubulin was analyzed in models reproducing HCV infection and ethanol treatment in HCC cells. Interestingly, HCV and alcohol increased the expression of Cyclin B, Aurora kinase A, and tyrosine-phosphorylated gamma-tubulin also in tissues from patients with HCV- or alcohol-related HCCs. *In vitro* models suggest that HCV requires the expression of PKR (RNA-activated protein kinase), as well as JNK (c-Jun N-terminal kinase) and p38MAPK (p38 mitogen-activated protein kinase) proteins; while, ethanol bypasses all these pathways.

Conclusions: Our results support the idea that HCV and alcohol may promote oncogenesis by acting through the same mitotic proteins, but via different signaling pathways.
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Abbreviations: HCCs, hepatocellular carcinomas; HBV, hepatitis B virus; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; p38MAPK, p38 mitogen-activated protein kinase; PKR, RNA-activated protein kinase; JNK, c-Jun N-terminal kinase; NF-kB, nuclear factor-kB; FAK, focal adhesion kinase; PI3K, phosphatydil-inositol-3 kinase; siRNA, small interference RNA.

Introduction

Each year, 550,000 new patients are diagnosed with hepatocellular carcinoma (HCC) worldwide, thus, at present, liver cancer is considered the fifth most frequent neoplasm and, because of its poor prognosis, the third leading cause of cancer death [1]. One of the most important clinical challenges associated with chronic hepatitis rises from the evidence that a considerable number of patients develop end-stage liver disease and HCC. HCC development and progression may depend on several etiologic factors, including hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse, and non-alcoholic steatohepatitis (NASH) [2,3]. HCV and alcohol consumption often coexist and may act synergistically, leading to a more rapid progression of liver disease and increasing the risk of HCC [4–6]. Accordingly, several studies from different geographic areas demonstrate that the risk for developing HCC is higher in patients with HCV infection and alcohol abuse than in patients with hepatitis C alone [6–8].

During HCC development, unrespectable to its etiologic origin, hepatic cells, committed to proliferate, may accumulate mutations in key cell cycle genes or bear epigenetic changes, microsatellite instability, and several chromosomal aberrations, including aneuploidy [9,10]. Chromosomal aberrations are frequently discovered in dysplastic nodules of cirrhotic livers and are often present in HCC, suggesting the occurrence of chromosomal defects at the early stages of hepatocarcinogenesis [9–11].

Although the mechanisms by which chromosomal aberrations arise during hepatocarcinogenesis are still unknown, it is known that deregulation of some mitotic events, usually found in other tumors, occurs. Alteration of expression and/or activity of mitotic regulatory components (cyclin B1, Aurora kinase A, etc.), as well as deregulation of continuous cycles of microtubule polymerization/depolymerization and phosphorylation of structural proteins (i.e. tubulins), radically influence the control of centrosome maturation and separation, bipolar spindle assembly, chromosome alignment and segregation, and cytokinesis in human cells, leading to aneuploidy [12–14]. Cyclin B1 was found over-expressed in HCC with advanced stage, portal invasion, and intrahepatic metastasis [15]. Recently, the role of aurora kinase A in HCC has moved into the focus of preclinical research. In fact, the up-regulation of the *Aurora-A* gene in HCC nodules compared with nontumorous liver tissues has been demonstrated [16].

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Moreover, modifications of beta-tubulin isotypes were found in liver cancer [17]. The close association between mitotic defects and hepatocarcinogenesis is also supported by *in vivo* and *vitro* studies demonstrating that both HCV and ethanol are able to interfere with the regular control of mitosis in hepatocarcinoma cell lines [18–20]. HCV is able to induce an accumulation of cells in G2/M phase and, concomitantly, an increase of cyclin B1/cdk1 complex nuclear translocation, through its effects on p38 mitogen-activated protein kinase (p38MAPK) and RNA-activated protein kinase (PKR), both molecules implicated in the control of G2/M phase progression [18,19]. Animal and human studies suggest that alcohol may be involved in initiation, promotion, and progression of HCC, through the activation of various molecular mechanisms including oxidative stress, changes in DNA methylation, immunosuppression, and genetic susceptibility [21,22]. Interestingly, it has been reported that HepG2 cells stably expressing alcohol dehydrogenase showed 6-fold increase in the percentage of cells in G2/M phase after ethanol exposure. The impairment in cell-cycle progression was due to the accumulation of the phosphorylated inactive form of Cdk1 [20,23].

Unfortunately, regarding the pathogenesis of HCV-associated HCC, it still remains controversial whether the virus plays a direct or an indirect role, and how alcohol operates in the acceleration of HCC development [24,25].

We postulate that HCV, acting in synergism with alcohol abuse, might promote mitotic aberrations through the activation/inhibition of different intracellular pathways; therefore, in this study we analyzed the changes in expression of some important mitotic regulators in HCC tissues derived from alcoholics or HCV infected patients.

Although several mitotic regulators have been implicated in cancer development and progression, we chose to study cyclin B1 and aurora kinase A as they form a network of interactions regulating the onset of mitosis, the centrosome biogenesis and microtubule nucleation, and the cytokinesis. Moreover, gamma-tubulin is a little known common partner of these two proteins [26,27].

Here, we identify the intracellular mechanism(s) potentially involved in mitotic aberrations, by the use of a cell-based *in vitro* model.

Materials and methods

Cells, plasmids, and polyclones

Human hepatocarcinoma cell lines (HepG2 and Huh7) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a 5% CO_2 incubator. Cells were grown to 70–80% confluence in 100-mm dishes and then transfected, using Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocols, with two different plasmids: an empty vector (pcDNA3-FLAG) or a unique construct encoding for entire HCV products (p34-9,7-HCVwt 1a) (gently provided by Dott. La Monica Nicola, IRBM-Pomezia, Italy) [28].

Control polyclones (with the empty vector) and polyclones stably expressing all HCV proteins were obtained by 21 days geneticin-selection of transfected cells. Polyclones were cryo-preserved at −80 °C and used at the occurrence.

Polyclones selected after transfection with the empty vector were used as internal experimental controls: preliminary experiments were performed to exclude the presence of substantial differences in comparison with non-transfected cells (see Supplementary Fig. 1).

For all reported experiments, polyclonal cells were alternatively synchronized in G1/S (by 2 mM thymidine) or G2/M transition (by 200 ng/ml nocodazole). See Supplementary Table 1 for FACS analysis.

Mitotic index

The mitotic index was calculated for G1/S-synchronized polyclones using double thymidine block [29]. After block, G1 cells were released to progress through the cell cycle over the next 15 h, and then treated or not with ethanol. Mitotic index was calculated by DAPI (40–60-diamino-2-phenylindole) staining, as already described [18]. After staining, the slides were extensively washed and mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA), before examination.

The mitotic index was expressed as the mean (%) of positive cells with respect to the total cell population per field, for a total of 20 fields. Cells were counted by fluorescence microscope (Nikon Eclipse E600 microscope, Nikon, Italy).

Western blotting

For Western blot analysis, equal amounts of proteins from tissue or cell extracts obtained after lysis in Ripa buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, SDS 0,1%, 2 mM phenylmethilsulfonyl fluoride, 1 g/ml aprotinin, 1 g/ml of leupeptin and phosphatase inhibitors), were electrophoresed on SDS–PAGE. Proteins were transferred to PVDF membrane (Millipore, Marlborough, MA, USA) and treated with specific primary antibodies overnight at 4 °C. Filters were then washed four times with PBS-Tween 20 and newly incubated with peroxidase-coupled secondary antibodies for 1 h at RT. After incubation, the blots were visualized by ECL (Amersham Pharmacia Biotechnology, Freiburg, Germany).

Immunoprecipitation

For immunoprecipitation, 0.1 mg of total lysate was incubated with 0.1 µg of anti-phosphotyrosine antibody (Santa Cruz) overnight at 4 °C. Samples were then incubated with protein A agarose (Amersham Pharmacia) for 1 h at 4 °C, washed three times with Ripa buffer, and resuspended in 10 µl of SDS-sample buffer. Each sample was then electrophoresed on SDS–PAGE, and Western blot to reveal gamma-tubulin was performed as described above.

Antibodies

The following antibodies were used: anti-core protein monoclonal antibody (Affinity Bioreagents, Denver, CO, USA); anti-NS5A mouse monoclonal antibody, anti-actin goat polyclonal antibody, anti-cyclinB1 mouse monoclonal antibody and anti-PKR rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phosphotyrosin mouse monoclonal antibodies (Upstate Biotechnology, Lake Placid, NY, USA); anti-aurora A rabbit polyclonal antibody, and anti-gamma-tubulin rabbit polyclonal antibody (GeneTex Inc., San. Antonio, TX, USA); peroxidase-conjugated goat anti-rabbit, goat anti-mouse, and rabbit anti-goat IgG (Santa Cruz).

Inhibitors treatment

Cells were pre-treated for 30 min with 10 µM SP600125 or SB203580 (Sigma–Aldrich, Milano, Italy) to inhibit the activity of c-Jun N-terminal kinase (JNK) and p38MAPK, respectively.

siRNA transfection

A cocktail of siRNA directed against several regions of *PKR* (si*PKR*) was designed by New England Biolabs (Beverly, MA, USA) and transfected using Lipofectamin 2000 reagent (Invitrogen), according to the manufacturer's protocols. Dosing experiments showed that optimal silencing was achieved using 10 nM *PKR* siRNA (see Supplementary Fig. 2). Cells were also transfected with siRNA double targeting *GFP* gene (Quiagen, Germantown, MD, USA), as a negative control.

Patients and liver samples

The study was performed on 60 archived liver tissues obtained from 10 patients with HCV-related HCC, 10 patients with alcohol-associated HCC, and control non-cirrhotic subjects obtained from 10 surgically treated patients during the course of routine clinical care at the Surgical Departments, University of "La Sapienza "Rome" and at the Azienda Ospedaliero-Universitaria di Parma. At the time of surgical resection the tumor area was separated by the surrounding tissue. Then, part of the resected sample was fixed in formalin and embedded in paraffin for histological diagnosis, another part of all tissues were snap frozen in liquid nitro-

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