Research Article

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Expression of HLA-G by mast cells is associated with hepatitis C virus-induced liver fibrosis

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Background & Aims: Infection with hepatitis C virus is a worldwide health problem. An inadequate Th2 cytokine response promotes the fibrosis-cirrhosis fate. Immune-modulating molecules favoring a Th2 profile, such as HLA-G molecules of the HLA class Ib family, may play a role in chronic hepatitis. HLA-G contributes to the escape of tumors, and their involvement in viral infections has been increasingly described. The aim of this work was to study the expression of HLA-G in the liver, its cellular source and its regulation in cases of chronic C hepatitis.

Methods: HLA-G cells in blocks of liver derived from patients infected with HCV were labeled by immunohistochemistry and enumerated. Double immunofluorescence allowed the identification of the cellular source. HLA-G secretion by a human mast cell line was quantified by ELISA after various stimulations. After treatment with IFN-α, real-time PCR was performed to determine the kinetics of cytokine expression profiles, followed by heat map clustering analysis.

Results: The number of HLA-G+ cells was significantly associated with the area of fibrosis. For the first time, we identify the HLA-G+ cells as being mast cells. HLA-G secretion was significantly induced in human mast cells stimulated by IL-10 or interferons of class I. The transcriptome of the secretome of this cell line stimulated by IFN-α revealed that (i) the *HLA-G* gene is upregulated late, and that (ii) T lymphocytes and NK cells are recruited.

Conclusions: These findings suggest an autocrine loop in the genesis of HCV liver fibrosis, based on mast cells expressing HLA-G.

Keywords: Hepatitis C; HCV; Liver; Fibrosis; HLA-G; Mast cell; IFN-α.

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Abbreviations: HCV, hepatitis C virus; TGF-β, transforming growth factor β; IL, interleukin; ILT, Immunoglobulin-like transcript receptors; HIV, human immunodeficiency virus; HCMV, human cytomegalovirus; HBV, hepatitis B virus; IFN, interferon; OSM, oncostatin; MC, mast cell; HSC, hepatic stellate cell; SCF, stem cell factor; HCC, hepatocellular carcinoma.

Introduction

Hepatitis C virus (HCV) infections are a worldwide public health problem. Infected patients may develop complications including cirrhosis, liver failure or hepatocellular carcinoma. The dynamics of fibrosis progression to cirrhosis is closely associated with chronic inflammation, which is related to the efficiency of the immune response. Cytokines play a central role in the immune response and thus in orienting the fibrosis-cirrhosis fate [1].

In cases of chronic hepatitis, cytokines, including TGFβ and some interleukins such as IL-4 and IL-13, directly induce the production of extracellular matrix proteins and promote fibrosis [2]. Immune escape by HCV and progression of the infection towards chronicity are associated with the development of a Th2 response. These observations suggest that immune-modulating molecules favoring a Th2 cytokine profile play a major role in chronic viral hepatitis. These factors may include HLA-G, a member of the HLA class Ib family, which has membrane-bound and soluble (sHLA-G) forms, which are immunomodulatory molecules. HLA-G was initially described at the fetal-maternal interface of the cytotrophoblast [3,4]. Its immunomodulatory function has since been described [5] and its involvement in various pathologies, especially in malignancies, has been demonstrated [6]. HLA-G exhibits suppressive properties in various cells by inhibiting (i) the cytolytic function of NK cells and CD8 T cells and also triggering their apoptosis, (ii) the allo response of T4 lymphocytes, (iii) the maturation and function of antigen-presenting cells, (iv) the proliferation and function of B cells and (v) angiogenesis. These functions are the consequence of the interaction between HLA-G and its receptors on immune cells, such as CD8, ILT2, ILT4, KIR2DL4 [7], or CD160 on endothelial cells [8]. HLA-G is also able to induce suppressor/regulatory cells [9,10]. Its suppressive action is amplified by trogocytosis, which allows effector cells to be converted into temporary regulatory cells [11]. HLA-G is involved in many malignant diseases, and participates in the escape of tumors by promoting a Th2 cytokine environment and by inhibiting immune effector cells. Also, there is increasing evidence of the involvement of HLA-G in viral infections. Increased HLA-G expression, as membrane

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and/or soluble forms, has been evidenced in various types of viral infection, including those of human immunodeficiency virus (HIV) [12], human cytomegalovirus (HCMV) [13], neurotropic viruses (herpes virus and rabies virus) [14], influenza A virus [15], and hepatitis B virus [16].

However, the expression and role of HLA-G in human chronic hepatitis C have not been thoroughly investigated. We therefore assessed HLA-G expression in liver tissues infected with HCV, and then identified its cellular source and studied its regulation. We show that HLA-G is expressed by some cells in hepatic tissue infected with HCV and that the number of HLA-G-positive liver cells is significantly correlated with the area of fibrosis. In addition, we identified the cell type expressing HLA-G as a subpopulation of mast cells. Finally, we show that HLA-G expression by mast cells is regulated by interferons of class I.

Material and methods

Tissues samples

Liver biopsies from twenty chronically HCV-infected patients were retrospectively selected from the Biological Resource Center (BRC) of Rennes University Hospital. They were classified according to the METAVIR classification evaluating the severity of fibrosis (stage F0 to F4) and the inflammatory activity (A0 to A3). All biopsies were reviewed by an experienced pathologist. The characteristics of the patients are summarized in Table 1. Non tumoral areas of the biopsies, away from the origin of the tumor, were used for all analyses.

Cell cultures

The following cell lines were used as a positive control for HLA-G expression: Jeg3 (American Type Culture Collection (ATCC)), and LCL.721.221-G5, a B lymphoblastoid cell line transfected with HLA-G5 (D. Geraghty, Fred Hutchinson Cancer Research Center) as previously described [17]. The human mast cell line used, HMC1.1 (a generous gift from Dr. Butterfield, Mayo clinic, Rochester), was established from a patient with mast cell leukemia as previously described [18]. HMC1.1 cells (10^6 cells/ml) were cultured in six-well culture plates in IMDM medium (Iscove Modified Dubelcco Medium, Gibco life technologies, Cergy Pontoise, France) at 37 °C with 5% CO_2 for 48 h. The effects of the presence of the following cytokines, supplied by Peprotech-Tebu Bio (Neuilly, France), were tested: IL-33 (100 ng/ml), IL-31 (50 ng/ml), IL-4 (50 ng/ml), IL-6 (50 ng/ml), TNF-α (10 ng/ml), Oncostatin M (OSM) (50 ng/ml), IL-1β (10 ng/ml), IL-10 (50 ng/ml), TGF-β (5 ng/ml), IL-22 (10 ng/ml), IFN-α (50 ng/ml), IFN-β (50 ng/ml), IFN-γ (50 ng/ml), IFN-ω (10 ng/ml), IFN-λ1 (50 ng/ml), and IFN-λ2 (50 ng/ml).

Immunohistochemistry

Standard histological staining, including Sirius red staining of collagen accumulation and HES coloration, was performed. Paraffin-embedded sections (4 μm thick) were prepared, subjected to an antigen retrieval protocol, and incubated with primary antibody in a Ventana CT 09/021 automated machine (Ventana Medical systems, USA). Primary antibodies and dilutions used were: monoclonal mouse anti-human hepatocyte (clone OCH1E5, Dako, 1:600), mouse monoclonal anti-human HLA-G (Exbio, 4H84, 2 μg/ml), monoclonal mouse anti-human HLA-G (Exbio, MEM-G/1, 1 μg/ml or 1:100), rabbit monoclonal anti-human CD3 (Thermo Scientific, SP7, 1:1500), mouse monoclonal anti-human CD163 recognizing liver macrophages (Novocastra, 10 D6, 1:500), polyclonal rabbit anti-human CD117/ckit recognizing myeloid cells (Dako, 1:200), and mouse monoclonal anti-human mast cell tryptase (clone AA1, Dako, 1:1000). Bound primary antibody was revealed with goat biotinylated anti-mouse or anti-rabbit IgG secondary antibody (Vector, ABCYS, Les Ulis, France, 1:700) and then diamino-benzidine (DAB Map Detection Kit, Roche, Meylan, France) and Mayer hematoxylin coloration.

After immunohistochemistry, the image of the whole surface of the section was digitized at 20× magnification using a NanoZoomer Digital Pathology 2.0 RS whole-slide scanner (Hamamatsu, Japan). An appropriate computer script analysis was developed and used for semi-automatic enumeration of HLA-G+ cells and MCs or CD3+ T cells. The area of fibrosis was evaluated by annotation. Fibrosis areas were drawn on the whole section and the surface area was calculated using "simple PCI" software. As direct HLA-G/human mast cell tryptase double immunofluorescence was not possible because of antibody incompatibility, two double indirect immunofluorescence experiments, sharing CD117 (CD117/HLA-G and CD117/anti human mast cell tryptase) were performed in parallel on serial sections of paraffin-embedded sections from the same liver block. For immunofluorescence detection, fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch, Cy5-affinipure donkey anti-mouse IgG and Cy3-affinipure donkey anti-mouse IgG) were incubated with the sections for 1 h at room temperature. Nuclei were counterstained with Hoechst stain (Molecular probe).

Table 1. Characteristics of the HCV patients.

#	Age	Sex	Metavir classification		Etiology	Pathology associated
			F stage	A stage		
1	49	M	F4	A1	HCV	HCC
2	73	F	F4	A1	HCV	HCC
3	72	M	F1	A3	HCV	HCC
4	46	M	F4	A1	HCV + HBV + HIV	HCC
5	56	F	F4	A1	HCV	HCC
6	56	F	F4	A0	HCV	HCC
7	62	M	F4	A1	HCV	HCC
8	47	M	F4	A1	HCV	Transplantation
9	73	F	F4	A1	HCV	HCC
10	84	M	F4	A1	HCV	HCC
11	55	M	F2	A1	HCV	HCC
12	51	F	F4	A1	HCV	HCC
13	83	F	F4	A1	HCV	HCC
14	40	M	F4	A1	HCV + HBV	HCC
15	49	F	F4	A0	HCV + HIV	HCC
16	65	F	F4	A0	HCV	HCC
17	55	M	F4	A1	HCV	HCC
18	75	M	F3	A1	HCV + alcohol	HCC
19	75	M	F4	A2	HCV	No
20	67	M	F4	A1	HCV	HCC

Specific soluble HLA-G enzyme-linked immunosorbent assay

Soluble HLA-G concentrations in HMC1.1 culture-conditioned media were measured by specific sandwich enzyme-linked immunosorbent assay (ELISA) using MEM-G/9 (Exbio, Prague, Czech Republic; 10 μg/ml) and rabbit anti human beta-2 microglobulin (Dako, Trappes, France) as capture and revelation antibodies, respectively, as previously described [19] with minor modifications: after the second Ab, DAKO envision system HRP was added and tetramethylbenzidine/peroxide (TMB, R&D system) was used as the substrate.

Quantification of chemokine gene expression by real-time quantitative PCR

After various times of stimulation of HMC1.1 cells with IFN-α, total RNA was extracted and purified with the Nucleospin RNA II Kit^R (Macherey-Nagel, Hoerdt, France). The High capacity cDNA archive Kit^R (Applied Biosystems, Cergy Pontoise, France) was used for reverse transcription of aliquots of 3 μg of total extracted RNA following the manufacturer's instructions. Primers having the same melting temperature (Tm) were designed using Primer3 software. A large panel of cytokines and chemokines was tested using a large-scale qPCR approach as shown in Fig. 4C. Real-time PCR for HLA-G was performed in parallel with the same samples and with the HLA-G-positive cell line, Jeg3, using primers designed according the same methodology (Supplementary Fig. 1). The ΔCT method was