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Journal of Hepatology 41 (2004) 319–326

Journal of Hepatology

www.elsevier.com/locate/jhep

A study of some hepatic immunological markers, iron load and virus genotype in chronic hepatitis C

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***Background/Aims*: Host factors that may influence progression of hepatitis C infection to chronic hepatitis include T-cell responses and iron accumulation. We evaluated the hepatic expression of immunological markers relevant for a cytotoxic response in relation to viral and HFE genotype.**

***Methods*: Frozen liver biopsies were obtained at diagnosis from 28 HFE genotyped patients. Sections stained for CD8, MHC-I, β_2m, HFE and CD68 were analyzed blind by morphometry. Response to therapy was available in 12 cases.**

***Results*: A negative correlation was found between the number of $CD8^+$ cells and fibrosis. $CD8^+$ cells localized as clusters in portal tracts and sinusoids and were seen interacting with MHC-I positive lining cells. MHC-I and β_2m were expressed mainly in the endothelial and Kupffer cells. HFE was expressed in most, but not all, round and dendritic $CD68^+$ cells. Patients with virus genotype 3a had higher hepatic MHC-I and HFE expression, and a better-sustained response to IFN therapy than other patients.**

***Conclusions*: In chronic hepatitis C virus infection MHC-I expression in the liver seems to relate to viral-genotype. In addition, the expression of MHC-I molecules by Kupffer cells places them as probable important players in the host response to HCV.**

***Keywords*: HFE; MHC-I; IFN; Hepatitis C virus; Liver**

1. Introduction

The hepatitis C virus (HCV) genotypes differ in world distribution and in resistance to IFN therapy [1]. The reasons for the differences in response to IFN are unclear. Possible modifiers include hepatic iron [2–7] and T-cell ($CD8^+$) reactivity against the hepatitis C virus [8–12].

A significant negative correlation between hepatic iron overload and the number of circulating and hepatic $CD8^+$ cells was reported in hereditary hemochromatosis (HH) patients homozygous for the C282Y HFE mutation [13]. HFE mutations may influence the course and outcome of chronic hepatitis C by affecting iron loading [14–19]. Since HFE is a non-classical MHC-I protein, it could affect host response to the virus and disease progression.

In this paper we examine the quantitative distribution of $CD8^+$ cells and other immunological markers relevant to a T $CD8^+$ host response by morphometry in liver biopsies from HFE and HCV genotyped patients with chronic hepatitis.

2. Patients and methods

2.1. Study design

Liver biopsies were prepared in the immunohistochemistry lab for the following markers: CD8, MHC-I, β_2m, HFE and CD68. The

Received 16 December 2003; received in revised form 10 March 2004; accepted 8 April 2004; available online 13 May 2004

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0168-8278/$30.00
doi:10.1016/j.jhep.2004.04.027

immunochemistry analysis was 'closed' when 30 biopsies were completed. The stained sections were seen 'blind' by two observers for the qualitative and quantitative assessment of the tissue distribution of the markers without knowledge of the patients' identity. The latter was only disclosed after the immunohistochemistry study was concluded. Correlations between histopathological and clinical parameters, HFE and HCV genotype were done. An analysis of response to therapy was possible in 12 cases (see Section 2.4). Six liver biopsies from C282Y homozygous patients, not infected with HCV, were used as controls.

Informed consent in writing was obtained from all patients. The protocol conformed to the ethical guidelines of the Helsinki Declaration of 1975. Chronic hepatitis C virus infection was diagnosed and treatment was implemented according to published guidelines [1]. Exclusion criteria were other viral hepatitis, autoimmune hepatitis, secondary causes of iron overload, and history of alcohol consumption one year before liver biopsy (men >60 g/day; women >40 g/day) (Table 1). Two cases had to be excluded for insufficient information.

2.2. Hepatitis C virus genotyping

Anti-HCV antibodies were detected by a commercial enzyme-linked immunosorbent assay (HCV 3rd generation ELISA and EIA, Ghent, Belgium) and by the 3rd generation recombinant immunoblot assay (RIBA, Inno-Lia, Ghent, Belgium) and confirmed by testing for HCV RNA using a reverse transcription polymerase chain reaction (Amplicor, HCV, Roche Diagnostic Systems, Basel, Switzerland). HCV genotypes were determined by the Inno-LiPA HCV II assay (Innogenetics, Bayer, Ghent, Belgium).

2.3. Treatment

Criteria for treatment were based on histological and biochemical parameters (ALT $>$ 40U/l) and PCR-RNA viral positive. Patients were treated with IFN-α (2a or 2b) 3 MU 3 times/week plus ribavirin 1 or 1.2 g/day (before October/2001) and after this date IFN-α Peguilated (2a or 2b) plus ribavirin. Genotype 1 and 4 patients were treated for 12 months, genotype 2 and 3 for 6 months (except before 1999 when patients with genotype 3a were also treated for 12 months).

2.4. Response to treatment

A positive treatment response was considered when a viral RNA negative test was detected by PCR, 6 months after the treatment was completed. Patients were followed up every 6 months. When patient identity was disclosed, it became apparent that 12 HFE and HCV genotyped patients had completed therapy one year before. A sustained response was observed in six patients. The other six were considered non-responders: five with HCV RNA detectable and ALT which failed to decrease throughout treatment; in one patient a complete virological and biochemical response was observed at the end of treatment, followed by re-emergence of the virus during the follow-up period.

2.5. Liver histopathology

Formalin-fixed paraffin-embedded liver biopsies were analyzed for siderosis, fibrosis, steatosis and necroinflammatory activity by one pathologist (Table 1). Hepatic iron concentration (HIC) was determined by atomic absorption in fresh biopsies or from formalin-fixed paraffin-embedded biopsies [20]. Hepatic iron index (HII) was calculated by dividing HIC (μmol/g of dry weight) by the patients' age (years).

2.6. Biochemical serum parameters

Fasting serum iron, total iron binding capacity, serum ferritin, ALT and AST were determined by standard assays, at the time of liver biopsy.

2.7. HFE genotyping

HFE gene genotyping was done using the 'Haemochromatosis Gene Mutation Assay' (Vienna Lab, Vienna, Austria), according to the manufacturer's specifications [21] as described [22].

2.8. Antibodies

Monoclonal antibodies (mAb) against the macrophage marker CD68 (KP1), the T-cell marker CD8 (C8/144B) and MHC-I (W6/32) were obtained from DAKO (Glostrup, Denmark). HFE mAb (2F5) was prepared by Ehrlich and collaborators [23]. $\beta_2$microglobulin was identified by an anti-β_2m mAb (TÜ99, BD PharMingen, Europe BD Biosciences, Belgium). Mouse mAbs DAK-GO1 (DAKO) and G155-228 (BD PharMingen), IgG and IgM, respectively, were used as negative controls. The antibody specificity of DAK-GO1 is directed towards *Aspergillus niger* glucose oxidase, an enzyme which is neither present nor inducible in mammalian tissues. G155-228 is specific for trinitrophenol, a hapten not expressed on human cells.

For immunofluorescence an anti-mouse Alexa 488-labelled secondary antibody was used. For signal amplification, the tyramide signal amplification (TSA)™ Kit (Alexa Fluor 568, Molecular Probes, Leiden, The Netherlands) was used. This Kit contained a HRP-conjugated secondary antibody and Alexa 568-labeled tyramide.

For immunohistochemistry rabbit anti-mouse immunoglobulins and APAAP conjugate were used (DAKO).

2.9. Immunohistochemistry

Cryostat sections or formalin-fixed, paraffin-embedded sections, 6 μm thick, were mounted on gelatin covered glass slides and dried overnight. Cryostat sections were fixed in acetone for 10 min, dried, wrapped in aluminum foil and stored at -20 °C until used. Slides were allowed to come to room temperature (RT) before immunohistochemical staining. Formalin-fixed paraffin-embedded sections were prepared as described in Ref. [13]. All sections were stained using the APAAP method. Cryostat sections were incubated for 30 min with the mAbs: 2F5 at dilution 1:6000, KP1 1:6000, W6/32 1:200, TÜ99 1:100; paraffin sections—overnight with C8/144B mAb at 1:200.

2.10. Double immunofluorescence

Double immunofluorescence was used to identify Kupffer cells positive for HFE and/or CD68. Detection of two primary mAbs raised in the same species (mouse, in this case) was performed by sequential tyramide signal amplification and conventional fluorescence detection (dilutional neglect) method [24]. All steps were performed at RT. Acetone-fixed cryostat sections were incubated in phosphate-buffer saline with H_2O_2 0.3% for 5 min, in the dark in order to quench endogenous peroxidase activity. Then, the specimens were blocked with 1% Blocking Reagent (TSA Kit) for 60 min. Tissues were labeled with the first primary antibody (2F5) diluted in 1% blocking reagent, for 60 min. After washing, the HRP-conjugated secondary antibody was applied at a 1:100 dilution, and incubated for 60 min. Additional washes were followed by the application on the specimen of the tyramide working solution at a 1:50 dilution. Finally, the second primary antibody (anti-CD68) was applied and incubated for 60 min, and subsequently, the Alexa 488-conjugated secondary antibody at a 1:2000 dilution (Molecular Probes) also for 60 min. Sections were mounted in Vectastain with DAPI (Vector Laboratories, Burlingame, CA) and analyzed in a Axioskop microscope (Zeiss, Göttingen, Germany) equipped with a SPOT II (Diagnostic Instruments, Sterling, Michigan) camera.

Stainings with TSA method using the standard (normal dilution used without TSA), $2\times$, $4\times$ and $8\times$ higher dilutions of the conventionally used primary antibody dilution were done. Thus, the dilution of the first primary antibody, which was consistently not recognized by a conventional Alexa 488-labeled secondary antibody, but was still clearly detectable by a simple fluorescent amplification method, was chosen. As controls, sections were also immunostained for either antigen separately.

Negative controls included incubations with DAK-GO1and leaving out the primary or secondary antibodies.

2.11. Morphometric microscopy analysis

2.11.1. $CD68^+$ and HFE^+ cells

Alternate sections were stained for CD68 or HFE. The positive cells in the two serial sections were counted by conventional microscopic analysis.

Table 1
Clinical characteristics of patients with chronic hepatitis C virus

Patient	Sex	Age[a] (yr)	HFE genotype	HCV genotype	Viral load[b] (UI/ml) × 10^6	Serum parameters at diagnosis					Histopathology[c]				HIC (μmol/g dry wt)	HII	Response to treatment
						ALT(U/l)	AST(U/l)	Iron μg/dl	TfSat (%)	Ft (ng/ml)	Fib	Sid	Ste	Inf			
1	F	31	C282Y/H63D	1a	0.36	64	41	216	53	13	0	0	0	1	19	0.61	NR
2	F	32	wt/H63D	NA	NA	NA	NA	NA	NA	NA	1	0	1	1	26	0.81	–
3	F	35	wt/wt	1a	0.39	114	57	99	32	150	1	0	1	1	5	0.14	–
4	F	38	wt/H63D	3a	1.70	48	32	222	96	273	1	2	1	1	28	0.74	–
5	F	41	wt/wt	3a	0.10	52	27	115	32	45	1	0	1	1	58	1.41	SR
6	F	50	wt/wt	3a	0.21	43	38	NA	NA	91	1	0	1	1	15	0.30	SR
7	F	60	wt/H63D	1b	0.85	50	38	89	28	85	0	0	0	1	14	0.23	–
8	F	63	wt/H63D	1b	0.54	67	66	94	30	73	1	0	0	2	10	0.16	NR*
9	M	29	wt/H63D	1a	NA	432	281	99	30	475	1	1	1	1	20	0.69	–
10	M	30	wt/wt	1a	2.28	84	39	123	43	290	1	0	0	1	15	0.50	–
11	M	30	wt/wt	NA	NA	219	78	117	42	NA	1	0	1	2	23	0.77	–
12	M	31	wt/wt	3a	NA	45	16	125	47	98	1	0	1	2	24	0.77	SR
13	M	31	wt/H63D	3a	NA	250	74	61	22	73	0	0	2	2	11	0.35	–
14	M	33	wt/H63D	1a	0.01	39	32	114	51	228	0	0	0	1	31	0.94	–
15	M	35	wt/H63D	3a	1.49	155	69	186	58	383	1	0	3	1	16	0.46	SR
16	M	35	wt/wt	NA	>1.00	111	62	115	27	58	1	0	0	1	9	0.26	–
17	M	37	wt/wt	4c/4d	0.29	60	46	75	25	332	1	0	0	2	72	1.94	–
18	M	40	wt/wt	NA	NA	49	31	91	37	96	1	0	1	1	18	0.45	SR
19	M	40	wt/wt	3a	0.30	38	39	217	61	146	1	0	0	2	14	0.35	–
20	M	42	wt/wt	3a	0.01	49	35	82	26	75	0	0	0	1	18	0.43	SR
21	M	43	C282Y/H63D	1b	1.08	NA	NA	NA	NA	NA	1	1	1	2	46	1.07	–
22	M	43	C282Y/wt	3a	0.21	75	38	238	98	214	3	1	2	1	48	1.12	–
23	M	43	wt/wt	4	>0.50	111	74	123	43	233	0	1	1	1	44	1.02	–
24	M	44	wt/wt	NA	0.94	109	85	107	40	202	1	0	0	1	8	0.18	–
25	M	47	wt/wt	1b	NA	58	38	103	33	NA	1	0	0	2	9	0.19	NR
26	M	47	wt/wt	1a/1b	0.48	53	47	106	29	152	2	0	0	2	11	0.23	NR
27	M	49	wt/wt	1b	0.71	83	40	79	22	544	0	1	1	1	14	0.29	NR
28	M	54	H63D/H63D	2a/2c	0.15	291	122	160	45	174	1	0	1	2	18	0.33	SR

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; F, female; Ft, ferritin; HIC, hepatic iron concentration; HII, hepatic iron index; M, male; NA, not available; NR, non-responder; NR*, relapser; SR, sustained responder; TfSat, transferrin saturation.

[a] Age at diagnosis.

[b] Pre-treatment viral load.

[c] Fib, fibrosis; Sid, siderosis; Ste, steatosis; Inf, necroinflammatory activity. Siderosis was scored by Perls' blue staining as follows: 0, no siderosis; 1, <25% of hepatocytes affected; 2, 25–50% of hepatocytes affected; 3, 50–70% of hepatocytes affected; 4, >75% of hepatocytes affected and Kupffer cells. Fibrosis staging was scored: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. Steatosis staging was scored: 0, no steatosis; 1, <30% of hepatocytes affected; 2, 30–70% of hepatocytes affected; 3, >70% of hepatocytes affected. Inflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity, 1, mild activity; 2 moderate activity; 3, severe activity.

Subsequently, the same sections were evaluated by computer-assisted image analysis. A Leica DMLB microscope (Leica Cambridge Lda., Cambridge, UK) equipped with a couple device color camera (3CCD) was used to examine the sections under a $\times 400$ magnification. In LeicaQWin image analyzer (Leica Cambridge, Lda.) a square image frame (with an area of 26276.5 μm^2) was used. In the first case, for each patient, 10 similar (i.e. in the same zone of the section) microscopic fields, in randomly chosen hepatic lobuli, were evaluated for both antibodies. The results were expressed as the mean ± SEM (no positive cells/mm^2). Computerized image analysis of CD68 and HFE was done using a routine developed in the LeicaQWin program (HFE.Q5R). Threshold levels of brightness were set and a temporary binary color image was super imposed on the digitalized image. The areas of interest (red, stained FastRed cells) were outlined in a pseudocolor. This permits checking the accuracy of the measured area. Each color is a unique combination of red, green and blue (RGB) ranging between levels of 0–255, allowing the detection of over 16×10^6 different colors. Thus to identify FastRed stained cells, selected RGB thresholds (RGB: 0–170, 0–49, 0–119), were obtained. Minimum size of cells was also identified (>4 μm). Mean positive area was expressed as μm^2 (mean ± SEM). For each biopsy 5 fields randomly chosen were analyzed. A good correlation between conventional microscope analysis and the computerized image analysis was obtained (HFE: $r = 0.632, n = 15, P = 0.011$; CD68: $r = 0.593, n = 15, P = 0.020$).

2.11.2. MHC-I and $\beta_2$microglobulin

MHC-I and β_2m expression was evaluated using a similar routine (MHC.Q5R), as the one for HFE and CD68, with different thresholds of RGB (0–180, 0–91, 0–158).

2.11.3. $CD8^+$ cells

$CD8^+$ cells were evaluated blindly using the conventional analysis. Intra-observer reproducibility was assessed by repeated measurement of this parameter in selected samples ($r = 0.985, n = 9, P < 0.001$).

2.12. Statistical analysis

Pearson's correlation coefficients were estimated to assess the relation between two continuous variables, while Spearman's rank correlation coefficients were used to determine the degree of association between two variables, when one of them was of an ordered categorical type. Independent-samples *T*-test was used for statistical comparison of means (± SD) between two independent groups. In this *T*-test analysis the variance of each variable was taken in consideration using the Levene's test for equality of variances. Paired-samples *T*-test was used to compare means of two variables for a single individual. Pearson χ^2 (with Yates's correction for 2×2 tables when one cell had expected count less than 5) was used for comparison of proportions between groups. *P*-values <0.05 were considered significant. The statistical analysis was performed using the SPSS 11.5 software (SPSS Inc., Chicago, IL).

3. Results

3.1. Hepatic expression of immunological markers

HFE protein was evenly distributed in the liver lobuli and was seen exclusively in non-parenchymal cells (Fig. 1B). HFE-positive cells were characterized by an irregular round shape or a dendritic shape, with protrusions of their cytoplasm towards sinusoidal spaces, typical of Kupffer cells. When an anti-CD68 mAb (a marker of Kupffer cells) was used, a positive immunoreactivity with a similar distribution pattern was observed (Fig. 1A). The negative control did not show any notable immune reactivity (not shown). However, although $CD68^+$ cells were morphologically identical to the sinusoidal lining cells that express HFE, in several patients the immune reactivity associated with the CD68 protein usually displayed a more intense staining and $CD68^+$ cells appeared to be more numerous than HFE^+ cells. Morphometric analysis of the density of HFE^+ cells and those exhibiting CD68 staining, an index of the Kupffer cell population, confirmed those differences: in approximately 50% of the patients the number of $CD68^+$ cells/mm^2 was higher than the number of HFE^+ cells (Table 2).

Double immunofluorescence, confirmed that HFE was expressed within the Kupffer cells, defined as CD68-positive cells. However, not all CD68-expressing cells were positive for HFE. Interestingly, in the majority of the cells these two proteins were in different cellular compartments (Fig. 2).

MHC-I (Fig. 1C) and β_2m (Fig. 1D) molecules were expressed mainly in endothelial and Kupffer cells. MHC-I expression was rare to occasional faint staining of hepatocellular membranes in the majority of the patients. Only 2 out of 27 patients showed strong 'honeycomb' positivity for MHC-I staining. $CD8^+$ cells were localized in the sinusoids and frequently as clusters in the portal tracts (Fig. 1F).

In general, HFE expression did not differ between HCV patients without ($n = 11$) or with any HFE mutation ($n = 11$) (mean ± SD positive cell area: 56 ± 37 and

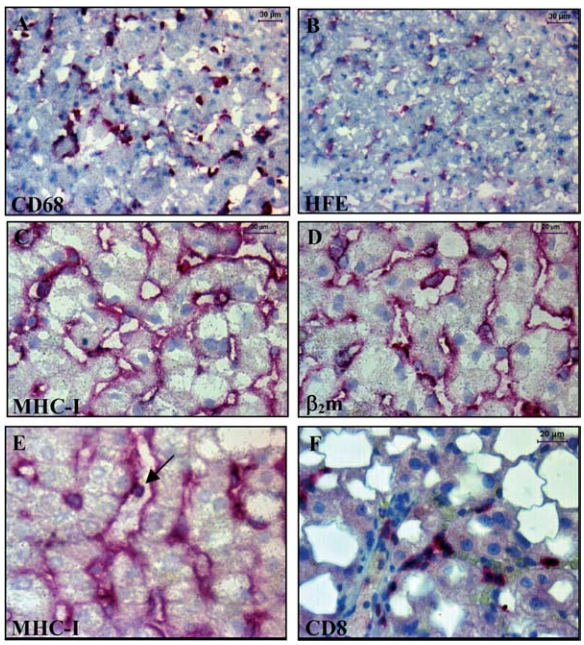

Fig. 1. Expression of different immunological markers in chronic HCV infection. Liver sections from a patient with chronic hepatitis C virus infection with a wt/wt HFE genotype stained using the APAAP method, and FastRed substrate A–E, cryostat sections; F, formalin-fixed paraffin-embedded section. $CD68^+$ (A) and HFE^+ (B) cells had a typical pattern of Kupffer cells, although the expression of HFE was less intense and abundant. MHC-I (C, E) and β_2m (D) molecules were expressed in endothelial and Kupffer cells. In E, a mononuclear cell (arrow) that is in close contact with a sinusoidal cell is also shown to be MHC-I positive. $CD8^+$ cells (F) were found in the sinusoids.

Table 2
Comparative morphometrical analysis of HFE$^+$ and CD68$^+$ cells in patients with chronic hepatitis C

Group	*n*	CD68[a]	HFE[a]
CD68 > HFE†	7	552 ± 68	300 ± 92
CD68 = HFE	8	463 ± 84	480 ± 119
CD68 < HFE‡	1	601	750

n, number of patients, $^\dagger P < 0.001$, $^\ddagger P < 0.0001$, paired-samples *T*-test.
[a] Mean ± SD.

42 ± 32 μm^2, respectively, $P = 0.369$). However, the one compound heterozygous (Patient #21, Table 1) and the only H63D homozygous (Patient #28, Table 1) had very low (7.1 μm^2) and null HFE expression, respectively. No HFE protein was expressed in the C282Y HH liver biopsies. The presence of any HFE mutation had no impact on the expression of all the other immunological markers analyzed (not shown).

3.2. Immunological markers, viral genotype, viral load and iron biochemistry

MHC-I expression in liver lobuli in biopsies from patients infected with HCV3a genotype (mean ± SD: 173 ± 105 μm^2, $n = 8$) was significantly higher ($P = 0.006$) than in patients with other viral genotype (32 ± 35 μm^2, $n = 13$, Fig. 3), and similar to the MHC-I expression in HH patients (207 ± 79 μm^2, $n = 6$, Fig. 3). HFE expression followed the MHC-I pattern in HCV patients: 3a genotype patients had significantly higher ($P = 0.036$) HFE expression (mean ± SD: 61 ± 32 μm^2, $n = 7$) than patients infected with other viral genotypes (30 ± 22 μm^2, $n = 10$). Expression of CD68, β_2m and the number of CD8$^+$ cells were not associated with virus genotype. None of the immunological markers studied was associated with viral load (not shown).

There was a negative correlation between the number of CD8$^+$ cells in the liver lobuli and the grade of fibrosis

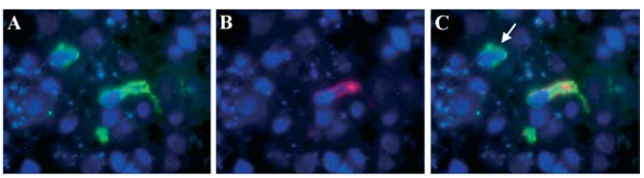

Fig. 2. Double immunofluorescent staining of CD68 and HFE molecules. Representative human liver section from a patient with chronic hepatitis C incubated with anti-CD68 (A) anti-HFE (B) monoclonal antibodies, detected with an Alexa 488-conjugated secondary antibody (green) and using the Tyramide Signal Amplification method with an HRP-conjugated secondary antibody and an Alexa 568-labelled tyramide (red), respectively. In (C) photos (A) and (B) were merged, demonstrating that these two proteins do not co-localize. The arrow indicates a Kupffer cell that is HFE negative (original magnification × 630).

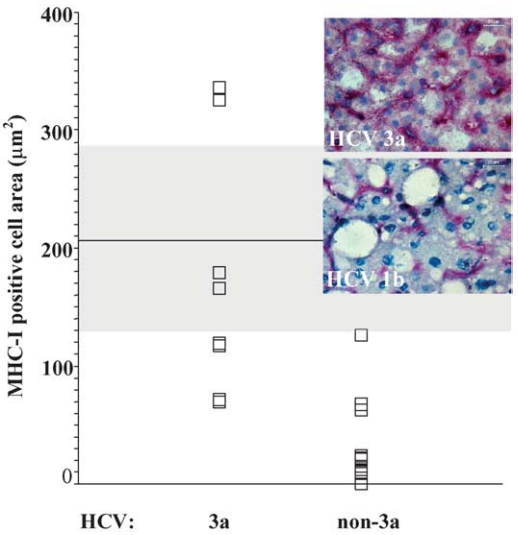

Fig. 3. Impact of HCV genotype on classical MHC-I expression. Sections from patients with chronic hepatitis C virus infection with 3a genotype had a significantly higher MHC-I expression (mean ± SD: 173 ± 105 μm^2, $n = 8$) than those infected with other viral genotypes (32 ± 35 μm^2, $n = 13$, $P = 0.006$, *T*-test). Insert shows two frozen liver sections stained for MHC-I with the W6/32 mAb, using the APAAP method and FastRed substrate, representative of a patient with 3a genotype and high MHC-I expression (upper figure) and a patient with 1b genotype and low MHC-I expression (lower figure). Shaded area: mean ± SD of MHC morphometry in liver biopsies from HH C282Y homozygous patients.

($r = -0.514, n = 19, P = 0.024$). In addition, the expression of CD68 correlated positively with serum iron ($r = 0.459, n = 19, P = 0.048$) and the expression of MHC-I correlated positively with HIC ($r = 0.669, n = 26, P < 0.001$), HII ($r = 0.667, n = 26, P < 0.001$), and transferrin saturation ($r = 0.486, n = 23, P = 0.019$). No differences in hepatic iron load levels were seen between viral genotypes.

3.3. Response to interferon treatment

Virus genotype was strongly associated with response to IFN treatment ($P = 0.019$). Five out of 6 patients with a sustained response had the 3a genotype (one had the 2a/2b genotype), while the non-responders had all the 1a or 1b genotype. Sustained responders had significantly higher levels of MHC-I (mean ± SD: 137 ± 111 μm^2, $n = 6$) than non-responders (14 ± 8 μm^2, $n = 6$; $P = 0.042$, Fig. 4).

4. Discussion

The starting purpose of the present study with basis on earlier work on HH [13] was to examine whether markers relevant to the development of a cytotoxic response were altered and/or related to hepatic iron load in patients with chronic HCV. The work focused on MHC-I, β_2m, HFE and

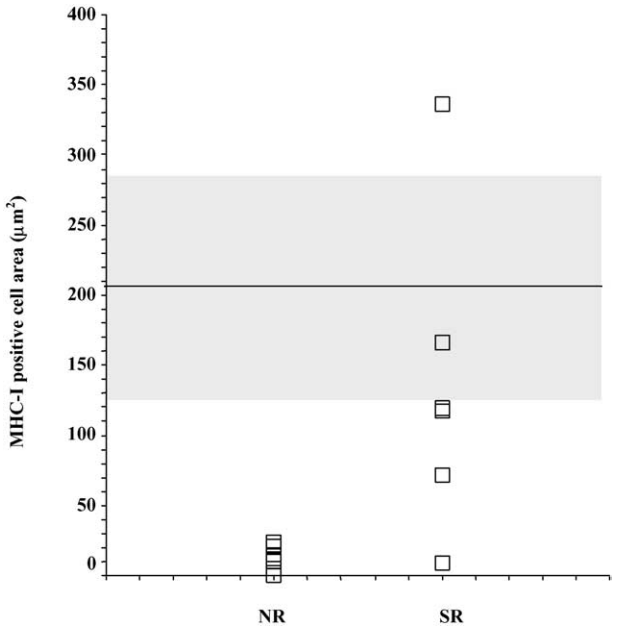

Fig. 4. Impact of MHC-I expression in the response to IFN therapy. Patients with chronic hepatitis C with a sustained response (SR) to IFN therapy had significantly higher expression of classical MHC-I molecules in the liver (mean ± SD: 137 ± 111 μm², $n = 6$) than non-responders (NR) (14 ± 6 μm², $n = 8$; $P = 0.042$, *T*-test). See text for viral genotype of the SR and NR. Shaded area: mean ± SD of MHC morphometry in liver biopsies from HH C282Y homozygous patients.

numbers of intra-hepatic $CD8^+$ cells. CD68 was used as a marker of Kupffer cells. A negative correlation was found between the number of $CD8^+$ cells and development of fibrosis confirming a recent report [12]. $CD8^+$ cells were localized as clusters in the portal tracts and in sinusoids. In the sinusoids, positive cells were frequently seen interacting with MHC-I positive lining cells. MHC-I and β_2m molecules were expressed mainly in the endothelial and Kupffer cells. Kupffer cells in chronic HCV have a phenotype of professional antigen presenting cells [25]. Their capacity to phagocytose infected hepatocytes and serum viral-antibody coated particles, together with their close contact with $CD8^+$ cells, both in sinusoids and in portal tracts, place them as possible important players in the host response against the viral infection. Although no correlation was seen between the level of MHC-I expression and the number of $CD8^+$ lymphocytes, the possibility that a stronger specific $CD8^+$ T-cell response occurs in the patients with higher MHC-I expression is attractive. The finding of much higher levels of MHC-I expression in 3a genotype patients may offer a molecular and immunological basis for the clinical experience reported by others [26]. This finding is in agreement with the earlier finding of a higher cytotoxic response in HCV3a genotype patients [27].

The results confirm that HFE is expressed in round and dendritic shaped cells, in $CD68^+$ Kupffer cells [28,29]. Interestingly, not all $CD68^+$ cells express HFE. This may indicate the existence of Kupffer cells in different metabolic states or that HFE identifies different subpopulations of Kupffer cells. The lowest levels of HFE expression were seen in a compound heterozygous for the two most common HFE mutations and in a liver biopsy from an H63D homozygous, suggesting contrary to current belief [30] that both mutations may affect HFE expression. In two recent studies of the rat HFE gene expression, others have found HFE in hepatocytes [31,32]. No immunohistochemistry data were presented in those studies. The present and earlier observations of Bastin et al. [28], were made in human liver biopsies. The differences reported may reflect differences in the species studied, in the detection methods, or the antibodies used.

The most significant observation of the present study, however, regards the measurement by morphometry of much higher levels of MHC-I expression in 3a genotype patients. The observation that HFE expression followed the same pattern of quantitative expression as that of classical MHC-I may indicate that a link may exist between the non-classical and the classical MHC-I molecules at the cellular level. Linkage disequilibrium between HLA-A alleles and HFE mutations has been reported previously [33].

Two possible explanations can be envisaged for the association with viral genotype. HCV viruses type 1 down-regulate MHC-I and HFE expression, similarly to reported findings with other viral proteins [23,34]. This explanation is supported by the recent finding of Konan et al., showing that the non-structural protein precursor NS4A/B from HCV 1b genotype reduces MHC-I cell surface presentation, by inhibiting global ER-to-Golgi traffic [35]. Two other studies showed that HCV virus might affect MHC-I expression [36,37]. These studies were done in vitro. It is of considerable interest that we now observe comparable differences in the expression of MHC-I in Kupffer cells and in endothelial cells in liver biopsies. A second explanation may relate to the finding that the major envelope protein of HCV (E2) of HCV-3a does not bind CD81 [38].

The statistically significant correlation between some iron parameters and MHC-I expression is of interest in the light of other emerging connections between iron load and MHC-I in mice [39].

In conclusion, the present study reinforces the evidence for the complexity of host–pathogen interactions taking place in the development of a chronic viral infection. The results also point to Kupffer cells and MHC-I as important players in the process.

Acknowledgements

We thank Dr Graça Porto for critical review of the manuscript. This study was supported by the EU QLG1-CT-1999-00665 project, the Calouste Gulbenkian Foundation/FCT Project on Hemochromatosis (Portugal) and the INNOVA Foundation/APBRF (USA).

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