Down-regulation of intrahepatic CD16+ and CD56+ immune cells in chronic *Hepatitis C* virus infection and HCV-related hepatocellular carcinoma

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Abstract

Introduction: The intrahepatic pool of natural killer (NK) and lymphocytes with NK receptors (NKRs) is thought to play a key role in determining whether host immune responses to hepatitis C virus (HCV) infection result in viral clearance or disease progression.
Material and methods: Liver biopsies were taken from 116 patients with chronic hepatitis C (CHC) infection, 44 of whom had liver cirrhosis (LC), and 36 patients had hepatocellular carcinoma (HCC). Twenty histologically normal livers served as controls. Tissue sections were stained with CD16 and CD56 monoclonal antibodies by avidin-biotin peroxidase complex method and were semi-quantitatively evaluated.
Results: The CD16 and CD56 immunoreactivity on intrahepatic NK cells and lymphocytes was significantly decreased in patients with CHC (34.8 ±1.9, 4.3 ±0.4, respectively), LC (22.4 ±0.3, 1.5 ±0.6, respectively) and HCC (11.5 ±1.1, 0.6 ±0.3, respectively) compared to controls ($p < 0.05$) with sequence order. Moreover, NK cells and lymphocytes positive for CD16 and CD56 in the liver of patients with high METAVIR scores for chronic hepatitis were significantly reduced compared to patients with low score ($p < 0.01$). The CD16+ Kupffer cells were, also, reduced in CHC compared to controls ($p < 0.01$), and the reduction was more pronounced with the progression of the disease.
Conclusions: These data show that CHC is associated with defects in NK cells and CD56+ lymphocytes that correlate with the degree of inflammatory activity and stage of hepatic fibrosis. The reduced CD16+ Kupffer cells in chronic HCV infection and HCC stress the important role of these cells in antiviral and antitumor immunity.

Key words: CD16, CD56, natural killer, *Hepatitis C* virus, chronic hepatitis C, liver cirrhosis C, hepatocellular carcinoma.

Introduction

Chronic hepatitis C (CHC) is one of the most serious liver diseases in Egypt [1]. It affects over 170 million people worldwide. About 80% of infected people develop a chronic course, which can lead to cirrhosis and/or hepatocellular carcinoma (HCC) [2]. The latter is one of the most common cancers in the world causing one million deaths a year [3], with

a rising incidence from 2 to 5.7% of total cancers in Egypt [4].

It is believed that hepatitis C virus (HCV) itself is not cytopathic, whereas the cellular immune response to infected hepatocytes may cause hepatocellular injury [5]. Natural killer (NK) cells constitute the first line of host defense against invading pathogens [6, 7]. They usually become activated in the early phase of viral infection [8]. The activated NK cells play an essential role in recruiting virus-specific T cells and inducing antiviral immunity in the liver [9]. They can, also, eliminate virus-infected hepatocytes directly by cytolytic mechanisms and indirectly by secreting cytokines that induce an antiviral state in host cells [10]. Therefore, the optimally activated NK cells are important in limiting viral replication in the liver [11].

Natural killer cells express a number of molecules (CD16 and CD56); often called co-receptors, which bind to their respective ligands on target cells [12]. The CD16 is a common marker on human NK cells [13]. It is involved in their activation pathway [14]. It is, also, expressed on a subset of monocytes/macrophages, neutrophil granulocytes and mast cells [15]. The CD56 surface antigen is typically expressed by NK cells [16]. It is an isoform of the human neural cell adhesion molecule that has been found on a subset of T lymphocytes and on cells derived from neural, muscle and embryonic tissue [15].

As prognosis of chronic HCV infection is largely dependent on histopathologic information gained by liver biopsy, there is a pressing need for immunomolecular markers that can help in early prediction of the outcome of HCV infection in order to reduce the disease morbidity and mortality. This study was designed to assess the hepatic expression of CD16 and CD56 molecules on NK cells, lymphocytes and Kupffer cells in CHC to determine whether alterations in these markers may influence the progression of HCV-mediated liver disease.

Material and methods

Patients and controls

This study was conducted on 152 patients with chronic liver disease admitted to Department of Hepato-Gastroenterology, Theodor Bilharz Research Institute, Egypt. Patients were subjected to thorough clinical examination, routine laboratory investigations including complete blood picture and liver function tests, abdominal ultrasound (Hitachi EuB-515A) and upper endoscopy whenever indicated. Core liver biopsies were taken by percutaneous ultrasound-guided Menghini needle for histopathological and immunohistochemical studies. Patients were included in the study if they had: (a) clinical and laboratory evidence of chronic HCV infection, (b) histological evidence consistent with HCV-induced chronic liver disease and (c) focal hepatic lesions suggestive of malignancy by abdominal ultrasound and confirmed by histology to be HCC.

Patients who had other causes of chronic liver disease than CHC and its sequelae; cirrhosis and/or HCC, were excluded from the study.

The control group included 20 wedge liver biopsies taken during laparoscopic cholecystectomy from age-matched healthy subjects. They all had clinical, biochemical, serological, ultrasonographic and histological findings within the normal range. Informed consent was obtained from all cases participating in the study according to Institution's Ethics Committee' rules.

Serological investigations

Liver function tests including albumin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were done for all cases.

Hepatitis viral markers including hepatitis B surface antigen, anti-HBs antibodies, total and IgM class antibodies against hepatitis B core antigen were detected using enzyme immunoassay kits (Murex Diagnostics, Dartford, England). Anti-HCV antibodies were detected using Version V anti-HCV ELISA kit (Murex Diagnostics, Dartford, England). Circulating HCV-RNA was assayed to confirm the presence of HCV antigenemia by nested RT-PCR using a set of primers within the 5' non-translated region according to Saber *et al*. [17].

Histological assessment

Liver biopsies were fixed in buffered formaldehyde, processed into paraffin blocks to obtain 4 μm-thick tissue sections that were stained with hematoxylin-eosin and Masson trichrome stains. As regard CHC, the degree of hepatic necro- inflammation and the stage of fibrosis were scored according to the METAVIR system [18]. Grade 1 inflammation as well as the first and second stages of fibrosis were considered low score, while, grades 2 and 3 inflammation as well as the third and fourth stages of fibrosis were considered high. Biopsies of HCC were graded according to the histological differentiation of Ishak [19].

According to the above mentioned clinical, serological and histopathological criteria, subjects included in this study were categorized into the following groups: (a) control (n = 20), (b) patients with CHC (n = 116), 44 of whom had liver cirrhosis (LC) and (c) patients with HCV-related HCC (n = 36).

Immunohistochemical studies

Avidin-Biotin peroxidase complex method [20] was applied on formalin-fixed, paraffin-embedded

tissue sections that were dewaxed and rehydrated. Endogenous peroxidase activity was quenched by incubation of the slides in 0.3% hydrogen peroxide in methanol for 10 min. Antigen retrieval was done to unmask the antigens by boiling the slides twice in 10 mmol/l citrate buffer solution (pH 6.0) (Zymed, USA), 5 min each. Tissue sections were treated with normal horse serum (Dako, Denmark) for 10 min to avoid non-specific immunoreactivity. Duplicate liver sections were incubated overnight at 4°C with mouse anti-human CD16 (Novacastra, UK) and CD56 (Santa Cruz Biotechnology, CA, USA) monoclonal antibodies at the optimal dilution (1 : 20 and 1 : 50 respectively). Sections were then incubated at room temperature with biotinylated goat anti-mouse antibody for 10 min followed by streptavidin horseradish peroxidase conjugate (all from Dako, Denmark). The CD16 and CD56 antigens were visualized by the addition of diaminobenzidine substrate solution (Dako, Denmark) followed by counterstaining with Mayers' hematoxylin. Positive and negative control slides were included within each session.

Interpretation

All immunostained slides were assessed and scored. The distribution of immuno-labeled NK cells, lymphocytes ± Kupffer cells in hepatic tissue was noted. The percentage of positive cells was evaluated semi-quantitatively in five fields of maximum staining intensity at 400× magnifi-cation and the mean value was obtained.

Statistical analysis

Results are presented as means ± SEM. Statistical analysis was conducted using analysis of variance (ANOVA test) for comparison between different groups and the least significance difference "*t*" test for comparison between 2 groups. Probability values less than 0.05 were considered significant.

Results

One hundred and fifty-two HCV-infected patients as well as 20 healthy controls were included in this study. The main demographic and laboratory data were summarized in Table I.

Hepatic expression of CD16

The pattern of CD16 immunoreactivity in both neoplastic and non-neoplastic hepatic lesions was that of diffuse cytoplasmic brown staining in both NK+ cells and Kupffer cells. Sections from histologically normal livers showed a moderate number of CD16+ NK cells (53.9 ±3.8) mainly scattered within the hepatic lobules as well as a high number of CD16+ Kupffer cells (97.6 ±2.1) (Figure 1A). However in CHC patients, the CD16+ NK cells (34.8 ±1.9) were found mainly in the regions of spotty necrosis and portal areas together with a moderate

Table I. Demographic and laboratory data of the studied groups

Parameters	Control (*n* = 20)	CHC (*N* = 116)		HCC (*n* = 36)
		without LC (*n* = 72)	with LC (*n* = 44)	
Age (mean ± SD)	38.5 ±8.1	47.4 ±7.2	48.9 ±9.3	61.3 ±5.9
Male/female ratio	9/16	41/31	26/18	29/7
Pallor	0	3 (4.2)	7 (15.9)	9 (25.0)
Jaundice	0	4 (5.6)	8 (18.2)	15 (41.7)
Palmer erythema	0	0	22 (50.0)	25 (69.4)
Spider naevi	0	0	20 (45.5)	20 (55.6)
Lower limb oedema	0	0	23 (52.3)	10 (27.8)
Child classification A		72 (100)	11 (25.0)	1 (2.8)
B		0	15 (34.1)	14 (38.9)
C		0	18 (40.9)	21 (58.3)
Albumin (mean ± SD)	4.3 ±0.5	3.8 ±0.3	2.9 ±0.7	2.8 ±0.4
AST (mean ± SD)	33.3 ±4.3	49.1 ±18.3	46.4 ±5.1	68.3 ±12.4
ALT (mean ± SD)	34.7 ±3.9	63.2 ±29.7	51.8 ±4.3	78.1 ±16.3

CHC – chronic hepatitis C, LC – liver cirrhosis, HCC – hepatocellular carcinoma
Number in parenthesis = %, normal range for albumin is 3.5-5.0 mg/dl, normal range for ALT and AST is up to 40 U/l

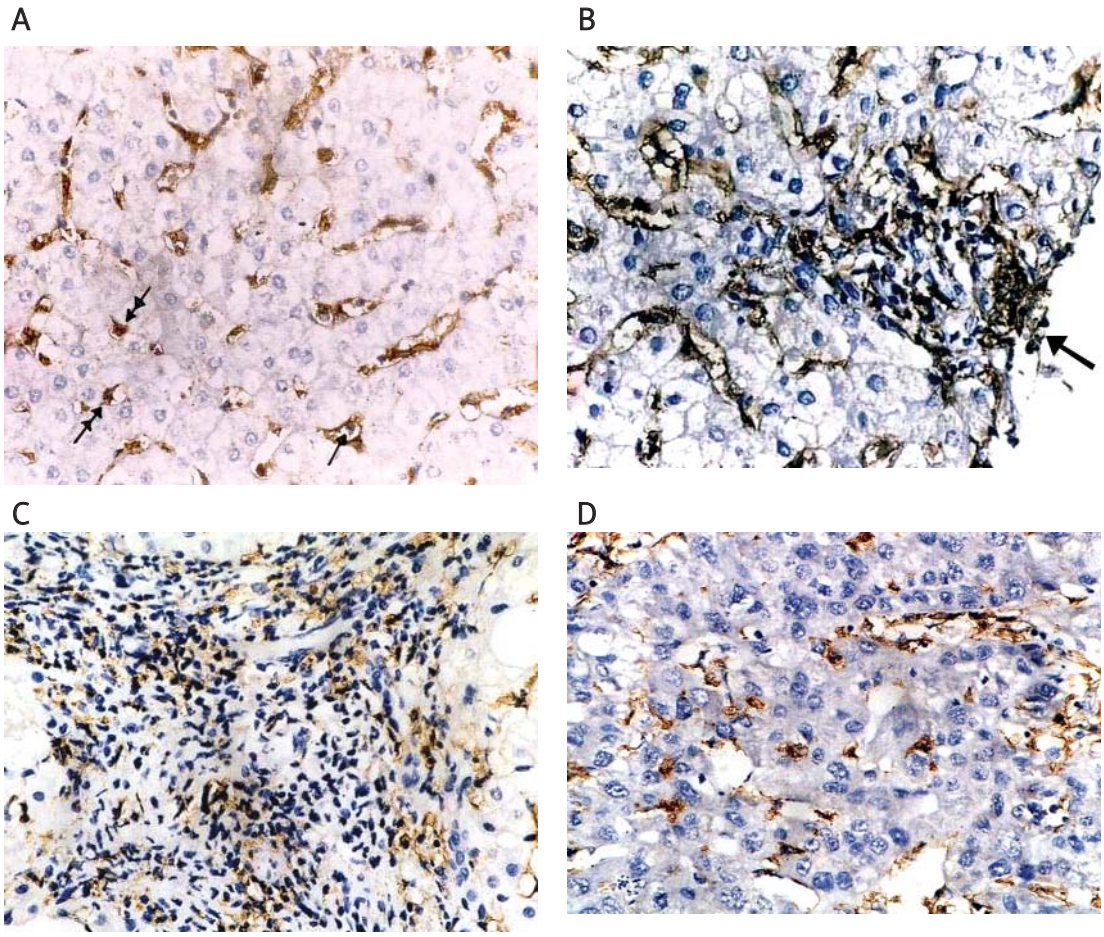

Figure 1. Liver sections from: **A**) a control case showing many CD16+ NK cells (single-head arrows) and CD16+ Kupffer cells (double-head arrows); **B**) a case of chronic hepatitis C with low METAVIR score showing mildly thickened portal tract infiltrated by a moderate number of CD16+ NK cells (arrow); **C**) a case with HCV-induced cirrhosis showing markedly thickened portal tract infiltrated by few CD16+ NK cells among numerous CD16− NK cells; **D**) a case of well differentiated hepatocellular carcinoma showing few CD16+ NK cells within the neoplastic growth (Immunostaining, 400× magnification)

number of CD16+ Kupffer cells (65.4 ±0.2). These values were significantly lower than those of controls ($p < 0.01$). Cases with lower grades of inflammation or stages of fibrosis showed significant increase ($p < 0.01$) of immuno-labeled CD16+ NK and Kupffer cells compared to those with higher scores (Figures 1B, 1C, Table II).

On the other hand, patients with HCC had the least number of CD16+ NK cells (11.5 ±1.1) and Kupffer cells (22.3 ±1.1) within the neoplastic growth (Figure 1D) with significant difference than those of controls or CHC patients ($p < 0.01$). All collected cases of HCC were associated with and developed on top of HCV-induced cirrhosis, the non-tumor regions expressed more CD16+ NK and kupffer cells than the tumor regions as in LC cases (22.4 ±0.3, 51.0 ±3.7 respectively) with significant difference between the two regions ($p < 0.01$) (Table II).

Moreover, it was found that CD16+ NK cells and Kupffer cells were significantly lower in poorly-differentiated than well-differentiated tumors ($p < 0.05$).

Hepatic expression of CD56

The pattern of CD56 immunoreactivity in both neoplastic and non-neoplastic lesions was that of diffuse cytoplasmic staining of NK cells and lymphocytes.

In histologically normal livers, few CD56+ NK and lymphocytes (7.9 ±1.9) were found scattered mainly within the lobules (Figure 2A). However, liver biopsies from patients with CHC showed infiltration of the parenchyma with a scanty number of CD56+ cells (4.3 ±0.4) mainly in the regions of spotty necrosis or in portal areas, which was significantly lower than that of controls ($p < 0.05$). These cells were significantly reduced in higher grades of inflammation or stages of fibrosis than lower ones ($p < 0.01$) (Figures 2B, 2C). In particular,

Table II. Hepatic expression of CD16 molecule on NK cells and Kupffer cells in different studied groups

Groups	Number of patients	CD16+ NK cells (mean ± SEM)	CD16+ Kupffer cells (mean ± SEM)
Control	**20**	**53.9 ±3.8**	**97.6 ±2.1**
CHC	**116**	**34.8 ±1.9[a,b]**	**65.4 ±0.2[a,b]**
low grade (A0 + A1)	76	41.3 ±2.2[c]	73.7 ±1.7[c]
high grade (A2 + A3)	40	24.4 ±3.2	49.0 ±4.0
low stage (F1 + F2)	72	45.3 ±2.6[c]	74.5 ±1.8[c]
high stage (F3 + F4)	44	22.4 ±0.3[b]	51.0 ±3.7[b]
HCC	**36**	**11.5 ±1.1[a]**	**22.3 ±1.1[a]**
well-differentiated	20	13.4 ±1.4[d]	30.4 ±2.2[d]
poorly-differentiated	16	9.2 ±1.9	19.8 ±2.2

NK – natural killer cells, CHC – chronic hepatitis C, HCC – hepatocellular carcinoma, A – necroinflammatory activity, F – stage of fibrosis
[a]p < 0.01 vs. control, [b]p < 0.01 vs. HCC, [c]p < 0.01 vs. high grade or stage CHC, [d]p < 0.05 vs. poorly differentiated HCC

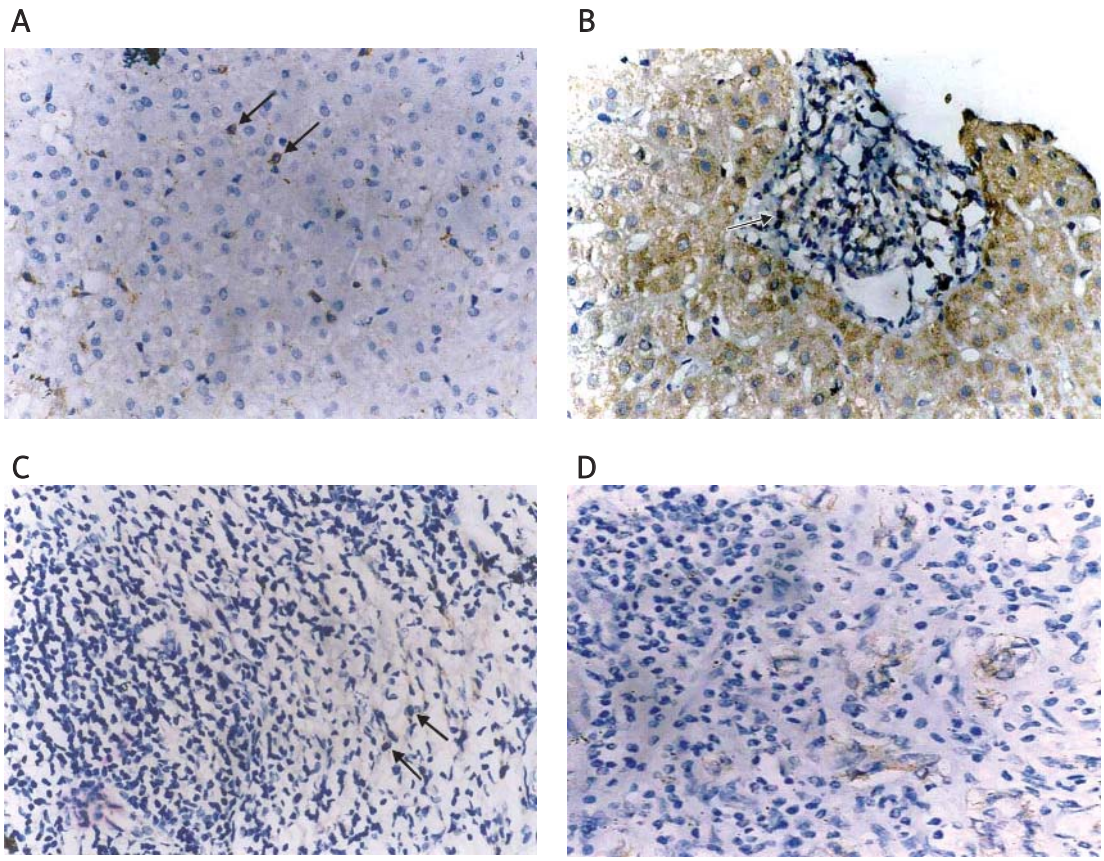

Figure 2. Liver sections from: **A**) a control case showing infiltration of the liver parenchyma with CD56+ NK (arrows); **B**) a case of chronic hepatitis C with low METAVIR score showing mildly thickened portal tract infiltrated by some CD56+ NK cells and lymphocytes (arrow); **C**) a case with HCV-induced cirrhosis showing markedly thickened portal tract infiltrated by few CD16+ NK cells and lymphocytes (arrows) among a huge number of CD56– cells; **D**) a case of poorly differentiated hepatocellular carcinoma showing infiltration of the neoplastic growth by numerous CD56– cells (Immunostaining, 400× magnification)

the expression of CD56 on NK cells was significantly decreased in the tumor regions of HCC compared with control and CHC groups ($p < 0.01$). It was also reduced than the adjacent non-tumor regions but without significant difference. It was found that there was significant reduction of positive cells in poorly differentiated than well-differentiated tumors ($p < 0.05$) (Figure 2D, Table III).

Table III. Hepatic expression of CD56 antigen on NK cells and lymphocytes with NK receptors in different studied groups

Groups	Number of patients	CD56+ cells (mean ± SEM)
Control	**20**	**7.9 ±1.9**
CHC	**116**	**4.3 ±0.4[a,c]**
• low grade (A0 + A1)	76	6.1 ±0.4[d]
• high grade (A2 + A3)	40	1.5 ±0.4
• low stage (F1 + F2)	72	5.6 ±0.5[d]
• high stage (F3 + F4)	44	1.5 ±0.6
HCC	**36**	**0.6 ±0.3[b]**
• well-differentiated	20	1.0 ±0.1[e]
• poorly-differentiated	16	0.1 ±0.4

NK – natural killer cells, CHC – chronic hepatitis C, HCC – hepatocellular carcinoma, A – necroinflammatory activity, F – stage of fibrosis [a]p < 0.05, [b]p < 0.01 vs. control, respectively, [c]p < 0.01 vs. HCC, [d]p < 0.01 vs. high grade or stage CHC, [e]p < 0.05 vs. poorly differentiated HCC

Discussion

Chronic hepatitis C patients often become victims of liver cirrhosis and subsequent HCC [5]. Resolution or persistence of HCV infection largely depends on the strength of intrahepatic immune responses that are generated at the early stages of acute hepatitis [21]. The intrahepatic immune system is characterized by a unique repertoire of immune cells. In addition to the conventional $CD4^+$ and $CD8^+$ T cells and B cells, the liver contains a large number of NK cells and T cells with NK stimulatory, co-stimulatory and inhibitory receptors (NKRs). These cells appear to play an important role in innate liver immunity [22].

The $CD56^+$ natural killer T (NKT) lymphocytes predominantly infiltrate the liver in viral infection and malignancies [23]. They can control virus-specific T cell differentiation and NK cell activation as they expand in response to hepato-tropic viruses [22].

In the present study, the expression of CD16 and CD56 on intrahepatic NK cells and lymphocytes was significantly down-regulated in patients with CHC, LC and HCC compared with control group. The reduction may be attributed to decreased production of these immunoreactive cells or hepatic infiltration with a high number of other cell populations. The low number of $CD16^+$ $CD56^+$ cells in the livers of patients with chronic HCV infection may contribute to inadequate elimination of HCV. This result is consistent with previous studies demonstrating a substantial decrease in intrahepatic $CD56^+$ NK and $CD56^+$ T cell numbers in chronically HCV-infected individuals compared to histologically normal donor livers [16, 22, 24]. NK cells can be divided into two subsets, dim and bright, according to CD56 surface density expression [25]. Lin *et al.* [26] found that HCV-infected patients had fewer activated NK cells and $CD56^{+dim}$ cells compared to controls. They suggested that chronic HCV infection may alter both the immunoregulatory and the cytotoxic function of NK cells towards enhancement of $CD56^{+dim}$ turnover.

Chronic hepatitis C is accompanied with NK cell stimulation which mediates hepatocyte injury [27, 28] and, thus, correlates with the grade of inflammatory activity and stage of liver fibrosis [29]. Furthermore, HCV develops a clever escape strategy from host immune response by diverting NK cells towards killing of uninfected hepatocytes. The virus enhances the expression of stress-inducible proteins on hepatocytes. These proteins act as ligands for NKG2D receptors on NK cells [30], which become auto-reactive and promotes killing of innocent "uninfected" bystander hepatocytes [31].

In this study, the expression of CD16 and CD56 on intrahepatic NK and lymphocytes was significantly decreased in patients with liver cirrhosis compared to those with lower stages of fibrosis. This finding is in accordance with data of Kawarabayashi *et al.* [5] and Doherty and O'Farrelly [32] who found that human hepatic $CD56^+$ NK cells progressively decreased in parallel with the progress of hepatitis C and diminished in liver cirrhosis as the development or proliferation of these cells may be inhibited. Deignan *et al.* [22] suggested that the decreased proportions of $CD56^+$ T cells may explain the susceptibility of chronically HCV-infected patients for further progression of liver disease. Also, Kawarabayashi *et al.* [5] suggested that the decrease in $CD56^+$ NK cell numbers in cirrhotic livers may be a risk factor for the development of hepatocellular carcinoma.

Also, this study showed that the intrahepatic CD16 and CD56 labeled NK cells and lymphocytes were diminished in neoplastic growth of HCC compared with controls and patients with CHC, LC as well as non-tumor regions. Cai *et al.*, [33] found a significant reduction of $CD56^{dim}$ $CD16^{pos}$ NK cells in tumor regions of HCC compared with non-tumor regions suggesting that local environment is more important for the regulation of NK cell functions in HCC patients. They reported that both peripheral and tumor-infiltrating NK cells exhibited functional deficiency in producing IFN-γ and killing K562 targets compared with healthy peripheral NK cells and non-tumor-infiltrating NK cells respectively. These findings suggest that the functional ampairment of NK cells might severely hinder the anti-tumor immune responses of HCC patients.

Kupffer cells are the resident hepatic macrophages that produce IL-12 required for

the activation of intrahepatic NK cells [21]. In this study, numerous CD16+ Kupffer cells were seen in control hepatic specimens. However in patients with CHC, the CD16 immunoreactivity on Kupffer cells was significantly decreased with progression of the disease. These findings matched those of Jinushi *et al.* [34] who found that dendritic cells, a subset of antigen presenting cells, were impaired and unable to activate NK cells for optimal function in chronic HCV infection.

In conclusion, chronic hepatitis C is associated with decreased number of CD16+ and CD56+ immunoreactive cells leading to defects in the host immune response thence disease progression. Although, the CD56 is a more specific marker for NK cell activation than CD16, the expression of both markers correlates inversely with the degree of inflammation and stage of fibrosis. The reduced CD16+ Kupffer cells in chronic HCV infection and HCC stress the important role of these cells in antiviral and antitumor immunity. Furthermore, the down-regulation of NK cells and CD56+ lymphocytes in cirrhosis may contribute to frequent emergence of HCC in cirrhotic livers.

Acknowledgments

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