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Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

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Review Article

Reactivation of hepatitis viruses following immunomodulating systemic chemotherapy

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Reactivation of hepatitis B virus (HBV) or hepatitis C virus (HCV) infection following anticancer chemotherapy and immunosuppressive therapy is a well-known complication. HBV reactivation has been reported to be associated with anti-CD20 monoclonal antibody rituximab-containing chemotherapy and tumor necrosis factor-α inhibitor-containing immunosuppressive therapy in HBV resolved patients (hepatitis B surface antigen negative and antibodies against hepatitis B core antigen positive and/or antibodies against surface antigen positive). On the other hand, HCV reactivation has been reported to be associated with liver damage or hepatic dysfunction, but fulminant hepatitis due to HCV reactivation is a rare complication. In this review, we describe the pathophysiology of the reactivation of HBV and HCV infection, as well as the clinical evidence and management of HCV reactivation.

Key words: chemotherapy, hepatitis B virus, hepatitis C virus, immunosuppressive, occult infection, reactivation

INTRODUCTION

REACTIVATION OF HEPATITIS B virus (HBV) or hepatitis C virus (HCV) infection following anticancer chemotherapy and immunosuppressive therapy is a well-known complication. In particular, HBV reactivation is a potentially fatal complication that needs to be followed up carefully. Most HBV reactivation occurs in hepatitis B surface antigen (HBsAg) positive patients prior to treatment; however, HBV reactivation has been observed increasingly in HBV resolved patients without HBsAg, but with antibodies against hepatitis B core antigen (anti-HBc) and/or HBsAg (anti-HBs). Moreover, HBV reactivation has been reported to be associated with anti-CD20 monoclonal antibody rituximab-containing chemotherapy and tumor necrosis factor (TNF)-α inhibitor-containing immunosuppressive therapy in patients with prior resolved HBV infection. On the other hand, HCV reactivation has been reported to be associated with liver damage or hepatic dysfunction, but fulminant hepatitis due to HCV reactivation is a rare complication.

Hematopoietic stem cell transplantation (HSCT) is often the chosen treatment for hematological malignancies and it has been suggested that the incidence and clinical characteristics of reactivation of HBV or HCV infection may depend on immune reconstitution, which may be associated with graft-versus-host disease (GVHD) and the combined immunosuppressant, especially in the allogeneic HSCT setting.

As several review papers about HBV reactivation had been already reported, we described here the pathophysiology of the reactivation of HBV and HCV infection, as well as the clinical evidence and management of HCV reactivation.

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PATHOPHYSIOLOGY OF REACTIVATION OF HBV AND HCV INFECTION

Immunity to HBV and HCV

BECAUSE HBV AND HCV are not cytopathogenic, it is widely accepted that both viral control and liver pathology are mediated by the host immune system (Table 1). Many studies of host genetics and

Table 1 Putative host immune system of HBV and HCV infection

	HBV	HCV
Innate immunity		
Hepatocytes	Stealth response	Type I and/or III IFN production
Main components	NK and NKT cells	DC
Critical cytokines	IFN-γ, TNF-α	Type I and/or III IFN
Adaptive immunity		
Components	T cells and B cells	T cell and B cells

DC, dendritic cells; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; NK, natural killer; NKT, natural killer T cells.

immunology demonstrate an important role for T lymphocytes in protective immunity against HBV and HCV.

The occurrence of HBV reactivation in patients with signs of resolved infection, particularly anti-HBc positive patients, relies on the existence of occult HBV infection. Patients with occult HBV infection are supposed to harbor HBV covalently closed circular DNA in the nuclei of their hepatocytes after the resolution of acute infection.[1] Most occult HBV infection individuals are infected with replicable viruses, whose replication and gene expression are strongly inhibited by the host immune system.[2] The exact mechanisms of inhibition have not yet been determined, but long-lasting specific host T-cell immune surveillance against HBV epitopes and epigenetic factors are presumably the major causes of long-term viral suppression.[3]

In contrast, although HCV reactivation following immunosuppressive therapy is rare,[4–8] fibrosing cholestatic hepatitis C (FCH) occurs in HCV positive liver transplant recipients with immunosuppressive therapy.[9–11] Whether immunosuppressive therapy leads to HCV reactivation in patients with cancer in whom the infection has cleared either spontaneously or secondary to therapy is uncertain. When HCV RNA clearance is achieved either spontaneously or in response to antiviral therapy in recipients of solid organ transplants, no relapse is observed in plasma, liver or peripheral blood mononuclear cells during chronic immunosuppressive treatment with agents such as calcineurin inhibitors, corticosteroids, antimetabolites, anti-thymocyte globulins, or anti-interleukin-2-receptor blockers.[12] This finding suggests the complete and permanent cure of HCV infection resulting from the elimination of HCV before transplantation.

Immunosuppression and viral replication in HBV reactivation

In general, there are three periods of HBV reactivation in patients with signs of resolved infection (Fig. 1).

The initial stage of HBV reactivation caused by chemotherapy-induced immune suppression is characterized by enhanced viral replication, as reflected by increases in the serum levels of HBV DNA, hepatitis B e-antigen (HBeAg) and HBsAg, indicating that suppression of a normal immunological response to HBV leads to enhanced viral replication and widespread infection of hepatocytes.[13] In particular, in cases of positive anti-HBs antibody, reactivation of HBV typically starts with a decrease of anti-HBs antibody titers. This may be related to the use of biologic therapy, such as anti-CD20 monoclonal antibody rituximab and anti-CD52 antibody alemtuzumab, which cause profound and long-lasting immunosuppression; however, a decrease of anti-HBs antibody titers is seen in all cases, including those on biologic drug-free chemotherapy, namely, tumor necrosis factor-α inhibitors.

There are at least two mechanisms by which immunosuppressive agents may increase HBV replication and expression. As the host immune response to the virus plays a crucial role in controlling HBV infection,[14] suppression of such immune responses should increase viral replication. Meanwhile, immunosuppressive agents may have a more direct stimulatory effect on viral replication. In fact, corticosteroid increases HBV DNA and RNA production *in vitro* by stimulating HBV transcription, by binding to the glucocorticoid responsive element and augmenting the HBV enhancer I;[15,16] however, it is controversial whether corticosteroid increases the secretion of HBsAg and HBeAg.[15–17] Although combinations of immunosuppressive agents may cause an increase in levels of intracellular HBV DNA, lower concentrations of prednisolone were presumably unable to stimulate HBV replication, so the doses of these compounds should be kept as low as practically possible when used clinically.

In the second stage of reactivation, functionality of the immune system is restored after chemotherapy is discontinued. Infected hepatocytes with recognizable viral antigens on their surface may then be exposed and would be cleared by T lymphocytes, leading to hepatic injury and necrosis. Clinically, this can lead to hepatitis with an increase in alanine aminotransferase (ALT) levels, hepatic failure and even death. Concurrently, HBV DNA levels may decrease

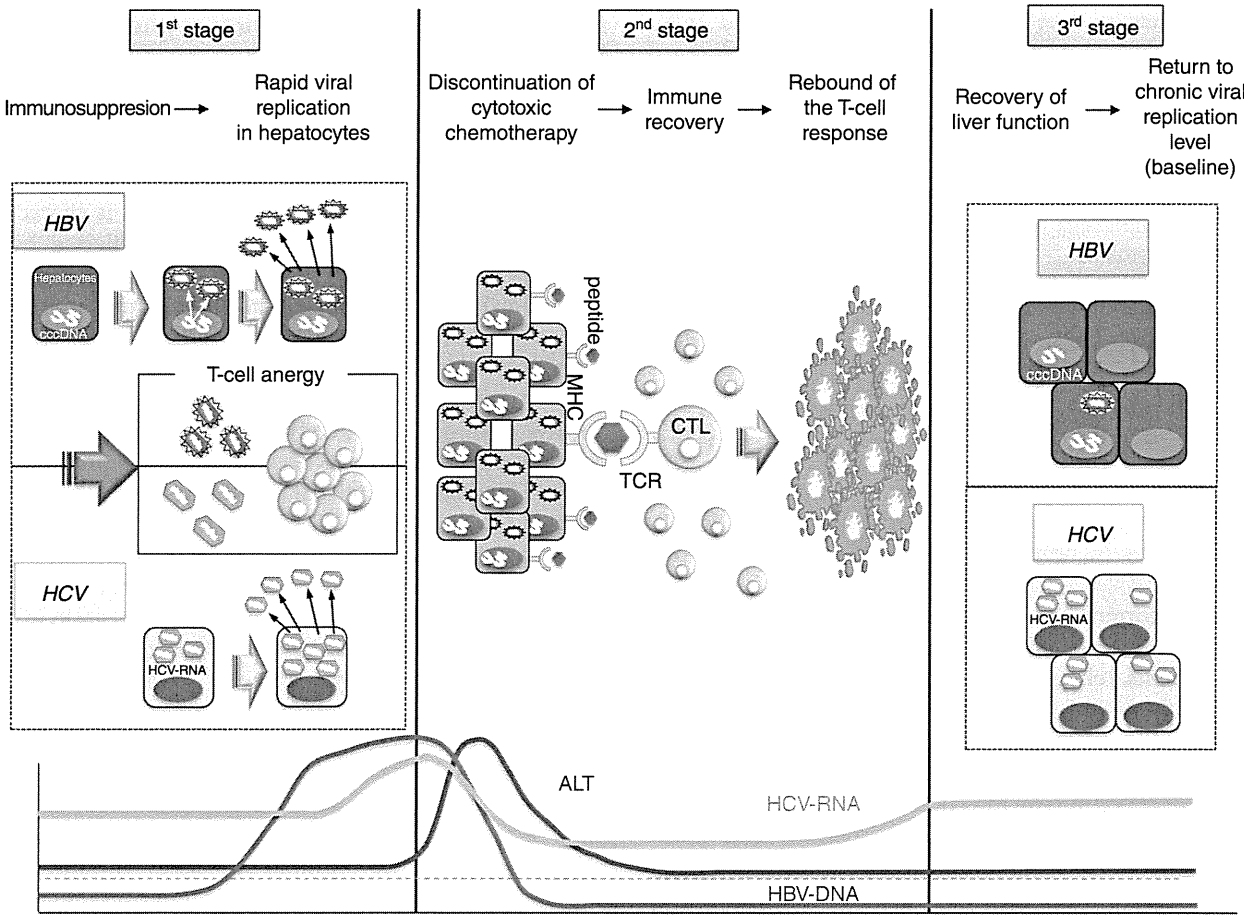

Figure 1 Pathophysiology of the reactivation of HBV and HCV infection. Reactivation of HBV or HCV as a result of chemotherapy can generally be divided into three stages. The first stage is characterized by enhanced viral replication, the second stage is the functional restoration of the immune system, and 3rd stage is the recovery stage. ALT, alanine aminotransferase; CTL, cytotoxic T lymphocyte; HBV, hepatitis B virus; HCV, hepatitis C virus; cccDNA, covalently closed circular DNA; MHC, major histocompatibility complex.

by improved cytopathic and non-cytopathic immune mechanisms.[18,19]

The third stage of reactivation is the recovery phase, during which clinical hepatitis resolves and HBV markers return to baseline levels.[20,21]

The retrospective and prospective studies of HBV reactivation in HBsAg negative patients with hematological malignancies were summarized in previous reviews.[22–24] As for the reason for considerable variation (1.0–23.3%) in the incidence of HBV reactivation in lymphoma patients with HBV resolved infection following rituximab-containing chemotherapy, there may be differences among institutions both in the study subjects (HBV serological status including baseline anti-HBs titer, steroid-containing chemotherapy, and salvage therapy including transplantation) and the assays used for HBV-related markers (cut-off values, sensitivity). Several guidelines for the management of HBV reactivation have been published by Asian, American and European societies (American Association for the Study of Liver Diseases, Asian Pacific Association for the Study of the Liver, and European Association for the Study of the Liver). In January 2009, the Japanese guideline was announced for HBV reactivation following immunosuppressive therapy and systemic chemotherapy.[25] Although the details of this guideline have been omitted from this review, in principle, antiviral prophylaxis is recommended for HBsAg

positive patients before treatment. For HBV resolved patients, monthly monitoring of HBV DNA levels is recommended during and for at least 1 year after the end of immunosuppressive therapy or chemotherapy. Preemptive antiviral therapy should be started as soon as possible if HBV DNA is detected during this monitoring; however, there is little evidence of HBV DNA monitoring to prevent hepatitis due to HBV reactivation in HBV resolved patients.

Reactivation of HCV infection

Although HCV reactivation is rare, hepatic toxicity related to chemotherapy is higher among patients with chronic HCV infection than in HCV uninfected patients,[26] suggesting that HCV reactivation occurred and can cause clinically relevant complications.

Hepatitis C virus-related liver dysfunction generally occurs 2–4 weeks after the cessation of chemotherapy.[27–30] A widely accepted hypothesis considering the pathogenesis indicates enhanced viral replication with a consequent increase in the number of infected hepatocytes following immunosuppressive treatment (Fig. 1). Withdrawal of immunosuppressive therapy leads to restoration of the host immune function, resulting in the rapid destruction of infected cells and hepatic injury.[27,31] Severe liver dysfunction was found to occur at a lower incidence in HCV positive patients than HBV positive patients.[5] The reason for this phenomenon is unknown; however, if severe hepatitis secondary to viral reactivation develops, mortality rates of HBV infected and HCV infected patients seem to be similar.[32–34]

CLINICAL EVIDENCE AND MANAGEMENT OF HCV REACTIVATION

Diagnosis for HCV reactivation

CHRONICALLY INFECTED PATIENTS have stable HCV RNA levels that may vary by approximately 0.5 $\log_{10}$ IU/mL;[35] therefore, an increase of the HCV viral load of more than 1 $\log_{10}$ IU/mL may be a sign of HCV reactivation. It was also reported that HCV reactivation showed an at least threefold increase in serum ALT in a patient in whom the tumor had not infiltrated the liver, who had not received hepatotoxic drugs and who had had no recent blood transfusions or other systemic infections besides HCV.[6,24] Changes in liver enzyme levels can be accompanied by the reappearance of HCV RNA or a sudden increase in the serum HCV RNA level.[6]

HCV reactivation after specific treatments

Patients with HCV infection who undergo HSCT or systematic chemotherapy including corticosteroids can experience severe hepatic dysfunction and fulminant hepatic failure (summarized in Table 2).

Corticosteroids have traditionally been associated with cases of HCV reactivation.[27,36] HCV reactivation has been associated with several immunosuppressive and chemotherapeutic agents, including rituximab, alemtuzumab, bleomycin, busulfan, cisplatin, cyclophosphamide, cyclosporin, cytarabine, dacarbazine, doxorubicin, etoposide, gemcitabine, methotrexate, vinblastine and vincristine;[27,37–44] however, many patients with HCV reactivation during treatment with one of these drugs were simultaneously treated with corticosteroids.[38,41,42,44,45] In a study by Zuckerman *et al.*,[46] 18 of 33 (54%) patients had mild to moderate increases of ALT, which occurred 2–3 weeks after the withdrawal of chemotherapy. HCV positive patients did not demonstrate a higher incidence of severe hepatic dysfunction during chemotherapy for malignancies than HCV negative patients; however, liver test abnormalities during therapy are very common and are seen in 54% HCV positive patients and in 36% HCV negative patients.

Whether corticosteroid therapy alone or in combination with other agents leads to reactivation of HCV infection and acute exacerbation of chronic HCV infection remains to be determined. A possible relationship between rituximab and HCV reactivation in patients with cancer has been reported.[41,44,45] Only the administration of rituximab-containing chemotherapy was associated with both acute exacerbation and reactivation of chronic HCV infection.[24]

Ennishi *et al.* also showed that the incidence of severe hepatic toxicity in HCV positive patients was significantly higher than in HCV negative patients, and HCV infection was determined to be a strong risk factor for this adverse effect in patients with diffuse large B-cell lymphoma (DLBCL) in the rituximab era.[44] These hepatic toxicities led to modification and discontinuation of immunochemotherapy, resulting in lymphoma progression. The study described that careful monitoring of hepatic function should be recommended for HCV positive patients, particularly those with high levels of pretreatment transaminase. More importantly, monitoring of HCV viral load demonstrated a marked enhancement of HCV replication, and it is suggested that increased HCV results in severe hepatic toxicity. Thus, HCV viral load should be

Table 2 Hepatic toxicity by HCV reactivation in HCV infected patients with hematological malignancies

Author	Year	Disease	Treatment	No. of cases with hepatic toxicity	Death from liver toxicity
Kanamori *et al.*	1992	AML	Allo-HSCT	2 patients	2
Maruta *et al.*	1994	AML, AA	Allo-HSCT	9 patients	2
Nakamura *et al.*	1996	Hematological malignancies	Various regimens	11 patients	5
Vento *et al.*	1996	B-cell NHL and HL	ABVD or CHOP- like regimen	2 patients	1
Luppi *et al.*	1998	B-cell NHL	Various regimens	20/35 patients	2
Zuckerman *et al.*	1998	Hematological malignancies	Various regimens	18/33 patients (55%)	0
Kawatani *et al.*	2001	Hematological malignancies	Various regimens	4/22 patients	1
Hamaguchi *et al.*	2002	Hematological malignancies	Allo-HSCT	40/58	9
Locasciulli *et al.*	2003	Hematological and solid malignancies	Allo-HSCT (21)/auto-HSCT (36)	21 6	2 1
Takai *et al.*	2005	Hematological malignancies	Various regimens	4/37 patients	0
Aksoy *et al.*	2006	DLBCL	Rituximab	0/1 patients	0
Besson *et al.*	2006	DLBCL	Various regimens	15/23 patients	3
Visco *et al.*	2006	DLBCL	CHOP-like, rituximab (35)	5/132 patients	1
Ennishi *et al.*	2008	DLBCL, MALT NHL	R-CHOP-like	1/5 patients	0
Hsieh *et al.*	2008	DLBCL	R-CNOP	1/1 patients	0
Ennishi *et al.*	2010	DLBCL	R-chemo	36/131 patients	6
Arcaini *et al.*	2010	NHL	R-chemo (28)	24/160 patients	3

AA, aplastic anemia; ABVD, doxorubicin hydrochloride (adriamycin), bleomycin, vinblastine and dacarbazine; Allo-HSCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; auto-HSCT, autologous hematopoietic stem cell transplantation; CHOP, cyclophosphamide, vincristine and prednisolone; DLBCL, diffuse large B-cell lymphoma; MALT, extranodal-marginal zone lymphoma of the mucosa-associated lymphoid tissue; NHL, non-Hodgkin's lymphoma; R-chemo, rituximab plus steroids combined chemotherapy; R-CHOP, rituximab, cyclophosphamide, vincristine and prednisolone; R-CNOP, rituximab, cyclophosphamide, mitoxantrone, vincristine and prednisolone.

carefully monitored in HCV positive patients who receive immunochemotherapy.

Severity of HCV reactivation versus HBV reactivation

The health consensus regarding HCV reactivation seems to be less severe than that of HBV reactivation (summarized in Table 3). Previous reports described that the incidence of post-chemotherapy liver injury in HBV carriers was significantly higher than that in HCV carriers,[5,31,32] namely, the incidence of post-chemotherapy liver injury in 25 HBV carriers (36%) was significantly higher than that in 37 HCV carriers (10.8%, $P = 0.026$),[31] and 44 (51.8%) of the 85 patients reported to have severe hepatitis along with hematological malignancies were HBV carriers, while only 11 (12.9%) were HCV carriers;[32] however, the mortality rates did not differ between HBV and HCV carriers (40.9% vs 45.5%) once severe hepatitis developed.

In a large Italian study of 57 HCV infected patients who underwent HSCT, patients undergoing autologous HSCT had a significantly lower risk of reactivation post-transplant than the allogeneic group (16% vs 100%, $P = 0.004$). In the allogeneic HSCT group, HCV reactivation occurred mainly within 6 months after HSCT, whereas in the autologous group, reactivation occurred within the first 3 months post-transplant. In this cohort, one HBsAg positive and three anti-HCV positive patients before HSCT died of liver failure. The risk of death from liver failure was not significantly different between HBsAg and anti-HCV positive patients, being 3% and 8% at 24 months, respectively ($P = 0.6$), or between recipients of autologous (5%) and allogeneic HSCT (7%) ($P = 0.34$).[33]

In a Japanese multicenter study of 135 patients with HBV or HCV infection who received allogeneic transplants, transient hepatitis was more common in HBV infected patients than in HCV infected patients, but the rates of fulminant hepatitis and death due to hepatic failure were similar in both groups.[34]

Table 3 Clinical state by HCV reactivation versus HBV reactivation

Severity or prognosis	Year	Survey period	No. of patients	Country	% of HBV	% of HCV	*P*	Comments	Reference
Liver injury	2005	1996–2002	601	Japan	36% (9/25)	10.8% (4/37)	0.026	Patients with hematological malignancies	31
Severe hepatitis	1996	1987–1991	Surveillance in 250 hospitals	Japan	51.8% (44/85)	12.9% (11/85)	ND	In 85 patients having severe hepatitis along with hematological malignancies	32
Death from liver failure	2003	1996–2000	90	Italy	3% (1/33)	8% (3/38*)	0.6	Patients with HBV or HCV receiving HSCT (during 24 months)	33
Hepatic failure	2002	1986–1998	135	Japan	10% (8/77)	12% (7/58)	ND	Patients with HBV or HCV receiving HSCT	34

HBV, hepatitis B virus; HCV, hepatitis C virus; HSCT, hematopoietic stem cell transplant; ND, not done.
*Fifty-seven were anti-HCV positive; of these, 38 were also tested for HCV RNA.

Outcome of HCV infected hematological patients

As previously highlighted, there is no significant short-term impact of HCV on the outcome after HSCT. Nevertheless, the long-term impact of chronic HCV infection can be deleterious in the liver, causing significant fibrosis progression, liver failure and increased risk of hepatocellular carcinoma (HCC). One study reported the rapid progression of hepatitis C in patients with humoral immunodeficiency disorders.[47] Another group has recently reported a more rapid rate of fibrosis progression after HSCT, with median time to cirrhosis of 18 years, as compared to 40 years seen in the control group. HCV disease progression ranked third, behind infections and GVHD, as a cause of late death after HSCT.[48] Long-term survivors after HSCT thus appear to be at higher risk for HCV-related complications and treatment of HCV becomes critical. A possible explanation for the genesis of cirrhosis could be an immune imbalance or impaired regulation of B and T cells.[47,48]

In various regimens for hematological malignancies, Ennishi *et al.* reported that hepatic disease progressed in four patients, and HCC was found to increase the risk of death from hepatic failure significantly in lymphoma patients receiving conventional chemotherapy, even during short-term observation.[44] Cox multivariate analysis showed that older age and advanced stage had significant adverse effects on overall survival (OS); however, HCV infection was not associated with poor progression-free survival (PFS) or OS. Besson *et al.* described that the overall proportion of subjects undergoing hepatic toxicity was 65% (15/23 patients). Outcome of HCV positive patients was poorer for OS ($P = 0.02$), but not for event-free survival ($P = 0.13$).[49] Visco *et al.* also described that only five of 132 patients (4%) had to discontinue chemotherapy due to severe liver function impairment.[50] Although previous papers mentioned that rituximab induced HCV reactivation after spontaneous remission in DLBCL,[45,51] the addition of rituximab did not seem to affect patients' tolerance to treatment. Five-year overall survival of the entire cohort was 72%, while 5-year PFS of the 132 patients treated with intent to cure was 51%. The prognosis of HCV infected patients with DLBCL is still controversial.

Recently, Arcaini *et al.*[43] studied 160 HCV positive patients with NHL (59 indolent NHL, 101 aggressive). Among 28 patients treated with rituximab-containing chemotherapy, five (18%) developed liver toxicity, and among 132 independent patients who received chemotherapy, only nine (7%) had hepatotoxicity, suggesting

that rituximab was related to a slightly higher occurrence of toxicity. Median PFS for patients who experienced liver toxicity was significantly shorter than median PFS of patients without toxicity (2 and 3.7 years, respectively, $P = 0.03$). HCV infected patients with NHL developed liver toxicity significantly, often leading to interruption of treatment.

Based on these findings, the impact of HCV infection on the outcome after HSCT or rituximab-containing chemotherapy seems to be deleterious for OS but not for event-free survival. Further studies are required in prospective multicenter cohorts.

Treatment of HCV infected patients with hematological malignancies

The long-term impact of chronic HCV infection can be deleterious to the liver, causing significant fibrosis progression, liver failure and increased risk of HCC. Interestingly, a more rapid rate of fibrosis progression was reported after HSCT.[48] Therapy for HCV infection in patients with hematological malignancy can be considered once a patient's immunity and bone marrow have recovered, immunosuppressive drugs have been stopped, and there is no evidence of GVHD, because the hematological adverse effects of anti-HCV drugs can exacerbate the toxicity of chemotherapy, which can involve complications such as severe cytopenias and potentially life-threatening infections.[52] Overall, antiviral therapy for HCV in patients (e.g. HIV, transplant) is often associated with poor response rates, even though patients with chronic HCV infection were treated with the combination of pegylated interferon-α and ribavirin.[53–55] The use of direct-acting antiviral drugs (such as recently approved inhibitors of nonstructural protein 3/4A [NS3/4A] protease [boceprevir or telaprevir], or NS5B polymerase inhibitors) has not been evaluated in patients with cancer. Boceprevir and telaprevir can inhibit hepatic drug-metabolizing enzymes such as cytochrome P450 (CYP)2C, CYP3A4 or CYP1A;[56] therefore, these agents potentially interact with various drugs that are co-administrated in patients with cancer. These new antiviral drugs should be used with caution in patients with cancer.

Large-scale studies are needed to better define which patients with cancer are most likely to benefit from simultaneous antiviral therapy and cytotoxic chemotherapy. Notably, antiviral treatment with pegylated interferon-α and ribavirin should not be used early in the post-transplant period (<2 years after transplantation) in patients who have undergone allogeneic HSCT as interferon-α therapy may precipitate or induce the development of GVDH.[57]

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ORIGINAL ARTICLE

Interleukin-1β enhances the production of soluble MICA in human hepatocellular carcinoma

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Abstract The production of soluble major histocompatibility complex class I–related chain A (MICA) is thought to antagonize NKG2D-mediated immunosurveillance. Interleukin-1β (IL-1β) is elevated in patients with chronic hepatitis C (CH), and this might contribute to the escape of hepatocellular carcinoma (HCC) cells from innate immunity. In this study, we investigated the immunoregulatory role of IL-1β in the production of soluble MICA of HCC cells. First, we investigated the correlation between the serum IL-1β levels and soluble MICA in CH patients. Serum IL-1β levels were associated with soluble MICA levels in CH patients. The serum IL-1β levels of CH patients with the HCC occurrence were significantly higher than those of CH patients without HCC. We next examined the MICA production of IL-1β-treated HCC cells. Addition of IL-1β resulted in significant increase in the production of soluble MICA in HepG2 and PLC/PRF/5 cells, human HCC cells. But soluble MICA was not detected in both non-treated and IL-1β-treated normal hepatocytes. Addition of IL-1β did not increase the expressions of membrane-bound MICA on HCC cells. These were observed similarly in various cancer cells including a gastric cancer (MKN1), two colon cancers (HCT116 and HT29) and a cervical cancer (HeLa). Addition of IL-1β also increased the expression of a disintegrin and metalloproteinase (ADAM)9 in HCC cells, and the knockdown of ADAM9 in IL-1β-treated HCC cells resulted in the decrease in the production of soluble MICA of HCC cells. These findings indicate that IL-1β might enhance the production of soluble MICA by activating ADAM9 in human HCC.

Keywords IL-1β · Hepatocellular carcinoma · Soluble MICA · ADAM9

Abbreviations

IL	Interleukin
HCC	Hepatocellular carcinoma
MICA	Major histocompatibility complex class I–related chain A
ADAM9	A disintegrin and metalloproteinase 9

Introduction

Interleukin-1β (IL-1β) is a proinflammatory cytokine with multiple biological effects [1]. Serum levels of IL-1β are elevated in patients infected with hepatitis C virus (HCV), suggesting the role of IL-1β in the inflammation of liver [2–4]. Several polymorphisms of the IL-1 gene have been reported to affect IL-1β production [5, 6]. A number of clinical studies suggested that polymorphisms of IL-1β gene are associated with diverse disease including cancer [5, 7]. IL-1β gene polymorphisms have also been reported to be associated with HCC in HCV- or HBV-infected patients [8–10]. While genetic studies have suggested an important role for IL-1β in cancer, direct evidence that IL-1β contributes to the pathogenesis of cancer has been lacking. Recently,

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Tu et al. [11] reported that stomach-specific expression of IL-1β in transgenic mice leads to spontaneous gastric inflammation and cancer that correlates with myeloid-derived suppressor cells to the stomach. However, no studies have been published on the direct effect of IL-1β on the HCC cells in patients infected with HCV.

MHC class I–related chain A (MICA), a ligand for NKG2D, is rarely expressed on normal cells, but frequently on tumor cells [12, 13]. The engagement of MICA and NKG2D strongly activates NK cells enhancing their cytolytic activity and cytokine production [14]. Thus, the MICA-NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells [15]. In addition to those membrane-bound forms, MICA molecules are cleaved proteolytically from tumor cells and appear as soluble forms in the sera of patients with malignancy including HCC [16–18]. The release of soluble MICA/B from tumor cells is thought to antagonize NKG2D-mediated immunosurveillance. We previously demonstrated that a disintegrin and metalloproteinase (ADAM)9 protease plays essential roles in the shedding of MICA molecules on HCC cells [19]. However, the mechanism of regulating the production of soluble MICA in HCC cells remains to be elucidated.

In this study, we investigated the immunoregulatory role of IL-1β in the production of soluble MICA from HCC cells. Of importance is the discovery that the serum IL-1β levels in chronic hepatitis patients with the HCC occurrence were significantly higher than those without HCC occurrence and that IL-1β enhances the production of soluble MICA via activating ADAM9 in human HCC cells. The present study sheds light on previously unrecognized immunological effects of IL-1β on HCC cells.

Materials and methods

HCC cell lines and normal hepatocyte cultures

HepG2 and PLC/PRF/5, human HCC cell lines, were purchased from American Type Culture Collection (Rockville, MD) and were cultured with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO_2 and 37°C. 2×10^5 HepG2 and PLC/PRF/5 cells or normal hepatocytes (ScienCell Research Laboratories, Carlsbad, CA) were cultured in 6-well tissue culture plates for 48 h in the presence or absence of human interleukin-1β (IL-1β) (50 ng/ml, PeproTech EC, London, UK), and the HCC cells were harvested and subjected to evaluating the expression of membrane-bound MICA and ADAM9 and the production of soluble MICA.

Flow cytometry

For the detection of membrane-bound MICA, HCC cells were incubated with anti-MICA-specific Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and stained with Goat $F(ab')2$ fragment anti-mouse IgG($H + L$) − PE (Beckman Coulter, Fullerton, CA) as a secondary reagent. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton–Dickinson, San Jose, CA).

Western blotting

The total cellular protein was electrophoretically separated by sodium dodecyl sulfate-12% polyacrylamide gels and transferred onto PVDF membrane. The membrane was blocked in Tris-buffered saline–Tween containing 5% skim milk for 1 h and then probed with anti-ADAM9 mAb (R&D Systems, Minneapolis, MN) at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit Ab and SuperSignal West Pico System (Pierce, Rockford, IL) were used for the detection of blots.

Real-time reverse transcription (RT) PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) and was reverse transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster city, CA). The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Ready-to-use assays (Applied Biosystems) were used for the quantification of ADAM9 (Hs00177638_m1), MICA (Hs00792195_m1) and β-actin (Hs99999903_m1) mRNAs according to the manufacturer's instructions. β-Actin mRNA from each sample was quantified as an endogenous control of internal RNA.

RNA silencing

The small interfering RNA (siRNA) method was used to knockdown ADAM9 as previously described [19]. At 24-h post-transfection, the cells were analyzed for specific depletion of the protein of ADAM9 by western blotting. The following siRNA were used: ADAM9, 5′-UGUCCAAAC ACAUUAAUCCCGCCUG-3′; an irrelevant siRNA as a control, 5′-UGUCGCACAAACACUUAACUCCCUG-3′.

ELISA

The sera from chronic hepatitis C patients ($N = 24$) with or without the occurrence of HCC were subjected to analysis of IL-1β and soluble MICA. Informed consent, under an Institutional Review Board–approved protocol, was obtained from all patients before sample acquisition.

The sera and the supernatants of cultured HCC cells were harvested, and the levels of IL-1β and soluble MICA were determined by human IL-1β ELISA set II (BD Biosciences, San Diego, CA) and DuoSet MICA eELISA kit (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions, respectively.

NK cell analysis

NK cells were isolated from human peripheral blood mononuclear cells by magnetic cell sorting using CD56 MicroBeads (Miltenyi Biotech, Auburn, CA) as previously described [19]. HepG2 and PLC/PRF/5 cells were treated with IL-1β (50 ng/ml) for 48 h. The cytolytic ability of NK cells against IL-1β-treated or non-treated HepG2 and PLC/PRF/5 cells was assessed by 4-hr ^{51}Cr-releasing assay as previously described [19].

Statistics

All values were expressed as the mean and SD. The statistical significance of differences between the groups was determined by applying Student's t test or two-sample t test with Welch correction after each group had been tested with equal variance and Fisher's exact probability test. We defined statistical significance as $p < 0.05$.

Results

Serum IL-1β levels were associated with soluble MICA in chronic liver disease patients

We first examined the IL-1β levels and soluble MICA levels of twenty-four chronic hepatitis C (CH) patients. Serum IL-1β levels in CH patients correlated with soluble MICA levels (Fig. 1a). We next examined the serum IL-1β levels of CH patients with or without the occurrence of HCC. We examined serum IL-1β levels of these 24 CH patients before HCC occurrence and followed these patients for 5 years. CH patients could be divided into two groups according to the occurrence of HCC (Table 1). As shown in Fig. 1b, the serum IL-1β levels of patients with the occurrence of HCC ($n = 11$) were significantly higher than those of patients without the occurrence of HCC ($n = 13$). These results suggested that the elevation of serum IL-1β levels might be associated with the occurrence of HCC in CH patients.

IL-1β increases the production of soluble MICA from HCC cells, but not from normal hepatocytes

We examined whether IL-1β treatment could induce MICA expressions on HCC cells (PLC/PRF/5 cells and HepG2 cells). Both PLC/PRF/5 cells and HepG2 cells were cultured for 48 h with IL-1β (50 ng/ml) and then subjected to analysis of the expression of membrane-bound MICA and mRNA of MICA. The expression of membrane-bound MICA of IL-1β-treated HCC cells was similar to that of non-treated HCC cells (Fig. 2a). IL-1β treatment induced significant increase of mRNA of MICA in PLC/PRF/5 cells, but this did not in HepG2 cells (Fig. 2b). We next examined the production of soluble MICA in the supernatants of the IL-1β-treated HCC cells. IL-1β treatment

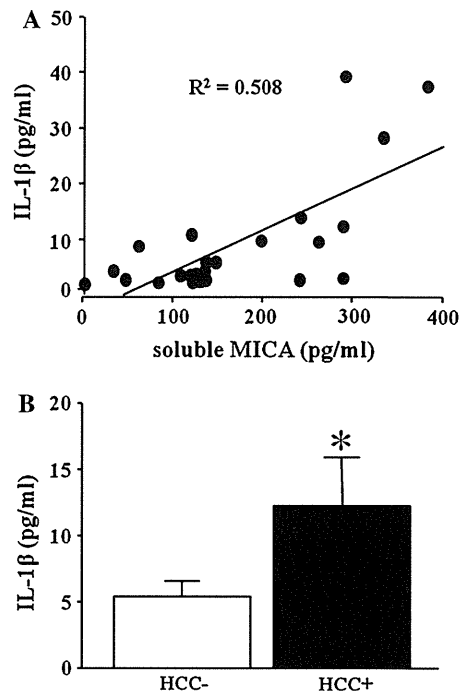

Fig. 1 The correlation between serum IL-1β and soluble MICA in patients with chronic liver disease and serum IL-1β levels in chronic liver disease patients with or without the HCC occurrence. **a** Correlation between serum IL-1β levels and soluble MICA levels in patients with chronic liver disease ($N = 24$). The serum IL-1β and soluble MICA were evaluated by specific ELISA, respectively. **b** Serum IL-1β levels in chronic hepatitis patients with HCC occurrence (HCC+, $N = 11$) or without HCC occurrence (HCC−, $N = 13$) were evaluated by specific ELISA. All patients were HCV-RNA-positive. *$p < 0.05$

Table 1 Clinical backgrounds

	HCC(+)	HCC(−)
Number	11	13
Age	61 ± 6	61 ± 8
Gender (M/F)	8/3	11/2
Platelet ($\times 10^4/\mu l$)	15 ± 5	14 ± 3
ALT (IU/l)	122 ± 109	89 ± 44

HCC(+) chronic hepatitis C patients with the occurrence of HCC, *HCC*(−) chronic hepatitis C patients without the occurrence of HCC, *M* male, *F* female, *ALT* alanine aminotransferase

resulted in the significant increase in the production of soluble MICA in both PLC/PRF/5 and HepG2 cells (Fig. 2c). These results demonstrated that the addition of IL-1β did not change the expression of membrane-bound MICA but resulted in significant increase in the production of soluble MICA in HCC cells. We also examined the effect of IL-1β on normal hepatocytes. As shown in Fig. 2d, normal hepatocytes did not produce soluble MICA and the addition of IL-1β did not result in its production.

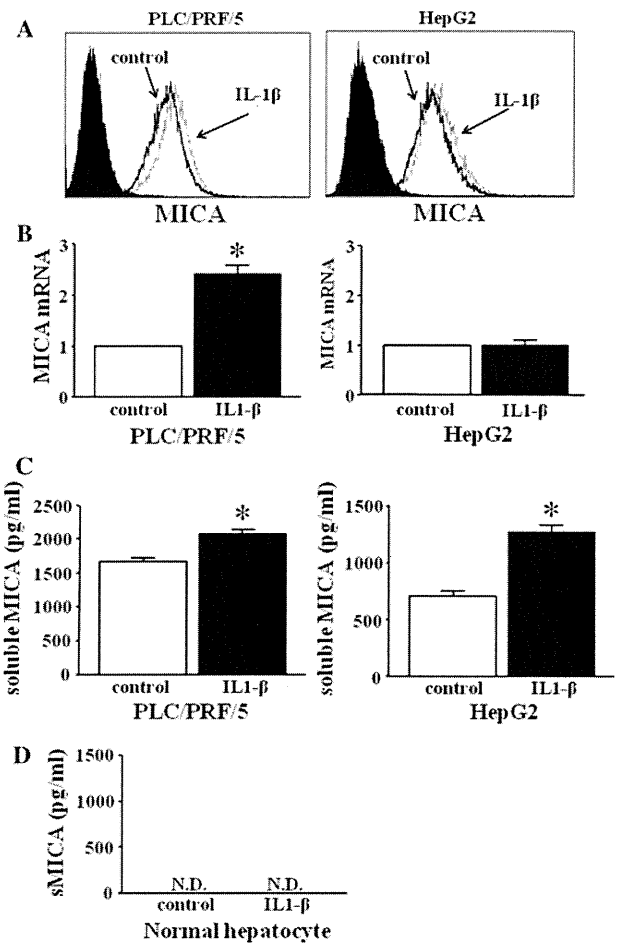

Fig. 2 Expression of membrane-bound MICA and the production of soluble MICA in IL-1β-treated HCC cells and normal hepatocytes. Both PLC/PRF/5 cells and HepG2 cells were treated in the presence or absence of IL-1β (50 ng/ml) for 48 h. The expression of membrane-bound MICA (**a**) and mRNA expression of MICA (**b**) in IL-1β-treated or non-treated HCC cells were evaluated by flow cytometry or real-time RT-PCR, respectively. *Black line histograms*, MICA staining of non-treated cells; *dotted line histograms*, MICA staining of IL-1β-treated cells; *shaded/black histograms*, control IgG isotype Ab staining. Similar results were obtained from two independent experiments. *$p < 0.05$. **c** We examined the production of soluble MICA on IL-1β-treated or non-treated HCC cells by specific ELISA. *$p < 0.05$. **d** Normal hepatocytes were treated in the presence or absence of IL-1β (50 ng/ml) for 48 h. The production of soluble MICA on IL-1β-treated or non-treated normal hepatocytes was examined by specific ELISA. *ND* not detected

These results demonstrated that IL-1β could induce the increase in the production of soluble MICA only from HCC cells, but not from normal hepatocytes.

IL-1β treatment increases the production of soluble MICA from various cancer cells

We also examined IL-1β-dependent MICA regulation on another cancer cells including a gastric cancer cell line (MKN1), colon cancer cell lines (HCT116, HT29) and a cervical cancer cell line (HeLa). The expressions of membrane-bound MICA on these cells did not change by the addition of IL-1β in all cancer cells. Interestingly, the addition of IL-1β resulted in significant increase in the production of soluble MICA in all cancer cells (Fig. 3).

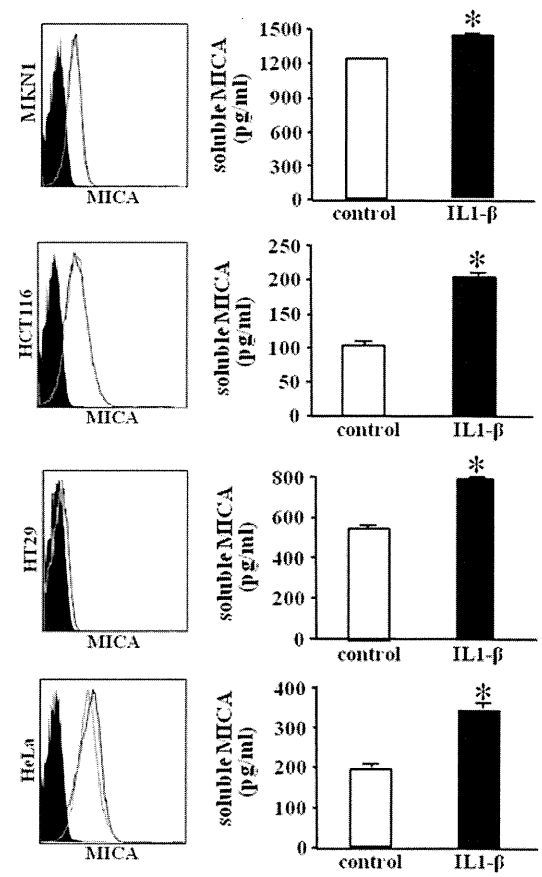

Fig. 3 Soluble MICA production of IL-1β-treated various cancer cells. Various cancer cells including MKN1, HCT116, HT29 and HeLa cells were treated in the presence or absence of IL-1β (50 ng/ml) for 48 h. Soluble MICA production of IL-1β-treated or non-treated various cancer cells was evaluated by specific ELISA (*right panel*). sMICA, soluble MICA. We also examined the expression of membrane-bound MICA on IL-1β-treated or non-treated various cancer cells by flow cytometry (*left panel*). *Black line histograms*, MICA staining of non-treated cells; *Gray line histograms*, MICA staining of IL-1β-treated cells; *shaded/black histograms*, control IgG isotype Ab staining. Similar results were obtained from two independent experiments. *$p < 0.05$

These results demonstrated that IL-1β could induce the increase in the production of soluble MICA not only from HCC cells but also from various cancer cells.

ADAM9 activated by IL-1β plays important roles in the production of soluble MICA from HCC cells

We next examined the mRNA of MICA and the production of soluble MICA in HCC cells treated with various doses of IL-1β. As shown in Fig. 4a, mRNA expression of MICA in IL-1β-treated PLC/PRF/5 cells significantly increased but that in HepG2 cells did not. The production of soluble MICA in IL-1β-treated PLC/PRF/5 cells significantly increased in a dose-dependent manner, and the production of soluble MICA significantly increased in 50 ng/ml IL-1β-treated HepG2 cells. Recently, members of the metzincin superfamily, such as ADAM proteins, have been reported to play essential roles in the proteolytic release of the ectodomain of transmembranous proteins, including MICA, from the cell surface [17, 20]. We previously reported that ADAM9 plays essential roles in MICA shedding in human HCC cells and that the activation of ADAM9 protease resulted in up-regulation of the production of soluble MICA from human HCC cells [19]. So we examined the involvement of ADAM9 in the up-regulation of soluble MICA production in IL-1β-treated HCC cells. As shown in Fig. 4b, mRNA levels of ADAM9 in IL-1β-treated PLC/PRF/5 cells significantly increased in a dose-dependent manner. mRNA of ADAM9 in IL-1β-treated HepG2 cells significantly increased in 10 ng/ml and 50 ng/ml IL-1β-treated HepG2 cells. The ADAM9 protein expression including both pro-form and active form also increased in IL-1β-treated HCC cells (Fig. 4c). To confirm the involvement of ADAM9 in IL-1β-treated HCC cells, we examined the soluble MICA production in IL-1β-treated ADAM9-knockdown (ADAM9KD) HCC cells. Both PLC/PRF/5 and HepG2 cells were transfected with ADAM9-siRNA or an irrelevant siRNA as a control. The expression of ADAM9 was clearly suppressed in PLC/PRF/5 cells and HepG2 cells at protein levels (Fig. 5a). In both PLC/PRF/5 and HepG2 cells transfected with control siRNA, the productions of soluble MICA in IL-1β-treated cells were significantly higher than those in non-treated HCC cells. In contrast, the production of soluble MICA in IL-1β-treated ADAM9KD-HepG2 cells was similar to that in non-treated ADAM9KD-HepG2 cells (Fig. 5b). The production of soluble MICA in IL-1β-treated ADAM9KD-PLC/PRF/5 cells also tended to decrease compared with that in non-treated ADAM9KD-PLC/PRF/5 cells (Fig. 5b). The decrease in soluble MICA production in ADAM9KD cells was different between PLC/PRF/5 cells and HepG2 cells. However, these results suggested at least that the increase in ADAM9 expression by IL-1β resulted in the increase in soluble MICA levels in IL-1β-treated HCC cells.

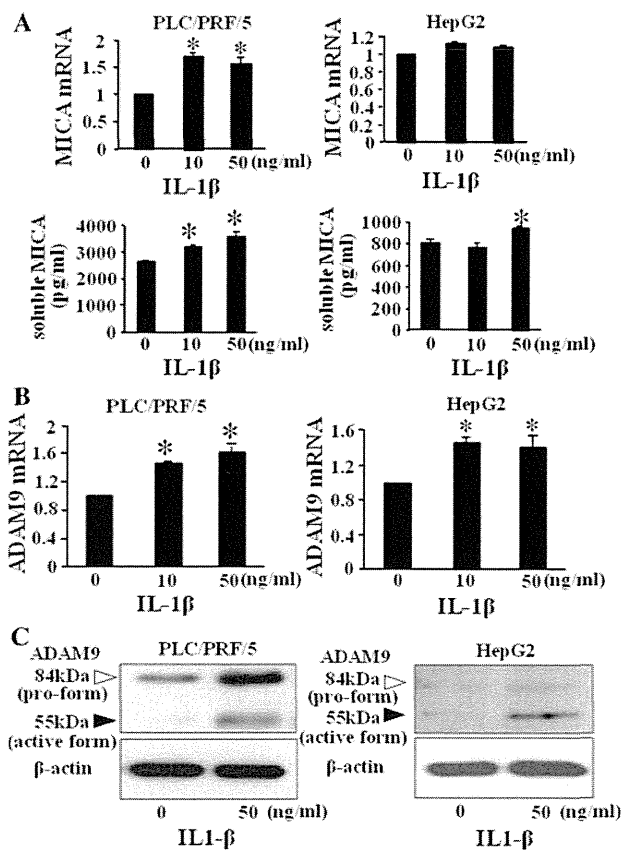

Fig. 4 IL-1β increased the ADAM9 expression of HCC cells and the production of soluble MICA. PLC/PRF/5 and HepG2 cells were cultured with 0, 10 and 50 ng/ml IL-1β for 48 h. **a** The production of soluble MICA from IL-1β-treated HCC cells was examined by specific ELISA, and mRNA levels of MICA of IL-1β-treated HCC cells were examined by real-time PCR. **b**, **c** mRNA and protein expression of ADAM9 by real-time RT-PCR (**b**) and western blotting (**c**), respectively. Representative results are shown. Similar results were obtained from 3 independent experiments. *$p < 0.05$

IL-1β-treated HCC cells are resistant to the cytolytic activity of NK cells

We next examined whether IL-1β could modify the NK sensitivity of human HCC cells. The cytolytic activities of NK cells against IL-1β-treated PLC/PRF/5 and IL-1β-treated HepG2 cells were lower than those against non-treated HCC cells (Fig. 5c). These results demonstrated that IL-1β treatment resulted in the increased resistance of HCC cells to NK cells.

Discussion

The liver contains a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells

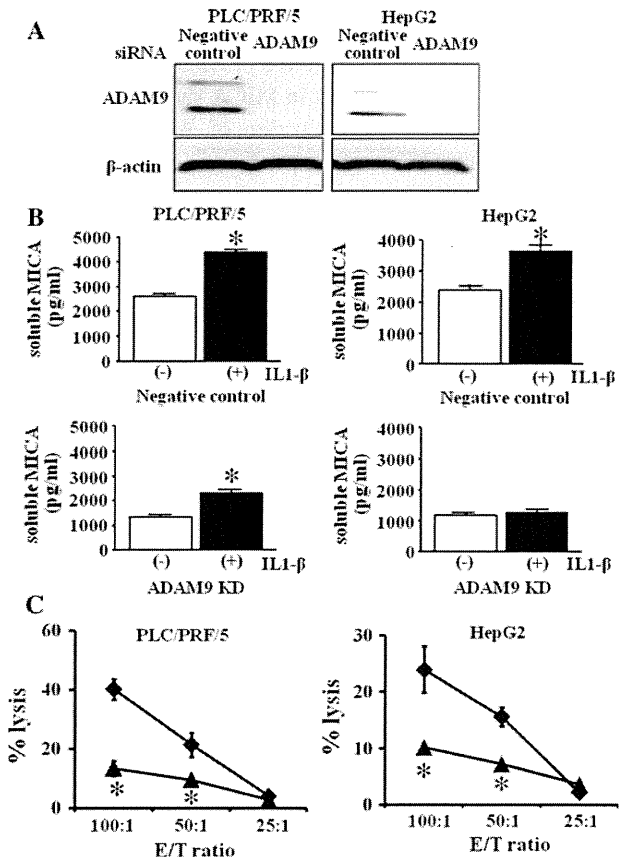

Fig. 5 The production of soluble MICA of ADAM9KD-HCC cells and the cytolytic activity against IL-1β-treated HCC cells. **a** Both HCC cells (PLC/PRF/5 and HepG2) were transfected with ADAM9 siRNA (ADAM9KD) or an irrelevant siRNA (negative control), and at 24-h post-transfection, the protein expression of ADAM9 was examined by western blotting. **b** Both HCC cells were cultured with (+) or without (−) 50 ng/ml IL-1β for 48 h. Soluble MICA production from ADAM9KD-HCC cells or negative control-HCC cells was evaluated by specific ELISA. $*p < 0.05$. Similar results were obtained from 3 independent experiments. **c** Both PLC/PRF/5 and HepG2 cells were cultured with or without IL-1β (50 ng/ml) for 48 h. The cytolytic activities of NK cells against IL-1β-treated or non-treated PLC/PRF/5 and HepG2 cells were evaluated by ^{51}Cr-releasing assay. Non-treated cells (*filled diamond*), IL-1β-treated cells (*filled triangle*). Representative results are shown. Similar results were obtained from three independent experiments. $*p < 0.05$ versus the cytolytic activity of non-treated cells. Similar results were obtained from 3 independent experiments

(T cells) [21, 22]. Recent study has demonstrated that innate immune system via NKG2D signal, expressing on NK cells, might play critical roles in tumor surveillance [23]. However, the escape mechanism of HCC cells from NK cells remains unclear. We previously demonstrated that membrane-bound MICA, activating molecule of NK cells, on HCC cells plays essential roles in the NK sensitivity of HCC cells [13, 24] and that the serum soluble MICA increase along the progression of chronic liver disease [18]. The production of soluble MICA in HCC patients is the highest compared with chronic hepatitis or liver cirrhosis patients without HCC [18]. These results suggest that unknown factors may accelerate the cleavage of MICA in HCC cells. IL-1β is produced mainly by local immune cells including activated Kupffer cells [25]. Because IL-1β increased in CH or LC patients [26–28], we focus on the possible role of IL-1β in the escape mechanism of HCC cells from NK cells.

Inflammatory cytokines including IL-1β and IL-6 increased in CH or LC patients [26–28], suggesting that both IL-1β and IL-6 might play roles in the HCC development. Recently high serum IL-6 level was an independent risk factor for HCC development in both chronic hepatitis C and B patients [29, 30], which suggested the possible roles of IL-6 in HCC development. However, the IL-1β levels in chronic liver disease, premalignant conditions, have been little reported. In this study, we demonstrated that serum IL-1β levels in chronic hepatitis C patients with HCC occurrence were significantly higher than those without HCC occurrence and that serum IL-1β levels correlated with soluble MICA which could inhibit NK activity. These results suggested that elevated IL-1β in CH patients might support the survival of HCC cells by changing local immunological environment.

MICA shedding is thought to be the principle mechanism by which tumor cells escape from NKG2D-mediated immunosurveillance [16]. In this study, we demonstrated that addition of IL-1β resulted in the increase in soluble MICA production from HCC cells. Interestingly, IL-1β treatment also resulted in the increase of soluble MICA in various cancer cells. Addition of other IL-1 family cytokines such as IL-1α, Il-18 and IL-33 did not result in the increase in soluble MICA production from both PLC/PRF/5 and HepG2 cells (Kohga, unpublished data). In addition to IL-1β, serum IL-6 and TNF-α are elevated in HCC patients. We compared IL-1β with IL-6 and TNF-α in the ability of the production of soluble MICA from HCC cells. IL-1β could increase the production of soluble MICA from HCC cells, but both IL-6 and TNF-α could not in PLC/PRF/5 cells and HepG2 cells. No synergistic effects of the combination of IL-1β, IL-6 and TNF-α were observed (Kohga, unpublished data). These results demonstrated that only IL-1β could induce the increase in the production of soluble MICA from HCC cells, suggesting that IL-1β might play an important role in the progression of HCC.

IL-1β treatment resulted in the increase in soluble MICA production but not the increase of mRNA in HepG2 cells. The production of soluble MICA depended on both the production of mRNA and the shedding of ADAM9. We previously demonstrated that ADAM9 plays an essential role in the shedding of MICA in HCC cells [19]. In the present study, we demonstrated that IL-1β treatment resulted in the increase in ADAM9 expression in HepG2

cells and that ADAM9 knockdown by siRNA resulted in the decrease in the production of soluble MICA from IL-1β-treated HepG2 cells. Our results suggested at least that the increase in ADAM9 might resulted in the increase in the shedding of soluble MICA in the IL-1β-treated HCC cells.

Recent studies have identified various metalloproteinases responsible for MICA/B cleavage in various cancers [31]. We previously found that ADAM9 plays critical roles in the shedding of MICA in human HCC. ADAM9 was directly associated with decreasing the expression of membrane-bound MICA and increasing the production of soluble MICA in human HCC [19]. Thus, it would be interesting to examine the activity of ADAM9 in IL-1β-treated HCC cells to understand how IL-1β regulates the production of soluble MICA from HCC cells. We demonstrated that IL-1β treatment could increase the mRNA and protein expression of ADAM9 in HCC cells and that ADAM9 knockdown in HCC cells resulted in decreasing of the soluble MICA production. These results suggested that ADAM9 played an important role in the increase in soluble MICA production from IL-1β-treated HCC cells. Both ADAMs and ADAMs with thrombospondin motifs (ADAMTS) are proteinases closely related to matrix metalloproteinases (MMPs). Structure of ADAMs and ADAMTS is highly conserved and involves metalloproteinase and disintegrin domains endowing them with features of both proteinases and adhesion molecules [32]. Several ADAMTSs including ADAMTS1 and ADAMTS9 were activated by IL-1β via NFATc1 transcription factor in chondrosarcoma [33, 34]. Although IL-1β may regulate such transcription factors in HCC cells, the detail mechanism of the activation of ADAM9 by IL-1β remains unclear. The concentration of IL-1β in our in vitro study was high compared with the serum IL-1β concentration level. However, the local IL-1β concentration in the liver tissues still remains unknown and may differ from the serum IL-1β concentration. Our in vitro study at least demonstrated that IL-1β could enhance the production of soluble MICA via up-regulating the expressions of ADAM9 in HCC cells, which might support the possible role of IL-1β in the survival of HCC cells.

Cai et al. [35] demonstrated that the numbers of CD56+ NK cells reduced in HCC tissues compared with healthy donors and CD56+ NK cells in HCC patients displayed impairments in cytotoxicity and IFN-γ production. This suggests that immunological microenvironment in liver tissues of CH patients might be favorable for the survival of HCC cells. We demonstrated that serum IL-1β levels correlated with soluble MICA in CH patients, which is consistent with our in vitro data. This suggests that the chronic elevation of IL-1β in CH patients might impair the function of NK cells by accelerating the production of soluble MICA. We also demonstrated that IL-1β treatment resulted in the inhibition of the cytolytic activity of NK cells against HCC cells. Intrahepatic activated macrophages and plasma cells could produce IL-1β inducing the inflammatory process in chronic liver disease [36]. If we could control the production of IL-1β with new reagents, it might be possible to develop a new therapeutic strategy against HCC. IL-1β receptor antagonist (IL-1RA) has been reported to apply clinically to the treatment of rheumatoid arthritis [37]. We believe the future clinical application of IL-1RA in HCC treatment as a new agent.

In spite of recent progress in the understanding of HCC, there remains to be unknown mechanism of the escape of HCC cells from innate immunity. We have shown here that ADAM9 was directly associated with increasing the production of soluble MICA in IL-1β-treated human HCC. These findings might indicate that IL-1β contributes to the survival of HCC cells by inhibiting innate immunity.

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