Hepatitis C Virus Disease

Emilio Jirillo
Editor

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Immunobiology and Clinical Applications

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Preface

Hepatits C virus (HCV) infection represents a worldwide disseminated disease, but despite numerous studies, its pathogenesis and medical treatment have not been completely elucidated.

As far as HCV pathogenesis is concerned, HCV genotypes and viral antigens have been investigated with the aim to find a correlation with disease severity and response to treatment. On the other hand, according to current literature, immunological response is implicated in disease progression rather than in host protection. Finally, use of interferon (IFN)-alpha alone or in combination with other antiviral drugs, e.g., ribavirin (RIB), at the moment represents the most effective treatment in chronic HCV disease, even if the percentage of cured patients is still low.

On these grounds, the present book, entitled *Hepatis C Virus Disease: Immunobiology and Clinical Applications*, will emphasize the most recent advances in HCV infection, moving from basic research to clinical application. In particular, in the first chapters of this volume, the full spectrum of immune responses to HCV is analyzed, taking into account either innate or adoptive immunity involvement. In this respect, the role of antigen-presenting cells (macrophages and dendritic celsl) and Toll-like receptors and that of T helper, T cytotoxic, natural killer, and T regulatory cells will be discussed in the course of HCV disease.

At the same time, deficits of innate immunity at the peripheral level with an easier access of microbes into the host will be described also in view of a putative interference of microbial products with IFN trreatment.

In the last part of this volume, a series of contributions elucidates the state of the art of IFN-alpha treatment in HCV patients and the effectiveness of therapy also in relation to HCV genotypes. Besides the combined treatment with IFN-alpha and RIB, the use and applications of pegylated IFNs aree the object of intensive speculation in specific chapters. Finally, the complicated HCV disease and its treatment are discussed.

In summary, this volume, written by various scientists with specific expertise in the field of HCV infection, should represent an efficacious up-to-date on the state of the art of HCV disease in diifferent geographical areas. Moreover, a clear description of disease pathogenesis, a detailed clarification of immune mechanisms, and a deep elucidation of the pharmacology of antiviral drugs should be very useful for

a large readership, even including medical students who may wish to learn basic principles of HCV infection.

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Innate Immunity in Type C Hepatitis

Tetsuo Takehara and Norio Hayashi

Hepatitis C Virus and Hepatocellular Carcinoma

As early as the days of Hippocrates, hepatitis has been described as a disease that occurs in the young and shows the cardinal symptom of jaundice, which sometimes develops into a critical condition. Ironically, research on hepatitis progressed rapidly during World War II because injuries and the terrible sanitary conditions of the battlefields caused serious hepatitis epidemics. People recognized that hepatitis could be classified into two types: infectious and serumal. The former became known as hepatitis A and the latter as hepatitis B. After the war, the hunt for hepatitis viruses had begun. First, the hepatitis B virus (HBV) was identified in 1967 by Blumberg, who was awarded a Nobel Prize in recognition of his discovery. Next, the hepatitis A virus (HAV) was discovered in 1973. These discoveries were thought to have clarified the causes of hepatitis, but by the following year, it was acknowledged that many cases of hepatitis were not caused by either HAV or HBV (Prince et al., 1974). Later, the hepatitis D virus (HDV) and the hepatitis E virus (HEV) were discovered in 1977 and 1983, respectively, but they were not the cause of hepatitis non-A, non-B, which is associated with blood transfusion. In 1989, HCV was identified by a molecular biological method where researchers induced the expression of cDNAs obtained from the blood plasma of a chimpanzee with hepatitis non-A, non-B and screened them with convalescent serum (Choo et al., 1989). HCV was the first virus to be discovered not by the previously used virological methods, but by a molecular biological method.

The discovery of HCV had a great impact on the treatment and prevention of liver diseases (Hayashi & Takehara, 2006). It turned out that not only did most patients who had been diagnosed as hepatitis non-A, non-B actually have hepatitis C, but also that there were quite a few hepatitis C patients among those who had been thought to have alcoholic liver disease or autoimmune hepatitis. As the natural history of hepatitis C was clarified, it became clear that the disease is a major risk factor for hepatocellular carcinoma (HCC) (Figure 1). The infection route of HCV is via the blood. Some patients who are exposed to the virus develop overt liver disease, but most of them remain in a latent state. Within six months of being infected, 30% of the patients expel the virus naturally while the remaining 70% enter a phase of persistent infection. Once patients enter in this latter phase, it is very rare for the

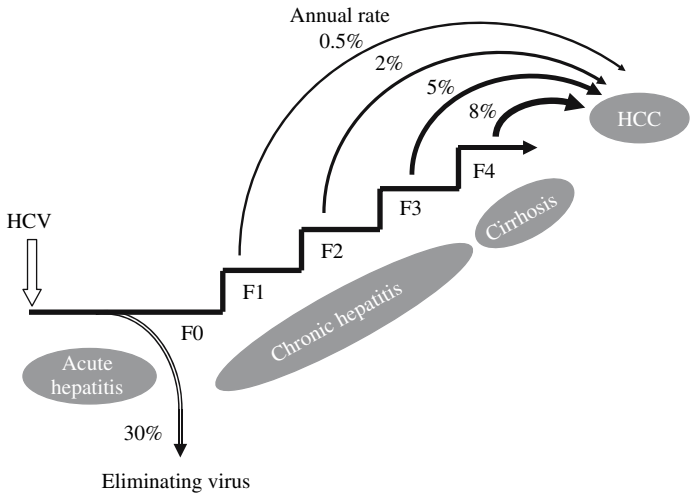

Fig. 1 Natural history of HCV infection and its incidence of HCC according to liver fibrosis stage

virus to be expelled naturally, with the estimated annual rate being less than 0.2% at most. Many of the patients with persistent infection show the medical conditions of chronic hepatitis and develop cirrhosis in 20 to 30 years. Patients with cirrhosis develop HCC at a very high annual rate of 8%, while patients with early chronic hepatitis do so at an annual rate of only 0.5%. The estimated number of patients with HCV is about 1.7 million in Japan and about 1.7 billion in the world. It is a serious public health problem as many of these patients belong to a high-risk group for HCC.

HCV does not fit into the classical definition of a tumor virus. The mechanism of carcinogenesis in patients with persistent infection of HCV is not fully understood, but it is usually explained from the virus and the inflammation viewpoints. From the virus viewpoint, the HCV core protein has an effect on the mitochondrial electron transport system and prompts the production of reactive oxygen species (ROS) (Moriya et al., 1998; Okuda et al., 2002). This process is thought to cause damage to the host gene. It has also been reported that the expression of HCV core protein activates *bcl-x*$_L$ transcription via the MAP kinase pathway as well as the activation of STAT3 (Otsuka et al., 2002). We have shown that high expression of *bcl-x*$_L$, observed in about one-third of HCC cases, is involved in the apoptosis resistance of cancer cells (Takehara et al., 2001; Takehara & Takahashi, 2003). From the inflammation viewpoint, it is thought that the inflammation itself induces oxidative stress and that hepatic regeneration in patients with hepatic disorder has an influence on the fixation of accumulated mutations (Kato et al., 2003). In any case, the larger clone size of the transformed hepatic cells caused by these factors leads to overt HCC. Generally, the innate immune system recognizes abnormality in autologous cells *in vivo*, and the immunological expulsion mechanism starts to function. Increasing evidence supports the possible involvement of innate immunity in the carcinogenesis of HCC and its development in patients with HCV infection.

Toll-like Receptor: Impact on the Study of Innate Immunity

The biological defense mechanism of higher organisms, including humans, is generally divided into innate immunity and adaptive immunity (Dranoff, 2004). In the adaptive immune response, gene rearrangement by T cells and B cells enables the establishment of a defense mechanism of high specification against "the molecular microstructure of a foreign substance," and this mechanism is immunologically memorized. However, as it takes a few days to induce adaptive immune responses, innate immunity works as an early defense mechanism. Innate immunity has existed as a biological defense mechanism from the earliest stages of evolution: for example, insects have only innate immunity as a defense mechanism. Cells involved in innate immunity include macrophages, neutrophils, NK cells, NKT cells, and γδT cells. Important humoral factors include complements, lectins, and interferons (IFNs) (Biron, 2001). Key notions in the paradigm of modern immunology are the presentation of the antigen by antigen-presenting cells (APCs) and the development of adaptive immune responses. Most research in immunology in a narrow sense has been focused in this area. In contrast, innate immunity has not been the focus of much attention because it is mainly involved in nonspecific phagocytosis and toxicity, which have been regarded as primitive immune responses. Recently, however, innate immunity has been receiving considerable attention for two major reasons. One is the discovery of Toll-like receptors (TLRs), beginning in 1996 (Lemaitre et al., 1996), and the other is a growing recognition that innate immune responses play a critical role not only in early immunity but also in determining the magnitude and direction of the subsequent adaptive immune responses.

TLRs are specific to structures peculiar to microbes, including bacterial and fungal compounds (such as LPS and flagellin) and microorganism-origin nucleic acids (such as double-stranded RNA and CpG DNA) (Akira et al., 2006). TLRs are molecules whose expression has been observed in non-hematopoietic cells as well as in hematopoietic cells such as dendritic cells (DCs). The discovery of TLRs was important because it showed that, at the molecular level, a living body recognizes the entrance of pathogens as "pathogen-associated molecular patterns" (PAMPs). This has shown that the innate immune system discriminates between self and not-self and recognizes abnormality via a mechanism that is different from the gene rearrangement of the adaptive immune system. As for the development of adaptive immunity, it had been thought to be a simple scheme where APCs (the most potent APC *in vivo* is a DC) trap antigens in peripheral tissues and present the antigens to T cells in secondary lymph nodes. However, it is now known that T cells cannot be activated by DCs without the process of DC maturation and that typical signals to induce DC maturation are sent by TLRs (Kaisho & Akira, 2003). Most of the adjuvants loosely recognized as activating factors of immunity have turned out to be TLR ligands. Thus, it can be said that the TLR has revealed the importance of innate immunity in adaptive immunization.

What is now clear is the existence of a scheme in which the recognition of pathogens by TLRs and DC maturation/activation are followed by adaptive immunization in immune responses to invading microbes. The questions then arise of

how abnormality *in vivo* is recognized in the process of carcinogenesis and how this recognition can lead to adaptive immunization. The development of cancer is a process in which a normal cell becomes abnormal. The mechanism of recognizing "abnormality in autologous cells" cannot be explained by means of TLRs, which recognize "pathogen-associated molecular patterns." We need to consider another important system.

NK Receptors: System of Recognizing Abnormality in Autologous Cells

Abnormality that occurs in autologous cells *in vivo* is generally reflected in the decreased expression of, or a deficiency of, MHC class I molecules (Smyth et al., 2002). NK cells are a group of cells originally defined based on their nonspecific cytotoxic activity to transformed cells (Trinchieri, 1989). The cytotoxic activity of NK cells to transformed cells has been suggested to depend on the decrease of MHC class I expression in those cells (missing-self hypothesis) (Karre et al., 1986; Ljunggren & Karre, 1990). As a series of inhibitory receptors expressed in NK cells has been identified recently, the molecular mechanism is now understood as follows (Figure 2) (Ravetch & Lanier, 2000). Inhibitory receptors of NK cells inhibit NK activity in normal cells, recognizing MHC class I molecules, which are constantly expressed in normal cells. For cells in which MHC class I expression has decreased, such as in tumor cells, the inhibition is released and the NK cells can display their cytotoxic activity. Inhibitory receptors are generally divided into two types according to their structures: the immunoglobulin superfamily of type I transmembrane proteins and C-type lectins, or type II transmembrane proteins. Inhibitory

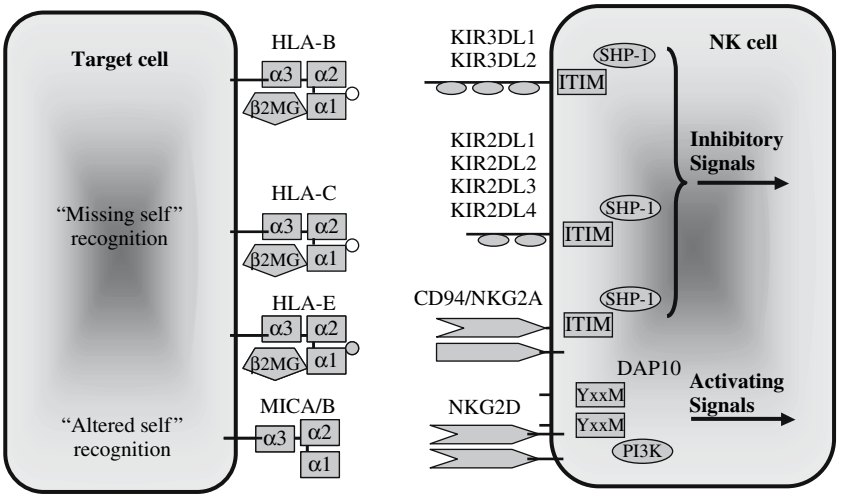

Fig. 2 NK-cell receptor and ligand interaction

receptors that belong to the immunoglobulin superfamily are called "killer cell immunoglobulin-like receptors" (KIRs), and more than 30 human cDNAs have been isolated. A number of these KIRs recognize HLA class I molecules present at the loci of HLA-B and -C, specifically genetic polymorphisms (for example, KIR2DL1 recognizes HLA-Cw4 and KIR2DL2 recognizes HLA-Cw3). Heterodimer receptors consisting of CD94 and NK group 2 (NKG2) are known to exist, being typical members of the C-type lectin family. Among them is CD94/NKG2A (NK group 2, member A), whose ligand is known to be HAL-E. CD94/NKG2A is thought to monitor the entire translation volume of HLA class I in a target cell by recognizing the leader sequence of HLA class I antigens presented by HLA-E. Inhibitory receptors belonging to the immunoglobulin superfamily and the C-type lectin family have a structure called "immunoreceptor tyrosine-based inhibitory motifs" (ITIM) in their intracellular domains. Tyrosyl residues of ITIM are phosphorylated by cross linking of ligands, and thus inhibitory signals are transmitted to NK cells.

Recently, NK cells have been shown to have activating receptors, which activate NK cell functions, as opposed to inhibitory receptors (Raulet, 2003). Among the molecules belonging to the NKG2 family of C-type lectin-like receptors, NKG2D (NK group 2, member D) is an unusual receptor. It has low homology with other NKG2 proteins. Structurally, it forms a homodimer with NKG2D and does not form a heterodimer with CD94. NKG2D forms a complex noncovalently with an adaptor protein called DAP10 and, through cross linking, recruits the p85 subunit of PI3 kinase, thus transmitting the activating signals to NK cells. The NKG2D-activating receptor has attracted attention because its expression in almost all NK cells presumably signifies its importance and because a ligand for it has been identified but not for many other activating receptors. Either MHC class I-related chain A or B (MICA or MICB), both of which are MHC-related molecules, is a ligand for NKG2D in humans (Bauer et al., 1999). MICA or MICB is not expressed in normal cells, being induced by the transformation of cells. This means that NK cells not only recognize abnormality in autologous cells as the missing self but also positively recognize the abnormal self, which is not expressed in normal cells as the altered self to regulate NK cell functions. In this manner, NK cells function as a system by which MHC class I or MHC-related molecules are recognized by the mediation of various NK receptors, leading to the recognition of abnormality in autologous cells.

MICA Expression in HCC and NK-Cell Sensitivity

MIC (MHC class I-related chain) genes make up a gene family identified in the HLA class I region, and seven MIC loci, from A to G, have been confirmed (Figure 3). C to G are pseudogenes, while both MICA and MICB encode 43 kDa proteins. MICA/B are glycoproteins expressed on the cellular membrane, and the structures of the extracellular domains composed of $\alpha 1$, $\alpha 2$, and $\alpha 3$ are similar to those of classic HLA class I molecules. However, their functions differ from those of classic HLA class I molecules because they lack the antigen-presenting function because their structures have no domain for peptides due to the narrow grooves formed by

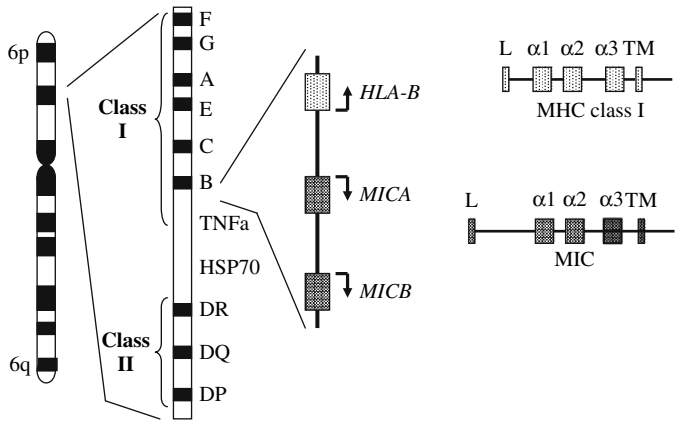

Fig. 3 MHC class I-related chain genes and their molecular structure

the α1 and α2 domains and because their expression is not induced by IFN as they do not need association with β2 microglobulin to be expressed on the membrane (Bahram et al., 1994). In general, MHC class I molecules are constantly expressed in cells while MICA/B are not expressed in normal cells, except in intestinal epithelial cells and some thymocytes. MICA/B expression is known to be induced in stressed cells and transformed epithelial cells. The functions of MICA/B have not been clarified since their discovery in 1994, although in 1999 they were found to be human ligands for NKG2D. Since then, their functional importance in the immune response has attracted attention (Jinushi et al., 2003a, 2003b).

The characteristics of cancer cells from various organs can be examined from the perspective of MHC class I molecule expression. For example, with colon cancer cells, the decreased expression or the deficiency of HLA class I molecules is observed in many mechanisms (Miyagi et al., 2003), and this is thought to be an immune evasion mechanism of colon cancer. By contrast, many hepatoma cells distinctively retain the expression of HLA class I molecules (Takehara et al., 1992). This works against hepatoma cells because of the development of an antigen-specific immune response, although this does help them evade NK cells. In order to clarify the molecular mechanism of NK-cell immune surveillance of HCC, we examined the MICA/B expression in HCC (Jinushi et al., 2003c). Immunohistological examination and PCR analysis of human HCC revealed that non-neoplastic liver tissue had no MICA/B expression, while about 50% of the HCC tissues had it. FACS analysis of hepatoma cell lines showed that many of them had MICA/B expression. When we examined the cytotoxic activity of CD56-positive cells (NK cells) separated from human peripheral blood, the target hepatoma cell lines were found to be susceptible to their cytotoxic activity in various degrees. What is important is that when anti-MICA/B antibody or anti-NKG2D antibody was added to mask these molecules, the cytotoxic activity of CD56-positive cells decreased. Therefore, it appears that the activation of NKG2D by MICA/B plays an important role in inducing the NK-cell sensitivity of HCC (Figure 4).

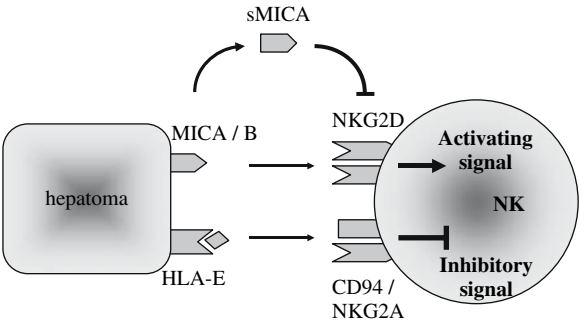

Fig. 4 HCC modulation of NK cells via NK inhibitory and activating receptors

NK Receptor Expression in Chronic Hepatitis C Patients

There is no established view of NK activity in patients with HCV: some researchers have reported decreased NK activity in HCV patients, while others have reported levels equivalent to those of healthy individuals (Ahmad & Alvarez, 2004; Golden-Mason & Rosen, 2006). In order to evaluate the function of NK cells in patients with HCV, we separated CD56-positive cells from the peripheral blood of patients with HCV and healthy donors and examined their cytotoxic activity. To K562 cells, a classic NK-sensitive target, CD56-positive cells in patients with HCV showed the same level of cytotoxic activity as those in healthy donors, but to hepatoma cell lines, the cytotoxic activity of CD56-positive cells decreased in patients with HCV. This suggests that the NK-cell receptor expression profile might differ between patients with HCV and healthy individuals. We next comprehensively analyzed NK receptor expression in CD56-positive cells using FACS. The results showed that for KIR, among the inhibitory receptors, there was no expression difference between the two groups, whereas for NKG2A and CD94, there was a significant increase of expression frequency in patients with HCV (Jinushi et al., 2004). On the other hand, as for NKG2D, one of the activating receptors, there was a trend toward decrease of expression frequency in patients with HCV, but it did not reach a significant level.

We further examined the expression of HLA-E, a ligand for CD94/NKG2A receptor. HLA-E was positive for primary hepatocytes and all hepatoma cell lines tested (HepG2, Hep3B, Huh7), while it was negative for K562. During the chromium release assay of NK-cell cytotoxic activity targeting hepatoma cell lines, we added anti-NKG2A neutralizing antibody, and the cytotoxic activity of NK cells was significantly increased. As for K562, by contrast, the addition of anti-NKG2A antibody made no difference in NK-cell sensitivity. Therefore, signals sent to NKG2A from HLA-E were thought to be inhibitory to NK-cell responsiveness in hepatoma cell lines. The addition of anti-NKG2A antibody led to a clearer increase in NK-cell sensitivity in patients with HCV. This demonstrated that the increase of NKG2A expression caused the decrease of NK-cell cytotoxic activity against hepatoma in patients with HCV.

These findings suggest that the responsiveness of human NK cells to hepatoma cell lines is regulated by a balance of activating signals from NKG2D and inhibitory signals from NKG2A (Figure 4) (Takehara & Hayashi, 2005). In patients with HCV, NKG2A expression frequency is increased and NK responsiveness to hepatoma cells is decreased. This suggests that NK cells, part of the innate immune system, act to recognize and expel hepatoma cells. Hepatocarcinogenesis in patients with HCV involves virus and inflammation factors, and it appears that the additional involvement of these immunological factors might result in a higher hepatoma incidence rate.

Control of DC Function by NK Cells

NK cells make up a cell family defined by an index of direct effector functions of being cytotoxic to transformed cells. Recently, NK cells have attracted attention for the possibility of having effects on the development of adaptive immunity through the modification of DC maturation, activation, and cell death (Gerosa et al., 2002). We have been conducting *in vitro* experiments on how NK cells are involved in DC maturation and activation (Jinushi et al., 2006). DCs that are inductively differentiated from peripheral blood monocytes of healthy donors using GM-CSF and IL-4 show an immature phenotype (IM-DC). In order to clarify how NK cells might be involved in the maturation of these IM-DCs, we conducted a mixed-culture test of IM-DCs and NK cells. The co-culture of IM-DCs with NK cells did not induce maturation, but a 48-hour co-culture of IM-DCs with hepatoma cell lines and NK cells led to increased expression of CD40, CD86, and HLA-DR in DCs and induced DC maturation. During the co-culture, we inserted a trans-well membrane between DCs and NK cells, but DC maturation was induced. This showed that DC maturation was induced via humoral factors, and not by direct cell-to-cell contacts. In fact, the stimulation of IM-DCs using a 24-hour mixed-culture supernatant of NK cells and hepatoma cells resulted in inducing maturation. This maturation was accompanied by functional activation, and allostimulatory capacity toward CD4-positive T cells from healthy donors was significantly enhanced compared with that of IM-DC.

Next, we examined DC maturation and functional activation resulting from co-culture with hepatoma cells and NK cells using NK cells from patients with HCV instead of those from healthy donors. The stimulation of IM-DCs using the supernatant of a 24-hour co-culture of NK cells from HCV patients with hepatoma cells resulted in suppressed DC maturation and allostimulatory capacity compared with the case in which we used NK cells from healthy donors. In order to examine whether NKG2A signals from HLA-E during a mixed culture of hepatoma cells and NK cells are involved in this inhibition of DC maturation and activation, we conducted an inhibition experiment by adding anti-NKG2A antibody during the mixed culture. DC maturation and activation resulting from culture supernatant stimulation were enhanced by adding anti-NKG2A antibody either in the case of

NK cells from healthy donors or in the case of those from patients with HCV. However, DC maturation and activation were more notably enhanced when NK cells from patients with HCV were used. Levels of various cytokines present in the culture supernatant were quantitatively analyzed in each case, and the levels of IFNγ and TNFα were high when NK cells from healthy donors were used, whereas the levels of IL-10 and TGFβ were high when those from patients with HCV were used. Results from experiments where a neutralizing antibody was added for each cytokine into the culture supernatant suggested that the change of the cytokine balance affected DC maturation and activation.

These observations suggest that in patients with HCV, excess NKG2A signals in NK cells not only have an inhibitory effect on the direct effector activity of NK cells but also have a negative effect on the subsequent DC maturation and activation. With improved methods to detect specific T cell responses, such as the ELISPOT assay, there have been reports of some T cell responses specific to cancer antigens in the case of HCC as well. Down-regulated adaptive immune responses from NK cells to DCs might inhibit development of the adaptive immunity.

Secretion of Soluble MICA into Serum in HCC and NKG2D Expression in NK Cells

Recently, it has been reported that some MICA expressed in tumor cells are truncated and their extracellular domains are secreted into culture solutions as soluble forms (Groh et al., 2002; Salih et al., 2002). It is known that soluble MICA (sMICA) is detected in the serum of patients with prostate cancer, colon cancer, brain neoplasm, and leukemia. The importance of this phenomenon is that MICA expression in tumor cells is decreased due to cleavage and NK responsiveness is decreased due to induced NKG2D internalization. These events might be involved in the ability to evade the immunomechanism.

In order to examine the importance of sMICA in liver disease, we conducted ELISA quantitation of serum sMICA from healthy donors and patients with chronic HBV/HCV and HCC (Jinushi et al., 2005). Only a small amount of sMICA was detected in a small number of cases of healthy donors and patients with chronic hepatitis, while notably larger amounts of sMICA were detected in some patients with HCC. We also conducted the test according to the HCC stage and observed that the number of sMICA positive cases was notably more frequent for advanced HCC than for early HCC. We conducted FACS analysis of NKG2D expression in CD56-positive cells from healthy donors and patients with HCC (sMICA positive/negative) and chronic HCV. The results showed that the level of NKG2D expression in patients with hepatitis or HCC (sMICA negative) was the same as that in healthy donors, while the level of NKG2D expression in patients with HCC (sMICA positive) was decreased. Next, in order to examine whether sMICA is involved in the decreased NKG2D expression, we treated CD56-positive cells from healthy donors

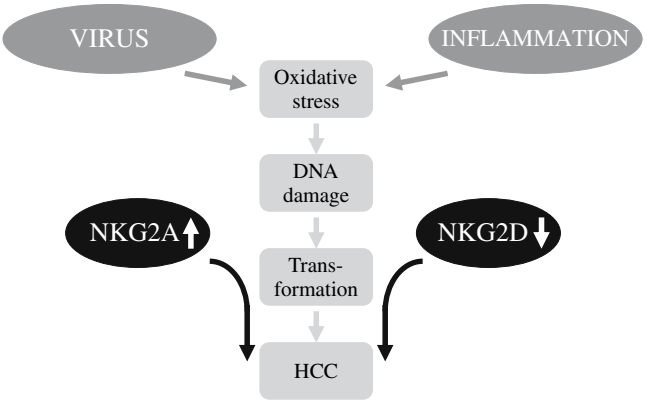

Fig. 5 Possible mechanisms of HCV-related liver carcinogenesis

with 10% patient serum for 48 hours and then determined the NKG2D expression. A significant decrease in NKG2D expression was noted after treatment with serum from patients with HCC (sMICA positive), while the expression remained the same after treatment with serum from other patients. Furthermore, when we added patient serum treated with an antibody that recognized α1 and α2 domains of MICA to NK cells, the decrease of NKG2D expression caused by the serum treatment was canceled. This showed that sMICA of the patient serum was involved in the decrease of NKG2D expression. Using the chromium release assay, we compared hepatoma cell line-specific cytotoxic activity of CD56-positive cells in healthy donors and patients with HCC (sMICA positive/negative) and found that the cytotoxic activity decreased most in patients with HCC (sMICA positive). These observations suggest that an increase of HCC tumor size leads to the release of sMICA into the blood, which triggers a decrease in the NKG2D expression in NK cells and the responsiveness of NK cells to hepatoma cells, thus further suppressing the immune response to tumors (Figure 5).

Immunotherapy for HCC

If HCC is in a limited area, hepatectomy or medical ablation treatment can be prescribed. If the reserve capacity of the liver is sufficient, the technical problems are resolved and the treatment can be conduced with an adequate margin of safety, then radical therapeutic measures can be taken. However, HCC possesses biological characteristics of multiple occurrence in both time and space dimensions, making its treatment extremely difficult. Topical treatment has no effect on multiple HCC spreading over both hepatic lobes. In such a case, transcatheter arterial embolization or arterial injection chemotherapy can be an option, but the effectiveness is limited. Even if topical treatment is successful, distant recurrence is likely to occur in many cases. Thus, there is an urgent need to establish ways to prevent recurrence. In order

to improve the prognosis of progressive HCC and the recurrence-free survival rate after topical treatment, we need to develop whole-liver treatment based on a new point of view. It is in this context that there are high expectations for immunotherapy to treat HCC (Butterfield, 2004; Palmer et al., 2005; Avila et al., 2006).

Immunotherapy for cancer has been developing from nonspecific to specific, from those using unknown mechanisms to those where the mechanism has been clarified. To holistically activate immune responses *in vivo*, immunomodulating therapy using bacterial compounds has been replaced by cytokine therapy. Adoptive immunotherapy using lymphokine-activated killer cells (LAK) has been replaced by therapy using tumor-infiltrating lymphocytes (TIL) that are tumor-specific, and nonspecific immunostimulation has been replaced by specific treatment using DC or tumor-specific antigens. In addition, conventional approaches have been reevaluated from an up-to-date point of view: bacterial compounds used for immunostimulation have been found to be ligands for TLRs, which activate innate or adaptive immunity by inducing DC maturation. To treat HCC, possibilities being explored include the application of cytokine therapy, adoptive immunotherapy, DC therapy, and tumor-derived peptide therapy.

The knowledge of the hepatoma recognition mechanism mediated by NK receptors should be useful for developing the immunotherapy for HCC based on new strategies. One possibility lies in the exploration of the method to induce MICA/B expression in hepatoma cells. We demonstrated that all-*trans*-retinoic acid inducement increases MICA/B expression in hepatoma cells and makes them more susceptible to NK cells (Jinushi et al., 2003c). The fact that this phenomenon is lost in the presence of synthetic retinoid, which functions as a competitive inhibitor of all-*trans*-retinoic acid receptors, shows that it is a specific action of all-*trans*-retinoic acid mediated by receptors, not a nonspecific response. It has been reported recently that DNA toxic antitumor agents (Gasser et al., 2005) or inhibitors of histone deacetylating enzyme (Armeanu et al., 2005; Skov et al., 2005) similarly induce MICA/B expression in neoplastic cells. It is also known that with respect to NKG2D expression in NK cells, cytokines such as IL-15 have the inducing capacity. As for the combined therapy of chemotherapeutic agents and cytokines, various combinations have been explored for the treatment of progressive HCC. New combinations should be examined from the perspective of NK receptors and the expression of their ligands.

Concluding Remarks

HCV infection and subsequent hepatocarcinogenesis and HCC progression can be summarized from the perspective of receptor expression in NK cells as follows. HCV patients show increased NKG2A expression, and patients with advanced HCC display decreased NKG2D expression under the influence of sMICA. There is a link between the staged change of NK-cell phenotypes and the decreased cytotoxic activity of NK cells to HLA-E positive/MICA positive hepatoma cell lines. This might have a disadvantageous effect on a living body in the ablation of transformed

liver cells or the growth of tumor. It appears that apart from well-known factors from the virus viewpoint and the inflammation viewpoint, the modulation of innate immunity like this is involved in the high rate of hepatocarcinogenesis and the following progression in patients with HCV (Figure 5).

In vivo, DCs are the most potent APC to activate naïve T cells, but to initiate adaptive immunity in this manner, DCs should be mature and activated. In the case of microbe infection, the recognition of molecular structures peculiar to microbes by TLRs induces DC maturation. In the process of carcinogenesis, on the other hand, NK receptors expressed in NK cells only recognize abnormality in autologous cells (altered self or missing self) and activate NK cells. Thus, NK receptors are involved in direct resistance to tumor cells. In addition, NK-cell activation has an influence on DC maturation via humoral factors. Therefore, NK receptors as well as TLRs play their part as an interface in transmitting information about abnormality arising *in vivo* to the immune systems (Figure 6). It appears that the aberrant expression of NK-cell receptors in patients with HCV or progressive HCC might have a negative influence on the development of not only innate immune responses but also adaptive immune responses.

The expression of NK-cell receptors and their ligands changes dynamically during the process from HCV infection to hepatocarcinogenesis to the progression of HCC. The expression kinetics of these molecules might have a close connection with the process. The modification of the expression of these molecules by drugs or cytokines might lead to the development of cancer immunotherapy based on a new perspective. There are great expectations for further progress of research in this field.

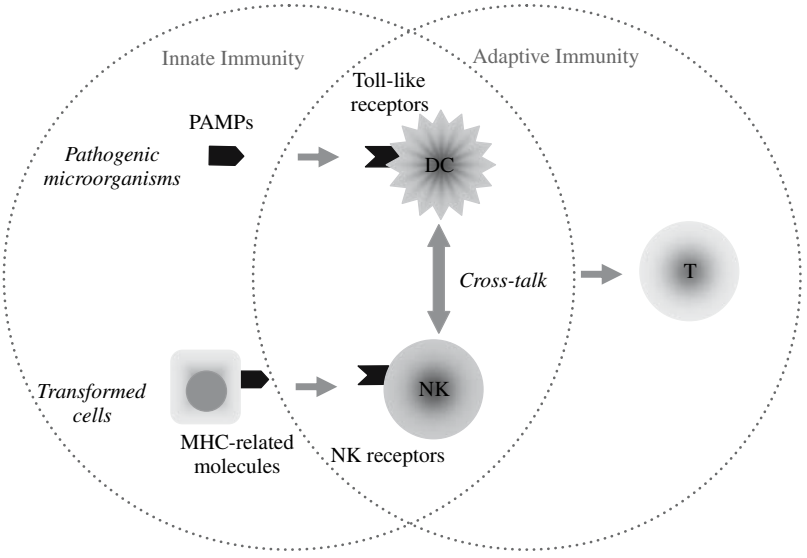

Fig. 6 Transmission of danger signals of pathogens and tumors to the immune system

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Mechanisms of Interferon Action and Resistance in Chronic Hepatitis C Virus Infection: Lessons Learned from Cell Culture Studies

Srikanta Dash*, Sidhartha Hazari, Robert F Garry, and Fredric Regenstein

Abstract Alpha interferon, usually in combination with ribavirin, is currently the standard care for patients infected with hepatitis C virus. Unfortunately, a significant number of patients fail to eradicate their infection with this regimen. The molecular details concerning the failure of many patients to achieve sustained clearance of the virus infection after interferon therapy are currently unknown. The primary focus of this chapter is to provide an overview of interferon action and resistance against hepatitis C virus (HCV) based on our understanding developed from *in vitro* experiments. Interferon first binds to receptors on the cell surface; this initiates a cascade of signal transduction pathways leading to the activation of antiviral genes. Using a cell culture model, we determined that the activation of an interferon promoter (interferon inducible genes) is important for a successful antiviral response against HCV. The level of activation of the IFN promoter by exogenous interferon appears to vary among different replicon cell lines. It was observed that a replicon cell line showing low activation of the IFN promoter frequently develops resistant phenotypes compared to cell lines with higher activation. Furthermore, interferon-alpha, -beta, and -gamma are each found to inhibit replication of HCV in the cell culture. The antiviral action of interferon is targeted to the highly conserved 5′ untranslated region (5′UTR) utilized by the virus to translate protein by an internal ribosome entry site (IRES) mechanism. This effect is the same among HCVs of other genotypes. Interferon inhibits translation of HCV by blocking at the level of formation of polyribosomes on the IRES containing mRNA. These *in vitro* studies suggest that differences in the regulation of IRES-mediated translation by interferon among hepatic cell clones may be directly related to the development of interferon resistance in chronic HCV infection.

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Introduction

Hepatitis C virus (HCV) infection is a major public health problem. At present approximately 170 million people worldwide have been infected with HCV. It is the principal virus accounting for chronic liver disease in patients previously designated as having non-A, non-B hepatitis. The hepatitis C virus was cloned and sequenced by a team of investigators from the Chiron Corporation, California, USA, in 1989 (Choo et al., 1989). During subsequent years, significant progress has been made in areas of clinical and molecular virology, development of cell culture models, antiviral therapy, and viral pathogenesis. The hepatitis C virus is an enveloped virus of the *Flaviviridae family* containing a single-stranded, positive-sense RNA genome approximately 9,600 nucleotides in length (Francki et al., 1991; Rice, 1996). The viral genome is organized into a 5′ untranslated region, followed by a large open reading frame and a 3′ untranslated region. The HCV RNA genome directly binds to host cell ribosomes and is translated into a large polyprotein of 3,010 amino acids. This polyprotein is subsequently processed in the endoplasmic reticulum of the infected cell into structural proteins (core, El and E2, P7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Reed & Rice, 2000). The structural proteins play important roles in the formation of complete virions, their export, and the infection of host cells. The nonstructural proteins provide the necessary enzymatic activities to replicate the HCV RNA genome. The viral genome persists in infected hepatocytes due to continuous replication of both positive- and negative-strand HCV RNAs in infected cells. The highly conserved structured RNA sequences located at the 5‘UTR and 3‘UTR are important in the viral translation and replication of the HCV genome (Friebe & Bartenschlager, 2002; Friebe et al., 2001; Yi & Lemon, 2003). Figure 1 demonstrates the structure of the positive-strand HCV genome and different mature proteins produced in the virus-infected cell.

Most people acquire HCV infection through direct contact with infected blood (e.g., blood transfusion, injection drug use). Following exposure to HCV, a robust host immune response is generated; however, in a majority of patients the response fails to eradicate the virus, leading to chronic infection. In chronically infected individuals, the virus preferentially replicates in the liver for prolonged intervals of time leading, both directly and indirectly, to potentially serious liver disease. It is now believed that long-standing chronic inflammation due to HCV infection triggers the development of hepatocellular carcinomas. The strong association between the chronic HCV infection and the development of hepatocellular carcinomas has been made in many parts of the world, including the United States, Japan, Australia, and Europe (El-Serag, 2004; Hoofnagle, 2004, Kiyosawa et al., 2004; Bosch et al., 2004). The mechanisms controlling the development of HCV into chronic infection and then evolving to cirrhosis and cancer appear to be complex.

Interferon-alpha alone, or in combination with ribavirin, is the standard therapy for acute and chronic HCV infection. Sustained virological response can be achieved in up to 90% of acute HCV infections and in approximately 50% of those chronic infections (Feld and Hoofnagle, 2005; Strader et al., 2004). HCV RNA

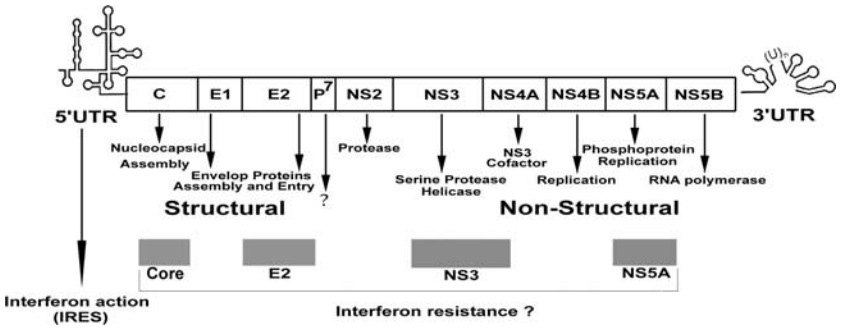

Fig. 1 Organization of the HCV RNA genome and protein translated from the single large open reading frame (ORF). The viral genome begins with a stretch of (1–341) untranslated sequences (5′UTR), followed by a large open reading frame (ORF), then another stretch of an untranslated region called 3′UTR. The 5′UTR that forms the complex secondary structure also initiates translation by directly binding to the host ribosome via internal ribosome entry site (IRES) mechanisms. Interferon specifically inhibits the IRES function in hepatic cells. Ten different mature proteins are generated due to the cleavage of large polyproteins. Among these (core, E1, E2) are structural proteins; NS2-NS5 are known as nonstructural proteins. The exact function of the p-7 protein is unknown. The core protein, envelope protein (E2), the nonstructural protein (NS5A), and the NS3/4A serine protease were reported to play an important role in the mechanisms of interferon resistance

levels in the blood and serum are used to monitor the response to interferon therapy in patients undergoing treatment. Approximately 60% of patients have undetectable levels of virus at the conclusion of therapy. Many chronic hepatitis C patients, particularly those infected with genotype 1, do not respond to interferon therapy. In most of these individuals, HCV RNA levels remain detectable throughout treatment. The reason why some patients become persistently infected, and why some respond to interferon therapy while others do not, is not clear. Understanding the mechanisms of interferon action and interferon resistance should open new directions to developing alternative strategies to improve the clinical efficacy of interferon therapy.

Interferon System

Interferons are the cytokines, which are produced initially to defend the host against infection, through mechanisms that inhibit the replication of a number of viruses. There are two main types of interferon. Type I interferons include interferon-alpha, interferon-beta, interferon-omega, and interferon-delta. Interferon-gamma is a Type II interferon. Interferon-alpha is mainly produced by leukocytes (dendritic cells and macrophages). Interferon-beta is produced by most of the epithelial cells and fibroblasts. Cells of immune system, including T cells and natural killer cells, produce interferon-gamma. Once stimulated by a viral infection, these cells go through a series of signaling events that leads to rapid production of interferons and other cytokines. Interferon is, therefore, an important cytokine during innate host defense

against viral infection. It is believed that the interferon system is transcriptionally activated intracellularly within a few hours of virus infection through a cascade of signaling pathways that involve NF-kB, ATF2-c-Jun, and interferon-regulatory factors (IRF 3) and IRF-7 (Kawai & Akira, 2006). In humans, Type I interferons are encoded by 14 functional genes that form the interferon-alpha family. Single genes encode for interferon-beta and -omega, and three genes encodes for interferon-lambda (Sen, 2001; Bekisz et al., 2004). The biological significance of having multiple genes for interferon-alpha and only one for interferon-beta is not clear. The genes for different Type I interferons are all located together on human chromosome 9 (Diaz et al. , 1994), and the Type II interferon gene is located on chromosome 12 (Schroder et al., 2004). The commercially available recombinant interferon used against HCV is interferon-α2a, interferon-α2b, or a consensus interferon (Blatt et al., 1996). The consensus interferon is a recombinant protein that has the most common amino acid sequences derived from several natural interferon-alpha subtypes (Heathcote et al., 1998). All Type I interferons bind to the human interferon-alpha receptor (IFNAR), which consists of an IFNAR-1 and IFNAR-2 subunit (Uze et al., 1990, 1995; Novick et al., 1994; Colamonici et al., 1994; Domanski and Colamonici, 1996; Platanias et al., 1996). IFNAR-1 has a relative molecular weight of 110 kDa, while IFNAR-2 occurs as two forms due to differential splicing of the same gene. These include the IFNAR-2c protein of molecular weight 90–100 kDa and the IFNAR2b protein of molecular weight 51 kDa. There are two distinct interferon-gamma receptors (IFNGR-1 and IFNGR-2). IFNGR-1 has a major binding subunit protein with a molecular weight of 90 kDa; IRNGR-2, a 62-kDa protein, plays a minimal role in ligand binding and is important in downstream signaling pathways (Stark et al., 1998; Bach et al. 1997; Hemmi et al., 1994). All interferons activate a cascade of signal transduction pathways through its receptors that stimulate synthesis of numerous antiviral genes. The differences and similarities between the signaling pathways of Type I interferon and Type II interferon are summarized in Figure 2. Interferon binding to the cell surface receptors activates the intracellular signaling pathways, which involve Janus kinase (JAK1) and tyrosine kinase 2 (TYK2) and signal transducer and activator of transcription (STAT1 and STAT2) proteins. The JAKs phosphorylate the STAT proteins, which either homo- or heterodimerize and then translocate to the nucleus to induce the expression of the IFN-stimulated genes (ISG). The phosphorylated STAT1 and STAT2 combine with IRF-9 (interferon regulatory factor 9) to form a trimeric ISGF-3 complex. This complex enters the nucleus and binds to a consensus DNA sequence [GAAAN (N) GAAA] called the "interferon stimulated response element" (ISRE) (Goodbourn et al., 2000). This regulatory sequence is present upstream of most interferon-alpha and interferon-beta responsive genes. These cascades of molecular signaling are essential for stimulation of interferon-mediated gene transcription. In contrast, binding of interferon-gamma to its receptors leads to tyrosine phosphorylation of STAT1, but not STAT2. The phosphorylated STAT1 protein forms a homodimer called "gamma-activated factor" (GAF) that translocates to the nucleus and binds to a consensus sequence [TTNCNNNAA] called "gamma activation sequence" (GAS) elements. This DNA sequence is present in the upstream regulatory region of the interferon-gamma inducible genes. These cascades of biochemical reactions

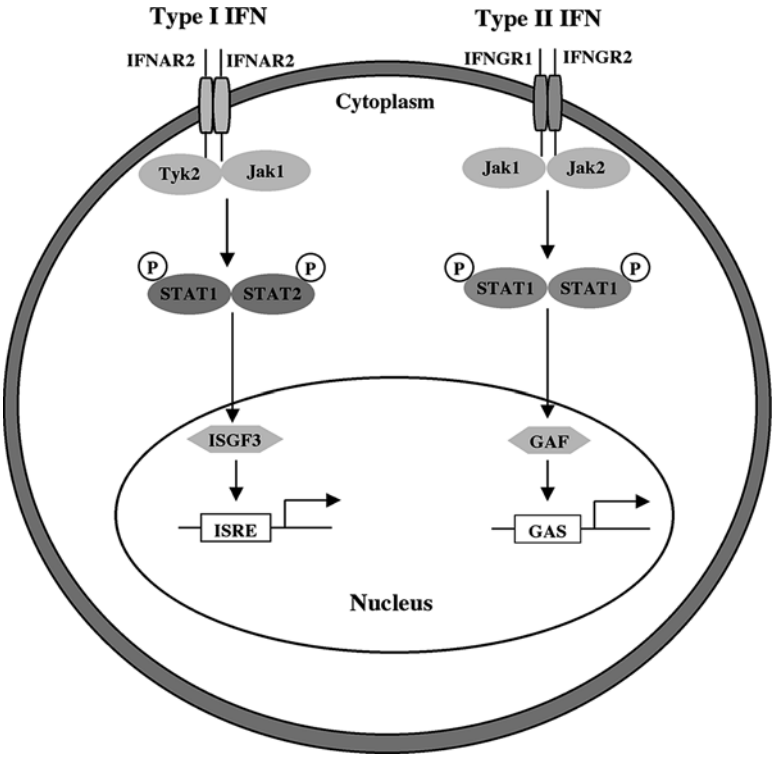

Fig. 2 Comparison of signaling pathways activated in a mammalian cell after addition of exogenous interferon. IFN-alpha/beta (type I IFN) and IFN-gamma (type II) binds to separate cell surface receptors. IFN-alpha or IFN-beta binding to their receptors activates two receptor associated tyrosine kinases, Jak1 and Tyk2, which then phosphorylate the STAT1 and STAT2 proteins. These two phosphorylated proteins combine with IRF-9 to form the trimeric ISGF3 complex. This complex enters the nucleus and binds to a regulatory consensus DNA sequence called ISRE (interferon sensitive response element) present in most of the type I interferon responsive genes, whereas IFN-gamma binding to its receptor leads to activation of Jak-1 and Jak-2 tyrosine kinases, resulting only in phosphorylation of STAT1 protein. The phosphorylated STAT1 protein forms a homodimer called "gamma-activated factor" (GAF). This complex enters the nucleus and binds to the consensus DNA sequence called the GAS (gamma activated sequence), which regulates the induction of type II responsive genes

occurring in normal cells due to interferon treatment have been termed the Jak-Stat pathways (Darnell, 1998). The Jak-Stat pathways activate a large number of genes in the IFN-treated hepatocyte, which are normally quiescent or present at low levels (William, 1991).

The roles of the interferon-stimulated genes have been well established while studying interferon action against different viruses (Katze et al., 2002). These include the double-stranded RNA-activated protein kinase PKR, which inhibits protein synthesis via eIF2alpha phosphorylation, the 2′-5′ oligoadenylate synthatase (2′-5′ OAS) (which activates RNAse L to degrade viral RNA), the MX GTPase (which blocks viral transport inside the cell), p56 (which inhibits translation via