Membrane IL1α Inhibits the Development of Hepatocellular Carcinoma via Promoting T- and NK-cell Activation

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Abstract

Hepatocellular carcinoma is a worldwide health problem with limited treatment options and poor prognosis. Inflammation associated with liver injury and hepatocyte regeneration can lead to fibrosis, cirrhosis, and eventually, hepatocellular carcinoma. IL1α is one of the most important inflammatory cytokines involved in inflammation and tumor development. IL1α presents as multiple forms in vivo, including precursor, propiece, membrane, and secreted forms, and their functions have been thought to be different. The role of membrane IL1α in hepatocellular carcinoma tumorigenesis is still not clear. Here, we examined the functions of membrane IL1α in murine hepatocellular carcinoma models. We found that membrane IL1α potently inhibited hepatocellular carcinoma tumor growth. Further studies showed that membrane IL1α promoted T- and natural killer (NK)-cell activation in vivo. IFNγ production by CD8⁺ T and NK cells was also increased as a result of membrane IL1α expression. Moreover, the cytotoxicity of the CTL and NK cells was also enhanced by membrane IL1α expression. Furthermore, in vitro studies demonstrated that membrane IL1α could directly activate T cells and NK cells in a cell contact-dependent manner. Conversely, depletion of both CD8⁺ T and NK cells suppressed the antitumor activity of membrane IL1α. Our studies demonstrated that membrane IL1α could promote antitumor immune responses through activation of T and NK cells. Thus, our findings provide new insights of IL1α functions during hepatocellular carcinoma development. Cancer Res; 76(11); 3179-88. ©2016 AACR.

Introduction

Hepatocellular carcinoma is one of the most common liver malignancies, and the third leading cause of cancer-related death worldwide (1). Hepatocellular carcinoma is caused by multiple risk factors, such as infection with hepatitis virus (HBV and HCV; ref. 2), nonviral causes (alcohol abuse, nonalcoholic steatohepatitis, type 2 diabetes mellitus; ref. 3), and environmental and dietary carcinogens ( aflatoxin B1, nitrosamines; ref. 4). The molecular mechanisms of hepatocellular carcinoma development are complex and not fully understood (3). Inflammation, tumor microenvironment, oxidative stress, and other events play important roles in liver tumor initiation, progression, and metastasis (6). Numerous studies have demonstrated that chronic inflammation can lead to carcinogenesis (7-9). Recently, many studies focused on the inflammation in the tumor microenvironment, which can describe and predict the phenotypic characteristics of cancer (10). Hence, understanding the role of inflammatory components and its signaling pathways in the tumor microenvironment could lead to the discovery of novel molecular therapeutic targets for hepatocellular carcinoma.

The IL1 cytokine family consists of two important agonistic subtypes, IL1α and IL1β, which are both synthesized as precursor proteins. Inactive IL1β precursor can be cleaved by caspase-1 into an active cytokine, whereas IL1α precursor (proIL1α) can be cleaved into mature form (secreted form) and N-terminal propiece (pPIL1α) by calpain. IL1α can also be myristoylated and expressed on the cell membrane possibly via a mannos-like receptor (11, 12). Membrane IL1α is constitutively expressed in resting cells under homeostatic conditions, whereas secreted IL1α cannot be detected in healthy human body fluids (13). Studies have suggested that membrane IL1α is immunostimulatory and may activate immune cells expressing IL1R1 including T cells and natural killer (NK) cells (14-16). However, the role of membrane IL1α in inflammation and tumor development has not been well studied.

Fibrosarcoma cells expressing IL1α intracellularly and on the cell membrane lost their tumorigenicity (14, 16). On the contrary, in smooth muscle cells, intracellular IL1α was shown to stimulate cell proliferation (17). Besides, in pancreatic ductal adenocarcinoma, tumor-associated IL1α was identified as the initiator of tumor growth by promoting the production of inflammatory factors (18). Overexpression of IL1α propiece in tumor cells
induced malignant transformation and increased tumor invasiveness to visceral organs (19). IL1α could induce expression of proteases in cancer cells (20, 21) and adhesion molecules in vascular endothelial cells (22, 23). In primary gastric carcinoma, IL1α expression was correlated with a higher incidence of liver metastasis (24). In addition, secreted IL1α plays a key proinflammatory role in models of many chronic inflammatory conditions (13, 25, 26). These reports suggest that IL1α plays an important role in tumor development. However, the functions of ppIL1α, membrane IL1α, and secreted IL1α cannot be distinguished in most of these studies.

Overexpressing IL1α precursor should result in the overexpression of all forms of IL1α. In the current study, we mutated the nuclear localization signal (NLS) and calpain cleavage site of IL1α. Therefore, the IL1α precursor cannot either translocate to the nucleus or be cleaved by calpain to be secreted. This methodology resulted in mainly membrane IL1α expressions, without the nuclear or secreted forms. We found that overexpression of membrane IL1α significantly inhibited hepatocellular carcinoma tumor growth. Membrane IL1α greatly increased numbers of activated CD4+ T, CD8+ T, and NK cells, as well as IFNγ production and cytotoxicity in CD8+ T and NK cells. Moreover, NK and T cells could be activated by membrane IL1α in a cell contact–dependent manner. Depletion of both CD8+ T and NK cells in vivo suppressed the antitumor activity of membrane IL1α. Taken together, our study demonstrated that membrane IL1α could inhibit hepatocellular carcinoma development by promoting T- and NK-cell activation.

Confocal microscopy

Hepa1-6–transfected cells were plated and observed in borosilicate cover glass (Thermo Fisher Scientific). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with rabbit anti-mouse IL1α antibody (Abcam) and PE goat anti-rabbit IgG secondary antibody (MultiSciences Biotech). DAPI (Sigma) was used to detect nuclei. Microscopy was performed with a Nikon A1 confocal microscope spectral detector (Nikon).

Cell proliferation assay

Cell proliferation was determined by using CCK-8 and Ki-67 assay. Hepa1-6–transduced cells (3,000 per well, 96-well plate) were plated and subjected to CCK-8 assay after 48 hours. CCK-8 solution was added to each well and incubated for 4 hours. The optical density value was detected at 450 nm in a microplate reader (BioTek). For Ki-67 assay, hepa1-6–transduced cells (3 × 105 per well, 6-well plate) were seeded and incubated for 48 hours. Cells were harvested for Ki-67 staining.

Murine hepatocellular carcinoma models

We generated three murine hepatocellular carcinoma models. In the subcutaneous hepatocellular carcinoma tumor model, hepa1-6 cells (1 × 106) were injected subcutaneously into C57BL/6 mice and tumor growth was monitored every 3 days. Mice were sacrificed 3 weeks after tumor inoculation. In the orthotopic hepatocellular carcinoma model, hepa1-6 cells (1 × 106) were injected into C57BL/6 mice by hydrodynamic cell delivery method. In brief, cell suspension in 2 mL PBS was injected into the tail vein within 5 to 8 seconds. Mice were sacrificed 3 weeks after tumor injection and the tumor nodules in the liver were counted. In a diethylaminoamine (DEN)-induced hepatocellular carcinoma model, 14-day-old C57BL/6 mice were injected with 25 mg/kg DEN (Sigma-Aldrich). After 8 months, mice were sacrificed, and their livers were removed and subjected to assessment of tumor growth. Mice were hydrodynamically injected with the MC plasmids every month in the last 4 months. Briefly, 50 μg/mouse DNA was diluted in 2.5 mL PBS and injected into the tail vein using a 25-gauge needle and syringe within a time period of 5 to 8 seconds.

Flow cytometry

Splenocytes and intraperitoneal leukocytes were harvested from mice and analyzed by flow cytometry (27). The antibodies used for FACS staining including anti-mouse CD3-PE/C594, CD44-PE, CD8-APC, CD4-APC/7H, CD69-FTTC, CD86-FTTC, CD11c-PE/Cy7, Gr1-PE, CD11b-APC, CD19-PE/Cy7, NK1.1-PerCP/Cy5.5, were purchased from BD Biosciences, and CD4-FTTC, CD62L-PerCP/Cy5.5, NKG2D-FTTC, F4/80-PerCP/Cy5.5 IFNγ-PE, and TNFα-PE/Cy7 were purchased from Biolegend. Anti-mouse CD16/32 FcR block antibody was purchased from Biolegend. The rabbit anti-mouse II.1R1 antibody and the chromo 488 goat anti-rabbit IgG secondary antibody were purchased from Abcam. Flow cytometric analyses were performed using a FACS-Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Cytokine analysis

Serum cytokine levels were analyzed using BD Cytometric Bead Array (CBA) Mouse Soluble Protein Master Buffer Kit and CBA mouse soluble protein flex set (BD Pharmingen) on a FACS Canto II Cytometer (BD Biosciences) and analyzed by BD FCAP Array.
software (BD Biosciences). The flex sets including mouse IL1α, IL1β, IL4, IL6, IL17A, TNF, and IFNγ were used for cytokine detection.

Cytotoxicity assay

Splenocytes were harvested from the tumor-bearing mice 7 days after tumor inoculation and stimulated with hepa1-6 cell lysates with rhIL2 (100 U/mL) for 4 days. CD8+ T cells were isolated from the cultured splenocytes by negative selection using immunomagnetic beads according to the manufacturer’s protocol (StemCell Technologies). CTL activity was assayed in a 4-hour cytotoxicity assay using hepa1-6 cells as target cells.

Splenocytes were harvested from the tumor-bearing mice 7 days after tumor inoculation and NK cells were isolated from splenocytes by negative selection using immunomagnetic beads (StemCell Technologies). NK cells killing activity was assayed in a 4-hour cytotoxicity assay using YAC-1 and hepa1-6 cells as target cells, respectively.

Effector CD8+ T cells and NK cells were added to target cells in different effector:target (E:T) ratio and incubated for 4 hours, respectively. The cytotoxic activity was determined by the CytoTox 96 nonradioactive cytotoxicity assay (Promega).

T-cell activation assay

Naïve T cells were sorted from splenocytes by FACSria III Flow Cytometer (BD Biosciences). Plates were coated with 2 μg/mL anti-CD3 and 0.4 μg/mL anti-CD28 antibodies (Biolegend) overnight before treatment. T cells and the transduced hepa1-6 cells were cocultured for 48 hours. In coculture system, $2 \times 10^5$ T cells and $1 \times 10^5$ hepa1-6 cells (irradiated at 50 Gy) were seeded per well in 96-well plate. In the transwell system (24-well), $1 \times 10^5$ hepa1-6 cells were placed in the top chamber and $4 \times 10^5$ T cells were used for cytokine detection.
were seeded in the bottom chamber. After incubation, T cells were analyzed by flow cytometry to determine the activation status of total T cells, CD8\(^+\) T, and CD4\(^+\) T cells.

**NK-cell activation assay**

NK cells were prepared as described previously (27). NK cells and the transduced hepa1-6 cells were cocultured for 24 hours. In coculture system, \(2 \times 10^5\) NK cells and \(1 \times 10^5\) hepa1-6 cells (irradiated at 50 Gy) were seeded per well in 96-well plate. In the transwell system (24-well), \(1 \times 10^5\) hepa1-6 cells were placed in the top chamber and \(4 \times 10^5\) NK cells were seeded in the bottom chamber. After incubation, NK cells were analyzed by flow cytometry to determine their activation status.

**Depletion of CD8\(^+\) T/NK cells**

To deplete CD8\(^+\) T and NK cells, the mice were injected intraperitoneally with anti-CD8a mAb (150 \(\mu\)g/mouse) and anti-NK1.1 (CD161) mAb (150 \(\mu\)g/mouse) once every week. Depletions of CD8\(^+\) T and/or NK cells were confirmed by flow cytometric analysis.

**Statistical analysis**

Statistical analyses were performed with Student \(t\) test (unpaired, two-tailed) with GraphPad Prism 5 software (GraphPad). Data were reported as mean ± SD. \(P\) value less than 0.05 was considered as statistically significant.

**Results**

**Membrane IL1\(\alpha\) expression in hepatocellular carcinoma hepa1-6 cells**

To study the role of membrane IL1\(\alpha\), we constructed the membrane IL1\(\alpha\) expression plasmid in which the NLS and calpain cleavage sites were mutated (Fig. 1A). Therefore, the precursor IL1\(\alpha\) could only be expressed on the cell surface as the membrane form without being able to translocalize into the nuclei or be secreted as the mature form. Membrane IL1\(\alpha\) expression was detected and confirmed by confocal microscopy (Fig. 1B). To assess the effect of membrane IL1\(\alpha\) overexpression on tumor cells, we examined the proliferation of the hepa1-6 cells stably expressing membrane IL1\(\alpha\). As shown in Fig. 1C and D, cell proliferation was slightly promoted by membrane IL1\(\alpha\) expression as determined by CCK-8 assay (Fig. 1C) and Ki-67 staining (Fig. 1D).

**Membrane IL1\(\alpha\) exerts an antitumor effect in murine hepatocellular carcinoma models**

To assess the role of membrane IL1\(\alpha\) in vivo, we established murine hepatocellular carcinoma models by subcutaneously or hydrodynamically injecting hepa1-6 cells stably expressing membrane IL1\(\alpha\) or vector control in C57BL/6 mice. In both subcutaneous and orthotopic hepatocellular carcinoma models, tumor growth was greatly reduced by membrane IL1\(\alpha\) expression (Fig. 2A and B). To further determine the role of membrane IL1\(\alpha\) in tumor development, we hydrodynamically injected the membrane IL1\(\alpha\) MC plasmids to overexpress membrane IL1\(\alpha\) in hepatocytes in a DEN-induced hepatocellular carcinoma model. We previously confirmed membrane IL1\(\alpha\) expression on the surface of hepatocytes after hydrodynamic injection and the expressed membrane IL1\(\alpha\) could not be secreted nor detected in the peripheral blood (28). The results showed that tumor growth...
in mice with membrane IL1α overexpression occurred at a much slower rate than in control mice (Fig. 2C). These data demonstrated that membrane IL1α inhibited hepatocellular carcinoma development in murine hepatocellular carcinoma models.

Membrane IL1α promotes T-cell and NK-cell activation in vivo

To understand the mechanisms of antitumor effect of membrane IL1α, we analyzed the percentage and number of different immune cell subsets and their activation status in orthotopic tumor-bearing mice on day 7 after tumor cell inoculation by flow cytometry. The percentages and numbers of CD4+ and CD8+ T cells in spleens and livers were not significantly affected by membrane IL1α expression (data not shown). However, the percentage of CD69+ CD4+ T cells was increased in both spleens and livers, with significant increase in numbers in the livers when membrane IL1α was overexpressed (Fig. 3A and B). The percentages and numbers of effector CD4+ and CD8+ T cells were upregulated in both spleens and livers, whereas the percentages and numbers of naïve CD4+ and CD8+ T cells were decreased in membrane IL1α group (Fig. 3A and B). These results suggested that overexpression of membrane IL1α promoted the activation of CD4+ and CD8+ T cells.

The percentage and number of NK cells were also higher in both spleens and livers in the membrane IL1α group (Fig. 3C and D). While the percentage of activated NK cells out of total NK cells was significantly decreased in the spleens, the numbers of activated NK cells were increased in spleens and livers due to membrane IL1α expression (Fig. 3C and D). These data demonstrated that NK-cell activation was also enhanced by membrane IL1α expression. In addition, the percentages of DCs and
macrophages were increased in the spleens and their numbers were increased in the livers with membrane IL1α overexpression (Fig. 3E and F).

Next, we examined TNFα and IFNγ production by T and NK cells. The percentages and numbers of CD8+ T and NK cells in the spleens were significantly increased in the spleens of the tumor-bearing mice overexpressing membrane IL1α (Fig. 4A). However, TNFα and IFNγ production by CD8+ T and NK cells in the livers were not significantly affected by membrane IL1α expression (Fig. 4B). Serum IL1α, IL1β, IL4, IL6, IL17A, TNF, and IFNγ levels were determined by CBA soluble protein flex sets (Fig. 4C). Serum TNF level was elevated, whereas IL6 level was decreased because of membrane IL1α expression. Moreover, CTLs and NK cells isolated from the tumor-bearing mice overexpressing membrane IL1α had increased killing capacity compared with those from control mice (Fig. 4D). While CTLs only exhibited cytotoxicity to hepa1-6 cells, NK cells exhibited killing capacity against both hepa1-6 and YAC-1 targets. These data suggested that membrane IL1α overexpression promoted antitumor immune responses through T- and NK-cell activation.

**Membrane IL1α directly promotes T- and NK-cell activation in vitro**

Previous studies have demonstrated that T cells and NK cells express IL1R1 (29–31). We observed the same results that about 70% of the NK cells and over 50% of the T cells in the liver of the
tumor-bearing mice expressed IL-1R1 (Supplemental Fig. S1A). We also found that IL1R1 was expressed on hepa1-6 cells (Supplemental Fig. S1B). To investigate whether membrane IL1α could directly promote T- and NK-cell activation, we stimulated T and NK cells with anti-CD3/anti-CD28 or IL2 and analyzed the activation status of T and NK cells cocultured with hepa1-6 cells expressing membrane IL1α or control vector. Flow cytometry analysis revealed that the percentage of activated NK cells was elevated by membrane IL1α expression (Fig. 5A), suggesting that membrane IL1α could directly activate NK cells. The percentages of both activated (CD69+) naive, effector, and memory CD4+ T cells and CD8+ T cells were analyzed by flow cytometry. D, naive T cells sorted from splenocytes were cocultured with hepa1-6 cells expressing membrane IL1α in separate chambers of transwell plate. Activated (CD69+), naive, effector, and memory CD4+ T cells and CD8+ T cells were analyzed by flow cytometry. The data shown are representative of three experiments. Values are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

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Figure 5. Membrane IL1α directly activates NK and T cells in vitro. A, NK cells were cocultured with irradiated hepa1-6 cells expressing membrane IL1α and the activation status was analyzed by flow cytometry. B, NK cells were cocultured with hepa1-6 cells expressing membrane IL1α in separate chambers of transwell plate, and the activation status was analyzed by flow cytometry. C, naive T cells sorted from splenocytes were cocultured with irradiated hepa1-6 cells expressing membrane IL1α in a plate coated with anti-CD5 and anti-CD28. Activated (CD69+), naive, effector, and memory CD4+ T cells and CD8+ T cells were analyzed by flow cytometry. D, naive T cells sorted from splenocytes were cocultured with hepa1-6 cells expressing membrane IL1α in separate chambers of transwell plate. Activated (CD69+), naive, effector, and memory CD4+ T cells and CD8+ T cells were analyzed by flow cytometry. The data shown are representative of three experiments. Values are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

Depletion both of CD8+ T and NK cells diminished the antitumor effect of membrane IL1α

To further evaluate the mechanisms of the antitumor activity of membrane IL1α, we depleted the CD8+ T and/or NK cells in orthotopic tumor-bearing mice in vivo. The depletions were confirmed by flow cytometry (Fig. 6A), and our results showed that depletions of either CD8+ T or NK cells did not affect the antitumor activity of membrane IL1α. However, the antitumor effect of membrane IL1α was totally diminished when both CD8+
T and NK cells were depleted (Fig. 6B). These results demonstrated that membrane IL1α exerted its antitumor effect through CD8+ T- or NK-cell activation.

**Discussion**

IL1α can be myristoylated and expressed on the membranes of various cells including monocytes and macrophages, by a lectin-like association (11, 12, 32). Hepatocytes can also express IL1α (15). Cell-associated IL1α expression could be detected in the liver samples of hepatocellular carcinoma patients, both in the tumor and adjacent normal tissues by confocal microscopy (Supplementary Fig. S2). Although the membrane localization could not be confirmed with the human samples, previous studies have shown that approximately 10% to 15% of translated IL1α is myristoylated and anchored to the cell membrane (13, 33). However, how the expression of membrane IL1α is regulated and its role during the hepatocellular carcinoma development are still not known and need further investigation. In the current study, we provided evidence that membrane IL1α inhibited tumor development in murine hepatocellular carcinoma models. We established hepatocellular carcinoma cell lines stably expressing membrane IL1α by mutating its NLS and calpain cleavage site and generated orthotopic hepatocellular carcinoma model by hydrodynamically injecting the stable cell line. As hepatocellular carcinoma tumor cells expressing membrane IL1α exhibited accelerated proliferation, the tumor-inhibiting effect of membrane IL1α was not due to cell-intrinsic mechanism. Furthermore, we demonstrated that membrane IL1α exerted antitumor effects by promoting T- and NK-cell activation, which required cell–cell contact.

In the tumor microenvironment, cytokines can modulate antitumor responses, yet they can also stimulate angiogenesis, tumor progression, and metastasis, and maintain tumor-promoting inflammation (34, 35). IL1α has been shown to be involved in inflammation and tumor development (36–38). Previously, IL1α was found to stimulate cell growth of several types of malignant cells in some cases without being secreted by the cells (13). Another study also demonstrated that IL1α released from necrotic hepatocytes promoted the development of hepatocellular carcinoma (15). Interestingly, membrane IL1α is thought to be the major source of the secreted IL1α from necrotic hepatocytes during chronic inflammation and tumor development. However, overexpression of membrane IL1α in hepatocytes in a DEN-induced hepatocellular carcinoma model did not increase the incidence of hepatocellular carcinoma, but rather, prevented tumor development. The serum IL1α levels were not elevated by membrane IL1α.
overexpression in DEN-induced (data not shown) or orthotopic tumor model (Fig. 4C), suggesting that the release of IL1α from necrotic cells may be an active process originating from IL1α precursor instead of passive release from membrane IL1α. Our results certainly suggested that the function of membrane IL1α was different from the IL1α released from necrotic hepatocytes during hepatocellular carcinoma development. We showed that membrane IL1α promoted the activation of T and NK cells. Potentially, membrane IL1α may serve as a cell-associated costimulatory molecule for activation of T cells and NK cells by binding to IL1R1. Fibrosarcoma cells expressing cell-associated IL1α lose their tumorigenicity and it is dependent on CD8+ T cells (14, 16). NK cells and activated macrophages also play a role in eradication of IL1α-positive fibrosarcomas. We showed in our in vitro studies that the promotion of T-cell activation by membrane IL1α required cell–cell contact. This result further supports the role of membrane IL1α as a cell-associated costimulatory molecule for T-cell activation. Many cell types in the liver express IL1Rα, including DCs and macrophages. IL1α could function on DCs and macrophages to upregulate the expression of cytokines that promote T-cell functions, including IFNγ and IL12. Previous studies indicate that tumor cell–associated IL1α promotes antigen presentation by the tumor cells themselves, possibly through IFNγ-induced MHC class II expression or cross-presentation by professional antigen-presentation cells (39). We observed increases in percentages and numbers of DCs and macrophages in spleen and liver by membrane IL1α expression (Fig. 3E and F). Therefore, DCs and macrophages may also be modulated by membrane IL1α to facilitate T-cell activation.

Membrane-associated cytokines expressed on engineered tumor cells, such as IFNγ, TNFα, M-CSF, and IL12, have been shown to increase the immunogenicity of malignant cells (33, 40, 41). Therefore, inducing the right inflammation in the tumor microenvironment may be an ideal strategy for cancer immunotherapy by increasing the immunogenicity of the tumor. The injection of fibrosarcoma-expressing cell-associated IL1α could generate long-term tumor-specific memory (14). Therefore, membrane IL1α could potentially be incorporated in certain tumor vaccines to promote antitumor immune responses. Further studies on the mechanisms of different IL1α molecules on malignant processes will be helpful for understanding tumor–host interactions and generating novel strategies for tumor immunotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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