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REVIEW ARTICLE

Inflammatory Immune Responses in Tumor Microenvironment and Metastasis of Hepatocellular Carcinoma

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Abstract Metastasis is a multistage process that requires cancer cells to escape from the primary tumor, survive in the circulation, seed at distant sites and grow. Each of these processes involves rate-limiting steps that are influenced by non-malignant cells of the tumor microenvironment. There are growing evidences that tumors are sustained and promoted by inflammatory signals from the surrounding microenvironment. This review describes experimental data demonstrating the role of the inflammatory immune responses of microenvironment in metastases of hepatocellular carcinoma (HCC), points out the prospective areas for future research and possible new therapeutic approaches to control the metastasis of HCC.

Keywords Cancer metastasis · Microenvironment · Inflammatory response · Th1/Th2 cytokines · Hepatocellular carcinoma (HCC) · Prognosis

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [1, 2]. Although during the past decades, much advances have been obtained in HCC studies including early detection, surgical resection and liver transplantation, the general prognosis of population with HCC still remains extremely dismal [3]. The natural survival time of patients with advanced HCC is only about 3 months [4]. The dismal outcome is attributed to the higher possibilities of metastatic recurrence after treatments which are resulted from the intrahepatic spreading, especially venous metastases, or multiple de novo tumors related to liver disease background [3]. A major hallmark of an aggressive solitary HCC is its ability to metastasize. Understanding the mechanisms underlying this process would allow for the development of effective approaches to reduce HCC mortality.

Metastasis is responsible for as much as 90 % of cancer-associated mortality, yet it remains the most poorly understood component of cancer pathogenesis [5]. Metastasis is a complex multistep process that involves alterations in dissemination, invasion, survival, and growth of new cancer cell colonies and the development of cancer-associated vasculature [5, 6]. Recently, this complex metastatic cascade was simplified into two major phases: (i) physical translocation of a cancer cell from the primary tumor to the microenvironment of a distant tissue and then (ii) colonization; both of them include the cross-talk between cancer cells and their surrounding microenvironments [5–7].

Cumulative evidence suggests that the cross-talk between tumor cells and the surrounding tumor microenvironments plays fundamental roles in the processes of tumor invasion and metastasis of HCC. The microenvironment of HCC is composed of hepatic stellate cells, fibroblasts, invading inflammatory/immune cells (including regulatory and cytotoxic T cells and tumor-associated macrophages), endothelial cells (ECs), pericytes adjacent to the ECs, and extensive extracellular matrix (ECM) components [8, 9]. These components of the microenvironment interact with each other and HCC cells directly or indirectly in order to acquire an abnormal phenotype and alter functions of HCC cells. Therefore, this aberrant microenvironment may play an important role in growth, invasion and metastasis of HCC cells. There have been many reviews that highlight the roles of tumor microenvironment in the development and progression of HCC [9]. In this review, we summarize the findings of our previous studies and focus on the significances of the

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immune/inflammatory responses of tumor microenvironment in the metastasis and molecular classification of HCC.

An Anti-Inflammatory Status in Tumor Microenvironment Facilitating HCC Metastasis

Accumulating evidence indicates that chronic inflammatory diseases result in a predisposition to various types of cancers including HCC [10]. An inflammatory component is present in the microenvironment of most neoplastic tissues, including those that are not causally related to an obvious inflammatory process. Inflammation has been regarded as a new hallmark of cancer [11]. HCC is usually present in inflamed fibrotic and/or cirrhotic liver due to chronic hepatitis. Thus, it is possible that HCC metastatic propensity may be determined and/or influenced by the local inflammatory response in tissue microenvironment [12].

The Inflammatory Response of the Surrounding Tumor Milieu Plays an Important Role in Promoting HCC Metastasis

Global gene expression profiling has revealed that the tumor microenvironment is an important component in the biologic and prognostic classification of HCC. To determine the role of the hepatic microenvironment in HCC metastasis, we collaborated with the National Cancer Institute of USA, to compare the gene expression profiles of noncancerous surrounding hepatic tissues from those with primary HCC with intra- or extra-hepatic metastases and those with HCC without detectable metastases, and demonstrated that livers bearing metastatic HCC have a significantly different gene expression profile compared with the livers bearing non-metastatic HCC [13]. Moreover, this unique change in the gene expression profiles associated with a metastatic phenotype from the noncancerous hepatic tissues is principally different from the tumor signature. Over 30 % of them are associated with either inflammation and/or immune response functions. These suggest that the metastatic potential and process of HCC may be influenced by the immune status of hepatic tissues [13].

Interestingly, this metastasis-related immune status change in the tumor-surrounding tissues is independent of the overall inflammation status of liver tissues based on histological activity index [13]. This indicates that besides the inflammatory response, some other factors including immune cells and the genetic constitution may also play an important role in affecting the levels and functional roles of cytokines. Several studies have identified cytokine gene polymorphisms, which are functionally associated with liver disease and/or HCC, and contribute to the susceptibility of certain individuals to cancer development [14, 15]. Some genetic alterations occurring in tumor cells are also able to activate an inflammatory program that has a profound impact on cancer development and aggressiveness [16]. Oncogenic transcription factors such as NF-κB and STAT3, together with inflammatory cytokines, have been defined as key orchestrators of the intricate dialogue among the components of the immune system present in the tumor microenvironment and cancer cells [16, 17]. Oncogenic β-catenin signaling produces an inflammatory microenvironment by inducing an inflammatory program in hepatocytes that involved direct transcriptional control by β-catenin and activation of the NF-κB pathway which determine the degree of tumor aggressiveness, and has an impact on HCC metastasis [18].

A Profound Switch of Th1-Th2 Cytokine Profiles in the Liver Microenvironment Promotes HCC Metastasis

A significant increase in the anti-inflammatory cytokines such as IL-4, IL-5, IL-8, and IL-10 (Th2 cytokines), a concomitant decrease in the pro-inflammatory cytokines such as tumor necrosis factor (TNF), interferon (IFN)-γ, and IL-1 (Th1 cytokines) was found in livers with metastatic HCC compared with the normal liver. Such a profound Th1-Th2 cytokine profile switch is unique to hepatic tissues from metastatic HCC patients and is not related to the degree of viral hepatitis or cirrhosis, nor is it a consequence of tumor burden. These imply that an anti-inflammatory status occurs in patients with metastatic HCC, and promote HCC metastasis [13].

IL-1 is a proinflammatory cytokine that promotes hepatic stellate cell (HSC) proliferation, activation, and transdifferentiation into the myofibroblastic phenotype in addition to activating HSCs to produce and activate MMPs, particularly MMP-9 [19]. TNF-α is a multifunctional cytokine produced mainly by Kupffer cells and other immune cells and is an essential cytokine for liver regeneration following liver injury due to the activation of its downstream NF-κB and Akt pathways [20]. IL-8 plays the critical role in the initiation of micro-environmental inflammation responsible for tumor growth and patient prognosis. In inflammatory microenvironment, HCC produces IL-8 through p38 MAPK, ERK and PI3K/Akt signaling pathways [21].

Besides, many other inflammatory cytokines have also been demonstrated to associate with HCC progression. IL-6 is a multifunctional inflammatory cytokine produced by Kupffer cells in the liver in response to hepatocyte death that contributes to compensatory hepatocyte proliferation [22]. Recent data show that parenchymal liver cells are an additional source of high levels of IL-6 in the HBV-infected liver microenvironment, and HBx could involve in HBV-mediated liver carcinogenesis through stimulating the production of IL-6 in a MyD88-dependent manner [23]. The antitumor effect of IL-12 is thought to be mediated by the

activation of tumor specific cytotoxic T lymphocytes and NK cells, and inhibition of angiogenesis [24]. IL-22, one of the cytokines secreted by Th17 cells, is a novel inflammation driver through STAT3 signaling activation. Excessive IL-22 is found in the HCC microenvironment, leading to tumor growth, inhibition of apoptosis, and promotion of metastasis due to STAT3 activation [25].

The Prognostic Values of Immune/Inflammatory Cytokine Signature for HCC Patients

Many studies have consistently revealed the significance of the tumor microenvironment in the biological and prognostic classification of HCC [26–29]. The importance of inflammatory cytokine profiles in the tumor microenvironment has also been recognized in gene expression profiling [30, 31]. The chemotaxis and humoral immune response genes were found to be associated with high risk of recurrence after HCC resection [30]. Very recently, a 14 immune-gene signature, which identifies molecular cues driving tumor infiltration by lymphocytes, has also been established and proved to accurately predict survival of patients with HCC especially in early disease. This signature includes the chemokine genes CXCL10, CCL5 and CCL2, whose expression correlates with markers of Th1, CD8 (+) T and natural killer (NK) cells. Inflammatory cytokines (TNF α, IFN γ) and Toll-like receptor 3 ligands stimulate intratumoural production of these chemokines which drive tumor infiltration by T and NK cells, leading to enhanced cancer cell death [32].

Using the different expressed genes in the liver tissues mentioned above, we constructed a refined expression signature containing 17 genes (12 Th1/Th2 cytokines, HLA-DR, HLA-DPA, ANXA1, PRG1, and CSF1) that could successfully predict both venous metastases and extrahepatic metastases by follow-up with >92 % overall accuracy. Kaplan-Meier survival or recurrence analysis based on the 17 gene prediction results indicated that the predicted metastatic group had a significantly shorter survival period and a significantly shorter period for recurrence when compared to the nonmetastatic group [13]. Moreover, the prognostic performance of this signature was superior to and independent of any of the clinical variables including patient age, tumor size, microvascular invasion, a-fetoprotein (AFP), albumin, Child-Pugh score, and several staging systems (TNM, CLIP, BCLC, and Okuda) [13].

Based on the 17-immune/inflammatory gene signature, we further evaluated the prognostic values of intratumoral and peritumoral Th1/Th2 cytokine protein levels in two independent cohorts of 453 HCC patients after curative resection using enzyme-linked immunosorbent assays (ELISA), and tissue microarrays and immunohistochemical staining. We found that higher IL-2 and IL-15 levels in peritumoral liver tissues, but not in tumor tissues, were significantly associated with a decreased incidence of intrahepatic tumor recurrence and a prolonged overall survival (OS). More importantly, the IL-2 and IL-15 can also predict outcome in patients with early-stage HCC without microvascular invasion for whom current clinical staging systems fail to provide an accurate prognostic assessment. Univariate and multivariate analyses indicated that the prognostic performance of peritumoral IL-2 and IL-15 was independent of other clinicopathological factors. These suggest that the peritumoral IL-2 and IL-15 levels are useful for stratifying patients, even those with early stage HCC, into subgroups with different prognoses after curative resection [33]. IL-2 and IL-15 belong to the group of cytokines whose receptors use the common γ chain and may induce a similar spectrum of biological activities, such as stimulating the proliferation of T cell subsets or augmenting cytotoxicity of T and NK cells. Both cytokines belong to Th1 families, suggesting that a stronger Th1-type immune response may, at least in part, inhibit tumor relapse [33].

HCC recurrences derive from residual intrahepatic metastasis or multicentric carcinogenesis. Based on the occurring time after HCC treatments, tumor recurrences are divided into early and late recurrence. Usually, early recurrence (occur within 2 years after HCC treatment) is mainly attributed to the intrahepatic dissemination of metastatic HCC cells; in contrast, late recurrence is thought to originate de novo in at risk liver [3]. Gene expression signatures from the adjacent benign tissue were reported to predict late recurrence of HCC. Okamoto et al. identified a specific gene profile in noncancerous liver tissue including 36 genes could predict multicentric occurrence or late recurrence of HCV-related HCC [29]. Hoshida et al. have also generated a 132-gene signature from noncancerous liver tissues for predicting late recurrences and prognosis in patients with relatively early HCC. This signature was characterized by inflammation-associated pathways and growth factors including NF-κB, TNF-α, and IL-6 [31]. In our study, we found that the peritumoral IL-2 level was associated with both early recurrence and late recurrence. These suggest that immune response-related factors, at least IL-2, play an important role in the prevention of HCC recurrences originating from the residual intrahepatic metastases, as well as the de novo cancer in cirrhotic liver [33].

The Significances of Imbalance of Immune Cells in Tumor Microenvironment in the Metastasis and Prognosis of HCC

The imbalance of immune cells in the tumor microenvironment is another important regulator of progression in many

cancers, including HCC. Increasing evidence indicates the immunosuppressive nature of the local environment in tumor, the tumor microenvironment of HCC is featured by the presence of multiple immunosuppressive factors [34]. Tumor-infiltrating lymphocytes (TILs), the primary immune component infiltrating solid tumors, are considered to be a manifestation of the host antitumor reaction. The majority of TILs in solid tumors are of the CD3+ T-cell phenotype. CD3+ T cells can be stratified further into CD4+ helper cells (including the Th1 and Th2 subtypes based on their cytokine profile), CD4+ regulatory T cells (Tregs), previously designated as suppressor cells, and CD8+ cytotoxic effector cells [35]. The role of CD4+ T cells in the host antitumor response is an area of considerable debate.

Regulatory T (Treg) Cells

Tregs, which accounts for 5–10 % of all CD4+ cells, is generating intense interest in tumor immunology, autoimmunity, and infectious disease. There is now considerable evidence that FOXP3 is a key control molecule for Treg development and function, and is an excellent marker for the study of Tregs. Tregs seem to be a detrimental factor in the generation of host-versus-tumor immunity via suppression of tumor specific effector T-cell responses and development of immune tolerance to neoplastic cells [35]. Increased numbers of Tregs infiltrating tumor cell nests have been demonstrated in many kinds of solid tumors including HCC, which impair cytotoxic CD8+ T (CTL) cell proliferation, activation, degranulation, and production of granzyme A, granzyme B, and perforin [35, 36]. Low intratumoral CTL and high Treg cell numbers are associated with a worse prognosis in HCC patients [36]. In the study from the author's institute, Gao et al. take a more comprehensive approach was took to determine the prognostic significance of Tregs, and demonstrated that the type (ratio of activated cytotoxic granzyme B–positive CD8+ T lymphocytes to FOXP3+ Tregs), density (high *vs.* low expression of cytotoxic or regulatory molecules), and location (lymphocytes within HCC tumor cell nests as opposed to the surrounding stroma) are all critical factors in the assessment of TILs and in the determination of their prognostic impact. And intratumoral imbalance toward Tregs (high Tregs/low activated CD8+ CTLs) is an independent predictor for recurrence and survival in HCC, the impact of this balance on patient survival was larger than that of the number of intraepithelial CD8+ or Treg cells alone [37]. Moreover, overexpression of human leukocyte antigen-G (HLA-G) which is a tumor-associated immunosuppressive molecule involved in tumor escape mechanisms add the prognostic power of Tregs/CD8+ ratio [38]. Chen et al. also found that FoxP3 (+) Tregs were highly aggregated and in an activated phenotype (CD69 (+) HLA-DR (high)) in the tumor site, where they can suppress the proliferation and INF-γ secretion of CD4 (+) CD25 (−) T cells. The increased tumor-infiltrating Tregs predicted poorer prognosis in HCC patients [39]. Recently, Gao et al. found that the intratumoral CD45RO (+)/peritumoral CD57 (+) (memory/senescent T cell) ratio is of vital importance in preventing HCC extrahepatic metastasis and in particular demonstrates its independent prognostic value in liver transplant recipients [40].

Accumulating evidence suggests a critical role of the increased Tregs in dampening the NK cell immune response [41]. Tregs selectively express membrane-bound transforming growth factor-beta (TGF-β), which downregulates NKG2D (natural killer group 2, member D) expression on NK cells, and suppresses NK cell effector functions, i.e. homeostatic proliferation, cytotoxicity, and IL-12-mediated IFN-γ production [42]. Recently, a complex differential regulation of human NK activity by tumor induced Treg (iTreg) cells in the tumor microenvironment was reported [43]. In contrast to the naturally occurring Treg cells, tumor iTreg cells inhibit IL-2-mediated NK-cell activity in the absence of target cells, whereas enhance the tumoricidal activity of NK cells by target cell contact [43].

Tumor-Associated Macrophages (TAMs)

Macrophages are a major component of the infiltrate of most tumors, which have multiple biological roles, including antigen presentation, target cell cytotoxicity, removal of foreign bodies, tissue remodeling, inflammation regulation, immunity induction, thrombosis, and endocytosis. Tumor-associated macrophages (TAMs) have been found to be associated with tumor progression. Recent immunological studies have identified two distinct states of polarized macrophage activation: the classically activated (M1) and the alternatively activated (M2) macrophage phenotypes. Bacterial moieties such as lipopolysaccharides and the Th1 cytokine interferon-γ polarize macrophages toward the M1 phenotype. The M2 polarization was discovered as a response to the Th2 cytokine IL-4. In general, M2 macrophages exert immunoregulatory activity, participate in polarized Th2 responses, and aid tumor progression [44].

Macrophages are attracted by chemokines, such as macrophage colony stimulating factors (M-CSF). M-CSF can also induce monocytes to produce more Th2 cytokines and fewer Th1 cytokines, after which the macrophages present the so-called M2 phenotype and secrete some growth factors that are essential for tumor growth and invasion, and facilitate colonization and growth of the micrometastasis [45]. TAMs have recently been found to play an important role in HCC progression. As the front line of defense to prevent tumor growth, the peritumoral liver tissue, which is endowed with abundant M-CSF and macrophages, plays an opposite

role by providing a fertilized soil (premetastasis niche) for subclinical metastatic tumor cells. High peritumoral M-CSF level and macrophage density were associated with HCC progression, a high incidence of intrahepatic metastasis, and poor survival after HCC resection, highlighting the importance of peritumoral tissue in the recurrence and metastasis of HCC [46]. In the gene expression profiling of peritumoral liver tissues, we have also found that the Th1 to Th2-like profile switch in livers bearing metastatic HCC are accompanied by an overexpression of CSF1, as well as MHC class II-related genes [13]. These provide further evidences to support that the peritumoral inflammation/immune environment is important in understanding the mechanism of intrahepatic metastasis of HCC and in shaping the postoperative strategy for prevention of recurrence after HCC resection [46].

Although Kupffer cells were initially thought to be involved in antitumor immunity, there is substantial clinical and experimental evidence that suggests that these TAMs enhance tumor progression by impairing cytotoxic CD8+ T cell immune responses, which is thought to be mediated by B7-H1/programmed death-1 interaction [47]. Kupffer cells, as well as stellate cells, when activated by inflammatory cytokines (IL-1, TNF-α, PDGF), produce excessive osteopontin (OPN) that plays a pivotal role in various cell signaling pathways that promote inflammation, tumor progression and metastasis [48]. In our previous studies, OPN is found to be a leading gene in HCC metastasis signatures [49], and has been proved to play important roles in HCC metastasis [50, 51]. These provided more evidences to support that a unique immunological condition regulated by Kupffer cells may promote HCC metastases. In Kupffer cells, NF-κB, the master regulator of inflammatory and immune responses, is an important pathway for the integration of signals from the tumor microenvironment that promote carcinogenesis.

New Approaches Targeting Tumor Microenvironment for HCC Metastasis

Biological agents that target components of the tumor microenvironment may provide an interesting alternative to traditional tumor cell-directed therapy. The unique interplay between the various aspects of the tumor cells and the microenvironment could be a molecular target for tumor treatment [52], and the postoperative adjuvant therapies should target not only the residual tumor cells, but also the soil to make it resistant to tumor growth [13].

Modification of the imbalance in immune/inflammatory response of tumor microenvironment represents a potential therapeutic option for patients with HCC, especially as secondary treatment to prevent recurrence. Current pro-inflammatory-based postoperative therapies to prevent tumor recurrence after surgical treatment of HCC show a beneficial effect. We speculate that postsurgical treatment with IFN-γ or perhaps other Th1-related cytokines in metastatic HCC patients may ameliorate the metastatic-related imbalance of cytokines toward that of nonmetastatic HCC patients. These adjuvant therapies may improve responses by selecting patients identified by the 17 gene predictor as those eligible and most likely to benefit from pro-inflammatory cytokine treatment [13]. We have found that postoperative IFN-α treatment is useful in decreasing HCC recurrence in patients after curative resection of HBV-related HCC [53], this effect is attributed to antiangiogenesis effect rather than the properties of anti-proliferation or anti-virus of IFN-α [54], and patients whose tumors had low miR-26 expression are more likely to benefit from IFN therapy [55]. Our recent study also indicates that IL-2 plays an important role in the prevention of HCC recurrences originating from the residual intrahepatic metastases, as well as the de novo cancer in cirrhotic liver [33].

Recently, physiological doses of estrogen, no matter endogenous or exogenous, are demonstrated to suppress metastasis of HCC through modulation of inflammatory tumor microenvironment by suppression of hepatocyte growth factor (HGF) and IL-6 production [56].

The inflammatory immune microenvironment within HCC tumors can also be an important means to control tumor progression via TIL activation and proliferation [57]. A combination of depletion of Tregs and concomitant stimulation of effector T cells may be an effective immunotherapy to reduce recurrence and prolong survival after surgery [37]. Since the CCL20-CCR6 axis mediates the migration of circulating Tregs into tumor microenvironment, thus, blocking CCL20-CCR6 axis-mediated Treg migration may be a novel therapeutic target for HCC [39].

Obviously, TAMs, particularly macrophages, could also be good targets for adjuvant therapy after HCC resection [46]. In one study from author's institute, Zhang et al. found that macrophages have an important role in tumor progression under sorafenib treatment, it induced a significant increase in peripheral recruitment and intratumoral infiltration of F4/80- and CD11b-positive cells, and elevation of CSF-1, stromal-derived factor 1alpha, and vascular endothelial growth factor (VEGF). Depletion of macrophages by zoledronic acid (ZA) in combination with sorafenib significantly inhibited tumor progression, tumor angiogenesis, and lung metastasis [58].

Summary and Prospects

There are growing evidences that tumors are sustained and promoted by inflammatory signals from the surrounding microenvironment. There have been substantial advances in understanding the importance of the inflammatory/immune

responses of tumor microenvironment in HCC progression, invasion, and metastasis over the past decade. Among of them, the profound switch ofTh1-Th2 cytokine profiles in peritumoral liver tissues, high peritumoral M-CSF level and macrophage density, and the intratumoral imbalance toward Tregs (high Tregs/low activated CD8+ CTLs) seem to be more important in facilitating HCC metastasis, and are useful in the prognostication of HCC.

Therapeutic targeting of cellular and molecular components of the HCC microenvironment, reverse the imbalances of both immune/inflammatory cytokines and immune cells provide interesting alternatives to traditional tumor cell-directed therapy. Current pro-inflammatory-based therapies and depletion of macrophages have been proven to be helpful in the prevention and control of HCC metastatic recurrence after hepatectomy.

HCC recurrences derive from residual intrahepatic metastasis or multicentric carcinogenesis. A better understanding of the important roles of immune/inflammatory response in these two kinds of HCC recurrence, and the biological and molecular interactions between each element of the tumor microenvironment and HCC cells is critical in the development of additional effective treatments of HCC metastasis and recurrences. Therapeutic vaccines are helpful strategy and deserve more attention.

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