Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma

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Background & Aims: Substantial evidence indicates that inflammation is a critical component of tumor progression. Hepatocellular carcinoma (HCC) is usually derived from inflamed cirrhotic liver with extensive leukocyte infiltration. Neutrophils are the common inflammatory infiltrate in tumors, but their nature and regulation in human cancers remain elusive.

Methods: A total of 238 HCC patients were enrolled randomly. Immunohistochemistry and SuperArray Real-Time PCR were used to analyze the distribution and clinical relevance of neutrophils in different microanatomical areas. The regulation and function of neutrophils were assessed by both in vitro and in vivo studies.

Results: Neutrophils were enriched predominantly in peritumoral stroma of HCC tissues and their levels could serve as a powerful predictor for poor survival in HCC patients. Proinflammatory IL-17 is a critical mediator of the recruitment of neutrophils into peritumoral stroma of HCC tissues by epithelial cell-derived CXC chemokines. The accumulated peritumoral neutrophils were the major source of matrix metalloproteinase-9 in HCC tissues; this secreted protein stimulated proangiogenic activity in hepatoma cells. Accordingly, high infiltration of peritumoral neutrophils was positively correlated with angiogenesis progression at tumor-invading edge of HCC patients. Furthermore, we found that selective depletion of neutrophils effectively inhibited tumor angiogenesis and growth, in vivo.

Conclusions: These data provide direct evidence supporting the critical role of neutrophils in human tumor progression and reveal a fine-tuned collaborative action between cancer cells and immune cells in distinct tumor milieu, which reroutes the inflammatory response into a tumor-promoting direction.

Introduction

Tumor progression is now recognized as the product of evolving crosstalk between different cell types within the tumor and its stroma [1,2]. There is substantial evidence that the proinflammatory response at the tumor stroma could be rerouted into a tumor-promoting direction by stimulating angiogenesis and tissue remodeling [3–5]. We have recently found that proinflammatory IL-17-producing cells accumulate in tumors from patients with hepatocellular carcinoma (HCC) and that their levels are positively correlated with microvessel density in tissues and poor survival in HCC patients [5,6]. However, the mechanisms that allow IL-17 to foster angiogenesis and promote tumor progression in humans are unclear.

Human neutrophils are the most abundant leukocytes and serve as key effectors in the first-line host defense against infectious microorganisms [7,8]. In addition to direct bactericidal activities, neutrophils can actively regulate angiogenesis and tissue remodeling by releasing multiple proteases [7,9]. Increased levels of neutrophils have been observed in several types of human tumor, and studies in mice indicate that, depending on microenvironment, tumor-infiltrating neutrophils are capable of being pro- or anti-tumorigenic [10–12]. However, direct evidence supporting a role for neutrophils in the immunopathogenesis of human cancers is still lacking, as is specific knowledge of the trafficking mechanisms utilized by neutrophils.

HCC is the fifth most common cancer worldwide, with an extremely poor prognosis [13–16]. By using HCC as a model sys-
tem, the present results from both clinical sample analysis and experimental studies showed that IL-17 recruited neutrophils into peritumoral stroma of HCC by epithelium-derived CXC chemokines. The accumulated peritumoral neutrophils were the major source of matrix metalloproteinase (MMP)-9, which in turn may stimulate proangiogenic activity of hepatoma cells at the adjacent invading edge. These data provide direct evidence supporting the important role of neutrophils in the immunopathogenesis of human cancers via rerouting of inflammation in a protumoral direction.

**Patients and methods**

**Patients and specimens**

Detailed information about the patients and specimens is provided in Supplementary Table 1 and Supplementary methods.

**Immunohistochemistry and immunofluorescence**

Paraffin-embedded samples were then processed for immunohistochemistry as previously described [17]. Detailed information is provided in Supplementary methods.

**Evaluation of immunohistochemical variables**

Analysis was performed by two independent observers, as previously described [4,6].

**Neutrophil isolation and culture**

Neutrophil isolation and culture is described in Supplementary methods.

**SuperArray Real-time PCR**

SuperArray Real-time PCR is described in Supplementary methods.

**Real-time PCR**

Real-time PCR is described in Supplementary methods and Supplementary Table 2.

**Neutrophil migration assay**

Neutrophil migration assay is described in Supplementary methods.

**Detection of MMP-2 and MMP-9 activity by gelatin zymography**

Gelatin zymography is described in Supplementary methods.

**Angiogenic tube formation**

The tube formation assay was done using HUVECs, as described previously [18], in the presence of serum-free conditioned media from neutrophils, HepG2 cells, or HepG2 cells exposed to neutrophil-conditioned medium.

**In vivo neutrophil inhibition**

In vivo neutrophil inhibition is described in Supplementary methods.

**Statistical analysis**

Statistical analysis is described in Supplementary methods.

**Results**

Accumulation of neutrophils in peritumoral stroma of HCC patients fosters disease progression and predicts poor survival

To evaluate the potential role of neutrophils in tumor immunopathology, we first investigated their infiltration and distribution in human HCC. The presence of neutrophils was visualized by immunohistochemical staining of CD15 in paraffin-embedded tissues from 200 untreated HCC patients. As shown in Fig. 1A, neutrophils were present throughout the tissue but often predominant in the peritumoral stroma rather than in the cancer nests (72.8 ± 3.9 and 8.7 ± 1.7 cells/field, respectively; n = 200 for both; p < 0.0001; Fig. 1B). The number of CD15+ cells in both nontumoral tissue and peritumoral stroma was significantly increased and correlated with disease progression in HCC patients (stage I [n = 146] vs. stages II-IV [n = 54]; p <0.01 for both tissue areas; Fig. 1B).

![Fig. 1. Accumulation of neutrophils in peritumoral stroma fosters disease progression and predicts reduced survival in HCC patients.](image-url)
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Based on the above observations, we predicted that the presence of peritumoral stromal CD15+ cells would have an adverse effect on survival. To test this assumption, 200 HCC patients, who had received curative resection with follow-up data, were divided into two groups according to the median value of CD15 density in different areas. There was a striking inverse association between CD15+ cell density in the peritumoral stroma and both overall survival (OS) and disease-free survival (DFS) (p < 0.001 for both; Fig. 1C). By contrast, CD15+ cells in the nontumoral or intratumoral area were unrelated to the prognosis of either OS or DFS (Fig. 1C). The peritumoral stromal CD15+ cell density was also associated with tumor size (p = 0.013), intrahepatic metastasis (p = 0.014), and TNM stage (p = 0.017) (Supplementary Table 3). Multivariate analysis revealed that the number of CD15+ cells in the peritumoral stroma was an independent prognostic factor for both OS and DFS (Supplementary Table 4).

To further evaluate the prognostic role of peritumoral stromal CD15+ cells in different subgroups, patients were stratified according to tumor size, TNM stage, and intrahepatic metastasis. As expected, a high density of peritumoral stromal CD15+ cells remained predictive of worse survival in all of the above subgroups (Supplementary Fig. 1 and Table 5) and could, therefore, serve as a powerful prognostic factor for HCC patients within different risk groups.

Peritumoral stromal IL-17+ cells recruit neutrophils through epithelial cell-derived CXC chemokines

At the site of inflammation, the accumulated neutrophils not only exert microbiocidal effects but also contribute to angiogenesis and tissue remodeling [7,8]. We recently found that proinflammatory IL-17+ cells are enriched in HCC tissue, where they promote disease progression by fostering angiogenesis [5,6]. Therefore, we next investigated the association between CD15+ cells and IL-17+ cells in human HCC, paying particular attention to the peritumoral stroma surrounding the cancer nests (Fig. 3A). We found that both CD15+ cells and IL-17+ cells accumulated in the peritumoral stroma (Fig. 2A), and a significant correlation between levels of CD15+ cells and IL-17+ cells was found in that area (r = 0.791, p < 0.001; Fig. 2A). Notably, we only detected marginal levels of CD15 and IL-17 signals in the cancer nests (Figs. 1A and 2A).

To investigate whether IL-17 signals are involved in the accumulation of neutrophils in HCC, we first established an in vitro system to examine the effect of IL-17 or liver microenvironment on neutrophil migration. IL-17 or the culture supernatants from normal liver cells, hepatoma cells alone, or a combination of the two had no direct effect on the migration of neutrophils across the tumor vasculature is mainly mediated by CXC chemokines that bind to and activate CXCR1 and/or CXCR2 [19]. Therefore, we used SuperArray Real-Time PCR to examine the chemokine profiles of IL-17-treated liver epithelial cells (LO2). As shown in Fig. 2C, exposure of LO2 cells to IL-17 markedly upregulated the expression of several CXC chemokines, including CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, and CXCL11. By contrast, IL-17 did not stimulate CC chemokine expression in LO2 cells (Fig. 2C). Moreover, neutrophil migration induced by IL-17-treated LO2 cells was markedly attenuated by specific mAbs that block the binding of CXC chemokines to CXCR1 and CXCR2 (Fig. 2D). Similar results were obtained when using specific siRNA (Supplementary Table 6) to inhibit the expression of corresponding CXC chemokines (Fig. 2D). Consistent with these in vitro data, significantly increased expression of several of these chemokines, including CXCL1, CXCL2, CXCL3, and CXCL5, was detected in peritumoral areas as compared with intratumoral areas in HCC patients, and their levels were positively correlated with a high degree of infiltration of IL-17+ cells in peritumoral tissues (Fig. 2E and F).

Tumor-educated neutrophils produce significantly more MMP-9

MMP-9 is known to participate in tumor invasion and metastasis by inducing an angiogenic switch [20–22]. In addition to its role in matrix remodeling, MMP-9 can trigger the generation of angiogenic regulatory molecules as well as the release or activation of sequestered growth factors [22]. We next investigated the expression of MMP-9 in serial sections of human HCC tissues. In all samples analyzed (n = 152), both CD15+ and MMP-9+ cells were often present throughout the tissue but were predominant in the peritumoral stroma surrounding the cancer nests (Fig. 3A). There was a positive association between the densities of CD15+ cells and MMP-9+ cells in the peritumoral stroma (n = 152; linear regression, r = 0.799; p < 0.01). Using confocal microscopy, we confirmed that most (85 ± 7.1%; n = 12) MMP-9 protein was expressed by CD15+ neutrophils in the peritumoral stroma; it was only weakly expressed by other stromal and hepatoma cells (Fig. 3B). By contrast, we only detected marginal expression of MMP-2, the other major gelatinase in the MMP family, in tumor cells, but not in tumor-infiltrating neutrophils (data not shown). Moreover, when cultured with tumor supernatant, neutrophils secreted significantly higher levels of MMP-9 than normal liver cells, hepatoma cells, macrophages or T cells (Fig. 3C). Exposure of neutrophils to 30% HepG2-derived supernatant resulted in a sustained release of MMP-9 that lasted for at least 72 h (Fig. 3D) as well as an inhibition of neutrophil apoptosis (Supplementary Fig. 2). Consistent with the findings in tumor samples, no MMP-2 was detected in tumor-exposed neutrophils or macrophages (data not shown). Collectively, these data indicate that neutrophils in the peritumoral stroma are major sources of MMP-9 in HCC tissues.

Peritumoral stromal neutrophils promote angiogenesis at invading tumor edge via MMP-9 signaling

To examine the role of MMP-9+ neutrophils in tumor angiogenesis, we initially examined the distribution of microvessels in HCC tissues by staining for the vascular endothelial marker CD34. As shown in Fig. 4A, angiogenesis was most active at the invading edge, which was situated close to the peritumoral stroma with abundant MMP-9+/CD15+ cells (Figs. 1A and 3A and B). We next investigated the effect of neutrophils on the angiogenic tube formation of HUVECs. Culture supernatants from neutrophils or untreated hepatoma (HepG2) cells had only a marginal effect on the tube formation of HUVECs. However, culture supernatants...
Fig. 2. Peritumoral stromal IL-17+ cells in HCC recruit neutrophils through epithelium-derived CXC chemokines. (A) Association of CD15+ and IL-17+ cells in the peritumoral stroma of HCC (n = 96). The micrographs at higher magnification show the stained nontumor (1), peritumor (2), and intratumor (3). Scale bar, 150 μM. (B) Soluble factors derived from IL-17-treated L02 cells significantly increased the neutrophil migration across endothelium. Neutrophils migrated across cultured monolayers of HUVECs in the presence of IL-17, the supernatant from L02 cells or IL-17-treated L02 cells (IL17_L02), L02 cell supernatant plus IL-17 (L02 + IL17), or medium alone in the lower chamber of the Transwell system. The illustrated results represent the mean ± SEM of four separate experiments. (C) Fold changes of chemokine mRNA levels in IL-17-treated L02 cells compared with untreated L02 cells were analyzed by SuperArray Real-Time PCR. A representative result from three separate experiments is shown. (D) Silencing of CXC chemokines in IL-17-treated L02 cells or blockade of CXC receptors in neutrophils attenuated the neutrophil migration across the endothelial monolayer. The illustrated results represent the mean ± SEM of four separate experiments. (E) Fold changes of CXC chemokine mRNAs in HCC peritumoral or intratumoral tissues (n = 6) were compared with those in paired nontumoral tissues by Real-Time PCR. (F) Fold changes of CXC chemokine mRNAs in HCC peritumoral tissues with high infiltration of IL-17+ cells (n = 5) were compared with those in peritumoral tissues with low infiltration of IL-17+ cells by Real-Time PCR. (D–F) *p <0.05 and **p <0.01 were considered statistically significant.
from HepG2 cells that had been exposed to neutrophils significantly promoted angiogenic tube formation (Fig. 4B); this effect was attenuated by the anti-MMP-9 antibody (Fig. 4C). These data indicate that soluble factors derived from neutrophils, including MMP-9, play an important role in the angiogenic tube formation.

The potential role of MMP-9+ neutrophils in tumor angiogenesis was further supported by SuperArray analysis for the expression of angiogenesis-associated genes in the invading edge of HCC tissues. Compared to invading tumor edges with no or few infiltrated peritumoral MMP9+ neutrophils, a marked upregulation of a set of proangiogenic genes was found in the invading edge of HCC tissues with a greater accumulation of peritumoral neutrophils (Fig. 4D). Among the 15 significantly upregulated genes (78.9% of 19 differentially expressed genes), all except for TIMP metallopeptidase inhibitor 2 (Timp2) were proangiogenic. Remarkably, transcript levels of CXCL3, CXCL5, and CXCL8 (IL8), which are known to function as both proangiogenic and neutrophil-chemotactic factors, were increased by over seven-fold in tissues showing high infiltration of peritumoral stromal CD15+ cells. Consistent with these results, exposure of HepG2 cells to neutrophils induced a rapid upregulation of several proangiogenic genes, with an expression profile comparable to those observed in the invading edges of HCC tissues (Fig. 4D and E).

**High infiltration of peritumoral stromal neutrophils is correlated with increased VEGF expression and sinusoidal vasculature in HCC**

Expression of vascular endothelial growth factor (VEGF) and concomitant high vessel densities are associated with poor prognosis in several tumor types [23]. Because VEGF was upregulated in HCC tissues with high neutrophil infiltration and in neutrophil-treated HepG2 cells (Fig. 4D and E), we next examined the association between the density of neutrophils and VEGF protein level, as well as the progression of angiogenesis in HCC. As shown in Fig. 5A, patients with high VEGF expression at the invading edge usually had more peritumoral stromal neutrophils than those with low or no VEGF expression (n = 45; p < 0.001; Fig. 5B). By analyzing the morphologic features of microvessels, we found that patients with high levels of peritumoral stromal neutrophils tended to develop a sinusoidal vasculature at the invading edge (n = 142; p < 0.001; Fig. 5C and D); this distinct microvascular architecture promotes blood-borne metastasis in both human and mouse tumors [24]. By contrast, the levels of CD15+ cells in intratumoral areas were unrelated to the expression of VEGF and formation of sinusoidal vasculature in HCC tissues (Fig. 5).

To test the effect of neutrophils on tumor angiogenesis and progression in vivo, we used a specific anti-Gr1 antibody to delete neutrophils in hepatoma-bearing mice. As expected, depletion of neutrophils reduced tumor size and microvessel density at the invading tumor edge, whereas the control antibody had no effect (Supplementary Fig. 3). These findings suggest that tumor-infiltrating neutrophils might regulate the progression of angiogenesis in the tumor-bearing host.

**Discussion**

Despite the generally immnosuppressed status of cancer patients, substantial evidence shows that inflammatory reactions at a tumor site can promote disease progression [3–5]. We recently found that proinflammatory IL-17-producing cells are enriched predominantly in the peritumoral stroma of HCC tissues [6], where they promote tumor progression by fostering angiogenesis [5]. The present study demonstrated that IL-17 induced the migration of neutrophils into HCC through epithelial cell-
Fig. 4. Peritumoral stromal neutrophils regulate tumor angiogenesis at the invading edge via MMP-9. (A) Analysis of capillary distribution in HCC samples by immunohistochemical staining for CD34. One out of 20 representative micrographs is shown. (B) Soluble factors derived from neutrophil-treated tumor cells induced angiogenic tube formation. The tube formation assay was done using HUVECs in the absence (medium) or presence of serum-free conditioned medium from neutrophils, HepG2 cells or HepG2 cells exposed to neutrophil culture supernatant. The illustrated results represent four separate experiments. (C) Anti-MMP9 antibody inhibited the tube formation of HUVECs. The tube formation assay was done using HUVECs in the absence (medium) or presence of serum-free conditioned medium from HepG2 cells exposed to neutrophil culture supernatant alone (control) or supplemented with a MMP-9-blocking antibody or a control antibody (isotype). The illustrated results represent the mean ± SEM of three separate experiments. (D) Fold changes of angiogenesis-related mRNA levels in HCC-invading tumor edges of tissues with high infiltration of peritumoral neutrophils (n = 5) were compared with those of invading tumor edge tissues with no or low infiltration of peritumoral neutrophils (n = 5) using SuperArray Real-Time PCR. Data with significant differences are shown. (E) Fold changes of angiogenesis-related mRNAs in HepG2 cells exposed to neutrophil culture supernatant for the indicated times were compared with those of untreated HepG2 cells using SuperArray Real-Time PCR. The illustrated results represent three separate experiments.
Neutrophils constitute an important component of the leukocyte infiltrate in the tumor stroma [13,19], but the nature and regulation of neutrophils in human tumors remain largely unknown. The present study shows that IL-17 is a critical mediator for recruitment of neutrophils into HCC based on the following findings. First, we observed that both IL-17+ cells and neutrophils accumulated in the peritumoral stroma, with a significant correlation between the densities of these two cell types in that area. Second, exposure of hepatocytes or hepatoma cells to IL-17 resulted in a marked upregulation of several CXC chemokines that are known to attract neutrophils, including CXCL8 (IL-8). Third, culture supernatants from IL-17-treated epithelial cells induced the transmigration of neutrophils across the endothelial monolayer, an effect that was inhibited by blockade of the binding of CXC chemokines to CXCR1 and CXCR2 or by silencing the expression of CXC chemokines. Fourth, consistent with these in vitro data, a high degree of infiltration of IL-17+ cells was associated with increased expression of CXC chemokines in HCC tissues. This notion is supported by a recent report showing that IL-17 could recruit neutrophils in the airway by releasing CXC chemokines [29].

By releasing preformed proteases, neutrophils can actively regulate angiogenesis, the crucial step for tumor progression [7,19]. Although MMP-9 can be produced by many types of cells, human neutrophils are reported to release TIMP-free MMP-9 that provides a potent catalytic stimulator of angiogenesis [30]. We found that neutrophils in the peritumoral stroma were major sources of MMP-9 in HCC. Tumor-activated neutrophils exhibited delayed apoptosis and sustained release of MMP-9. The neutrophil-derived factors, including MMP-9, stimulated proangiogenic activity in hepatoma cells. Consistent with these results, high neutrophil infiltration was correlated with increased VEGF expression and sinusoidal vasculature in HCC tissues, and the depletion of neutrophils markedly inhibited tumor growth and angiogenesis in mice. In addition, exposure to neutrophils upregulated several neutrophil chemoattractants in tumor cells, which could lead to a positive-feedback loop to recruit more neutrophils into the tumor.

In addition to having proangiogenic activity, neutrophils are also capable of being anti-tumorigenic by releasing their toxic granules against tumor cells [31]. Recent research in a mouse

Fig. 5. Accumulation of peritumoral stromal neutrophils coincides with increased VEGF expression and angiogenesis progression at the invading tumor edge of HCC. (A and B) Paraffin-embedded hepatoma samples stained with an anti-VEGF antibody. Different levels of VEGF expression can be seen along the invading tumor edge: I, negative, \( n = 10 \); II, low expression, \( n = 12 \); and III, high expression, \( n = 23 \). Scale bar, 150 \( \mu \text{M} \). (B) The association between VEGFA expression at the invading edge and CD15+ cell densities in nontumor, peritumoral stroma, or intratumor. (C and D) Paraffin-embedded hepatoma samples stained with an anti-CD34 antibody. Different levels of tumor capillaries can be seen along the invading tumor edge: I, capillary, \( n = 91 \); and II, sinusoid, \( n = 51 \). Scale bar, 150 \( \mu \text{M} \). (D) The association between capillary progression at the invading edge and CD15+ cell densities in nontumor, peritumoral stroma, or intratumor.

derived CXC chemokines, and by that subsequently, neutrophils stimulated the proangiogenic activity of tumor cells at adjacent invading edges. These findings reveal a fine-tuned collaborative action between cancer cells and different types of immune cells in distinct tumor microenvironments, which reroutes the inflammatory response into a tumor-promoting direction.

In contrast to the immunosuppressive micromilieu in most intratumoral areas, the peritumoral stroma contains a significant number of infiltrated leukocytes with potent proinflammatory properties [4,17,25]. We and others have recently shown that most monocytes/macrophages in the peritumoral stroma exhibit an activated phenotype that favors the generation of IL-17-produ-
model showed that neutrophils can have anti-tumorigenic or pro-tumorigenic functions, depending on the presence of TGF-β in the tumor microenvironment. Such a mechanism may also contribute to the pro-tumorigenic role of neutrophils in HCC tissues, which usually contain TGF-β produced by both tumor and stromal cells. Therefore, the specific nature of inflammation and the tissue context may determine the ability of inflammatory response to facilitate or prevent tumor growth. Studying the mechanisms that can selectively modulate functional activities of neutrophils might provide a novel strategy for anticancer therapy.

Conflict of interest

The authors declare that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.08.041.

References