Research Article

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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.

Keywords: Neutrophils; Inflammatory response; Angiogenesis; Hepatocellular carcinomas.

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Abbreviations: CI, confidence interval; DFS, disease-free survival; HbsAg, HBV surface antigen; HCC, hepatocellular carcinoma; HR, hazard ratio; MMP, matrix metalloproteinases; OS, overall survival; TIMP2, TIMP metallopeptidase inhibitor 2; TNM, tumor node metastases.

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).

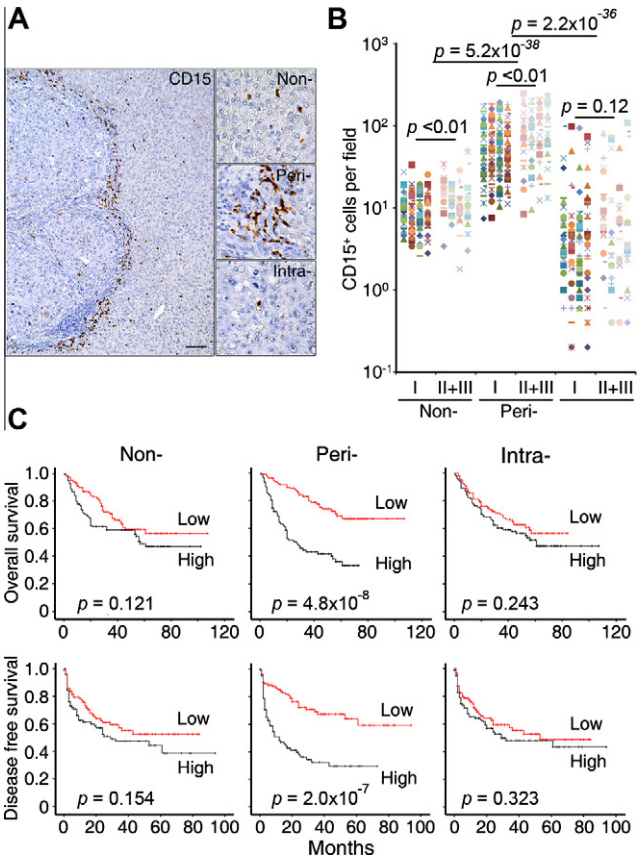

Fig. 1. Accumulation of neutrophils in peritumoral stroma fosters disease progression and predicts reduced survival in HCC patients. Paraffin-embedded HCC samples ($n = 200$) were stained with an anti-CD15 antibody. (A and B) Distribution of $CD15^+$ cells in nontumoral (Non-), peritumoral stromal (Peri-), and intratumoral (Intra-) areas of HCC samples. The samples used were derived from 127 stage I to 73 stages II and III HCC patients. Scale bar, 150 μM. (C) Cumulative overall and disease-free survival curves of patients. The patients were divided into two groups according to the median value of $CD15^+$ neutrophil density in nontumoral (median = 10), peritumoral stromal (median = 54), and intratumoral (median = 4) tissues: red lines, low density ($n = 101$); black lines, high density ($n = 99$). Cumulative overall and disease-free survival time were calculated using the Kaplan–Meier method and analyzed by the log-rank test.