Gastroenterology 2016;150:1646–1658

BASIC AND TRANSLATIONAL—LIVER

Tumor-Associated Neutrophils Recruit Macrophages and T-Regulatory Cells to Promote Progression of Hepatocellular Carcinoma and Resistance to Sorafenib

Shao-Lai Zhou,[1,2,3,*] **Zheng-Jun Zhou**,[1,2,3,*] **Zhi-Qiang Hu**,[1,2,*] Xiao-Wu Huang,[1,2] Zheng Wang,[1,2] Er-Bao Chen,[1,2] Jia Fan,[1,2,3] Ya Cao,[4] Zhi Dai,[1,2] and Jian Zhou[1,2,3]

[1]Liver Surgery Department, Liver Cancer Institute, Zhongshan Hospital, Fudan University, [2]Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Fudan University, [3]Institute of Biomedical Sciences, Fudan University, Shanghai, China; [4]Cancer Research Institute, Central South University; Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Changsha, China

BACKGROUND & AIMS: Neutrophils can either promote or inhibit tumor progression, depending on the tumor microenvironment, via release of cytokines. Neither the factors produced by tumor-associated neutrophils (TANs) nor their effects on tumor progression have been characterized. We investigated the roles of TANs in progression of hepatocellular carcinoma (HCC) using cell lines and immune cells isolated from patients. **METHODS:** We performed studies with HepG2, PLC/PRF/5, MHCC97H, and HCCLM3 human and Hepa1-6 and H22 mouse HCC cell lines; expression of chemokines and cytokines were knocked down with small hairpin RNAs. Cells were analyzed in chemotaxis assays and as growth as tumors in mice. HCC tissues and peripheral blood were collected from 20 patients undergoing curative resection or 20 healthy individuals (controls) in 2012 at Zhongshan Hospital in China. TANs and peripheral blood neutrophils (PBNs) were isolated and exposed to conditioned media from HCC cell lines; reverse-transcription polymerase chain reaction was used to quantify the expression of cytokines and chemokines. We collected neutrophils from another 60 patients undergoing curative resection for HCC in 2012 to measure the production of C-C motif chemokine ligand 2(CCL2) and CCL17. Patients were followed up until March 15, 2014. For immunohistochemical analyses, we collected HCC tissues and paired, adjacent, nontumor cirrhotic liver tissues from 832 HCC patients undergoing curative resection from 2006 through 2008. All patients were followed up until March 15, 2013. To study the effects of sorafenib, we collected clinical and pathology data from 46 patients who underwent curative resection in 2010. **RESULTS:** CCL2 and CCL17 were the cytokines most highly expressed by TANs and HCC cell-activated PBNs. Levels of *CCL2* and *CCL17* messenger RNAs and proteins were significantly higher in TANs than in PBNs, and increased in patients with HCC recurrence. *CCL2* and *CCL17* messenger RNA and proteins also increased when PBNs were exposed to conditioned media from HCC cell lines. Immunohistochemical analysis of a tissue microarray showed that CCL2+ and CCL17+ cells, which also expressed the neutrophil marker CD66b, were distributed throughout the HCC stroma, but not in tumor cells or the adjacent nontumor liver cells. The number of CCL2+ or CCL17+ TANs correlated with tumor size, microvascular invasion, tumor encapsulation, tumor differentiation, and stage. Patients whose tumors had lower levels of CCL2+ or CCL17+ cells had longer survival times than those with higher numbers of these cells. TAN-conditioned media, as well as recombinant CCL2 and CCL17, increased the migratory activity of the macrophages and T-regulatory (Treg) cells from patients or mice with HCC to a greater extent that PBN-conditioned media. Neutralizing antibodies against CCL2 and CCL17, or their receptors C-C chemokine receptor 2 and C-C chemokine receptor 4, reduced the migratory activities of macrophage and Treg cells. HCC cell lines injected into mice formed larger tumors when they were co-injected with TANs and formed more pulmonary metastases; these tumors were infiltrated by Ly6G+ cells, F4/80+ macrophages, and Foxp3+ Treg cells. In a phosphokinase array of human PBNs, levels of phosphorylated AKT and P38 increased after exposure to conditioned media from all 4 HCC cell types. Pharmacologic inhibitors of AKT and P38 inhibited secretion of CCL2 and CCL17 by these PBNs. In tumor-bearing mice, sorafenib increased the numbers of TANs and levels of CCL2 and CCL17 in tumors. HCC tissues from patients who received sorafenib before surgery contained more TANs than tissues from patients who did not receive sorafenib. In knockdown cells, HCC cell–derived CXCL5 was the strongest effector of neutrophil migration under hypoxic conditions. In mice, the combination of sorafenib and TAN depletion inhibited tumor growth and neovascularization to a greater extent than sorafenib alone. **CONCLUSIONS:** TANs recruit macrophages and Treg cells to HCCs to promote their growth, progression, and resistance to sorafenib.

Keywords: Liver Cancer; Mouse Model; Immune Response; Neovascularization.

Cancers rely on the tumor microenvironment, which comprises a variety of nonmalignant stromal cells for growth, invasion, and metastasis.[1,2] Recent evidence has indicated that there is a complex and multidirectional

*Authors share co-first authorship.

Abbreviations used in this paper: CCR, C-C chemokine receptor; HCC, hepatocellular carcinoma; HIF1, hypoxia-inducible factor 1; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; MVD, microvascular density; nPBN, normal peripheral blood neutrophil; PBN, peripheral blood neutrophil; PCR, polymerase chain reaction; PI3K, phosphoinositide 3 kinase; pPBN, peripheral blood neutrophil from hepatocellular carcinoma patients; shRNA, short hairpin RNA; TAN, tumor-associated neutrophil; Treg, T-regulatory.

Most current article

0016-5085
http://dx.doi.org/10.1053/j.gastro.2016.02.040

interplay between tumor cells and immune or nonimmune stromal cells during cancer development and progression. The interaction between tumor and stromal cells may polarize stromal cells to favor tumor promotion.[2,3] Moreover, unlike tumor cells, stromal cells within the tumor microenvironment are genetically stable and represent an attractive therapeutic target because they are less likely to develop drug resistance.[2]

Recently, there has been emerging interest to study the role of neutrophils in cancer. This interest stems from the fact that cancer-related inflammation now is recognized as a new hallmark of cancer.[4] In the tumor microenvironment, tumor-associated neutrophils (TANs) have been proposed to support tumor development by promoting cellular transformation, tumor progression, and antitumor immunity.[5] TAN infiltration is prognostic in several human cancers,[6] including hepatocellular carcinoma (HCC).[7] Neutrophils may influence tumor progression through the paracrine release of cytokines and chemokines with protumor or antitumor functions, depending on the tumor microenvironment.[8] Until now, a full characterization of the factors released by TANs has not been performed, and it is unclear whether there are factors released exclusively by TANs that drive tumor progression. Moreover, the role of TANs in HCC progression and their effect on the microenvironment remain undefined.

A multikinase inhibitor called sorafenib is the only drug that is approved to treat patients with advanced HCC.[9] Sorafenib inhibits Raf, as well as the kinase activity of vascular endothelial growth factor receptor and platelet-derived growth factor receptor.[10] Despite an apparent survival benefit, the response rate to sorafenib is low, with only a modest prolongation of survival.[11] Therefore, it is crucial to identify the molecular mechanisms mediating sorafenib resistance to improve clinical outcomes for HCC patients.

We designed this study to examine the role of TANs in the progression of HCC, particularly to uncover their effects on the tumor microenvironment. We also tested the role of TANs in sorafenib resistance. Finally, we validated our results in clinical HCC samples.

Materials and Methods

Cell Lines, Animals, and Lentiviral Vectors

Four human and 2 mouse HCC cell lines were used in this investigation. MHCC97H and HCCLM3 (highly metastatic human HCC cell lines) were established at our institute.[7] HepG2, PLC/PRF/5 (low-metastatic human HCC cell lines; American Type Culture Collection, Manassas, VA), Hepa1-6, and H22 (mouse HCC cell lines) were procured through the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China) and transfected with pGCSIL-Green Fluorescent Protein (GFP) lentiviral vectors. These cell lines all routinely were maintained. For experiments under hypoxic conditions, cells were exposed to 1% O_2 in an Invivo$_2$ 200 hypoxia chamber (Ruskinn Technologies, Leeds, UK). Male C57BL/6 and BALB/c mice (4–6 weeks old, obtained from the Shanghai Institute of Material Medicine, Chinese Academy of Science) were housed in pathogen-free conditions. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, revised 1985).

The pGCSIL–GFP–short hairpin RNA (shRNA)–hypoxia-inducible factor 1 (HIF1)α/p65/CXCL5/CXCL16/CCL24/interleukin 12α lentiviral vectors were procured from Shanghai GeneChem Co (Shanghai, China), and the target shRNA sequences are listed in Supplementary Table 1. pGCSIL-GFP lentiviral vectors were used as controls. Transduction was performed as previously described.[7]

Patients and Follow-Up Evaluation

For neutrophil isolation and RT^2 profiler polymerase chain reaction (PCR) array analysis, tumor tissues and peripheral blood from 20 HCC patients undergoing curative resection in February 2012 and peripheral blood from 20 healthy donors who had a medical check-up during the same period in Zhongshan Hospital were collected. For examination of CCL2 and CCL17 expression on neutrophils, another set of 60 HCC patients undergoing curative resection between February and March of 2012 were collected. All of these patients were monitored postsurgically until March 15, 2014.

For immunohistochemical and prognostic analysis, 2 independent cohorts totaling 832 HCC patients were enrolled (Supplementary Table 2). The 452 tumor tissues and paired adjacent nontumor cirrhotic liver tissues were obtained from patients undergoing curative resection between 2007 and 2008 at the Liver Cancer Institute, Zhongshan Hospital, Fudan University (cohort 1, snap-frozen tissues and corresponding paraffin-embedded tissues). In the other cohort, paraffin-embedded tissues were obtained randomly from HCC patients undergoing curative resection in 2006 (cohort 2, n = 380). All patients were monitored postsurgically until March 15, 2013. The histopathologic diagnosis was based on the World Health Organization criteria. The tumor grade was determined in accordance with the classification proposed by Edmondson and Steiner.[12] The Child–Pugh scoring system was used to assess liver function. Tumor stage was determined according to the TNM classification system established by the 2010 International Union Against Cancer. The Research Ethics Committee of Zhongshan Hospital approved the ethical use of human subjects for this study, and informed consent was obtained from each patient. Postsurgical patient surveillance was performed as previously described.[13,14] Overall survival was defined as the interval between surgery and death or between surgery and the last observation point. For surviving patients, the data were censored at the last follow-up evaluation. Time to recurrence[15] was defined as the interval between the surgery date and the date of any diagnosed relapse (intrahepatic recurrence and extrahepatic metastasis).

For patients enrolled in the sorafenib study, 46 patients who underwent curative resection in 2010 at the Liver Cancer Institute were included, and these patients received sorafenib treatment after recurrence. Clinical and pathologic data were collected (Supplementary Tables 3 and 4), and all of these