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Tumor-Associated Macrophages Produce Interleukin 6 and Signal via STAT3 to Promote Expansion of Human Hepatocellular Carcinoma Stem Cells

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BACKGROUND & AIMS: Cancer stem cells (CSCs) can contribute to hepatocellular carcinoma (HCC) progression and recurrence after therapy. The presence of tumor-associated macrophages (TAMs) in patients with HCC is associated with poor outcomes. It is not clear whether TAMs interact with CSCs during HCC development. We investigated whether TAMs affect the activities of CSCs in the microenvironment of human HCCs. **METHODS:** HCCs were collected from 17 patients during surgical resection and single-cell suspensions were analyzed by flow cytometry. $CD14^+$ TAMs were isolated from the HCC cell suspensions and placed into co-culture with HepG2 or Hep3B cells, and CSC functions were measured. The interleukin 6 (IL6) receptor was blocked with a monoclonal antibody (tocilizumab), and signal transducer and activator of transcription 3 was knocked down with small hairpin RNAs in HepG2 cells. Xenograft tumors were grown in NOD-SCID/*Il2*Rg^{null} mice from human primary HCC cells or HepG2 cells. **RESULTS:** $CD44^+$ cells from human HCCs and cell lines formed more spheres in culture and more xenograft tumors in mice than $CD44^-$ cells, indicating that $CD44^+$ cells are CSCs. Incubation of the $CD44^+$ cells with TAMs promoted expansion of $CD44^+$ cells, and increased their sphere formation in culture and formation of xenograft tumors in mice. In human HCC samples, the numbers of TAMs correlated with the numbers of $CD44^+$ cells. Of all cytokines expressed by TAMs, IL6 was increased at the highest level in human HCC co-cultures, compared with TAMs not undergoing co-culture. IL6 was detected in the microenvironment of HCC samples and induced expansion of $CD44^+$ cells in culture. Levels of IL6 correlated with stages of human HCCs and detection of CSC markers. Incubation of HCC cell lines with tocilizumab or knockdown of signal transducer and activator of transcription 3 in HCC cells reduced the ability of TAMs to promote sphere formation by CD44+ cells in culture and growth of xenograft tumors in mice. **CONCLUSIONS:** $CD44^+$ cells isolated from human HCC tissues and cell lines have CSC activities in vitro and form a larger number of xenograft tumors in mice than $CD44^-$ cells. TAMs produce IL6, which promotes expansion of these CSCs and tumorigenesis. Levels of IL6 in human HCC samples correlate with tumor stage and markers of CSCs. Blockade of IL6 signaling with tocilizumab, a drug approved by the Food and Drug Administration for treatment of rheumatoid arthritis, inhibits TAM-stimulated activity of $CD44^+$ cells. This drug might be used to treat patients with HCC.

Keywords: Liver Cancer; Mouse Model; IL6 Receptor; Cancer Biology.

Hepatocellular carcinoma (HCC) is a leading cause of death among solid malignancies and the incidence is increasing in the United States.[1,2] HCC is linked to the incidence of liver disease, with viral hepatitis being one of the strongest risk factors.[1] HCC outcomes are poor,[3,4] and one medication, sorafenib, has marginal efficacy.[5] Cancer stem cells (CSCs) may account for tumor recurrence after therapy and for tumor development and metastasis. CSCs may not be a fixed cell population and may show plasticity regulated by tumor microenvironmental factors (reviewed by Gupta et al[6] and Magee et al[7]). Such regulation has been shown with colon cancer tumor–associated fibroblasts and with breast cancer bone marrow mesenchymal stem cells.[8,9]

The immune cell component has prognostic importance in HCC and other malignancies.[10–12] Effector T cells, T-regulatory cells, and suppressive tumor–associated macrophages (TAMs) all balance tumor immunity in HCC patients.[10,13,14] We and others have shown TAMs in HCC to be particularly important to this balance of immune response and overall prognosis. However, the immune infiltrating cell influence on CSC functionality, particularly in human HCC, is not completely defined. We tested the hypothesis that HCC-infiltrating TAMs promote HCC CSC function. We show that $CD44^+$ CSCs are expanded by human HCC TAMs by their secretion of interleukin (IL)6 and activation of signal transducer and activator of transcription (STAT)3 signaling in HCC cells. This effect was blocked by the IL6-receptor blocking antibody, tocilizumab, a Food and Drug Administration (FDA)-approved drug, highlighting a novel therapeutic strategy for targeting CSCs in HCC.

Materials and Methods

Human Subjects

HCC tissues were obtained from patients undergoing surgical resection as described.[13] All patients provided written

Abbreviations used in this paper: **CSC, cancer stem cell; EMT, epithelial mesenchymal transition; EpCAM, epithelial cell adhesion molecule; FDA, Food and Drug Administration; HCC, hepatocellular carcinoma; IL, interleukin; IP, intraperitoneally; NSG, NOD-SCID/IL2Rg^{null}; shRNA, small hairpin RNA; STAT, signal transducer and activator of transcription; TAM, tumor-associated macrophage.**

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informed consent. The study was approved by and followed the University of Michigan Institutional Review Board guidelines.

Additional Methods can be found online in the Supplementary information.

Results

HCC Cells Express Multiple Potential CSC Markers

Many cell surface markers have been described to define human HCC CSCs, including CD24, CD44, CD90, CD117, CD133, and epithelial cell adhesion molecule (EpCAM).[15–17] To test whether these markers identify CSCs in a Western HCC patient cohort, surgically resected HCCs (n = 17 patients) (Supplementary Table 1) underwent single-cell suspension and flow cytometry by gating on 7-AAD$^-$CD45$^-$ (excluding nonviable and immune cells), and were stained for putative CSC markers (Figure 1*A* and *B*). HCC patients had an extremely low percentage (<1%) of tumor cell populations of CD117, CD133, and EpCAM. However, CD24$^+$, CD44$^+$, and CD90$^+$ cells reproducibly were observed, with CD44$^+$ being present in all HCCs. Cells that were co-positive for CD44 and other markers rarely were identified, with occasional CD44$^+$/CD90$^+$ and CD44$^+$/CD24$^+$ populations noted in some tumors; however, these were less than 1% of the total population (Supplementary Figure 1*A*). CD44, when determined by immunohistochemistry, was primarily in a membranous pattern on HCC cells with some staining noted on stromal cells such as infiltrating leukocytes (Supplementary Figure 1*B*). Early passage HCC patient xenograft tumors and HCC cell lines next were examined for CSC markers (Figure 1*C* and *D*). All HCC patient-derived xenografts and cell lines consistently possessed a CD44$^+$ population (2%–6%), whereas CD90 (0%–6%), CD133 (0%–98%), and EpCAM (0%–98%) populations varied in their presence considerably. Thus, a small discrete population of CD44$^+$ HCC cells was identified reliably in all fresh HCC patient specimens, patient-derived xenograft tissues, and HCC cell lines.

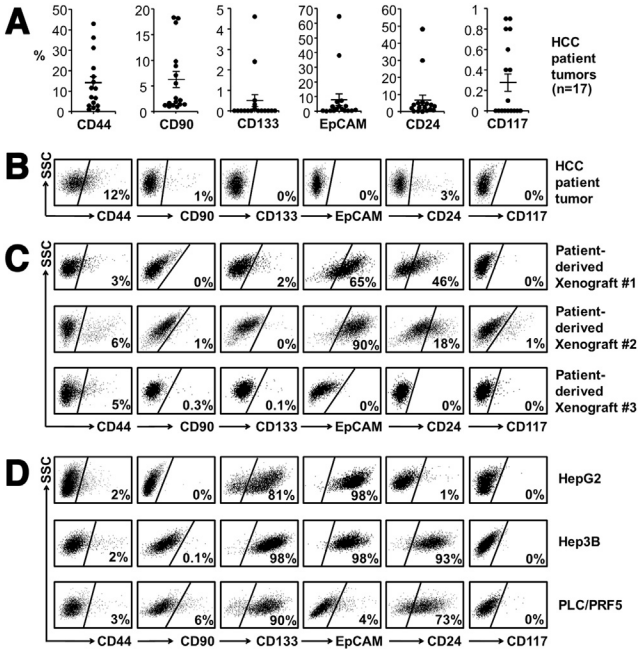

Figure 1. CSC markers in human HCC. (*A*) Flow cytometry analysis of putative CSC markers in primary HCC tumors (n = 17 patients, means ± SEM). Results are the percentage of a marker-positive population in 7-AAD$^-$CD45$^-$ (viable nonimmune) cells. The CD44$^+$ percentage was significantly higher than other markers ($P < .05$). (*B*) Representative HCC patient flow cytometry analysis in panel *A*. (*C*) Representative patient-derived HCC xenografts flow cytometry analysis (n = 3 patients) gated on mCD45$^-$H2Kd$^-$ to exclude murine infiltrating cells. (*D*) Representative flow cytometry analysis for CSC markers in HCC cell lines. Results are the percentage of a positive population in viable cells relative to negative isotype controls.

CD44$^+$ HCC Cells Have CSC Properties

Because the CD44$^+$ population was detected in all HCCs (Figure 1) and is a CSC marker in other malignancies,[18,19] we examined whether the CD44$^+$ subset was enriched for CSC function. CSCs have self-renewal capacity and form tumor spheres in serum-free and anchorage-independent conditions. HepG2 cells, when in sphere-forming conditions, were able to generate spheres that could be passaged (Supplementary Figure 2*A*). HepG2 spheres were enriched for CD44$^+$ cells more than 4-fold compared with conventional cultured HepG2 cells (Figure 2*A*), and had increased stem cell–associated *POU5F1* gene expression (previously known as *OCT-4*) and *CD44* (Supplementary Figure 2*B*). We then sorted CD44$^+$ and CD44$^-$ HepG2 cells and found that CD44$^+$ cells formed almost 2-fold more spheres and had higher *POU5F1* gene expression than CD44$^-$ cells (Figure 2*B*). Likewise, HCC patient xenografts sorted for CD44$^+$ cells showed increased POU5F1 expression (Supplementary Figure 2*C*).

To compare the in vivo tumor-forming capacity of CD44$^+$ and CD44$^-$ cells, limiting dilution tumor-initiating assays of sorted CD44$^+$ and CD44$^-$ HCC cells from HepG2 or HCC patient-derived xenograft tumors were performed in immune-deficient NOD-SCID/IL2Rg^{null} (NSG) mice (Figure 2*C* and *D*). CD44$^+$ cells had higher tumorigenicity than CD44$^-$ cells. As few as 100 CD44$^+$ HepG2 cells and 1000 CD44$^+$ cells from 2 HCC patient-derived xenografts showed a 50%–100% tumor formation rate, whereas no tumor was formed with the same number of CD44$^-$ cells. Although CD44$^-$ cells occasionally formed tumors at higher cell numbers, tumor volumes were smaller compared with those from CD44$^+$ cells (Supplementary Figure 2*D*). Tumors formed from CD44$^+$ cells had similar histology to the original unsorted (parental) HCC xenograft (Supplementary Figure 2*E*) and had similar stem cell marker expression percentages (Supplementary Figure 2*F*), showing recapitulation and differentiation of the parental tumor heterogeneity by the CD44$^+$ subset. A described HCC gene expression data set[20] showed that CD44 expression correlated with tumor progression and stemness gene expression (Figure 2*E*). CSCs are resistant to chemotherapeutic agents and other cytotoxic agents. To test if CD44$^+$ HCC CSCs showed this feature, HepG2 cells were exposed to the