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Inflammatory models drastically alter tumor growth and the immune microenvironment in hepatocellular carcinoma

Geoffrey J. Markowitz · Gregory A. Michelotti · Anna Mae Diehl · Xiao-Fan Wang

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Abstract Initiation and progression of hepatocellular carcinoma (HCC) is intimately associated with a chronically diseased liver tissue. This diseased liver tissue background is a drastically different microenvironment from the healthy liver, especially with regard to immune cell prevalence and presence of mediators of immune function. To better understand the consequences of liver disease on tumor growth and the interplay with its microenvironment, we utilized two standard methods of fibrosis induction and orthotopic implantation of tumors into the inflamed and fibrotic liver to mimic the liver condition in human HCC patients. Compared to non-diseased controls, tumor growth was significantly enhanced under fibrotic conditions. The immune cells that infiltrated the tumors were also drastically different, with decreased numbers of natural killer cells but greatly increased numbers of immune-suppressive $CD11b^{+}$ $Gr1^{hi}$ myeloid cells in both models of fibrosis. In addition, there were model-specific differences: Increased numbers of $CD11b^{+}$ myeloid cells and $CD4^{+}$ $CD25^{+}$ T cells were found in tumors in the bile duct ligation model but not in the carbon tetrachloride model. Induction of fibrosis altered the cytokine production of implanted tumor cells, which could have far-reaching consequences on the immune infiltrate and its functionality. Taken together, this work demonstrates that the combination of fibrosis induction with orthotopic tumor implantation results in a markedly different tumor microenvironment and tumor growth kinetics, emphasizing the necessity for more accurate modeling of HCC progression in mice, which takes into account the drastic changes in the tissue caused by chronic liver disease.

Keywords Hepatocellular carcinoma · Fibrosis · Tumor microenvironment · Carbon tetrachloride · Bile duct ligation · Cytokines

1 Introduction

Liver cancer is the fifth most common cancer in men, seventh most common cancer in women, and the third most deadly cancer worldwide [1, 2]. Liver cancer, particularly hepatocellular carcinoma (HCC), almost always develops on the background of a chronically diseased liver tissue, induced by long-term exposure to an inflammatory stimulus, such as hepatitis viral infections, excessive alcohol consumption, or metabolic syndrome [1, 3]. These stimuli lead to the induction of hepatocyte death and compensatory proliferation, hepatic stellate cell activation, and immune infiltration [3]. This process frequently results in a fibrotic and ultimately cirrhotic liver microenvironment; in this context, the cycling hepatocytes accumulate genetic alterations and eventually undergo malignant transformation [3]. This is in stark contrast with many other cancer types, in which the tissue from which the tumor arises is generally quite normal and functional [4, 5]. Indeed, 80 %–90 % of patients, whom have developed liver cancer after a long history of liver illness, have cirrhosis of the liver or late-stage fibrosis [2]. The fibrotic liver, with its characteristic scarring of the tissue,

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G. J. Markowitz · X.-F. Wang (✉)
Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA
e-mail: xiao.fan.wang@duke.edu

G. A. Michelotti · A. M. Diehl
Division of Gastroenterology, Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA

comes with a concurrent production of numerous cytokines, chemokines, and growth factors, all of which shape the microenvironment and its constituents during hepatocarcinogenesis and progression [6–9]. With this common theme of inflammation and fibrosis accompanying and driving malignant transformation, a better understanding of the microenvironment and its effects on tumor progression can yield valuable insights into these processes as well as produce potential targets for therapeutic intervention.

To investigate the development and progression of HCC, mouse models are essential tools to dissect the complex roles of tumor cells and stromal components in the tumor microenvironment. Although numerous mouse models have been created in which liver cancer is induced either by carcinogens or by transgenic activation of oncogenes combined with inactivated tumor suppressor genes, it has been difficult to mimic the pathological process of the most common HCC types, those initiated by hepatitis viral infections. Thus, orthotopic transplantation of cancer cells, via direct injection into the liver or inoculation into the spleen that drains into the liver, has been a very valuable experimental approach for evaluating both primary tumor growth and metastatic potential [10–12]. However, in most experimental settings, the tumor cells have been implanted into a healthy liver in the animal host, a tissue background hardly resembling those in patients where HCC emerges and progresses, consequently leading to the generation of incomplete or even misleading information on the mechanistic aspects of the pathological process. In this regard, several studies have shown that manipulation of the fibrotic process could result in dramatic effects on tumorigenesis; however, they have primarily focused on the hepatic stellate cells themselves, with limited probes into the accompanying immune changes in the experimental systems [10, 13–15].

In this study, we addressed the question of whether common fibrotic stimuli could result in alterations to tumor growth in the context of orthotopic implantation models, with accompanying changes in both the immune components and tumor responses to the fibrotic tumor microenvironment. Our findings reveal a markedly different tumor microenvironment and tumor growth kinetics in a clinically relevant fibrotic context, emphasizing the necessity for more accurate modeling that takes into account the drastic changes in the tissue caused by chronic liver disease.

2 Materials and methods

2.1 Mice

All experimental procedures described here were approved by the Duke University Animal Care and Use Committee. C57BL/6 breeding mice were purchased from The Jackson Laboratory.

2.2 Fibrosis induction

For the carbon tetrachloride model of liver fibrosis, 6- to 7-week-old male C57BL/6 mice were treated with either carbon tetrachloride diluted 1:20 in olive oil or olive oil alone at a volume of 600 μL kg^{-1}. Mice received biweekly intraperitoneal injections for 6 weeks and were sacrificed 2 days following the 12th injection.

For the bile duct ligation (BDL) model of liver fibrosis, 12-week-old male C57BL/6 mice were anesthetized, and the common bile duct isolated and transected between two ligations. Mice were sacrificed 14 days post-surgery.

2.3 Orthotopic implantation

Implantation occurred either 2 days after the eighth injection of carbon tetrachloride or olive oil, or immediately following BDL. Mice were anesthetized, or maintained under anesthesia in the case of the BDL, and the abdomen opened to expose the liver. 3×10^6 Hepa1-6 cells expressing a GFP-labeled luciferase reporter were suspended in 30 μL of growth factor-reduced Matrigel and injected into the left lobe of the liver. Following implantation, the peritoneum and the skin were closed using surgical suture. Mice were monitored for health and sacrificed 14 days after implantation. In the case of mice with liver fibrosis induced by carbon tetrachloride or their controls, injection of the ninth dose of carbon tetrachloride or olive oil occurred 2 days after surgery, and the 12th dose occurred 12 days after surgery, with sacrifice of the mice occurring 14 days after surgery.

2.4 Sample harvest and preparation

Blood was harvested from the inferior vena cava to examine serum circulating alanine aminotransferase (ALT) and circulating aspartate aminotransferase (AST). Samples were incubated at room temperature for 10 min then centrifuged at 3000 r min^{-1} for 10 min, following which serum was aspirated and transferred to a new microcentrifuge tube and stored at –80 °C until assay.

Livers were harvested and either immediately post-fixed in 10 % phosphate-buffered formalin at 4 °C on an orbital shaker for 1 day before being washed with 70 % ethanol and embedded in paraffin blocks, or processed for flow cytometry or fluorescence-activated cell sorting (FACS). Tissue processed for flow cytometry was isolated from the mouse, diced, and ground through a 40-μm-pore filter into DMEM with 10 % FBS and 12 μL DNase I per 2 mLs of DMEM, incubated on an orbital shaker at 37 °C for 1 h, resuspended in ACK Lysing Buffer to clear red blood cells, and resuspended in FACS buffer (PBS with 2 % FBS and 2 mmol L^{-1} EDTA). Samples were subsequently blocked with anti-mouse CD16/