Research in Veterinary Science 96 (2014) 315–322

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Identification of tumor-initiating cells in a canine hepatocellular carcinoma cell line

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ARTICLE INFO

Article history:
Received 5 September 2012
Accepted 26 January 2014

Keywords:
Dog
Flow cytometry
Hepatocellular carcinoma
Tumor-initiating cells
Xenograft

ABSTRACT

Tumor-initiating cells (TICs) or cancer stem cells (CSCs), a small subset of tumor cells, are involved in tumor initiation, progression, recurrence and metastasis. In human hepatocellular carcinoma (HCC), TICs are enriched with cell surface markers and have the ability to self-renew and differentiate tumors at a high frequency. We established a canine HCC cell line, HCC930599, and analyzed it for stem and progenitor cell marker expression using flow cytometry. HCC930599 showed high CD44 and CD29, moderate CD90, and low CD133, CD34, CD24, CD117, and CD13 expression. $CD90^{+}CD44^{+}$ and $CD90^{-}CD44^{+}$ cells were characterized using the *in vitro* sphere assay and an *in vivo* transplant model. $CD90^{+}CD44^{+}$ cells acquired enhanced self-renewal capacity, proliferative activity and tumourigenicity compared with $CD90^{-}CD44^{+}$ cells, suggesting that TICs exist in the HCC930599 cell line and that CD90 is a marker for enriched TICs. Understanding TIC characteristics may help elucidate hepatic carcinogenesis and HCC therapy development.

1. Introduction

Hepatocellular carcinoma (HCC) has become a clinically important disease in both veterinary and human medicine and its histologic features are extremely similar in both species (Ramos-Vara et al., 2001). The majority of hepatic tumors in humans are superimposed on a background of chronic hepatitis with hepatitis virus B or C infection and hepatic cirrhosis (Macdonald, 2001). HCC is the most common primary liver tumor in dogs (Patnaik et al., 1981); however, the etiology of spontaneous HCC in dogs remains unknown. Canine hepatic tumors are grossly classified into massive, nodular and diffuse types (Patnaik et al., 1980). The massive type generally has a good prognosis because it is confined to a single liver lobe, whereas the nodular and diffuse types have poor prognoses because they involve several liver lobes and frequently metastasize to the lungs and regional lymph nodes (Liptak et al., 2004). Unfortunately, there is no effective treatment for dogs with metastatic, non-resectable, incompletely excised HCC, although liver lobectomy is the best treatment in the early stage of the disease (Balkman, 2009).

There is increasing evidence that only a small fraction of cancer cells, which have stem cell properties, and are referred to as cancer stem cells (CSCs) or tumor-initiating cells (TICs), are involved in tumour initiation, progression, recurrence, and metastasis. These cells are reportedly less sensitive to chemo- and radiotherapy (Visvader and Lindeman, 2008). TICs are capable of self-renewal and differentiation into multiple phenotypes, and are the source of several solid tumors, including breast cancer and HCC (Al-Hajj et al., 2003; Visvader and Lindeman, 2008; Yang et al., 2008a,b). In human HCCs, TICs are identified using side population (SP) analysis, the sphere assay, and detection of surface markers, such as CD133, CD13, and CD90. Sphere assay is a useful method for the evaluation of self-renewal capacity as well as the identification of tumor-initiating cells. $CD133^{+}$, $CD90^{+}CD44^{+}$, and $CD13^{+}$ cells have a high capacity to form tumors in immunodeficient mice (Chiba et al., 2006; Ma et al., 2007; Yang et al., 2008a,b; Haraguchi et al., 2010; Uchida et al., 2010; Cao et al., 2011). Differentiation capacity is able to assess by detection of both TICs and non-TICs in the xenograft tumors derived from TICs using the flow cytometry (Michishita et al., 2012).

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http://dx.doi.org/10.1016/j.rvsc.2014.01.004
0034-5288/

In human HCCs, TICs are immunohistochemically distinguishable from other neoplastic cells using several markers, such as CD133, CD13 for TICs, HepPer1 for hepatocytic lineage, and cytokeratin 7 for cholangiocytic lineage (Mishra et al., 2009).

In dogs, a small population of cells with potential progenitor-like characteristics, referred to as SP cells, has been identified in normal liver tissue, and has a high capacity to exclude the dye Hoechst33342 (Arends et al., 2009). The existence of TICs in HCCs, however, is supported only by immunohistochemistry using stem cell markers, including CD133, CD44, and Keratin 19 (Cogliati et al., 2010). Cells with stem cell properties have not yet been isolated from canine HCCs, although they have been identified by sphere assay in solid tumors, including osteosarcoma, glioblastoma, and mammary carcinoma (Wilson et al., 2008; Penzo et al., 2009; Stoica et al., 2009; Michishita et al., 2011). Using flow cytometric analysis, TICs have also been identified in canine solid tumors using the stem/progenitor marker CD44, the efflux capacity of ATP-binding transporters, and aldehyde dehydrogenase (Blacking et al., 2011; Nemoto et al., 2011; Barbieri et al., 2012; Michishita et al., 2012). Tumorigenicity by transplantation into immunodeficient mice is evaluated to demonstrate the presence of TICs.

CD133 and CD13 are specific markers of a subset of TICs in human liver cancer. $CD133^+$ and $CD13^+$ cells are not only responsible for tumor initiation or progression but also have stem cell properties (Ma et al., 2007; Haraguchi et al., 2010). In addition, CD13 is known as a candidate therapeutic target because the combination of a CD13 inhibitor and fluorouracil (5-FU) was shown to drastically reduce tumor progression in xenograft models (Haraguchi et al., 2010). CD90 (Thy-1) is a glycosylphosphatidylinositol-anchored glycoprotein associated with cell–cell and cell–matrix interactions (Rege and Hagood, 2006). In addition, CD90 is a potential marker of CSCs in HCC, as well as normal hepatic stem/progenitor cells in healthy liver, leukocytes, and mesenchymal stem cells (Dennis et al., 2007; Herrera et al., 2006; Rege and Hagood, 2006; Yang et al., 2008a,b). $CD90^+$ cells isolated from HCC cell lines show high tumorigenic capacity in immunodeficient mice, and $CD90^+CD44^+$ cells prominently contribute to tumor aggressiveness and metastasis (Yang et al., 2008a,b). In dogs, CD90 expression has been identified in adipose-derived stem cells and T lymphocytes; however, its expression has not yet been evaluated in hepatic TICs using flow cytometry (Vieira et al., 2010; Weiss, 2001).

In this study, an established canine HCC cell line was evaluated by immunocytochemistry and flow cytometry using various cell surface markers, including CD90, CD34, CD133, CD24, CD29, CD44, CD13, and CD117, followed by *in vitro* and *in vivo* analyses of $CD90^+CD44^+$ and $CD90^-CD44^+$ cells. This is the first report to demonstrate the existence of TICs in a canine HCC cell line.

2. Materials and methods

2.1. Cell culture

A canine HCC cell line was established from the tissue of a 12-year-old female Maltese dog with various small-size masses occurring only in the lobus hepatis sinister lateralis. The masses were diagnosed as solid HCC by histopathology. In brief, the dissected tumor tissue was gently minced, digested with 0.2% trypsin in phosphate-buffered saline (PBS) for 30 min at 32 °C and filtered through gauze. After hemolysing with Tris–HCl-buffered solution (pH 7.65), the collected cells were re-suspended with Eagle's minimum essential medium (EMEM; Gibco) supplemented with 15% fetal bovine serum (FBS; Nichirei), 0.24 mg/mL tryptose phosphate broth (Wako) and 0.8 mM sodium bicarbonate ($NaHCO_3$), and subsequently cultured at 37 °C in an atmosphere containing 5% CO_2. The medium was renewed twice weekly, and the cells were maintained in the same medium once a week. The established cell line was designated as HCC930599. HepG2 cell line obtained from American Type Culture Collection (ATCC) was cultured in Dulbecco's modified Eagle's medium (DMEM; Wako) supplemented with 10% FBS, and maintained at 37 °C in an atmosphere containing 5% CO_2.

2.2. Morphology and immunocytochemistry

Cell suspensions (2×10^5 cells) from HCC930599 and HepG2 cell lines were inoculated on two-well chamber slides (Nalgene Nunc) and stained with May–Grunwald–Giemsa. For immunocytochemistry, the cultured cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and subsequently treated with 0.03% hydrogen peroxide (H_2O_2) in 33% methanol for 15 min at room temperature. After blocking with Block Ace (Dainippon Sumitomo Pharma) for 60 min at room temperature, the cells were immunostained using a labeled streptavidin–biotin–peroxidase technique with rabbit polyclonal antibodies against alpha-fetoprotein (AFP, 1:100; Dako) and albumin (1:2000; Dako) for hepatocyte markers. The validation of antibodies was confirmed by a positive reaction with HepG2 cell line expressing AFP and albumin. Normal rabbit immunoglobulin G (Dako) was used as a negative control. Cells were visualized with 3,3′-diaminobenzidine–4HCl (DAB; Dojindo) and counterstained with hematoxylin.

2.3. Flow cytometric analysis and cell sorting (FACS)

The cells were washed with PBS, and enzymatically dissociated with 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA; Invitrogen). The cells were then resuspended with PBS containing 2% FBS and incubated with phycoerythrin (PE)-conjugated CD90 (clone 5E10; BD Biosciences), CD34 (clone 1H6; R&D), CD133 (clone 13A4; eBioscience), CD24 (clone M1/69; BD Biosciences), CD29 (clone TS2/16; BioLegend), allophycocyanin (APC)-conjugated CD44 (clone IM7; BD Biosciences), CD13 (clone WM15; eBioscience), and CD117 (clone 2B8; BD Biosciences), in combination with CD90 and either CD44 or CD13 at 4 °C for 40 min. After washing, the labeled cells were analyzed using FACSAria (BD Biosciences), and the data were analyzed using Flow Jo software or FACSDiva software (BD Biosciences). The $CD90^+CD44^+$ and $CD90^-CD44^+$ cells were sorted using a FACSAria cell sorter (BD Biosciences) and subsequently analyzed.

2.4. Cell proliferation assays

Cell proliferation assays were performed using a Cell Counting Kit-8 assay (CCK-8; Dojindo) according to the manufacturer's instructions. In brief, sorted $CD90^+CD44^+$ and $CD90^-CD44^+$ cells were plated onto 96-well, flat-bottomed plates at 5×10^3 cells/well. After 24 and 48 h, CCK-8 reagent was added to each well, and samples were incubated at 37 °C for 2 h. Absorbance was measured for each well at a wavelength of 450 nm.

2.5. Sphere assay

Individual unsorted parent cells and sorted $CD90^+CD44^+$ and $CD90^-CD44^+$ cells were plated in ultra-low attachment six-well plates (Corning) at a density of 1×10^3 cells/well. The cells were grown in serum-free DMEM/F12 (Invitrogen) supplemented with 10 ng/mL basic fibroblast growth factor (bFGF; Invitrogen), 10 ng/mL epidermal growth factor (EGF; Invitrogen), 4 μg/mL heparin (Sigma) and B27 (Invitrogen) for 10 days. Spheres were counted using microscopy. For serial passages, all spheres were collected, enzymatically dissociated into single cells, and seeded again in ultra-low attachment plates.