Expression of Intercellular Adhesion Molecule 1 by Hepatocellular Carcinoma Stem Cells and Circulating Tumor Cells

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BACKGROUND & AIMS: Intercellular adhesion molecule 1 (ICAM-1) is believed to be involved in metastasis of hepatocellular carcinoma (HCC) cells. Cancer stem cells promote tumor relapse and metastasis. We investigated whether ICAM-1 is a marker of HCC stem cells. **METHODS:** Sphere formation and tumor formation assays were performed to investigate the stem cell properties of ICAM-1$^+$ cells in vitro and in vivo. A specific targeting system that inhibits ICAM-1 expression and hepatitis B virus transgenic mice (*M-TgHBV*) were used to investigate whether inhibition of ICAM-1 reduced tumor incidence and metastasis in vivo. We used real-time polymerase chain reaction and immunoblot analysis to assess ICAM-1 and Nanog expression in tumor cell lines, and flow cytometry analysis was used to investigate ICAM-1 expression in HCC and blood samples. **RESULTS:** ICAM-1 was expressed on a minor cell population in HCC tumor cell lines, as well as in tumor tissues and circulating tumor cells isolated from patients and transgenic mice. ICAM-1$^+$ tumor cells had greater sphere-forming and tumorigenic capacities and increased expression of stemness-related genes compared with ICAM-1$^-$ tumor cells. The specific inhibition of ICAM-1 reduced formation and metastasis in *M-TgHBV* mice. ICAM-1 was found to be a marker of circulating tumor cells from patients and *M-TgHBV* mice. Increased numbers of CD45$^-$ICAM-1$^+$ cells in blood samples of patients with HCC correlated with worse clinical outcomes. The stem cell transcription factor Nanog regulated expression of ICAM-1 in HCC stem cells. **CONCLUSIONS: ICAM-1 is a marker of HCC stem cells in humans and mice; ICAM-1 inhibitors slow tumor formation and metastasis in mice. ICAM-1 expression is regulated by the stem cell transcription factor Nanog.**

Keywords: Mouse Model; Liver Cancer; Cell-Surface Protein; Cell Migration.

Hepatocellular carcinoma (HCC) is the 5th most common cancer in the world and accounts for more than 90% of human liver cancers. Hundreds of thousands of deaths result from HCC worldwide every year, and as many as 90% of these cancer-associated deaths are related to metastasis.[1]

Intercellular adhesion molecule 1 (ICAM-1; CD54), a 90-kilodalton cell surface glycoprotein of the immunoglobulin superfamily, is believed to be responsible for HCC metastasis.[2] Previous studies have shown that ICAM-1 is expressed on hepatocytes in cancerous areas but not on hepatocytes in noncancerous areas.[3] The expression of ICAM-1 has been reported to mediate adhesion-dependent cell-cell interactions and facilitate the movement (or retention) of cells through the extracellular matrix,[4–6] and it has been shown to be positively correlated with tumor size and poor prognosis in HCC.[7,8] Recently, it was observed that ICAM-1 is expressed on stem cells, such as bone marrow mesenchymal stem cells, adipose stem cells, periodontal ligament stem cells, and placenta mesenchymal stem cells,[9–12] in addition to endothelial cells and epithelial cells.[2] Based on these findings, ICAM-1 is now considered a mesenchymal stem cell and periodontal ligament stem cell marker.[12,13] Although it is known that cancer stem cells (CSCs) have crucial roles in cancer relapse and metastasis, whether ICAM-1 is expressed on HCC CSCs remains unclear.

CSCs, a subset of cancer cells with features of stem cells such as self-renewal ability and pluripotency, are believed to be responsible for tumor relapse and metastasis.[14–16] In HCC, CSCs were first defined as a side population. Haraguchi et al identified side population cells that possess stem cell properties such as self-renewal, pluripotency, and chemoresistance.[17] Recently, CSCs were characterized based on their expression of various surface molecules, such as CD133, CD90, EpCAM, and CD44.[18–21] Using these cell surface molecules, HCC CSCs were sorted from HCC cell lines, tissues, and blood samples and found to be capable of self-renewal and tumor initiation and to be resistant to chemotherapeutic drugs. Experimental procedures targeting cells expressing CSC markers have been shown to reduce tumor incidence and metastasis in vivo, indicating that eliminating CSCs may be an efficient therapeutic strategy.

In the present study, we detected ICAM-1 expression in a minor cell population found in HCC tumor cell lines, tumor tissues, and circulating tumor cells. ICAM-1$^+$

Abbreviations used in this paper: AFP, α-fetoprotein; ChIP, chromatin immunoprecipitation; CSC, cancer stem cell; CTC, circulating tumor cell; HCC, hepatocellular carcinoma; ICAM-1, intercellular adhesion molecule 1; K19, cytokeratin 19; mRNA, messenger RNA; PCR, polymerase chain reaction; P#, patient number; Sc, pAFP–ICAM-1–scramble; SD, standard deviation; Sh, pAFP–ICAM-1–short hairpin RNA; shRNA, short hairpin RNA.

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tumor cells displayed enhanced sphere-forming and tumorigenic capacities and elevated expression of stemness-related genes, including Nanog and Oct4, compared with ICAM-1$^-$ tumor cells. Moreover, the inhibition of ICAM-1 reduced tumor initiation and metastasis in vivo. Additionally, we found that Nanog transcribes ICAM-1 expression in CSCs. Based on these findings, we propose that ICAM-1 is a functional CSC surface marker in HCC that is regulated by the stem cell transcription factor Nanog.

Materials and Methods

Samples

Human tumor tissues and blood samples were obtained from patients with HCC at the Eastern Hepatobiliary Surgery Hospital after obtaining informed consent. The follow-up procedures applied to these patients have been described in a previous report.[22] Overall survival and disease-free survival were defined as previously described.[23] Female nude mice (4–6 weeks old) were purchased from the Transgenic Animal Research Center, Second Military Medical University. All mice were maintained in a pathogen-free facility and used in accordance with the institutional guidelines for animal care.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from cell lines and clinical samples with TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Reverse-transcription reactions were conducted with oligo(dT) 18 primers and random primers according to the instructions of the manufacturer of the M-MLV Reverse Transcriptase Kit (Invitrogen). Real-time polymerase chain reaction (PCR) was performed with SYBR Premix Ex Taq (Takara Bio Inc, Otsu Shiga, Japan) using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers used in these assays are listed in Supplementary Table 1. The gene expression levels were calculated relative to the expression of β-actin in tumor cell lines or clinical samples using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

The total soluble proteins (100 μg) extracted from the samples were resolved in 10% sodium dodecyl sulfate/polyacrylamide gels and transferred electrophoretically to a polyvinylidene fluoride membrane. The blots were blocked with 5% skim milk and then incubated with primary antibodies (Supplementary Table 2). The blots were then incubated with an anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by enhanced chemiluminescence.

Single-Cell Sorting

Single-cell suspensions were obtained from clinical samples by digesting tumor tissues with type IV collagenase (Gibco BRL, Grand Island, NY) for 1 to 3 hours at 37°C, followed by filtration through a 100-μm cell strainer (BD Biosciences, San Jose, CA). To isolate ICAM-1$^+$ cell populations, single cells from cell lines or clinical samples were stained with a PE-conjugated ICAM-1 antibody (eBioscience, San Diego, CA) and with the corresponding isotype control. The samples were analyzed and sorted on a FACSAria cell sorter (BD Biosciences). The positive and negative populations were selected for the following experiments.

Flow Cytometric Analysis

The antibodies used in these analyses are listed in Supplementary Table 2. The cells were incubated with the antibodies in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide. The corresponding isotype immunoglobulins were used as controls. The data were analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Chromatin Immunoprecipitation Assay

Cells were processed for chromatin immunoprecipitation (ChIP) assays using a chromatin immunoprecipitation assay kit (Millipore Corp, Billerica, MA) according to the manufacturer's protocol. Briefly, the cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C and lysed with sodium dodecyl sulfate lysis buffer. The lysate pellets were sonicated with a sonic dismembrator (Fisher Scientific, Waltham, MA). Protein-DNA complexes were immunoprecipitated with the appropriate antibodies (Supplementary Table 2). The immunoprecipitates were dissolved in 20 μL water for PCR analysis. Standard PCR amplifications were performed using Taq PCR Master Mix (Takara Bio Inc) with the specific primers listed in Supplementary Table 1.

Statistical Analysis

Student *t* tests were used to compare 2 groups unless otherwise indicated (χ^2 test). Categorical data were analyzed using Fisher exact test, and quantitative variables were analyzed using *t* tests or Pearson's correlation test. Survival was calculated with the log-rank test. The Cox regression model was used to perform multivariate analysis. $P < .05$ was considered statistically significant.

For a description of other materials and methods used in this study, see Supplementary Materials and Methods.

Results

ICAM-1$^+$ Tumor Cells Possess Characteristics of Stem/Progenitor Cells

To investigate whether ICAM-1 can be used as a CSC marker, we first determined whether an ICAM-1$^+$ cell population was present in tumor cell lines. Flow cytometry analysis showed that approximately 5% of Huh7 cells and 7% of Hep3B cells expressed ICAM-1 (Figure 1*A*). We next examined whether the ICAM-1$^+$ cells exhibited intrinsic properties of stem cells. For this purpose, ICAM-1$^+$ cells were isolated from tumor cell lines, and the expression of stemness-related genes, including *sox2*, *nanog*, *oct4*, and *β-catenin*, was assessed at the messenger RNA (mRNA) level. Compared with ICAM-1$^-$ cells, ICAM-1$^+$ cells expressed higher levels of these 4 genes, of which *β-catenin* was increased the least (Figure 1*B*, *upper left panel*). This finding was further confirmed with real-time PCR assays (Figure 1*B*, *right panel*) and Western blotting (Figure 1*B*, *lower left panel*).

To investigate the CSC properties of ICAM-1$^+$ cells in vitro, a sphere formation assay was performed. Isolated cells were cultured in serum-free epidermal growth factor/basic fibroblast growth factor–supplemented medium. One week later, many hepatospheres were observed in cultures of ICAM-1$^+$ cells isolated from both Huh7 and