Human Cancer Biology

Human Hepatocellular Carcinoma Tumor–derived Endothelial Cells Manifest Increased Angiogenesis Capability and Drug Resistance Compared with Normal Endothelial Cells

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Abstract

Purpose: Increasing evidence indicates that tumor-derived endothelial cells (TEC) possess a distinct and unique phenotype compared with endothelial cells (NEC) from adjacent normal tissue and may be able to acquire resistance to drugs. The aim of this study was to investigate the angiogenesis activity and response to drug treatment of TECs and NECs derived from human hepatocellular carcinoma (HCC).

Experimental Design: TECs or NECs were isolated from HCC or adjacent normal liver tissue using anti-CD105 antibody coupled to magnetic beads. The phenotypic and functional properties of endothelial cells were characterized by testing the expression of CD105, CD31, CD144, vascular endothelial growth factor receptor-1, vascular endothelial growth factor receptor-2, and von Willebrand factor, and the ability of Dil-Ac-LDL-uptake and tube formations. CD105+ TECs were compared with CD105+ NECs and human umbilical vein endothelial cells (HUVEC) by examining their ability to proliferate, motility, ability to adhere to tumor cells, response to tumor conditioned medium, and reactions to the chemotherapy drugs Adriamycin and 5-fluorouracil and the antiangiogenic drug sorafenib.

Results: Compared with CD105+ NECs and HUVECs, CD105+ TECs showed increased apoptosis resistance and motility and proangiogenic properties. Meanwhile, CD105+ TECs had a greater ability to adhere to tumor cells and survive in the tumor environment. Moreover, CD105+ TECs acquired more resistance to Adriamycin, 5-fluorouracil, and sorafenib than CD105+ NECs and HUVECs.

Conclusions: TECs possessed enhanced angiogenic activity and resistance to chemotherapeutic drugs and an angiogenesis inhibitor, and may provide a better tool for studying tumor angiogenesis and antiangiogenesis drugs in HCC.

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide (1). It is characterized by a high propensity for vascular invasion, and the angiogenic activity of HCC correlates with the risk of vascular invasion and prognostic significance (2). Recent studies showed a survival advantage can be achieved by treatment with angiogenesis inhibitors, including sorafenib (3) and bevacizumab (4).

Angiogenesis is important for supporting the rapid growth of a tumor beyond 1 to 2 mm in diameter and serves as the route for a tumor cell to escape and enter into the circulation (5–7). It is well documented that tumor vasculature differs morphologically from normal vessels, with formation of incomplete and irregular vessels with fenestrations, irregular blood flow, and increased permeability (8–11). Also, evidence indicates that there are many differences at the molecular and functional levels between tumor and normal endothelial cells (TEC and NEC, respectively). Many tumor-specific endothelial markers were found by comparing the gene expression profiles between TECs and NECs immediately after isolation from tissues (12–14). Strikingly, TECs were cytogenetically abnormal (15); they had structural aberrations such as nonreciprocal translocations, missing chromosomes, marker chromosomes, and double minutes by multicolor fluorescent in situ hybridization analysis. Our previous study showed that overexpression of platelet-derived growth factor receptor α in TECs immediately after being isolated from xerographic HCC tissue is associated with high...
Translational Relevance
Recent studies showed that survival benefits in patients with cancer can be achieved by treatment with an angiogenesis inhibitor. However, most functional studies of endothelial cells were conducted in normal endothelial cells, such as human umbilical vein endothelial cells. Our results showed that tumor-derived endothelial cells possess enhanced angiogenic activity and resistance to chemotherapeutic drugs and an angiogenesis inhibitor, sorafenib. These findings suggest that tumor-derived endothelial cells are more appropriate for studying angiogenesis mechanisms in tumors and for exploring antiangiogenesis drugs.

metastatic potential of HCC (16). However, the functional change is often impossible to determine because these studies, similar to angiogenesis ability and reaction to different drug treatments, cannot be accomplished in primarily isolated ECs. There are only a few studies that have shown successful isolation and subsequent culture of tumor ECs until now, and none in HCC-isolated ECs with anti-CD105 antibody (Ab; refs. 17–19). On the other hand, most functional studies have been conducted in established cell lines such as human umbilical vein ECs (HUVECs) because these have been widely characterized and thus offer a reproducible and reliable in vitro system. But with regard to complex and tissue-specific angiogenesis patterns, this model has obvious disadvantages because HUVECs are derived from large vessels that lack the typical characteristics of tumor vessels.

Unlike ECs in normal quiescent endothelium, TECs have a rapid turnover rate and have therefore been termed “activated” (20). Compared with traditional vascular markers, such as CD31 and von Willebrand factor (vWF), endoglin (CD105, also known as transforming growth factor-β receptor) is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic ECs (21). Anti-CD105 Ab seems to react only with ECs in the newly formed vessels, and in particular, the immature tumor blood vessels including HCC (22–24); furthermore, its presence in the vasculature of HCC has prognostic value (25). There is no specific biomarker expressed in all types of ECs that can be used in isolation of all types of ECs. One study reported that isolation of TEC by CD31 approach was suboptimal because it cross-reacts with hematopoietic cells; therefore, CD105 approach was used to isolate TEC from brain and liver cancer (13).

In the present study, we isolated, passed, and characterized the phenotypic and functional properties of ECs derived from human HCC. Our results showed that CD105+ TECs display increased angiogenesis activity and more resistance to drug treatment, compared with CD105+ NECs and HUVECs.

Materials and Methods

Patients. Seven patients who had positive α-fetoprotein (AFP) received resection for HCC in our hospital. None of these patients received any preoperative anticancer treatment including chemotherapy or antiangiogenesis drugs. The study was approved by the Zhongshan Hospital Research Ethics Committee. Informed consent was obtained according to the committee's regulations.

Cell lines and culture conditions. HUVECs (ScienCell Research Laboratory) were grown in EBM-2 medium (Cambrex BioScience) at 5% CO2. NECs and TECs were obtained from surgical HCC specimens and normal-surrounding liver tissue right after removal from patients. Specimens were minced with scissors and digested by incubation for 1 h at 37°C in high-glucose DMEM (Life Technologies) containing 0.1% collagenase IV (Sigma). After being washed in medium plus 10% fetal bovine serum (FBS; Life Technologies), the cell suspension was forced through a graded series of meshes to separate the cell components from stroma and aggregates. ECs were isolated from cell suspension using anti-CD105 Ab coupled to magnetic beads (Miltenyi Biotech) and magnetic cell-sorting using the MACS system (Miltenyi Biotech). To increase the purity of isolated ECs after positive magnetic bead isolation, the cell pellets underwent a second isolation with anti-CD105 Ab. Cells were grown in complete EBM-2 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. ECs between passages 1 and 6 were used in the following studies. Stable green fluorescent protein (GFP)-expressing HCCCLM3 cells (HCCCLM3-G), a human HCC cell line established at our institute (26), was maintained in high-glucose DMEM supplemented with 10% FBC in a humidified atmosphere of 5% CO2 at 37°C.

Flow cytometric analysis of cell surface protein. For flow cytometric analysis, cells were detached from plates with a nonenzymatic cell detachment factor (BD Biosciences) and analyzed with an FACS cell sorter (Becton Dickinson). We used primary murine monoclonal antibodies against human CD105 conjugated to phycoerythrin (PE), human CD34 conjugated to PE-Cy5 (5E-Cy5), human CD31 conjugated to allophycocyanin (APC), human CD144 conjugated to PE, human VEGFR-1 conjugated to APC, human VEGFR-2 conjugated to FITC, and human CD68 conjugated to FITC. Directly conjugated mouse immunoglobulin G1 were used for isotype controls. All antibodies were purchased from BD Pharmpingen with the exception of VEGFR-1-APC, VEGFR-2-FITC, and CD31-APC (R&D Systems), and CD68-FITC (eBioscience).

Reverse transcription-PCR analysis. Total RNA from NECs (cells without CD105 labeling), CD105+ TECs, and CD105+ NECs were extracted and reverse transcribed into single-stranded cDNA using ReverTra Aid Moloney Murine Leukemia Virus Reverse Transcriptase (MBI Fermentas) according to the manufacturer's instructions. To assess the purity of the isolated ECs, PCR was done on cDNA generated from NCs, CD105+ TECs, and CD105+ NECs. HCC cell–specific transcript of AFP was analyzed to exclude the contamination of tumor cells. Forward primer 5-GGGACGGCGCTGACATTAT-3 and reverse primer 5-GTGATGGGACACATTAT-3 were used for primer 5-GGAGTCAACGGATTTGGT-3 and reverse primer 5-GTGATGGGACACATTAT-3 were used for reverse transcription of AFP.

Immunocytochemistry. For immunocytochemistry, ECs were cultured on attachment factor–coated slide wells (Sonic Seal Slide Well, Nalg Nunc International). When they reached subconfluence, cells were preincubated with 5% normal donkey serum (Dako Corporation) for 30 min to avoid nonspecific binding. Rabbit anti–human polyclonal Ab against factor VIII–related antigen (Dako) was used as the first Ab (1:100 dilution), and the bound antibodies were detected with FITC-conjugated anti-rabbit secondary Ab (Santa Cruz Biotechnology). Samples were analyzed with an inverted fluorescence microscope (Olympus IX51) equipped with an Olympus Qcolor 3 digital camera (Olympus) using digital camera (Olympus).

Internalization of acetylated low-density lipoprotein. The isolated ECs were incubated in serum-free EBM-2 medium containing 10 μg/mL rhodamine-labeled 1,1′dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine acetylated low-density lipoprotein (DiI-Ac-LDL;
Biomedical Technologies, Inc.) for 4 h at 37°C. The cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Samples were analyzed with an inverted fluorescence microscope (Olympus IX51) equipped with an Olympus Qcolor 3 digital camera. All images were processed with ImagePro Plus (Media Cybernetics).

**Wound healing assay.** ECs ($4 \times 10^5$) were plated onto gelatin-coated six-well plates to create a confluent monolayer. Cells were allowed to adhere and spread completely for ~4 h under proper incubation. A wound was created by manually scraping the cell monolayer with a p1000 pipette tip. The cells were washed once with 1 mL of desired media, which was then replaced with 2 mL of the same media. Dishes were incubated for 18 h at 37°C in a humidified atmosphere containing 5% CO$_2$. Cells were periodically observed with an inverted fluorescence microscope (Olympus IX51) equipped with an Olympus Qcolor 3 digital camera. All images were processed with ImagePro Plus (Media Cybernetics).

**EC-tumor cell interaction assay.** Cells were cultured in gelatin-coated 24-well tissue culture plates. Before the assay, the monolayers were rinsed twice with EBM-2 containing 0.2% BSA and overlaid with 200 μL of HCCLM3-G tumor cells ($10^5$ cells) in DMEM supplemented with 0.2% BSA. The plates were incubated at 37°C in 5% CO$_2$ for 20 min. Nonattached cells were eliminated by washing twice with DMEM supplemented with 0.2% BSA, and the adherent HCCLM3-G tumor cells in each individual well were counted.

**Survival assays.** Cells ($2 \times 10^3$) were plated in triplicate into 96-well plates in complete EBM-2 medium; after 10 h, cells were washed...
once with serum-free EBM-2. For proliferation assay, cells were cultured for 18, 24, 48, and 72 h in complete EBM-2 medium; for apoptosis resistance assay, cells were cultured for 72 h in serum-free EBM-2 medium; for detecting cells' response to treatment with doxorubicin hydrochloride (Shenzhen Main Luck Pharmaceuticals, Inc.), 5-fluorouracil (5-FU; Sigma), and sorafenib (Bayer HealthCare Pharmaceuticals), cells were cultured for 48 h in complete EBM-2 medium containing different drugs at indicated concentrations. The supernatant was carefully removed and discarded without disturbing the cell pellet and the cell pellets were frozen at -70°C. Then the viable cells grown in microplates were detected with CyQUANT Cell Proliferation Assay kit (Molecular Probes-Invitrogen).

**Tube formation assay.** In vitro formation of capillary-like structures was studied on growth factor-reduced Matrigel diluted 1:1 in ice with cold DMEM in a 96-well plate. Cells (2 × 10⁴ cells per well) were seeded onto Matrigel-coated wells in EBM-2 medium containing 0% or 2% fetal bovine serum. Cells were periodically observed with an inverted fluorescence microscope (Olympus IX51) equipped with an Olympus Qcolor 3 digital camera. All images were processed with ImagePro Plus (Media Cybernetics).

**Spheroid assay of angiogenesis in vitro.** ECs were seeded in U-bottomed nontissue culture-treated 96-well plates (Greiner) at a density of 500 cells per well in medium containing 0.25% methylcellulose (Sigma) and incubated for 24 h to form a spheroid. Then spheroids were collected and pooled by centrifugation at 500 × g for 3 min. Eight volumes of collagen I stock (BD Biosciences) was mixed with 1 volume of 10× EBSS (Sigma) and 1 volume of 0.1 N NaOH up to 10 volumes; spheroids were resuspended in medium containing 0.25% methylcellulose, mixed 1:1 with collagen solution and transferred to prewarmed 48-well plates. Polymerization was induced by incubation at 37°C for 1 h, after which 200 μL medium was pipetted on top of the gel. The gels were incubated at 37°C for 36 h and fixed by addition of 500 μL 8% paraformaldehyde, and then gels were incubated overnight with TritC-conjugated phalloidin (Sigma) and Hoechst (Sigma). Photos were acquired using an inverted fluorescence microscope (Olympus IX51) equipped with an Olympus Qcolor 3 digital camera. All images were processed with ImagePro Plus (Media Cybernetics).

**Western blot analysis.** Cells were harvested in a lysis buffer (Pierce, Rockford, IL) and equal amount of proteins was subjected to 12% SDS-PAGE. After gel electrophoresis, the proteins were transferred to the polyvinylidene difluoride membranes (Immobilon PVDF; Millipore). The membranes were blocked for 1 h at room temperature in 5% nonfat dry milk in TBS containing 0.05% Tween 20, followed by an overnight incubation at 4°C with primary antibodies. The membranes were then incubated with horseradish peroxidase–labeled anti-rabbit secondary Ab (Chemicon) for 1 h at room temperature. Peroxidase activity was detected via chemiluminescence (SuperSignal West Femto luminol substrate and peroxide buffer; Pierce). Primary antibodies used include anti-Akt, anti–phospho-Akt, anti–signal transducers and activators of transcription 3 (STAT3), anti–phospho-STAT3, anti-mitogen-activated protein kinase, anti–phospho-mitogen-activated protein kinase (MAPK), and anti–β-Actin (Cell Signaling Technology), and anti-DLL4 (Abcam).

**Results**

**Isolation and characterization of EC from tumor tissues.** Seven strains of CD105+ TECs and three strains of CD105+ NECs were obtained from seven patients. Primary isolated CD105+ TECs had a flat appearance, with abundant cytoplasm and fibroblast-like structures, and grew in a monolayer without the typical cobblestone-like EC morphology (Supplementary Fig. S1). CD105+ NECs seemed the same as HUVECs and displayed the typical cobblestone-like morphology. Reverse transcription-PCR analysis confirmed that AFP expression can be detected in NCs but not in TECs and NECs (Fig. 1C). Negative expression of CD68 in isolated Ecs excludes the contamination of macrophages (Supplementary Fig. S1). Fluorescent-activated cell sorter analysis showed CD105 expression in 98 ± 1% of immunosorted Ecs. Ecs were further characterized by cytofluorimetric analysis for the expression of a panel of endothelial markers, including CD105, CD31, VE-cadherin (CD144), VEGFR1, VEGFR2 (Fig. 1A). The expression level of endothelial markers was tested at the first, third, and fifth passages, and similar results were confirmed during cell culture, as showed in Table 1. More than 95% of Ecs were positive for vWF staining and uptake of Dil-Ac-LDL (Fig. 1B). Then, two CD105+ TEC clones (TEC-B, TEC-F) and two CD105+ NEC clones (NEC-A, NEC-B) within six passages were used in the following experiments.

**Proliferation and apoptosis assays.** Cell proliferation was measured over a period of 72 hours under identical conditions. Proliferation of CD105+ TECs was significantly more active than CD105+ NECs and HUVECs when cultured in the serum-supplemented medium for 24, 48, and 72 hours (P < 0.05; Fig. 2A). To determine resistance to apoptosis, CD105+ TECs, CD105+ NECs, and HUVECs were cultured in serum-free medium. After 72 hours, CD105+ TECs continued proliferating, whereas CD105+ NECs and HUVECs stopped growth and underwent apoptosis (Fig. 2A).

**Motility and tube forming assays.** A wound healing assay was used to compare the migration capability of CD105+ TECs, CD105+ NECs, and HUVECs. Cultured for 18 hours after

### Table 1. Expression of endothelial markers by three different clones of ECs

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<tr>
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<th>TEC-A</th>
<th>TEC-B</th>
<th>TEC-C</th>
<th>TEC-D</th>
<th>TEC-E</th>
<th>TEC-F</th>
<th>TEC-G</th>
<th>NEC-A</th>
<th>NEC-B</th>
<th>NEC-C</th>
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<td>56 ± 10</td>
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<td>96 ± 2</td>
<td>90 ± 1</td>
<td>99</td>
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<td>20 ± 5</td>
<td>28 ± 6</td>
<td>36 ± 6</td>
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<td>68 ± 3</td>
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<tr>
<td>VEGFR2</td>
<td>15 ± 5</td>
<td>10 ± 3</td>
<td>42 ± 12</td>
<td>3 ± 1</td>
<td>10 ± 3</td>
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<td>60 ± 10</td>
<td>71 ± 9</td>
<td>64 ± 16</td>
<td>76 ± 6</td>
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NOTE: Marker expression was evaluated by fluorescence-activated cell sorting analysis and results expressed as percentage of positive cells. Results are the mean of three different experiments.

Abbreviation: ND, not done.
TEC Cells Increase Drug Resistance

Fig. 3. Increased drug resistance of CD105+ TECs to cytotoxic drugs 5-FU (5-Fu) and Adriamycin compared with CD105+ NECs and HUVECs. CD105+ TECs, CD105+ NECs, and HUVECs were seeded at 1 x 10^5 cells/mL in a 96-well plate, and cell proliferation assay was used to determine the percentage of viable cells after exposure of cells to various concentrations of 5-FU (A) and Adriamycin (B) and incubation for 48 h. Columns, mean from three individual experiments with three to four samples per group; bars, SE. *, P < 0.05; #, P < 0.01, versus CD105+ TECs groups.

Fig. 2. The increased ability of proliferation, apoptosis resistance, motility, tubulogenesis, and interaction with tumor cells of CD105+ TECs with respect to CD105+ NECs and HUVECs. A, increased proliferation of CD105+ TECs with respect to CD105+ NECs and HUVECs when cultured in full medium, and increased apoptosis resistance of CD105+ TECs with respect to CD105+ NECs and HUVECs after incubation without serum for 72 h. B, wound healing assay revealed increased migration ability of CD105+ TECs with respect to CD105+ NECs and HUVECs. C, representative micrograph of the tubes formed by CD105+ TECs and CD105+ NECs in the absence of serum; HUVECs did not form tubes in the absence of serum. D, representative micrographs of fluorescence microscopy detection of adherent HCCLM3-G cells on CD105+ TECs, CD105+ NECs, and HUVECs monolayers after coculture for 20 min. Adherent LM3-GFP cells appear as bright spots. Inset, the fluorescent imaging of adherent LM3-GFP cells. The data illustrated are representative of one of at least three independently done experiments. B to D, original magnification, ×100. Columns, mean from three individual experiments with three to four samples per group; bars, SE. *, P < 0.05; #, P < 0.01, versus CD105+ TECs groups except the top right chart in Fig. 2D.

scraping, horizontal migration was observed in the CD105+ TECs, whereas cellular migration was apparently slower in the CD105+ NECs and HUVECs (Fig. 2B). Also the proliferation of CD105+ TECs was more significant compared with CD105+ NECs (Supplementary Fig. S2), which is one of factors contributing to the ability of wound healing. In the absence of serum, CD105+ TECs and CD105+ NECs, but not HUVECs, were capable of forming tubes when planted onto Matrigel (Fig. 2C).

EC and tumor cell adhering assay. After being cocultured with HCCLM3-G tumor cells for 20 minutes, the adhered cells were counted. The results showed CD105+ TECs adhered significantly more HCC cells than CD105+ NECs or HUVECs (P < 0.001; Fig. 2D), and CD105+ NECs adhered few HCC cells.

Resistance to chemotherapeutic and angiogenesis inhibitor. The response of CD105+ TECs, CD105+ NECs, and HUVECs to Adriamycin and 5-FU was compared. As shown in Fig. 3, Adriamycin and 5-FU inhibited proliferation of CD105+ TECs, CD105+ NECs, and HUVECs in a dose-dependent manner after 48 hours. The average IC_{50} values of CD105+ TECs to Adriamycin and 5-FU were approximately 0.06 μg/mL and 37 μmol/mL, respectively, whereas the average IC_{50} values of Adriamycin and 5-FU were approximately 0.025 μg/mL and 4.9 μmol/mL, respectively, for CD105+ NECs, and approximately 0.02 μg/mL and 5 μmol/mL, respectively, for HUVECs.

To compare the effect of antiangiogenesis drugs on CD105+ TECs, CD105+ NECs, and HUVECs, cells were treated with different dosages of sorafenib. HUVECs and CD105+ NECs were sensitive to sorafenib in a dose-dependent manner when the concentration was over 2 μmol/L, whereas CD105+ TECs were resistant to sorafenib at the higher dosage of 10 μmol/L (Fig. 4A).

ECs and NECs could hardly form branching tubes at 1 μmol/L; CD105+ NECs could form branching tubes at 2 μmol/L and underwent apoptosis at 5 μmol/L, but CD105+ TECs formed tubes and branching networks well at 2 μmol/L and partly at 5 μmol/L (Fig. 4B). Similarly, the EC-spheroid assay showed CD105+ TECs presented with a more active sprouting in length and number than NECs and sprouted well when added with 5 μmol/L sorafenib; CD105+ NECs can hardly produce sprouts at 5 μmol/L sorafenib; HUVECs cannot maintain the EC-spheroid and spread to a plane structure at 2 μmol/L sorafenib (Fig. 4C).

Sorafenib inhibits MAPK, ATAT3, and Akt signaling pathways. We investigated the expression levels of total and phosphorylated STAT3, Akt, and MAPK (p42/p44) proteins in ECs. Total protein levels of STAT3 and AKT were not significantly changed by 24-hour sorafenib treatment in all types of ECs, whereas phosphorylated STAT3 (p-STAT3) and p-Akt were substantially reduced in HUVECs and NECs, but not in TECs. The total MAPK level was not significantly changed by sorafenib treatment in all three types of ECs; however, before sorafenib treatment, CD105+ TECs had a lowest p-MA pyruvate kinase level, compared with HUVECs and CD105+ NECs; p-MA pyruvate kinase level was down-regulated in the beginning of sorafenib treatment, and then recovered at the end of treatment, whereas in CD105+ TECs, p-MAPK level was significantly higher than that before treatment (Supplementary Fig. S2; Fig. 4D). Moreover, the expression of DLL4 was higher in CD105+ TECs than in CD105+ NECs.

Discussion

In the present study, we isolated, passaged, and characterized CD105+ TECs from human HCC. These cells did not undergo cell senescence in serum-free medium and showed enhanced...
Fig. 4. Increased drug resistance of CD105+ TECs to antiangiogenesis drug sorafenib compared with HUVECs and CD105+ NECs. A, CD105+ TECs, CD105+ NECs, and HUVECs were seeded at 1 × 10^4 cells/mL in a 96-well plate, and cell proliferation assay was used to determine the percentage of viable cells after exposure of cells to various concentrations of sorafenib and incubation for 48 h. Columns, mean from three individual experiments with three to four samples per group; bars, SE. *, P < 0.05; #, P < 0.01, versus CD105+ TEC groups. B and C, tube formation assay and spheroid assay showed inhibition of HUVECs ability to form tubes or produce sprouts by 1 μmol/L sorafenib and inhibition of CD105+ NECs ability to form tubes or produce sprouts by 5 μmol/L sorafenib, whereas CD105+ TECs showed tube formation capabilities or sprouting behaviors at 5 μmol/L sorafenib. D, sorafenib inhibits MAPK, ATAT3, and Akt signaling pathways in ECs after sorafenib treatment for 24 h and 30 min. Each experimental condition was repeated thrice. The data illustrated are representative of one of at least three independently done experiments. Original magnification, ×100.
proliferation, motility, proangiogenesis properties, and resistance to drug treatment. For the first time, we showed CD105+ TECs are more resistant to sorafenib, compared with CD105+ NECs and HUVECs.

Antiangiogenesis is a promising cancer treatment modality and has been shown to be beneficial to patients (4, 27, 28). However, it has become clear from clinical trials that some patients do not benefit from the treatment (29, 30). In addition to the redundancy of angiogenesis-stimulating factors in tumor microenvironment, many studies also suggest that TECs possess some different characteristics from NECs including morphologic (31), pathophysiological (32), cytogenetic (15), epigenetic (33), gene expression (12), and atypical multipotent plasticity (34). In the present study, we used anti-CD105 Ab to isolate TECs from human HCC. Confirmed by the EC-specific surface markers, LDL test, tube forming assay, and expression of AFP and CD68, we have obtained ECs without contamination with tumor cells and macrophages. We confirmed that CD105+ TECs, at least a portion of the TECs, possess an enhanced proliferation ability compared with CD105+ NECs and HUVECs. Furthermore, CD144 (VE-cadherin), a tight junction protein that plays an important role in the integrity of the blood vessels, leads to abnormal capillary junctions and the formation of aberrant tubules when it is blocked (35). Therefore, the reduced expression of CD144 found in the present study may contribute to leakiness in tumor blood vessels, a typical feature of tumor vasculature. Moreover, this type of TEC was more resistant to apoptosis in the serum-free medium and had enhanced motility and increased ability to adhere more HCC cells, and could form capillary-like structures on Matrigel in the absence of serum, compared with NECs and HUVECs. These functional characteristics of this type of TEC indicate increased proangiogenesis activity and usefulness as a better model for study of tumor vasculature.

Recently, it has been reported that human TECs from melanoma, liposarcoma, renal cancer, and breast cancer are resistant to apoptosis induced by chemotherapeutics (15, 18, 36). In the present study, we also found that human CD105+ TECs derived from HCC exhibited increased resistance to apoptosis induced by Adriamycin and 5-FU when compared with CD105+ NECs and HUVECs. These data suggest that the reported drug resistance of CD105+ TECs may not be a tumor type-specific characteristic, but rather a more common phenomenon, which also implies that the cytotoxic drugs may induce more damage to normal tissue than to tumor vasculature.

Some research suggests that TECs would be more appropriate for screening antiangiogenesis drugs than normal ECs (37), but there is still no direct evidence probably due to the unavailability of cultured TECs. The present study showed CD105+ TECs were more resistant to sorafenib, compared with CD105+ NECs and HUVECs. As a multikinase inhibitor with activity against the Ser/Thr kinase Raf and several receptor tyrosine kinases involved in angiogenesis including VEGFR2 and PDGFR, sorafenib inhibits cell proliferation and survival with inhibition of MAPK, STAT3, and Akt signaling pathways (38, 39). The present study showed sorafenib inhibited STAT3, Akt, and MAPK signaling pathways in all types of ECs, but the expression of p-STAT3 and p-Akt in CD105+ TECs before and after sorafenib treatment were higher than NECs. It is also noted that the baseline level of p-MAPK associated with response of ECs to sorafenib treatment, which is in consistence with other reports from clinical and experimental studies (3). We also found an up-regulation of p-MAPK in the late phase of sorafenib treatment, whereas in CD105+ TECs, p-MAPK expression was significantly higher than untreated cells. Currently, we do not know the underlying mechanism of this biphasic change of p-MAPK, but it may attribute to a unique feature of CD105+ TECs. Moreover, the expression of DLL4, a Notch receptor reported as one factor contributing to resistance to anti-vascular endothelial growth factor therapy (40), was higher in CD105+ TECs than in CD105+ NECs. On the other hand, the present study also implied that CD105+ TECs possess similar basic characteristics but with an enhanced angiogenic and survival ability compared with normal ECs. These characteristics probably result from genetic instability of TECs (15) or viral infection, as suggested by human pancreatic islet ECs that express Coxsackievirus and adenovirus receptors being activated by Coxsackie B virus infection (41). In addition, a multistep process involving mutations of both the epithelial and mesenchymal compartment has been reported in breast carcinoma (42), suggesting a possible reciprocal interaction between a tumor and surrounding cells. Alternatively, it can be speculated that TEGs may originate from endothelial precursors, which are known to display an enhanced angiogenic potential (43). Finally, it has been reported that tumor cells may constitute part of the tumor microvessels (44, 45). However, considering that CD105+ NECs were more sensitive to sorafenib compared with CD105+ TECs, the more clinically relevant implication is that sorafenib treatment may do more damage to the normal liver parenchyma, especially in patients with cirrhosis, in whose cases angiogenesis is important for maintaining the liver function (46, 47).

The present study also suggests there may be several subpopulations of TECs within one tumor in terms of cell surface markers. Although the expression rates of CD105 were high (>98%) in isolated ECs from seven patients and HUVECs, CD34, CD31, and CD144 expression was significantly lower (48); another showed CD34 is usually present on ECs in large vessels, but absent on ECs from tumor microvessels (49). Therefore, it is easy to speculate that tumor vasculature consists of CD105+ TEC and CD105+ NEC. Benetti and colleagues (50), meanwhile, confirmed that only part of cultured CD31+ TEC expressed CD105 and CD105 expression correlated with the grade of HCC malignancy. The present study also showed VEGFR1 and VEGFR2 expression was lower in CD105+ TECs compared with CD105+ NECs or HUVECs. Although the underlying mechanism is still under investigation, the discrepancy in VEGFR1 and VEGFR2 expression in TECs, NECs, and HUVECs constitutes one explanation for the different response to sorafenib.

In conclusion, the present study showed that CD105+ TECs isolated from human HCC specimens maintained a proangiogenesis phenotype, and presented enhanced resistance to apoptosis in the absence of a tumor microenvironment and increased drug resistance to cytotoxic therapy. Moreover, CD105+ TECs acquire more resistance to antiangiogenic therapies than normal ECs. Therefore, CD105+ TECs are more appropriate for studying angiogenesis mechanisms of tumors and exploring antiangiogenesis drugs, compared with CD105+ NECs.
and HUVECs. Finally, there were two CD105+ TECs clones and two CD105+ NECs clones were studied, which may not represent the heterogeneous features of CD105+ TECs and NECs; therefore, more investigation is necessary to confirm that increased angiogenesis capability and drug resistance is a general phenomenon of CD105+ TECs.

References


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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Human Hepatocellular Carcinoma Tumor–derived Endothelial Cells Manifest Increased Angiogenesis Capability and Drug Resistance Compared with Normal Endothelial Cells

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