Culture and Characterization of Circulating Endothelial Progenitor Cells in Patients with Renal Cell Carcinoma

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Abbreviations and Acronyms

Ac-LDL = acetylated low density lipoprotein
CAC = circulating angiogenic cell
CEPC = circulating endothelial progenitor cell
CFU-EC = colony forming unit-endothelial cell
DiI = 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate
ECFC = endothelial colony forming cell
FCM = flow cytometry
FITC = fluorescein isothiocyanate
HC = healthy control
HUVEC = human umbilical vein endothelial cell
MACS = magnetic activated cell sorting
PBMNC = peripheral blood mononuclear cell
RCC = renal cell carcinoma
SSC-A = side scatter
UEA-I = Ulex europaeus agglutinin I
VEGF = vascular endothelial growth factor
VEGF-R2 = VEGF receptor 2

Purpose: Although emerging evidence demonstrates increased circulating endothelial progenitor cells in patients with solid tumors, to our knowledge it is still unknown whether such cells can be cultured from patients with highly angiogenic renal cell carcinoma. We cultured and characterized circulating endothelial progenitor cells from patients with renal cell carcinoma.

Materials and Methods: The circulating endothelial progenitor cell level (percent of $CD45^{-}CD34^{+}$ $VEGF\text{-}R2^{+}$ cells in total peripheral blood mononuclear cells) was quantified in 47 patients with renal cell carcinoma and 40 healthy controls. Peripheral blood mononuclear cells were then isolated from 33 patients with renal cell carcinoma and 30 healthy controls to culture and characterize circulating endothelial progenitor cells.

Results: The circulating endothelial progenitor cell level was significantly higher in patients with renal cell carcinoma than in healthy controls (0.276% vs 0.086%, $p < 0.001$). A colony of circulating endothelial progenitor cells first emerged significantly earlier in patient than in control preparations (6.72 vs 14.67 days, $p < 0.001$). The culture success rate (87.8% vs 40.0% of participants) and the number of colonies (10.06 vs 1.83) were significantly greater for patients than for controls (each $p < 0.001$). The circulating endothelial progenitor cell level correlated positively with the number of patient colonies ($r = 0.762$, $p < 0.001$). Cells cultured from patients and controls showed a similar growth pattern, immunophenotype, ability to uptake Ac-LDL and bind lectin, and form capillary tubes in vitro. However, significantly more $VEGF\text{-}R2^{+}$ circulating endothelial progenitor cells were found in preparations from patients with renal cell carcinoma than from healthy controls (21.1% vs 13.4%, $p < 0.001$).

Conclusions: Earlier emergence of circulating endothelial progenitor cell colonies, a higher cell culture success rate and more colonies were found for patients with renal cell carcinoma than for healthy controls. Results indicate the important significance of $VEGF\text{-}R2^{+}$ circulating endothelial progenitors in patients with renal cell carcinoma.

Key Words: kidney; carcinoma, renal cell; stem cells; vascular endothelial growth factor A; endothelial progenitor cells

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CIRCULATING endothelial progenitor cells, which are $CD45^{-}CD34^{+}VEGF\text{-}R2^{+}$ mononuclear cells in peripheral blood derived from bone marrow, were originally identified in 1997 by Asahara et al.[1] Accumulated evidence indicates that CEPCs can be mobilized from bone marrow under ischemia, wound healing and tumor conditions.[2,3] CEPC levels are increased in patients with breast cancer,[4] glioma,[5] hepatocellular carcinoma[6] and gastric cancer.[7] Our previous study also showed increased CEPCs in patients with RCC, which positively correlated with the serum VEGF level.[8] Further studies revealed that CEPCs have an important role in promoting the earliest phases of tumor vasculogenesis,[9] facilitating tumor growth[10] and controlling the angiogenic switch of metastasis development.[11] Blocking CEPC mobilization with antiangiogenic drugs enhanced tumor sensitivity to therapy.[12]

To better understand the role of CEPCs in tumor neovascularization it is necessary to culture and characterize these cells from patients with tumors. Methods of isolating and culturing CEPCs were established for several pathological conditions.[2,3] CEPC colonies in culture were decreased in pregnant woman with intrauterine growth restriction[13] and preeclampsia[14] but increased in patients with rheumatoid arthritis.[15]

However, to our knowledge it is still unknown whether CEPCs can be isolated and cultured from patients with RCC. The characteristics of CEPC in these patients are not yet well documented. We cultured CEPCs from patients with RCC and compared CEPC characteristics between patients with RCC and HCs.

MATERIALS AND METHODS

Participant Eligibility

A total of 47 patients with RCC and 40 age matched HCs were enrolled in analysis between January 2013 and May 2014 according to previous study inclusion and exclusion criteria (table 1).[8] All patients with RCC were newly diagnosed and peripheral blood was harvested before any surgical procedure. The study was approved by the institutional review board and informed consent was obtained from each participant. Briefly, participants with a known condition that might influence the CEPC level were excluded.[8] The characteristics and laboratory results of each participant, including renal function, blood cell count, liver biochemistry, tumor size, Furhman grade and 2002 AJCC (American Joint Committee on Cancer) stage, were entered in a protected database.

Circulating Endothelial Progenitor Cells

Quantification. FCM was performed to quantify the CEPC level using our previous protocol in 100 μl of unselected peripheral blood cells from each of the 47 patients with RCC and each of the 40 HCs.[8] CEPCs were identified as $CD45^{-}CD34^{+}VEGF\text{-}R2^{+}$ mononuclear cells and the percent of CEPCs among the total number of PBMNCs was calculated. Quantitative fluorescence analysis was done using the 2-laser, 6-color configuration FACSCanto™ II flow cytometer with FACSDiva™ 6.1.2.

Culture. Heparin anticoagulated peripheral blood samples were collected from 33 patients with RCC and 30 HCs (20 ml per participant). PBMNCs were isolated by Ficoll-Paque®™ PLUS gradient centrifugation.[16] They were seeded in 4 wells of a 6-well culture plate precoated with human fibronectin (Chemicon®) and maintained in complete EGM-2 medium composed of EBM-2 supplemented with MV SingleQuots™ at 37C in a humidified atmosphere with 5% CO_2. After 24 hours in culture nonadherent cells were removed. Medium was refreshed daily for the initial 7 days and then every other day until CEPC colonies, identified as well circumscribed monolayers of cobblestone-shaped cells, needed to be subcultured.

Cell Growth Kinetics

Early CEPCs (passages 2 and 3) from patients with RCC or from HCs were plated in quintuplicate on 96-well plates at 2.5×10^3 per well and cultured in complete EGM-2 medium for 1, 2, 3, 4, 5, 6, 7 or 8 days. At the indicated time the cell number was determined by a calibration curve generated using CCK-8 (Dojindo Laboratories, Kumamoto, Japan).[17] Population doubling time was calculated according to the equation, population doubling time $= t \times [lg2/(lgNt - lgN0)]$, where t represents culture time, Nt represents the number of cells at the indicated time and N0 represents the initial number of seeded cells.[16] Cell growth assay was repeated in 5 participants in 1 colony per participant.

Cultured CEPC Immunophenotyping

FCM was performed to determine the cultured CEPC immunophenotype. Briefly, 1×10^5 CEPCs at passages 2 and 3 in 100 μl phosphate buffered saline containing 1% bovine serum albumin were incubated with primary cell surface antibodies or isotypes at 4C for 20 minutes, washed twice and analyzed by flow cytometry (supplementary table, http://jurology.com/). Average cell surface marker expression was determined in 10 colonies from patients with RCC or from HCs in 1 colony per participant.

Table 1. *Characteristics of patients with RCC and HCs*

	RCC	HC
Mean age (range)*	58 (33–84)	56 (30–84)
No. male (%)	25 (53.2)	20 (50)
No. female (%)*	22 (46.8)	20 (50)
No. AJCC stage (%):		—
I-II	21 (44.6)	
III-IV	26 (55.5)	
No. Furhman grade (%):		—
I-II	27 (57.4)	
III-IV	20 (42.6)	
No. clear cell Ca (%)	47 (100)	—

* Age and gender did not statistically differ in 2 groups ($p > 0.05$).