Significance of Circulating Endothelial Progenitor Cells in Hepatocellular Carcinoma

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This study evaluated the significance of circulating bone marrow-derived endothelial progenitor cells (EPCs) in patients with hepatocellular carcinoma (HCC), a solid tumor with rich neovasculature. Eighty patients with HCC were recruited for the study, and 16 patients with liver cirrhosis and 14 healthy subjects were also included for comparison. Blood samples were taken before treatment. Total mononuclear cells were isolated from peripheral blood, preplated to eliminate mature circulating endothelial cells, and colony-forming units (CFUs) formed by circulating EPCs were counted. To validate the CFU scores, FACS quantification of EPCs using CD133, VEGFR2, and CD34 as markers was performed in 30 cases. Our study showed significantly higher mean CFU scores in patients with HCC compared to patients with cirrhosis and healthy controls (P = .001 and .009, respectively). Furthermore, the CFU scores of patients with HCC positively correlated with levels of serum α-fetoprotein (r = .303, P = .017), plasma VEGF (r = .242, P = .035), and plasma interleukin-8 (IL-8) (r = .258, P = .025). Patients with unresectable HCC had higher CFU scores than patients with resectable tumors (P = .027). Furthermore, for those who underwent curative surgery, higher preoperative CFU scores were observed in patients with recurrence within 1 year compared with those who were disease-free after 1 year (P = .013). In conclusion, higher circulating levels of EPCs are seen in patients with advanced unresectable HCC as compared to patients with resectable HCC or those with liver cirrhosis. Our evidence supports the potential use of circulating level of EPCs as a prognostic marker in patients with HCC.

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Hepatocellular carcinoma (HCC) is one of the most aggressive solid tumors associated with poor prognosis.1 Wider application of screening program for high-risk populations has led to increasing detection of early tumors amenable to curative surgical treatment.2 However, most patients present with symptomatic disease, often with bilobar tumors, portal vein invasion, or metastasis, and are therefore incurable by surgery.3 Nonsurgical strategies such as transarterial chemoembolization and systemic chemotherapy are the palliative treatment options.1

Little is known of the tumor biology of HCC that leads to its rapid growth and metastasis. One of the striking characteristics of HCC is its rich tumor neovascularization, which can be clearly observed in hepatic angiography.4 Angiogenesis is essential for the development, progression, and invasion of solid tumors.5,6 Tumor neovascularization requires recruitment and proliferation of endothelial cells. Traditionally, it was thought that the original source of the recruitment of endothelial cells came from neighboring endothelial cells in adjacent normal tissues.7,8 However, recent studies have overturned such paradigm by demonstrating that endothelial progenitor cells (EPCs) are mobilized from bone marrow (BM) by cytokines such as vascular endothelial growth factor (VEGF), migrate via peripheral blood, and home to the site of tumor neovascularization.8-13 In animal studies, the contribution of such BM-derived EPCs to tumor neovascularization may be as high as 35%-45%.14 A recent study
on human tumors provided data supporting the concept of EPCs incorporation in the tumor vessels by demonstrating that BM-derived endothelial cells could indeed be found in the tumor endothelium.15

Thus far, there have been only a few clinical reports on the detection of circulating EPCs, and most of these studies evaluated the significance of EPCs in noncancer conditions.16-18 The significance of circulating EPCs in cancers has only been reported in a hematological malignancy, namely multiple myeloma.19,20 Furthermore, available reports of clinical studies are largely limited to the detection of circulating endothelial cells, which may not only include BM-derived EPCs, but also include endothelial cells from other sources such as those shedded from activated or damaged vessels.11-21-24 Therefore, the circulating level of EPCs in cancer patients and its clinical significance are still unclear.

Recently, Hill et al.25 reported the use of a short-term culture assay to score the colony formation units (CFUs) by EPCs in the peripheral blood. With this assay, the circulating levels of angioblast-like EPCs can be specifically quantified by their ability to form a “blood island-like” colony. They demonstrated that the circulating EPCs in men can be a useful predictor of cardiovascular risks because of the recruitment of EPCs from BM for repair in ischemic cardiovascular diseases. Since then, others have also used this assay to study EPCs in the circulation.26-28 However, no report has been published on the quantification of EPCs in solid tumors using this assay method.

In this study, we hypothesized that the circulating level of BM-derived EPCs was elevated in patients with HCC and might be correlated with the aggressiveness of the tumor. To test the hypothesis, we assessed the circulating levels of EPCs in patients with HCC and studied the correlation of the EPC levels with clinical data including the circulating level of α-fetoprotein (AFP). Furthermore, because both VEGF and interleukin-8 (IL-8) have been shown to be the biomarkers of tumor angiogenesis in HCC in our previous studies,29-31 we also evaluated the correlation of EPC levels with the levels of VEGF and IL-8 in the circulation.

Patients and Methods

Sample Collection. Blood samples were taken from 80 consecutive patients with confirmed HCC who were seen in the HCC clinic of the Department of Surgery, Queen Mary Hospital, Hong Kong, China. None of the patients had received chemotherapy treatment before blood sampling. All blood samples that were taken for this study were pretreatment samples. The study was approved by the Institutional Review Board of our institution and informed consent was obtained from the patients. Sixteen patients with liver cirrhosis and 14 normal healthy subjects were included for comparison and as controls, respectively.

EPC Culture Assay. The assay method for EPC as described by Hill et al.25 was employed. In brief, 20 mL of venous blood was taken from the subject and placed in tubes with sodium citrate. The samples were diluted with phosphate-buffered saline before Ficoll density-gradient centrifugation at 500g for 30 minutes at room temperature within 3 hours after collection. The recovered cells were washed twice in phosphate-buffered saline and once in growth medium containing Medium 199 (GIBCO BRL Life Technologies, Grand Island, NY) supplemented with 20% fetal bovine serum (GIBCO BRL Life Technologies), penicillin (100 U/mL), and streptomycin (100 U/mL). The same lot of fetal bovine serum was used for the entire study to avoid variations in culture condition caused by lot-to-lot variations. All assays were performed by the same investigator, and the working concentrations of fibronectin were diluted from the same stock batch. The cells were counted using the same cell counting chamber throughout the study. Five million peripheral blood mononuclear cells per well were preplated into fibronectin-coated 6-well plates to eliminate the adhering mature circulating endothelial cells. After 48 hours, nonadherent cells were replated into 24-well fibronectin-coated plates with 1 million cells per well. The numbers of colonies formed were counted 5 days after plating.

One CFU consisted of a single colony with an appearance of a blood island-like cell cluster where round-shaped cells were clustered in the center while elongated spindle-shaped cells were radiating out from the cluster. The number of CFUs was counted manually in a minimum of 4 wells using phase contrast light microscopy under 200X magnification. The CFU scores in the resulting sessions reflected the mean number of CFU per well. Confirmation of endothelial cell lineage was performed in samples of 5 subjects by the colony’s positive uptake of Dil-labeled acetylated low-density lipoprotein (Dil-ac-LDL, Molecular Probes, CA) and FITC-labeled Ulex europaeus agglutinin-1 (ulex-lectin, Sigma-Aldrich, St. Louis, MO).25,27,28 The expressions of Di-ac-LDL and lectin were observed under inverted fluorescent microscopy (Olympus, Japan). Reproducibility of the assay was assessed by taking samples 2 times at least 1 week apart from the same subjects. The consistency in terms of the quality of the assays was controlled by having all the samples and assays processed and executed by 1 individual who was blinded to the subjects’ cancer status. The CFU
scores were counted by 2 individuals independently, and the final 2 sets of scores were found to not differ statistically.

**FACS Analysis.** In previous reports, circulating mononuclear cells with CD34+/CD133+/VEGFR2+ were quantified as tentative EPCs, with differential expression of these surface markers during early (CD133+/VEGFR2+) and mature (CD34+/VEGFR2+) phenotypes. We therefore analyzed the subpopulation of cells expressing CD133+/VEGFR2+ and CD34+/VEGFR2+ by flow cytometry. We stained 100 µL of peripheral blood for 30 minutes with monoclonal antibody against VEGFR2 (Sigma, St. Louis, MO) followed by a FITC-conjugated anti-mouse F(ab')2 (BD Bioscience, San Jose, CA) in darkness for 15 minutes. Cells were then either incubated with phycoerythrin (PE)-conjugated CD34 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) or PE-conjugated CD34 (Beckman Coulter, Mississauga, Canada) at room temperature in darkness for 20 minutes. FITC-conjugated CD45 (Beckman Coulter) was stained for identification of progenitor cells. Combinations of isotypic controls were used as negative controls based on the species and IgG subclass of each antibody (Beckman Coulter). After incubation with the antibodies, cells were washed with 2 mL phosphate-buffered saline and centrifuged at 500g for 5 minutes. Red cells were lysed with 500 µL of optiLyse C (Beckman Coulter), vortexed, and resuspended in 750 µL phosphate-buffered saline. Samples were then subjected to a 2-dimensional side scatter-fluorescence histogram analysis with a FACS instrument (Beckman Coulter). After appropriate gating with low cytoplasmic granularity and selection of progenitor cells, cells expressing CD133+/VEGFR2+ and CD34+/VEGFR2+ were identified and quantified out of the progenitor population. The quantity of each of the cell subpopulation was expressed as a percentage of the total mononuclear cells.

**Quantification of Plasma VEGF and IL-8 Levels.** Whole blood samples collected in tubes containing sodium citrate were centrifuged for 15 minutes at 1500g at room temperature after collection. Plasma levels of VEGF and IL-8 were measured by ELISA assays (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Statistical Analysis.** Data were expressed as mean ± standard deviation with range in parentheses. For the continuous variables, LSD (Least Significant Difference) test was used for intergroup comparisons of greater than 2 groups, while independent sample t test was used for comparison between 2 groups. Correlation analysis was performed using the Spearman rank correlation for nonparametric analysis, and Pearson’s correlation for parametric analysis. All statistical analyses were performed using the SPSS statistical software (SPSS/PC+, SPSS Inc., Chicago, IL). A P value of less than .05 was considered statistically significant.

**Results**

**Patient Data.** Clinical data of all patients were collected prospectively in a computer database. There were 44 male and 36 female patients with a mean age of 57.6 (31-82) years. Among the 80 HCC patients, 27 patients had resectable HCC and underwent curative surgery shortly after collection of blood samples for the study. Half the patients underwent minor hepatic resection of 2 segments of liver or fewer, and the other half underwent major hepatic resection of 3 segments or more. Seventeen of these 27 patients were subsequently followed for at least 12 months for their disease-free survival. Serum AFP level, chest x-ray, and contrast computer tomography scan were performed every 3 months to detect tumor recurrence. The other 53 patients were unresectable cases and they received various nonsurgical palliative treatments, such as transarterial chemoembolization.

**EPC Colony Formation.** Distinct colonies could be identified in the 24-well plates after culturing peripheral blood mononuclear cells. Only those colonies with elongated spindle-shaped cells sprouting out from the center cluster of round-shaped cells were counted (Fig. 1Aa-b). The appearance and organization of these cell clusters resembled the characteristic blood island-like cell clusters observed in dissociated quail epiblast culture, which gave rise to endothelial cells and vascular structures in vitro. The uptake of Dil-ac-LDL and ulex-lectin by these colonies matched that described in the literature and confirmed their EPC phenotype (Fig. 1A, c and d). In 30 cases of HCC, we further demonstrated significant correlations between CFU scores with the FACS quantification of EPCs via labeling of early stage (CD133+/VEGFR2+) and mature (CD34+/VEGFR2+) EPC populations from peripheral blood mononuclear cell samples (Fig. 1B).

**Significantly Higher CFU Scores in HCC Patients.** While the mean CFU score in HCC patients (n = 80) was 21.9 ± 20.1 (range 1.1-91.0), the mean CFU scores in healthy subjects (n = 14) and subjects with cirrhosis (n = 16) were 8.3 ± 4.0 (range 4.0-16.0) and 6.2 ± 6.6 (range 0-16.0), respectively. Intergroup comparison by 1-way ANOVA showed significantly higher CFU scores in HCC patients (n = 80) compared to healthy subjects (n = 14, P = .001) and patients with cirrhosis (n = 16, P = .009).
Significantly Higher CFU Scores in Subgroups of Patients With HCC. With 1-way ANOVA, we found significantly higher CFU scores in the patient group with unresectable HCC (n = 53) compared to the patient group with resectable HCC (n = 27) (P = .027, Fig. 2A). Furthermore, with the available recurrence data for the 17 of 27 patients in the resectable group who had been followed for at least 1 year, we found the preoperation CFU score for those patients with recurrence within 1 year of operation was much higher (P = .013) (Fig. 2B).

Table 1 shows that the CFU scores were not significantly different when patients with HCC were categorized by sex, age, white blood cell count, hemoglobin level, platelet count, degree of liver function impairment (total bilirubin and albumin levels), and presence of hepatitis B surface antigen.

Positive Correlation of CFU Scores With Circulating Levels of AFP. Because circulating AFP is a specific biomarker of HCC, we analyzed the correlation between the circulating AFP levels for those patients with AFP greater...
than 20 ng/mL (n = 62) at the time when CFU scores were obtained. Figure 3 shows that patients with higher CFU scores correlated positively with higher circulating levels of AFP (n = 62, r = .303, P = .017). The mean CFU score of these patients with serum AFP greater than 20 ng/mL was 21.28 ± 19.9 (range 1.1-91.0). Among the other 18 patients with serum AFP less than or equal to 20 ng/mL, the mean CFU score was 23.9 ± 21.4 (range 4-81.8). In the later subgroup, there was no significant correlation between CFU scores and serum AFP levels.

Table 1. CFU Scores and Clinical Features in Patients With HCC

| Clinical Features | Mean CFU Score (± SD) | P Value |
|------------------|-----------------------|---------|
| Sex              |                       |         |
| Male (n = 44)    | 24.9 ± 22.2           | 0.138   |
| Female (n = 36)  | 18.2 ± 16.9           |         |
| Age              |                       |         |
| ≤60 (n = 46)     | 20.0 ± 18.9           |         |
| ≥60 (n = 34)     | 24.5 ± 21.8           | 0.328   |
| White blood cell |                       |         |
| ≤4 × 10^9/L (n = 10) | 21.4 ± 19.0 |         |
| ≥4 × 10^9/L (n = 70) | 21.9 ± 20.4 | 0.940   |
| Hemoglobin       |                       |         |
| ≤12 g/dL (n = 46) | 22.6 ± 15.8           |         |
| ≥12 g/dL (n = 34) | 21.6 ± 21.6           | 0.860   |
| Platelet         |                       |         |
| ≤150 × 10^9/L (n = 31) | 22.2 ± 18.7 |         |
| ≥150 × 10^9/L (n = 49) | 21.7 ± 21.2 | 0.923   |
| Albumin          |                       |         |
| ≤40 g/L (n = 52)  | 22.1 ± 20.0           |         |
| ≥40 g/L (n = 28)  | 21.4 ± 20.7           | 0.890   |
| Total bilirubin  |                       |         |
| ≤20 μmol/L (n = 52) | 20.7 ± 22.8           |         |
| ≥20 μmol/L (n = 28) | 24.1 ± 13.9           | 0.468   |
| HBsAg            |                       |         |
| Negative (n = 14) | 17.8 ± 22.0           |         |
| Positive (n = 66) | 22.8 ± 19.8           | 0.404   |

Abbreviations: HBsAg, hepatitis B surface antigen; CFU, colony-forming units; HCC, hepatocellular carcinoma.

Positive Correlation of CFU Scores With Circulating Levels of IL-8 and VEGF. Both VEGF and IL-8 are important cytokines that have been reported to be involved in tumor angiogenesis in HCC.29-31 We there-
fore examined the circulating levels of these cytokines in plasma samples that were extracted at the time when CFU scores were obtained. The levels of cytokines obtained by ELISA were correlated with the CFU scores. As shown in Fig. 4, significant positive correlation was observed between plasma VEGF levels and CFU scores \( (r = 0.242, P = 0.035) \), and positive correlation was also observed between plasma IL-8 levels and CFU scores \( (r = 0.258, P = 0.025) \).

**Discussion**

The isolation of EPCs in adult human peripheral blood was first reported almost a decade ago.\(^9\) Subsequently, using transgenic mouse models, Asahara et al. demonstrated that the BM-derived EPCs contribute to pathological neovascularization.\(^{40}\) There is now mounting evidence in animal studies in support of the hypothesis that BM-derived EPCs facilitate tumor growth by direct incorporation into the sprouting vascular endothelium.\(^{36,41,42}\) This led to a proposed paradigm that in cancer patients, EPCs are mobilized from the BM by cytokines secreted by the tumor, migrate via circulation to the site of tumor growth, and are incorporated into tumor neovascularity.\(^{28}\)

While the monitoring of BM-derived EPCs in the circulation should be the most convenient approach to study this proposed paradigm in cancer patients, clinical reports on the significance of BM-derived EPCs in cancer patients are scarce. As mentioned in recent reviews on endothelial cells,\(^{27,28}\) the lack of accurate phenotypic definition for EPCs is largely confounded by the presence of other circulating endothelial cells in the blood circulation. Furthermore, the extremely low number of EPCs present in the circulation also makes isolation and characterization of EPCs in cancer patients difficult. The specific culture method used in our study is currently the only reliable approach to quantify the level of BM-derived circulating EPCs.\(^{27}\) This culture method avoids contamination of EPC culture with the monocytes, hematopoietic progenitors, and circulating endothelial cells in human peripheral blood. Many recently published reports used this method as their fundamental basis for EPC studies.\(^{27,28,41,43}\)

Unlike the use of FACS for selection of cells with EPC markers, which is phenotypic by nature, the CFU shows that the selected EPCs have proliferative capacity. Indeed, it has been reported that a subset of cells derived from such CFU cell clusters can divide.\(^{27}\) Our study, as well as others, observed that the center core of the CFUs disappeared in a few days in culture, leaving behind some spindle-shaped cells, which represent cells that previously radiated out from the center core of the CFU.\(^{27,28}\) In the literature, it has been well defined that these adherent spindle-shaped cells express endothelial cell antigens and they can divide, with the total number of these cells increasing by 8-fold to 90-fold within a period of 3-10 days.\(^{27,44,45}\) Although we have not counted the fold increase of this subset of cells with time in our assay, we could also observe that once the core of CFU disappeared, the spindle-shaped cells that were left behind in the cell culture became more confluent after a few more days of culturing. It would be of interest to perform further studies for phe-
nototypic characterization of this subset of cells that can divide.

In contrast to the high levels of circulating EPCs that have been reported in patients with multiple myeloma, a study reported that circulating EPC levels in patients with gastric and breast cancers were not higher than controls. Our study, by using a more specific culture method for EPCs, demonstrated significantly higher CFU scores in patients with HCC compared with controls and patients, and therefore illustrated that circulating EPCs could also be elevated in a solid cancer, namely HCC. This finding provides clinical evidence in support of hypotheses from experimental observations in animal studies regarding mobilization and migration of BM-derived EPCs via circulation into tumor neovasculature. Furthermore, with the circulating level of AFP being a specific biomarker for disease progression in HCC, the direct positive correlation of circulating levels of EPCs with serum AFP levels further supports the clinical relevance of the elevated circulating EPCs levels in patients with HCC.

To avoid any treatment-associated interference, such as change in cytokine levels caused by possible increase in angiogenesis activity during wound healing after surgery or after transarterial chemoembolization, blood samples for the assays in this study were taken before any treatment. Although it is clear from this study that the mean CFU score was higher in patients with HCC compared to both healthy subjects and patients with cirrhosis, we observed a wide range of CFU scores (range 4-91) among the 80 patients with HCC. Interestingly, as shown in Fig. 2A, segregation of the patients into surgical treatment group and unresectable group revealed higher scores in the latter group. Furthermore, among patients with surgical resection, those who were disease-free 1 year after resection had lower preoperative CFU scores compared with patients who had disease recurrence within 1 year. These findings could suggest a possible relationship between more aggressive tumor and higher circulating level of EPCs.

In experimental settings, VEGF appears to be the most important mediator for mobilization of BM-derived EPCs. However, reports on other solid tumors have so far failed to demonstrate a positive correlation between circulating VEGF and EPC levels. Interestingly, we observed a significant positive correlation between circulating EPC and VEGF levels. A previous study from our group demonstrating that circulating VEGF levels correlated with VEGF expression by tumor cells. It is tempting to speculate that HCC tumor cells might be able to mobilize BM-derived EPCs via VEGF signaling, but the correlation between plasma VEGF levels and CFU scores in this study was weak ($r = .242$). Therefore it remains to be determined whether VEGF recruits EPC from BM in patients with HCC. Furthermore, our study also demonstrated a positive correlation between circulating IL-8 levels and circulating EPC levels. Interestingly, a recent report by He et al. described that compared with secretion by mature endothelial cells, significantly higher levels of IL-8 were secreted by EPCs, and such IL-8 appeared to play as an important mediator of EPCs’ paracrine mitogenic effect on the mature endothelial cells. Furthermore, because both circulating level of IL-8 and VEGF have been reported to be highly associated with angiogenesis and prognosis of HCC, the finding in our study provides additional evidence for the possible role of EPCs in tumor angiogenesis.

In hematological malignancies such as multiple myeloma, circulating level of EPCs has already been shown to have close association with disease activity, and is thus implicated as a useful prognostic biomarker. The results of this study illustrate significant correlations of the circulating EPC level with known HCC biomarkers such as AFP, VEGF, and IL-8. Furthermore, higher EPC levels were associated with more advanced HCC tumors. Taken together, our study suggests that EPC may have a significant implication in HCC in terms of prognosis. Previously, we demonstrated that HCC tumors with high tumor angiogenesis activity were associated with a higher tumor recurrence rate after surgical resection. It would be justified to further investigate the prognostic significance of circulating EPC level with a study of a larger sample of patients with longer follow-up. Because CFU score correlated with AFP level, which is a known prognostic factor of HCC, it would be helpful to perform a multivariate analysis of known prognostic variables together with CFU and AFP levels to assess if CFU score has independent prognostic value in the management of patients with HCC when long-term follow-up data are available. The clinical significance of circulating EPC level could be better assessed by study of the longitudinal evolution of CFU scores in the same patient who undergoes resection of HCC to see if circulating EPC levels increase at the time of tumor recurrence. In this study, blood samples at the time of tumor recurrence were not available for assay of circulating EPC levels. A much larger cohort in the surgical group with serial collection of blood samples over a longer follow-up period should be carried out to further confirm the prognostic significance of HCC.

In conclusion, our study shows that circulating EPC levels were significantly elevated in patients with advanced HCC compared to patients with early resectable HCC, patients with liver cirrhosis only, and healthy controls. Furthermore, there were significant positive correlations of circulating EPC levels with circulating AFP, VEGF, and IL-8 levels. Our data suggest that further studies are worthwhile to validate the prognostic value of
circulating EPC levels in patients with HCC, and to explore possible therapeutic strategies against HCC by targeting bone marrow mobilization of EPCs.

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