Distribution of endothelial progenitor cells in tissues from patients with gastric cancer

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Abstract. It is accepted that endothelial progenitor cells (EPCs) are recruited into tumor sites and take part in the neovascularization of tumors. However, few articles have discussed the specific distribution of EPCs in vivo in tissues of gastric cancer patients. For this reason, the present study sought to elucidate EPC distribution in vivo in tissues of patients with gastric cancer. Fresh tumor tissues were collected from 26 newly diagnosed patients with histologically confirmed gastric cancer (mean age, 51 years; range, 21-81 years; 7 females, 19 males). All patients were treated surgically with curative intent. One portion of the fresh tissues was prepared for flow cytometric analysis and another was immediately snap frozen in liquid nitrogen and stored at -80°C for later use in quantitative polymerase chain reaction. The analysis was based on two groups of tissues, namely the cancer group and cancer-adjacent group. The presence of CD34+/CD133+ double-positive cells was determined in cancer-adjacent and cancer tissues by flow cytometry. The analysis revealed that the total number of EPCs in cancer tissue was slightly greater than the number in the cancer-adjacent tissue, but not to the point of statistical significance. The number of EPCs in cancer-adjacent and cancer tissues of patients with early-stage gastric cancer was higher than the EPC count in late-stage gastric cancer patients, and significant differences were identified in the number of EPCs in cancer tissue between patients of different tumor stages. Levels of cluster of differentiation (CD)34, CD133 and vascular endothelial growth factor receptor 2 were not significantly different in cancer-adjacent tissue compared with cancer tissue. These results suggest that cancer-adjacent and cancer tissue of gastric cancer patients may be used as a reference index in the clinical and pathological staging of tumors.

Introduction

It is well known that the growth of new blood vessels is a component of certain pathological conditions, including tumor growth and metastasis. Previous experimental studies have suggested that bone marrow-derived circulating endothelial progenitor cells (EPCs) migrate to neovascularization sites and differentiate into endothelial cells in situ, a process termed vasculogenesis (1,2). Whether bone marrow-derived EPCs participate in the progression of gastric cancer has not yet been evaluated.

Bone marrow-derived EPCs were first isolated from whole blood using magnetic microbeads coated with cluster of differentiation (CD)34 antibody by Asahara et al in 1997 (2). EPCs are a group of immature endothelial cells with proliferation and differentiation potential, and are derived from hematopoietic stem/progenitor cells, which are also the precursor of hematopoietic cells. It is widely accepted that CD34+, CD133+ and vascular endothelial growth factor receptor 2+ (VEGFR-2, also known as kinase insert domain receptor or Flk1) cells are EPCs (3). EPCs are important initiators of vasculogenesis in the process of tumor neovascularization. Increased levels of EPCs in peripheral blood were identified in patients with pancreatic carcinoma (4), malignant gliomas (5), and ovarian (6), non-small cell lung (7) and gastric (8) cancer. Consequently, the level of EPCs has been proposed as a novel biomarker for the diagnosis and monitoring of these lesions. Although these studies have prompted trials to use EPCs in this way, the specific distribution of EPCs in vivo, and whether the number of EPCs is associated with tumor stage in cancer tissue, has seldom been discussed. The present study investigated the distribution of EPCs in vivo, providing valuable information for clinical diagnosis, detection and treatment of cancer.

Materials and methods

Patients. Patients were recruited from the Lanzhou Military Command General Hospital of the People's Liberation Army (Lanzhou, China). The ethics committee of Lanzhou Military
Table I. Primer sequences used for qPCR.

| Primer | Sense, 5'-3' | Antisense, 5'-3' |
|--------|-------------|----------------|
| CD34   | CTTCACCTGTACTCTTCC | CAGCTGTTGATAAGGGTTA |
| CD133 (9) | TGGATGCAGAACTTGACAACGT | ATACCTGTACGACAGTCGGTT |
| VEGFR-2 | CACCACCTGAACGCTAGATGA | GCTCGTGGCAGCACCTTT |
| β-actin | TCTGGCACACACCTTTTCTAC | CTCCTAAATGTCACGCAGATTTTC |

CD, cluster of differentiation; VEGFR-2, vascular endothelial growth factor receptor 2.

Command General Hospital of the People's Liberation Army (Lanzhou, China) approved the study, and written informed consent was obtained from all study participants. Fresh tumor tissues from 26 newly diagnosed patients with histologically confirmed gastric cancer were collected. All patients were treated surgically with curative intent. The patients had no additional malignant, inflammatory or ischemic disease, or wounds or ulcers that could influence the number of EPCs. One portion of the fresh tissues was prepared for flow cytometric analysis and another was immediately snap frozen in liquid nitrogen and stored at -80°C for later use in the study. The entire group of patients included 19 male cases and 7 female cases aged 21-81 years (mean, 51 years; median, 55 years; ≥55 years of age in 13 cases). No patient had received radiotherapy or chemotherapy prior to tumor excision. In addition, normal gastric cancer tissue 5 cm from the tumor margin was obtained from each patient for comparison.

**Sample preparation.** Fresh tissue (50 mg) was washed with saline (10% heparin), then soaked in saline for ~40 min. With ophthalmic scissors the tissue was cut into small pieces ~1 mm² and digested with 1 ml trypsin at 37°C for 15 min, gentle agitation every 3 min during the process. Digestion was terminated with 2 ml 10% fetal calf serum (Clonetics, Cambrex, MD, USA). Large clumps of tissue and connective tissue were removed using a 200-mesh filter and the cell suspension was collected. The solution was centrifuged at low speed (1,300 x g) for 10 min, the cells were collected and the supernatant was discarded. The precipitate was washed with 1 ml PBS buffer containing CD34 (BD Biosciences, San Diego, CA, USA), CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany), VEGFR-2 (R&D Systems Inc., Minneapolis, MN, USA) and 5 µl monoclonal fluorescent antibody. The mixture was incubated for 30 min according to the manufacturer’s recommendations (BD Biosciences). Samples were fixed in 1% formaldehyde and analyzed using a FACSCalibur flow cytometer (BD Biosciences). The control and experimental groups used the same processing methods.

**Flow cytometric analysis.** EPCs were identified by the expression of CD34, VEGFR-2 and CD133. A volume of 200 µl single cell suspension was incubated for 30 min in the dark with fluorescein isothiocyanate-labeled monoclonal antibodies from mouse ascites against human CD34, allophycocyanin-labeled monoclonal antibodies from mouse ascites against human CD133 and phycoerythrin-labeled monoclonal antibodies from mouse ascites against human VEGFR-2. Mouse isotype-identical antibodies served as controls (BD Biosciences). For analysis, 200,000 cells within the leukocyte gate were acquired using a FACSCalibur analyzer and data were processed using FACSDiva software (both purchased from BD Biosciences). The percentage of cancer-adjacent and cancer tissue EPCs was determined using the three-color antibody panel previously described and an appropriate gating strategy. CD45-dim cells positive for VEGFR-2 with low to medium forward- and side-scattered light and positive for CD34 and CD133 were considered EPCs. The absolute number of cells (cells/µl) was calculated with the following formula: Percentage of cells x total nucleated cells/100 (10).

**Quantitative polymerase chain reaction (qPCR).** Total RNA was extracted from tissue that had been frozen in liquid nitrogen immediately following surgery, using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA), and cDNA was synthesized from each tissue sample with M-MLV reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer’s instructions. qPCR (20 µl reactions) with SYBR GreenER qPCR SuperMix Universal (Invitrogen Life Technologies) was performed in triplicate using a 7300 Fast Real Time PCR system (Stratagene, La Jolla, CA, USA). A no-template reaction (RNA replaced with water) was used as a negative control. Target gene expression was determined using the 2-ΔΔCt method and normalized using β-actin as an internal control. To determine PCR amplification efficiency, standard curves were constructed using different concentrations of template cDNA for VEGFR-2, CD34, CD133 and β-actin. For all genes, the correlation coefficient of the standard curve was ≥0.96, and the amplification efficiency was almost 1.0. The primer sequences used for qPCR are listed in Table I.

**Statistical analysis.** Results are presented as mean values ± standard deviation. Statistical analyses were performed using SPSS software (version 17.0; SPSS Japan Inc., Tokyo, Japan). Differences between groups were calculated using the Mann-Whitney U test and two-way analysis of variance, and these were later evaluated by post hoc analysis. P<0.05 was considered to indicate a statistically significant difference.


Results

EPCs and clinical data. Although no clear definition of EPC exists, based on previous studies using flow cytometry, this study determined the numbers of CD34/CD133 double-positive cells in cancer-adjacent and cancer tissue of gastric cancer patients (Fig. 1A). Additionally, the number of VEGFR-2+/CD133+ cells was measured, corresponding to a subfraction of immature EPCs. However, as VEGFR-2+/CD133+ and CD34+ EPC counts did not differ from each other significantly (P>0.1 for all analyses; data not shown), in further experiments, only the levels of EPCs with the latter phenotype were evaluated, in accordance with previous studies (11).

For the 26 patients, the results revealed that the mean number of EPCs in cancer tissue was marginally greater than the number in the cancer-adjacent tissue, but with no statistical significance in the age and gender groups (Table II). The number of EPCs in cancer-adjacent tissue of patients with early-stage gastric cancer was lower than the number in patients with late-stage gastric cancer (TNM stage I, n=14, 340±105.8 cells/mm²; stage II, n=7, 821±197.3 cells/mm²; stage III, n=7, 1,360±196.6 cells/mm²; stage IV, n=5, 2,455±163.5 cells/mm²). However, the number of EPCs in cancer-adjacent tissue at each TNM stage was no higher than that in cancer tissue. Further analysis revealed that Borrmann stage and histological type were also associated with the number of EPCs (P<0.05) (Fig. 1B).

EPC markers in cancer-adjacent and cancer tissue determined by qPCR. Cancer tissue CD34, CD133 and VEGFR-2 mRNA levels were determined by qPCR. Levels of CD34, CD133, VEGFR-2 were not significantly different in cancer-adjacent tissue compared with cancer tissue in the gastric cancer patients (Fig. 2).

Discussion

Gastric cancer is the fourth most common type of cancer worldwide (12). According to Parkin et al (13), gastric cancer has the second and fourth highest mortality rate for men and women, respectively (13). The prognosis of gastric cancer patients is poor, with a five-year survival rate of ~20% (12,14). Surgical resection with curative aim is the principal treatment for gastric cancer, and the suitability of surgical resection is decided based on the tumor stage of the patient (15).

At present, the role of EPCs in tumors is a major focus in the field of oncology. However, few articles have discussed the specific distribution of EPCs in vivo. For this reason, the present study sought to elucidate EPC distribution in vivo by assessing the number of EPCs in cancer tissue excised from gastric cancer patients. In addition, the study analyzed the association between EPCs and tumor stage in an attempt to identify more reliable diagnostic methods for tumors.

| Data                  | Patients, n | Endothelial progenitor cells, n | P-value |
|-----------------------|-------------|---------------------------------|---------|
| Age, years            |             | Cancer-adjacent | Cancer |       |
| <55                   | 13          | 984±618.4          | 988±622.5 | >0.05 |
| ≥55                   | 13          | 1318±889.7          | 1189±833.5 |       |
| Gender                |             | Cancer-adjacent | Cancer |       |
| Male                  | 7           | 1102±777.8          | 1042±715.5 | >0.05 |
| Female                | 19          | 1169±787.2          | 1106±751.2 |       |
| TNM stage             |             | Cancer-adjacent | Cancer |       |
| I                     | 7           | 299±98.2            | 340±105.8 | <0.001 |
| II                    | 7           | 817±206.4           | 821±197.3 |       |
| III                   | 7           | 1364±367.8          | 1360±196.6 |       |
| IV                    | 5           | 2187±415.7          | 2455±163.5 |       |
| Borrmann stage        |             | Cancer-adjacent | Cancer |       |
| II                    | 9           | 398±150.9           | 378±225.7 | <0.001 |
| III                   | 7           | 976±166.7           | 1018±442.9 |       |
| IV                    | 10          | 1951±552.2          | 2010±427.4 |       |
| Differentiation status|             | Cancer-adjacent | Cancer |       |
| Well differentiated    | 7           | 364±119.3           | 319±106.3 | <0.001 |
| Moderately differentiated | 7   | 1047±291.7          | 986±258.8 |       |
| Poorly differentiated  | 12          | 2068±556.3          | 1986±429.8 |       |

Cells numbers are expressed as mean ± standard deviation.
Since EPCs were first reported (2), it has been recognised that EPCs correlate closely with neovascular formation. Preliminary reports have demonstrated that circulating EPCs may be incorporated into tumor vascularization and may correlate with neovascularization (1). The existence of a BM reservoir and its contribution to neovascular formation are of
great interest (16) and may be used as an index in order to
detect cancer progression (15). However, it remains uncertain
as to whether EPCs are present in patients with cancer and
what roles they may play.

EPCs are derived from BM-derived hematopoietic cells,
which may be induced into forming ECs which in turn
contribute to neovessel formation (18). Tumor cytokines,
evolved in the formation of CEPs, are derived from EPCs
in the peripheral blood circulation. Subsequently the EPCs
gradually infiltrate the tumor vascular bed and are incorpo-
rated into neovessels (16).

In the present study, EPCs were measured by fluores-
cence-activated cell analysis of fresh cancer and cancer-adjacent
tissue and defined by the expression of surface markers
CD34+/VEGFR-2+ and CD133+/VEGFR-2+ (19). Experiments
on the migration of EPCs toward the site of neovasculariza-
tion were carried out in order to examine the significance
of EPCs in cancer tissue. The present study provides evidence
that BM-derived EPCs, defined by the cell surface expression
of CD34 and CD133, differentiate into mature endothelial cells
and contribute structurally and functionally to tumor neovas-
cularization.

The purpose of the study was to observe whether the
number of EPCs in gastric cancer and paracancerous tissue
differed. The results revealed no significant differences
between gastric cancer tissue and paracancerous tissue.
According to previous reports, hypoxia in the tumor tissue
micro-environment is the initiating factor for EPCs to par-
ticipate in tumor growth. Tumors may produce high levels
of hypoxia-inducible factor (HIF) 1α, which induces the
production of VEGF and stromal derived factor 1α. VEGF
is one of the most important target genes of HIF-1α and it
is also a main factor in the creation of new blood vessels
in tumors. Tumor cells, alongside immune cells and tumor
fibroblasts, can secrete VEGF directly. VEGF mobilizes
VEGFR-2-positive EPCs to the peripheral blood circulation,
which then migrate to the tumor site to assist in the formation
of new blood vessels (20). This may suggest that changes in
the number of EPCs in gastric cancer tissue and paracan-
cerous tissue may not be significantly different.

The stage of the tumor is extremely important for treat-
ment and prognosis, and this study demonstrated incidentally
that EPC levels correlate with tumor clinical and pathological
staging. The number of EPCs is significantly correlated with
tumor TNM stage, Bormann stage and degree of differen-
tiation. Thus, testing the number of EPCs in gastric cancer
tissue and paracancerous tissue may provide indicators for the
clinical and pathological diagnosis of gastric cancer.

To date no unique marker for EPCs has been reported.
Additionally there is no consensus on the definition of EPCs.
Therefore, building a functionally characterized dataset rare
putative EPCs based on FACs phenotypes is difficult, making
comparisons with other published work difficult as there is
no standard. Therefore, it is necessary to locate an effective
method for the enumeration of circulating EPCs (21,22). With
a better understanding of EPCs, we can approach the role of
EPCs in tumor progression. The present study demonstrates
that EPC levels are significantly increased and are correlated
with cancer stage in the cancer tissue and paracancerous tissue
of gastric cancer patients. Furthermore, although our data
suggest the participation of EPCs in tumor growth in gastric
cancer, it is not clear whether these cells are essential for this
process. Further investigation is warranted for the potential
application of EPCs in monitoring disease progression or as
targets for gastric cancer treatment.

These results suggest that EPC count in cancer-adjacent
and cancer tissue of gastric cancer patients can be used as
a reference index in the clinical and pathological staging of
tumors. Additional prospective investigations in a large popu-
lation are required to confirm these findings.

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References

1. Asahara T, Masuda H, Takahashi T, et al: Bone marrow origin
of endothelial progenitor cells responsible for postnatal vascu-
logenesis in physiological and pathological neovascularization.
Circ Res 85: 221-228, 1999.

2. Asahara T, Murohara T, Sullivan A, et al: Isolation of putative
progenitor endothelial progenitor cells for angiogenesis.
Science 275: 964-967, 1997.

3. Gehling UM, Ergin S, Schumacher U, et al: In vitro differen-
tiation of endothelial cells from AC133-positive progenitor cells.
Blood 95: 3106-3112, 2000.

4. Vizio B, Novarino A, Giacobino A, et al: Pilot study to relate
clinical outcome in pancreatic carcinoma and angiogenic
plasma factors/circulating mature/progenitor endothelial cells:
Preliminary results. Cancer Sci 101: 2448-2454, 2010.

5. Rafat N, Beck GCh, Schulte J, Tuettenberg J and Vajkoczy P:
Circulating endothelial progenitor cells in malignant gliomas. J
Neurosurg 112: 43-49, 2010.

6. Yu Y, Zheng L, Wang Q, et al: Quantity and clinical relevance
of circulating endothelial progenitor cells in human ovarian cancer.
Journal Exp Clin Cancer Res 29: 27, 2010.

7. Dome B, Timar J, Dobos J, et al: Identification and clinical
significance of circulating endothelial progenitor cells in human
non-small cell lung cancer. Cancer Res 66: 7341-7347, 2006.

8. Ahn JB, Rha SY, Shin SJ, et al: Circulating endothelial progenitor
cells (EPC) for tumor vasculogenesis in gastric cancer patients.
Cancer Lett 288: 124-132, 2010.

9. Susman LK, Upalakalin JN, Roberts MJ, Kocher O and
Benjamin LE: Blood markers for vasculogenesis increase with
tumor progression in patients with breast carcinoma. Cancer Biol
Ther 2: 255-256, 2003.

10. Cesari F, Caporale R, Marcucci R, et al: NT-proBNP and the
anti-inflammatory cytokines are correlated with endothelial
progenitor cells’ response to cardiac surgery. Atherosclerosis
199: 138-146, 2008.

11. Ha X, Zhao M, Zhao H, et al: Identification and clinical
significance of circulating endothelial progenitor cells in gastric
cancer. Biomarkers 18: 487-492, 2013.

12. Kamangar F, Dorex GM and Anderson WF: Patterns of cancer
incidence, mortality, and prevalence across five continents:
defining priorities to reduce cancer disparities in different
graphic regions of the world. J Clin Oncol 24: 2137-2150, 2006.

13. Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics,
2002. CA Cancer J Clin 55: 74-108, 2005.

14. Jemal A, Siegel R, Ward E, et al: Cancer statistics, 2008. CA
Cancer J Clin 58: 71-96, 2008.

15. Hartgrink HH, Putter H, Klein Kranenburg E, Bonenkamp JJ, and
van de Velde CJ; Dutch Gastric Cancer Group: Value of palliative
resection in gastric cancer. Br J Surg 89: 1438-1443, 2002.

16. Rafii S, Lyden D, Hattori K and Heissig B: Vascular
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18. Kopp HG, Ramos CA and Rafii S: Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. Curr Opin Hematol 13: 175-181, 2006.

19. Wong CY, Qiuwaxi J, Chen H, et al: Daily intake of thiamine correlates with the circulating level of endothelial progenitor cells and the endothelial function in patients with type II diabetes. Mol Nutr Food Res 52: 1421-1427, 2008.

20. Liu LX, Lu H, Luo Y, et al: Stabilization of vascular endothelial growth factor mRNA by hypoxia-inducible factor-1. Biochem Biophys Res Commun 291: 908-914, 2002.

21. Timmermans F, Plum J, Yöder MC, Ingram DA, Vandekerckhove B and Case J: Endothelial progenitor cells: identity defined? J Cell Mol Med 13: 87-102, 2009.

22. Duda DG, Cohen KS, Scadden DT and Jain RK: A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. Nat Protoc 2: 805-810, 2007.