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·Original Article·

The recruitment of exogenous endothelial progenitor cells in lung tumor model of nude mice

Qiang Peng[1], Ming Liu[1], Shu-Min Song[1], Xian-Hua Li[2], Yi-Hua Du[1], Yong Zhi[1], Min-Yong Wang[3]

[1] Department of Surgery, Affiliated Hospital of Luzhou Medical College, Luzhou, Sichuan 646000, P. R. China; [2] Physiology Laboratory, Luzhou Medical College, Luzhou, Sichuan 646000, P. R. China; [3] Central Laboratory, Luzhou Medical College, Luzhou, Sichuan 646000, P. R. China

[Abstract] Background and Objective: Endothelial progenitor cells (EPCs) play an important role in hypoxia-triggered tumor vasculogenesis. However, the homing of exogenous EPCs in tumors is still unclear. In this study, we investigated the recruitment of exogenous EPCs in human lung adenocarcinoma model of nude mice. **Methods:** EPCs labeled with green fluorescence protein (GFP) were transplanted into nude mice bearing human lung adenocarcinoma. The growth of tumor was observed. After the mice were killed, GFP-EPCs in different tissues were examined by fluorescence. The tumor tissues were stained for CD133, hypoxia-inducible factor-1alpha (HIF-1α), stromal cell-derived factor-1α (SDF-1α), and vascular endothelial growth factor receptor (KDR). Real-time polymerase chain reaction of CD133, HIF-1α, SDF-1α, and VEGF-1 were also performed. **Results:** The growth of tumor in EPC group was significantly faster than that in saline solution group ($P < 0.05$). Under fluorescence microscope, GFP-EPCs were strongly expressed in both tumor and bone marrow. EPCs were recruited to the tumor periphery to participate in tumor vasculogenesis. The expression of CD133, HIF-1α, and SDF-1 mRNA in tumor and bone marrow were significantly higher than that in the liver, spleen, and skin ($P < 0.05$). **Conclusions:** Exogenous EPCs can be recruited to tumor and accelerate tumor growth. Except tumor, bone marrow can also recruit EPCs.

Key words: Endothelial progenitor cell, tumor, recruitment, exogenous, nude mice

Endothelial progenitor cells (EPCs) play an important role in the growth of tumor. However, whether circulating EPCs participate in tumor vasculogenesis remain controversial. Some studies[1-3] show that, although circulating vascular EPCs can be recruited to the tumor vessel wall, it does not constitute a part of tumor vessel and has no contribution to tumor growth. Other studies[4,5] showed that exogenous EPCs not only participate in angiogenesis of tumor but also have significant influence on the growth of tumor. Many cytokines such as hypoxia-inducible factor (HIF), vascular endothelial growth factor (VEGF), and stromal cell-derived factor (SDF) play crucial roles in the recruitment of EPCs. To understand the action of exogenous EPCs to tumor, this study established a human lung adenocarcinoma model in nude mice to investigate the recruitment of EPCs.

Correspondence to: Qiang Peng; Tel: +86-830-3165431; Fax: +86-830-3165437; Email: pengqiangpq@sina.com.cn

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Materials and Methods

Isolation and cultivation of EPCs

The protocol was approved by the Affiliated Hospital of Luzhou Medical College. Informed consent was obtained from 3 donors. All of them were healthy pregnant women administrated by Caesarean birth, age from 22 to 26. Human umbilical cord blood sample (50 mL) was collected from fresh umbilical cord with placenta. After centrifugation (280 × *g* for 10 min), the cell pellets were resuspended in 5 mL Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) containing 10% fetal calf serum (FCS, Sigma, USA), then added to the top of 15 mL 20% Percoll (Sigma, USA) and centrifuged at 350 × *g* for 15 min. After the supernatant

was removed, the cell pellets were collected and washed twice with phosphate buffered saline (PBS). Mononuclear cells were resuspended in DMEM containing 10% FCS and seeded in gelatin-coated 6-well plates (Santa Cruz, USA). After incubation at 37℃ for 1 h, non-adherent cells were collected and resuspended in DMEM containing 10% FCS, 2 mmol/L L-glutamine, 100 μg/mL antibiotics (penicillin/streptomycin), 100 μg/mL endothelial cell growth supplement (Sigma, USA), and 40 U/mL heparin (Santa Cruz, USA). The cells were cultured in gelatin-coated T25 flasks, and the medium was changed every other day.

Cell amplification was observed every day. EPC was detected by CD133 immunofluorescence-histochemical staining and flow cytometry for CD133, CD34 and KDR (Cyagen, USA). Phosphate-buffered saline stain was used as negative control instead of primary antibody.

EPCs transfected with GFP

EPCs digested by 0.02% EDTA were collected, washed and centrifuged at 100 × *g* for 10 min. The cells were cultured in EGM-2 medium and counted. A total of 8 × 10^6 EPCs were transfected with 1 × 10^7 GFP (packaged by lentivirus, Cyagen Inc, USA) and co-cultured at 37℃, 5% CO_2 for 24 h. GFP-EPCs were observed under fluorescence microscope.

Establishment of human lung tumor model in nude mice and implantation of EPCs

After resuscitation, human lung adenocarcinoma A549 cells (provided by the Affiliated Hospital of Luzhou Medical College) were cultured at 37℃, 5% CO_2 for 24 h. Tumor cells were made into cell suspension and inoculated subcutaneously into 48 nude mice (1 × 10^6 cells/mouse). Nude mice inoculated with tumor cells were fed 14 days while the tumor grew up to 5 mm × 4 mm × 2 mm volume. Subsequently, 48 mice were randomly divided into two groups: 40 in EPC group and 8 in control group. GFP-EPCs were injected into the tail vein of the mice in EPC group (1 × 10^5 cells/mouse). Meanwhile, 0.5 mL saline solution was administered to control mouse. All nude mice were fed at 20–22℃, relative humidity 40%–60%, kept and complemented by clean laminar flow cabinet under specific-pathogen free (SPF) environment. The mice in EPC group were killed separately at the 1st, 3rd, 7th, 14th, and 21st day after EPCs injection. Control mice were killed at the 21st day.

Measurement of tumor volume

The tumor volumes of all surviving mice were measured through the longitudinal and vertical length by vernier caliper at the 1st, 3rd, 7th, 14th, and 21st day after EPCs injection. The growth curve of the tumor was analyzed statistically by Graphpad Prism5 software.

After the mice were killed, the tumor, bone marrow, skin, liver, and spleen were resected for following tests.

GFP-EPCs examination under fluorescence microscope

The tissues of the tumor, bone marrow, skin, liver, and spleen were frozen rapidly into sections (4 μm). GFP-EPCs were examined under fluorescence microscope.

Immunohistochemistry assay of samples

Tissues were fixed in 10% neutralized formalin, dehydrated, embedded in paraffin, and sliced into 4 μm sections. Subsequently, the sections were deparaffinized and stained with hematoxylin and eosin (HE) for histological analysis. All sample sections were stained by standard SP immunohistochemistry process using mouse anti-human monoclonal antibodies against CD133, HIF-1α, SDF-1 and KDR (Cyagen, USA). Phosphate-buffered saline was used as negative control instead of primary antibody.

Real-time polymerase chain reaction (PCR)

The mRNA of CD133, HIF-1α, SDF-1, and VEGF expressed in samples were isolated by Trizol reagent and reversely transcribed into cDNA. The cDNA was amplified according to standard PCR. The abundance of transcripts in the cDNA samples was measured by real-time PCR with specific primers (Shanghai Bioengineering Corporation, China) according to the manufacturer's instructions. Samples were submitted to an initial cycle of 94℃, and 45 cycles of 94℃ for 30 s and 55℃ for 1 min in a Themocycler PTC2000 (Funglyn, Canada). Polymerase chain reaction products were subjected to 1% agarose gel electrophoresis with ethidium bromide staining.

Statistical analysis

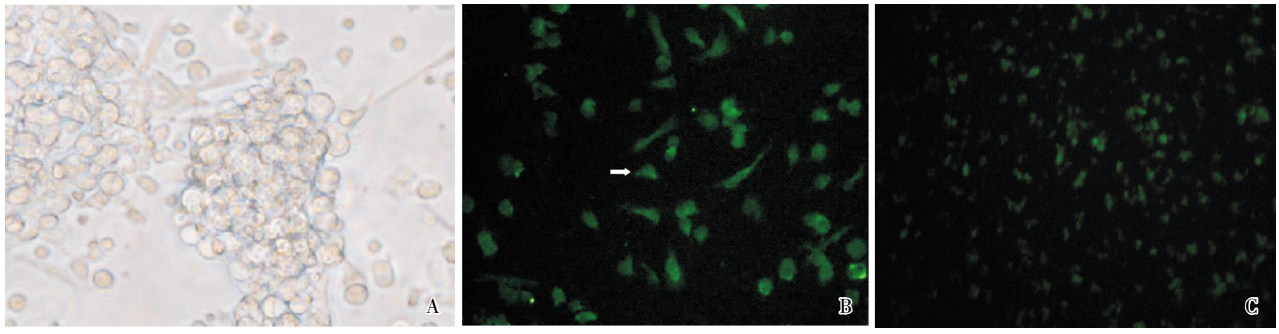
All data are presented as the mean ± standard deviation (SD). Student's *t* test and one-way ANOVA analysis were used to evaluate the significance of differences between groups. $P < 0.05$ were considered significant. All data were analyzed by SPSS version 13.0 soft.

Results

Culture and identification of EPCs

When cultured for 3 days, many mononuclear cells

showed typical "cobble stone"-like appearance (Figure 1A). These cells were CD133-positive (Figure 1B), and the purity was 96%. After seven days, confluent cells were observed and the shape of cells changed from round to spindle. By flow cytometry analysis, the purity of $CD133^+KDR^+$ EPCs was 87.8%; the purity of $CD133^+CD34^+$ cells was 90.1% (Figure 2A, B).



Figures 1 Culture, identification, and transfection of endothelial progenitor cells (EPCs)

Put the mononuclear cells isolated from human umbilical cord blood in culture. After 3 days, light microscopy of adherent cells revealed a cobblestone morphology (×400)(A). The cells were analyzed by fluorescence microscope for the expression of CD133 (green, ×400) and the CD133 positive rate was 96% (B). The cells were transfected by GFP gene. Analyzed by fluorescence microscope, the positive rate of GFP-EPCs was 95% (green, ×200)(C). It symbolized that GFP gene was transfected successfully into endothelial progenitor cells and expressed GFP protein in cells.

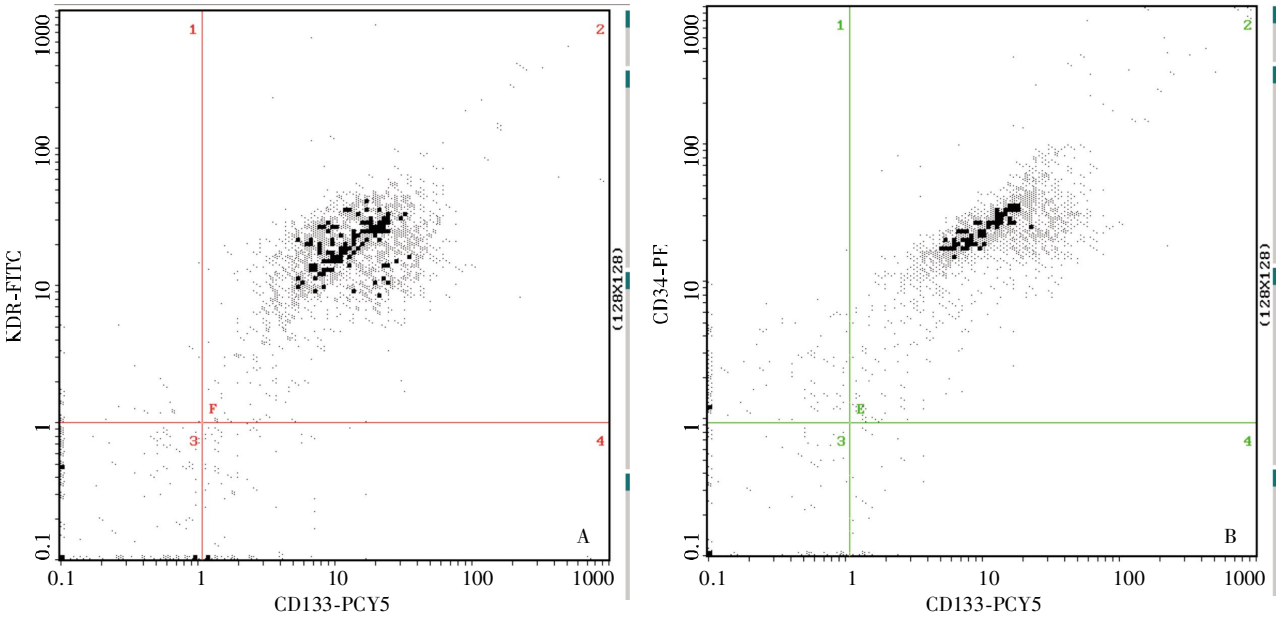


Figure 2 Detection of EPCs by flow cytometry

After 7 days' culture, the cells were harvested by EDTA solution and stained for CD133, KDR and CD34 primary antibody. By flow cytometry test, the positive rate of $CD133^+$ KDR^+ cells is 87.8% (A). The positive rate of $CD133^+$ $CD34^+$ cells is 90.1% (B). $CD133^+$ KDR^+ cells are regarded as endothelial progenitor cells. It confirmed that most of the cultured cells were endothelial progenitor cells.

Transfection and transplantation with GFP-EPCs

It was observed under fluorescence microscope that GFP was expressed strongly in most EPCs at 24 h after transfection, with a transfection rate of more than 95% (Figure 1C).

All tumors in the 48 nude mice grew up to 5 mm × 4 mm × 2 mm volume at the 14th day. GFP-EPCs were infused through the tail vein of mice in EPC group [1×10^5 cells (0.5 mL)/mouse]. Meanwhile, 0.5 mL of 0.9% physiological saline was administered to control mice.

Measurement of tumor volume

At the 1st day after EPCs injection, the volume of tumor had no difference between EPC group and control

group ($P > 0.05$). However, after 7 days, tumor volume in EPC group was significantly bigge r than that in control group ($P < 0.05$). As time went by, this difference became more and more distinct (Figure 3).

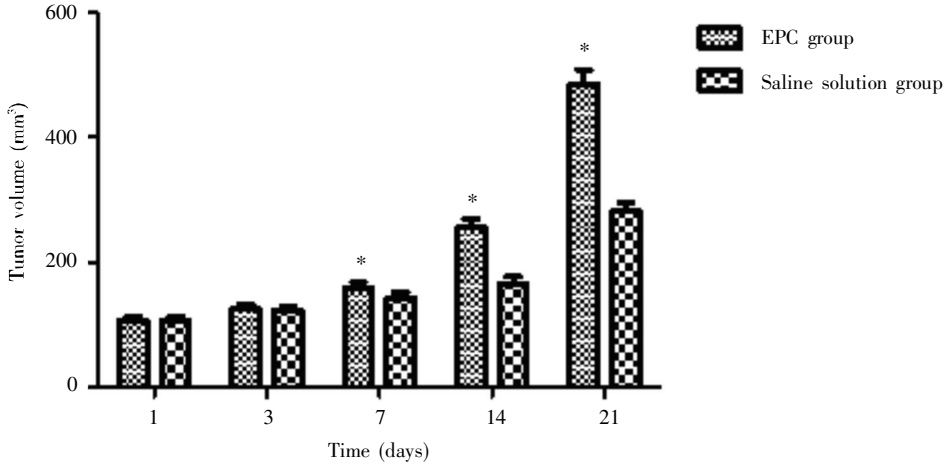


Figure 3 Tumor growth in different groups

At the 1 day after EPCs injection, the tumor volume between EPC group and control group had no significant difference ($P > 0.05$). From the beginning of 7 days after EPCs injection, tumor volume in EPC group was significantly bigger than that in control group ($P < 0.05$). *Compared with saline solution group, P< 0.05. It indicated exogenous endothelial progenitor cells expedited the growth of tumor.

Fluorescence microscope test and immunohistochemistry assay

At the third day after EPCs injection, many GFP-EPCs were observed in both tumor and bone marrow (Figure 4A, B). CD133 and HIF-1α were positive in both tumor and bone marrow (Figure 4C-F).

PCR assay of CD133, HIF-1α, SDF-1α, and VEGF

After EPCs administration, the expression level of CD133 mRNA in tumor and bone marrow increased

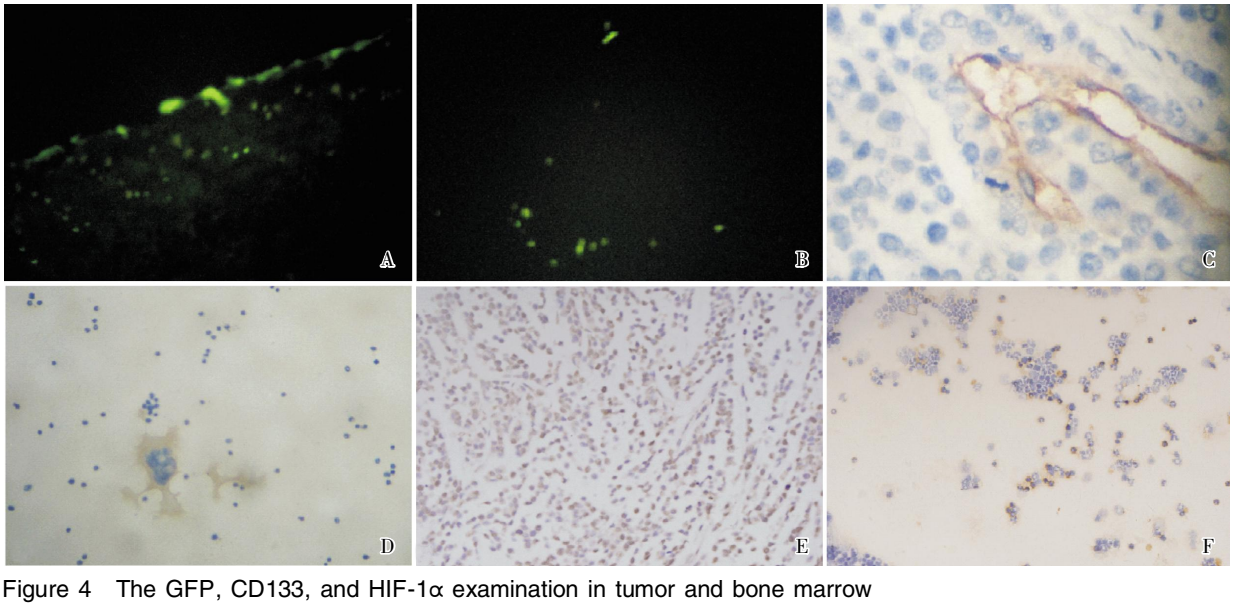


Figure 4 The GFP, CD133, and HIF-1α examination in tumor and bone marrow

By fluorescence microscopy, green fluorescence proteln (GFP)-EPCs (green) were observed mainly at the periphery of tumor tissue (GFP, ×200)(A). It was in accord with vasculogenesis in tumor. GFP-EPCs (green) were also recruited to the bone marrow (GFP, ×200)(B). It indicated bone marrow also had the capability to induce the homing of EPCs. CD133 positive cells (yellow) were observed in lung adenocarcinoma which showed EPCs participated in the formation of tumor vessel (SP, ×400)(C). CD133 positive cells (yellow) were also found in the bone marrow under light microscope (SP, yellow, ×200)(D). By immunohistochemistry test, HIF-1α (yellow) is confirmed to express in tumor tissue (E) and bone marrow (F)(SP, ×200).

gradually and reached the peak at the 7th day which was far higher than that in the skin, liver, and spleen ($P < 0.01$). After that, it was declining. Finally, it reached the low point at the 21st day, but it was still higher than that in the skin, spleen, and liver ($P < 0.05$). Simultaneously, the expression level of CD133 mRNA in bone marrow increased to the maximum at the 7th day, but it kept a relatively stable level without distinct decline after that point (Figure 5).

At the 3rd day, the expression level of HIF-1α mRNA in tumor, bone marrow, liver, spleen, and skin were respectively 1.88 ± 0.61, 1.68 ± 0.51, 1.16 ± 0.32, 1.20 ± 0.35, and 1.04 ± 0.15. The expression level of HIF-1α mRNA in tumor and bone marrow were significantly higher than that in the liver, spleen, and skin ($P < 0.05$). In tumor, bone marrow, liver, spleen, and skin, SDF-1α mRNA was 1.60 ± 0.42, 1.56 ± 0.32, 1.03 ± 0.06, 1.09 ± 0.08, and 1.01 ± 0.06, respectively; VEGF mRNA was 1.58 ± 0.36, 0.56 ± 0.12, 1.07 ± 0.08, 1.11 ± 0.09, and 1.03 ± 0.05, respectively. VEGF mRNA in bone marrow was significantly lower than that in tumor ($P < 0.01$).

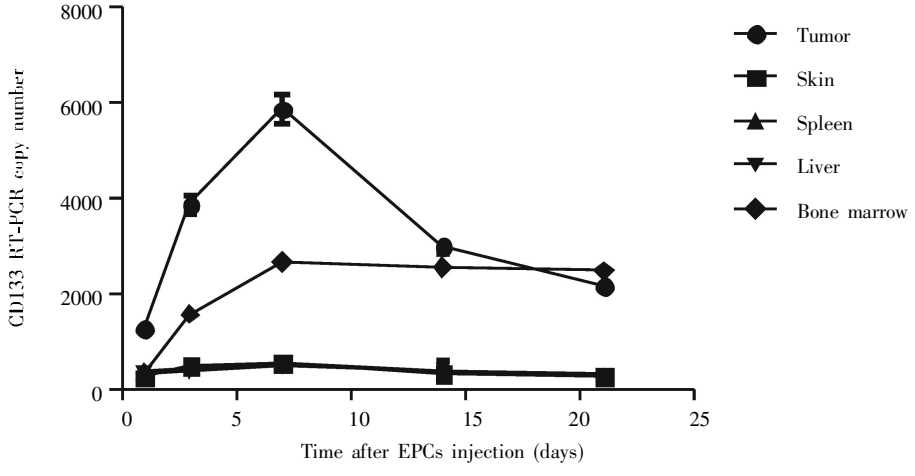


Figure 5 The expression level of CD133 mRNA in different tissues

CD133 mRNA in tumor, skin, spleen, liver, and bone marrow was analyzed separately by RT-PCR ($P < 0.01$). After EPCs administration, the express of CD133 mRNA increased significantly in tumor and bone marrow and reached to the peak at 7 days. After 7 days, CD133 mRNA in tumor declined gradually. However, CD133 mRNA in bone marrow kept in a stable level. It shows that EPCs can not only be recruited to bone marrow but also keep a steady level in it. However, this phenomenon doesn't exist in tumor.

Discussion

EPCs are a group of immature endothelial cells which have proliferation and differentiation potentials. They are derived from hematopoietic stem / progenitor cells (HSPC) which are also the precursor of hematopoietic cells. EPCs can be marked by CD133, CD34, and KDR[6]. Peichev *et al.*[7] reported that mature endothelial cells did not express CD133, a special cell membrane receptor expressed in EPCs. Therefore, the $CD133^{+}KDR^{+}$ cells are identified as EPCs. EPCs have a strong capability of migration. It can not only be recruited to the ischemic area but also participate in tumor angiogenesis[4].

The mobilization and recruitment of EPCs are multi-step and multi-factor events in tumor angiogenesis. This complex process requires the participation of growth factors.

Hypoxia is a common phenomenon in tumor growth. Hypoxia can affect the synthesis of adenosine triphosphate in tumor cells, and down-regulate their aerobic metabolism which enhances tumor cell proliferation and angiogenesis at the transcriptional level. HIF is a class of transcription factor, which is expressed in mammalian cells under low oxygen pressure and regulates cell growth and apoptosis. Studies[8,9] demonstrated that HIF-1α and HIF-2α were two positive regulatory factors, and could promote cell proliferation on a stabilizing hypoxia environment. HIF-3α is considered a negative regulator factor which enhances cell apoptosis[10].

Because of fast growth, tumor is often severely lack of oxygen. Hypoxic micro-environment can induce HIF-1α expression and up-regulate the expression level of VEGF which enhances tumor angiogenesis in cancer growth [11]. HIF-1α is the crucial mediator of the cellular response to hypoxia, regulating the expression of over 60 genes that affect cell survival and metabolism in adverse conditions [12]. Activated by tyrosine kinase, HIF-1α transcription factor is transferred to the nucleus to form functional dimmer and up-regulate the expression level of RNA transcription factor. Over-expression of HIF-1α can increase the expression of VEGF, a specific vascular endothelial cell stimulating factor which promotes the proliferation of endothelial cells and

angiogenesis to alleviate hypoxia. Ceradini *et al.*[13] have found a new mechanism in which hypoxia stimulated the endothelial expression of chemokine SDF-1/CXCL12 through HIF-1α and consecutively resulted in the homing of CXCR4-positive EPCs to hypoxic areas. Ceradini *et al.*[14] investigated the potential impact of reduced oxygen tension on SDF-1 regulation in vivo by using a model of soft tissue ischemia in athymic nude mice. They observed a marked increase in SDF-1 mRNA in ischemic tissue after surgery that was directly proportional to the reduction of tissue oxygen tension and resulted in a similar increase in SDF-1 protein expression. Nomura *et al.*[15] demonstrated that SDF-1 mRNA level in pituitary adenoma cells increased significantly in 1% oxygen compared with that in 21% oxygen. SDF-1 secretion was inversely related to oxygen levels. It strongly suggests that SDF-1 is a crucial angiogenic factor in pituitary adenomas, which acts as a homing agent to mediate the mobilization of CD34-positive EPCs to the tumor parenchyma in hypoxic condition.

These results show that SDF-1 expression induced by hypoxia is crucially important in the selective homing and migration of CXCR4+ progenitor cells to ischemic tissues and tumor. Hypoxia-specific transcriptional element such as HIF-1α is a primary control mechanism for SDF-1 expression.

In this study, tracked by GFP, EPCs were recruited to the periphery of the tumor. Microvessel density at the periphery of tumor in EPC group was significantly higher than that in control group. The result is consistent with other investigations that demonstrated the contribution of EPCs in tumor neo-angiogenesis by MRI and histology[16]. In response to tumor cytokines, including HIF, VEGF, and SDF, exogenous EPCs are mobilized to the peripheral blood circulation of tumor, subsequently, move to the tumor vascular bed and incorporate into neo-vessels. SDF-1 and HIF-1 are intensely expressed in $CD133^+$ cells. EPCs differentiate into mature endothelial cells and incorporate luminally into a subset of sprouting tumor neo-vessels in various tumors[17]. Selective ablation of EPCs in vivo results in a marked delay in tumor growth associated with distinct vascular defects. BM-derived EPCs are critical components of the earliest phases of tumor neo-angiogenesis[18]. Our results showed that tumor growth in EPC group was more rapid than that in control group. It can be deduced that exogenous EPCs can be recruited to tumor and enhance its growth.

Reddy *et al.*[19] found that SDF promoted the growth of Ewing's tumors, even in the face of markedly reduced VEGF. Aghi *et al.*[20] demonstrated that tumor SDF-1, not VEGF, recruited vascular progenitor cells to mitotic neovasculature and increase tumor vasculogenesis. Expression of VEGF in the bone marrow was significantly lower than that in tumor ($P < 0.05$), suggesting that VEGF may play a different role in the mobilization of EPCs in different situations.

Impaired recruitment of endothelial and hematopoietic precursor cells is known to block both tumor angiogenesis and growth[21]. Arafat *et al.*[22] proposed the use of genetically modified cells combined endothelial lineage cells to induce anti-tumor effect. Muta *et al.*[23] demonstrated that IL-12-transfected EPCs suppressed tumor growth through the secretion of IL-12 which activated cytotoxic lymphocytes and natural killer cells. Then, a new strategy of tumor therapy was advanced that EPCs were used as a carrier and modified by special antitumorigenic gene to suppress tumor growth through the homing of EPCs to tumor[24]. Unfortunately, our investigation indicated that tumor was not the unique region in where EPCs congregated. The bone marrow, even other ischemic position could recruit EPC too. Heterogeneous regions of hypoxia in the bone marrow microenvironment can be explained by constitutive and regional expression of SDF-1 in the bone marrow and the EPC tropism to the bone marrow[25]. It reminds us of the danger of the bone marrow damage in target therapy of tumor EPCs. It still has a long way to go before the clinical application of tumor EPCs target therapy.

In conclusion, exogenous EPCs can be recruited significantly to tumor and boost its growth. Hypoxia plays an important role in the recruitment and migration of EPCs to tumor. Moreover, it must be pointed out that tumor is not the only region which recruits EPCs; the bone marrow can also induce the homing of EPCs in hypoxia microenvironment.

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