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Purification and Growth of Endothelial Progenitor Cells From Murine Bone Marrow Mononuclear Cells

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Abstract This study reports the culture and purification of murine bone marrow endothelial progenitor cells (EPCs) using endothelial cell-conditioned medium (EC-CM). Endothelial-like cells appeared at day 5 in culture of bone marrow mononuclear cells in the presence of EC-CM in the culture system, and these cells incorporated acetylated low-density lipoproteins (Ac-LDL) and reacted with endothelial-specific *Ulex Europaeus Lectin*. Continued incubation of these cells at low density with EC-CM for longer than 10 days resulted in the formation of endothelial cell colonies which gave rise to colonies of endothelial progeny and can be passed for many generations in the EC-CM culture system. Cells derived from these colonies expressed endothelial cell markers such as vWF and CD31, incorporated Dil-Ac-LDL, stained positive for *Ulex Europaeus Lectin*, formed capillary-like structures on Matrigel, and demonstrated a high proliferative capacity in culture. These bone marrow-derived adherent cells were identified as EPCs. The purification and the formation of EPC colonies by using EC-CM were associated with the cytokines secreted in the EC-CM. VEGF, bFGF, and GM-CSF in the EC-CM stimulated the proliferation and growth of EPCs, whereas AcSDKP (tetrapeptide NAc-Ser-Asp-Lys-Pro) in EC-CM suppressed the growth of mesenchymal stem cells (MSC) and fibroblasts. This approach is efficient for isolation/purification and outgrowth of bone marrow EPCs in vitro, a very important cell source in angiogenic therapies and regenerative medicine. J. Cell. Biochem. 103: 21–29, 2008.

Key words: endothelial progenitor cells; murine bone marrow; endothelial cell-conditioned medium; GM-CSF; VEGF; AcSDKP; cytokines

Bone marrow of adults contains a small subpopulation of endothelial progenitor cells (EPCs) that can proliferate and differentiate into mature endothelial cells. Although located pre-dominantly in the bone marrow, EPCs were also identified in human peripheral blood [Ingram et al., 2004], fetal liver [Cherqui et al., 2006], and umbilical cord blood [Ingram et al., 2004]. Clinical applications employing EPCs have tremendous therapeutic potential because of their growth potential and their ability for vasculogenesis [Hristov et al., 2003; Shirota et al., 2003; Schmidt-Lucke et al., 2005].

The definition of EPCs has been complex due to the lack of a single specific marker. Early functional EPCs, located primarily in bone marrow, are characterized to express three cell markers of CD133, CD34, and VEGFR 2 [Hristov et al., 2003]. EPCs can also be defined as adherent cells derived from peripheral blood- or bone marrow-derived mononuclear cells demonstrating ac-LDL uptake and isolectin-binding capacity [Liew et al., 2006];

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alternatively, EPCs have been identified by their high clonogenic and proliferative potential in cell culture [Ingram et al., 2004]. EPCs from human bone marrow and periphery blood had been isolated and expanded by immunomagnetic sorting of CD 133+ and CD34+ cells, followed by culture of the cells with appropriate cytokines [Schweitzer et al., 1995; Shi et al., 1998; Quirici et al., 2001]. However, the viability after sorting the antibody-coated magnetic bead cells could be an issue; more importantly, the very small percentage of EPCs in bone marrow [Gehling et al., 2000] or peripheral blood [Peichev et al., 2000] makes it difficult to have large EPC population to start with. Therefore, in vitro purification and expansion of EPCs could be an excellent alternative. With controlled condition, EPCs could also be differentiated along an endothelial lineage requested for respective applications.

To date, in vitro purification and growth of EPCs from bone marrow mononuclear cells have been challenging [Wang et al., 1998]. The primary difficulty was the overgrowth of mesenchymal stem cells (MSC) and fibroblasts in the EPC culture and monocytes contamination. The purpose of this study was to develop an efficient approach for the in vitro purification and growth of EPCs from murine bone marrow, and characterize the EPCs in vitro. This study showed that the purification and outgrowth of bone marrow-derived EPCs were dependent primarily on the angiogenic cytokines and MSC inhibitors in the endothelial cell-conditioned medium (EC-CM), an enriched conditioned medium from a murine bone marrow endothelial cell line [Wang et al., 1998]. The data also demonstrate the autocrine regulation of endothelial cells and the paracrine regulation of endothelial cells and fibroblasts in bone marrow microenvironment.

MATERIALS AND METHODS

Purification and Growth of Murine Bone Marrow EPCs and EPC-Derived EC

Femurs from Balb/c mice of 8–12 weeks of age were flushed three times in DMEM with 26G needle to collect bone marrow mononuclear cells. Bone marrow cells, BMCs ($1-2 \times 10^6$/ml), were suspended in the EPC medium [DMEM containing 20% fetal bovine serum (FBS) and 20% EC-CM (see below) supplemented with 100 U/ml penicillin G, and 100 μg/ml streptomycin SO_4], and $1-2 \times 10^6$ cells were seeded onto a fibronectin-coated well of a 24-well tissue plate. FBS was screened from a group of different lot numbers, the selected FBS has a favorable effect on the growth of endothelial cells rather than fibroblasts. Culture medium was replaced every 24 h. After 5 days in culture, adherent cells were trypsinized and replated at low density of 1×10^4 cells/ml/well (24-well) or 10 cells/200μl/well (96-well) in EPC medium for over 10 days for the formation of endothelial cell colonies. Only the wells (96-well plate) with a single cell attached were selected for the analysis of EPC clonogenic potential. The culture medium was replaced each week. After 18 days incubation, the endothelial-like colonies (>50 cells) or the endothelial-like clusters (<50 cells) were characterized respectively for EC markers and growth features (see below).

Preparation of EC-CM

The murine bone marrow-derived endothelial cell line was established in our laboratory as described previously [Wang et al., 1998]. The immortalized endothelial cells were cultured in DMEM for 48 h without serum. The conditioned medium (EC-CM) was collected from the subconfluent endothelial cell culture. EC-CM was ultrafiltrated in a series of sizes of filtrators. EC-CM was initially separated into two fractions by a Centriprep-10 concentrator (Amicon, Inc., Beverly, MA), resulting in a >10 kDa fraction and a <10 kDa fraction. The latter was ultrafiltrated further with a Centriprep-3 concentrator of cutoff molecular weight of 3 kDa (Amicon, Inc.) to obtain the fraction of mw 3–10 kDa, and the other fraction of mw <3 kDa. Each fraction of EC-CM was filter-sterilized and kept at −20°C before use.

Immunofluonescence and Uptake of DiI-ac-LDL

Adherent cells were incubated with 2.5 μg/ml DiI-acLDL (Biomedical Technologies, Inc.) at 37°C for 3 h. Cells were subsequently fixed with 3% paraformaldehyde and counterstained with *Ulex Europaeus Lectin* conjugated to fluorescein isothiocyanate (UEA-1-FITC) (Biomeda) for 2 h at RT. Nuclei were stained with Hoechst 33258 (4 μg/ml; Molecular Probe). The cells were also immunostained with anti-murine CD31 IgG (Sigma), anti-vWF IgG (Dako), and anti-mouse alpha-smooth muscle actin (SMA) IgG (Sigma), and anti-mouse F4/80 IgG (a murine macrophage marker; Serotec), respectively,

using a standard procedure described before [Yan et al., 1996].

Matrigel Assay

Growth-factor reduced Matrigel (BD Bio Sciences) was added to the wells of a 96-well plate in 30 μl for 1 h at 37°C to allow solidification. The third passage cells-derived from EPC colonies were suspended in EPC medium at a density of 5×10^4 cells/ml, 50 μl of which were plated onto the Matrigel-coated wells. The cells were incubated in tissue incubator for 48 h. Cells were photographed under an inverted phase-contrast microscopy (Leica). The cells derived from MSC colony were used as a control.

Bone Marrow MSC Colony

Bone marrow-derived MSC colonies were obtained by culturing of 1×10^6 murine BMC per 35 mm dish in DMEM supplemented with 20% FBS (unselected serum). Triplicate culture dishes were incubated at 37°C for 9 days in a humidified chamber in tissue culture incubator containing 5% CO_2. The cells were then stained with Wright's–Giemsa or immunostained with antibodies of EC markers and alpha-SMA described above. MSC colonies were counted under an inverted microscopy at 5 and 9 days culture. Colonies containing more than 50 cells was counted as a MSC-colony.

Growth Kinetics of EPCs and Analysis of EC-CM

EPC cells were formed at day 5 after initial BMC culture in EPC medium, these early EPCs were collected and replated with 10,000 cells per well of a 24-well plate or 10 cells per well of a 96-well plate with EPC medium for 10–30 days. Clonogenic formation and proliferative capacity of EPCs were studied by analyzing one EC colony per well in a 96-well plate. The effects of cytokines and EC-CM on growth of EPCs were evaluated by analyzing endothelial colony (En-col) formation. VEGF (50 ng/ml; mouse recombinant, R & D Systems), GM-CSF (25 ng/ml; mouse recombinant, R & D Systems), bFGF (5 ng/ml; mouse recombinant, R & D Systems), or AcSDKP (10^{-9} M; Sigma) was tested individually to the En-col culture condition (DMEM containing 2% FBS and 2% EC-CM). To characterize the effects of various factors in EC-CM, EC-CM was divided into three fractions by molecular weight: >10 kDa fraction, 3–10 kDa fraction, and <3 kDa fraction. Each fraction was also individually tested in the En-col culture system. Medium was replaced every week. Cultures were incubated for 18 days at 37°C in a humidified atmosphere containing 5% CO_2 in air. Colony numbers were counted under an inverted microscope. An aggregation containing more than 50 cells was defined as an EC or MSC colony.

AcSDKP Antibody Neutralization Assay

The <3 kDa fraction of EC-CM was pre-incubated with antibody against AcSDKP [Huang and Wang, 2001]. After pre-incubation, <3 kDa fraction was added to MSC-col culture system. DMEM pre-incubated with this antibody was included as a control.

Statistical Analysis

Student's *t*-test was used to determine statistical differences on cell numbers or colonies between treated groups and controls. *P*-values <0.05 were considered significant.

RESULTS

Identification and Characterization of EPCs Derived From BM

The BMC were plated on FN-coated wells in EPC medium or 20% FBS/DMEM as a control. The rounded cells (Fig. 1A) or rounded cells surrounded by spindle-shaped cells (Fig. 1B) were typically observed after 5 days culture in wells with EPC medium. These BM-derived adherent cells were positive for *UEA-1* staining and ac-LDL uptake (Fig. 1C,D). These cells were replated and incubated for cell colony formation for an additional 15–18 days. The colony cells showed a typical endothelial-like cobblestone morphology (Fig. 1H), and the colony-derived cells showed positive immunoreactivities with antibodies against vWF (Fig. 1E) and CD31 (Fig. 1F); again, they incorporated Dil-Ac-LDL and were positive for *UEA-1* staining, and formed capillary-like structures when plated on Matrigel (Fig. 1I). More importantly, these cells showed a high growth and clonogenic potential in culture (see below). Taken together, the BM-derived adherent cells described above were identified as murine bone marrow-derived EPCs. In contrast, MSC/fibroblasts that exhibited irregular morphology (Fig. 1J) and expressed none of the EC markers but were positive for SMA-alpha (Fig. 1K), or monocytes that showed immunoreactivity with anti-F4/80 IgG (Fig. 1L), were limited in the EPC culture

system but abundant in control cultures (20% FBS/DMEM, Fig. 1J). These cells did not form any tube-like structures when plated on Matrigel (Fig. 1M).

Among the EPC colonies resulted from a single cell assay in 96-well plate, some (Fig. 2A,B) displayed higher proliferative potential which gave rise to large secondary colonies (Fig. 2a,b). These colonies were designated as high-proliferative potential-endothelial progenitor cells (HPP-EPCs); some colonies have less proliferative potential (Fig. 2C) which only gave rise to clusters (Fig. 2c). These colonies were designated as low-proliferative potential-endothelial progenitor cells (LPP-EPCs). Some aggregations which contained less than 50 EC were designated as endothelial cluster-forming cell (Fig. 2D). EC in clusters have limited

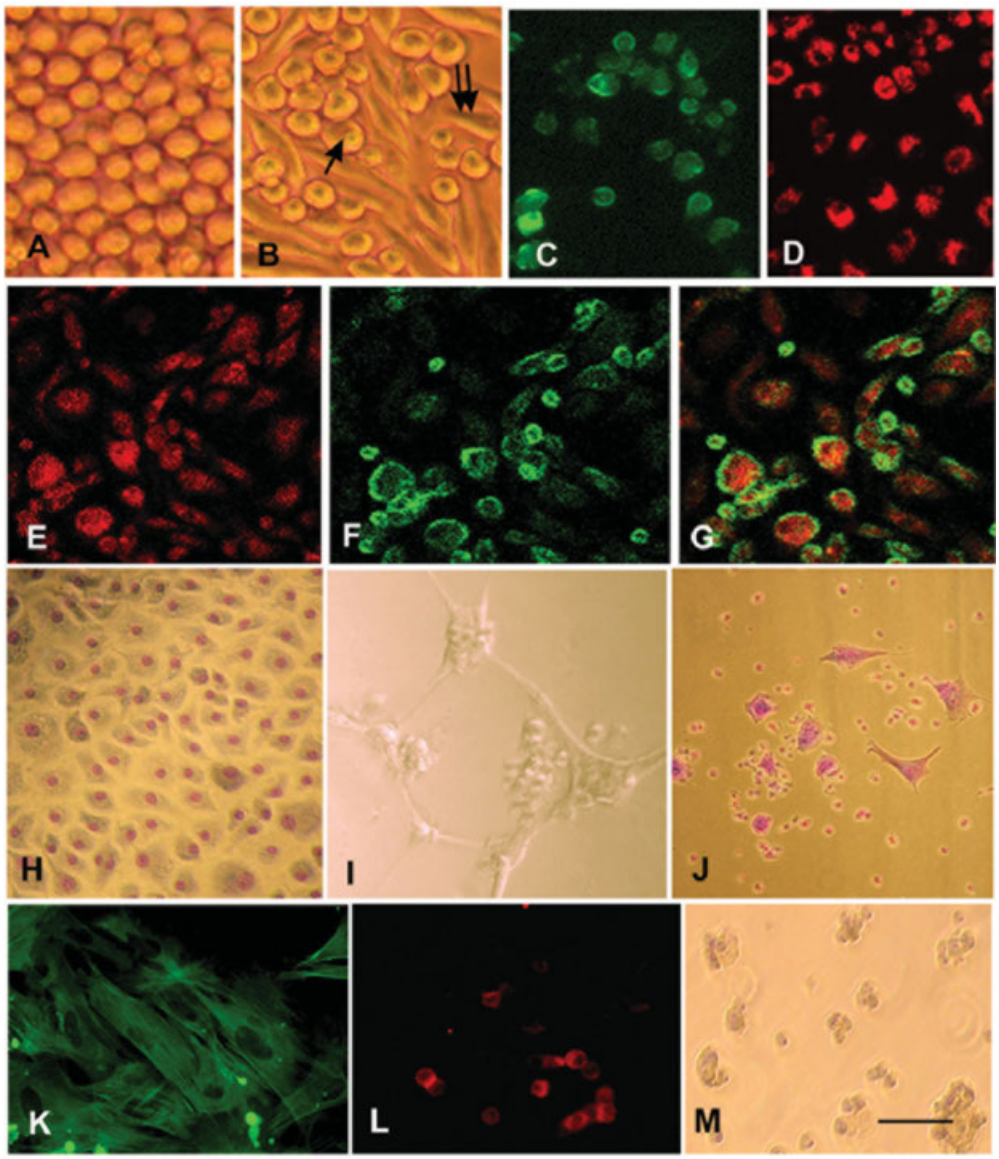

Fig. 1. Murine bone marrow-derived adherent cells were identified to be EPCs. After 5 days culture of BMC with EPC medium, round cells (**A**) or round cells (**B**, arrow) surrounded by spindle-shaped cells (B, double arrows) were observed under phase-contrast microscope. These cells were positive for *UEA-1* staining (**C**) and DiI-acLDL uptake (**D**). They were passed and replated on coverslips for immunostaining. They showed positive for vWF (**E**) and CD31 (**F**); (**G**) merge of E + F; The En-col-derived cells showed cobberstone morphology (**H**, Wright's–Giemsa staining), and formed tube-like structures when plated on Matrigel (**I**). Control cultures (20% FBS/DMEM, no EC-CM) showed abundant MSC/fibroblasts and monocytes (**J**, Wright's–Giemsa staining), positive for SMA-alpha (**K**) and F4/80 (**L**), and did not form tube-like structures when plated on Matrigel (**M**). Scale bar, I, J, and M = 50 μm; others = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proliferative potential (Fig. 2d). These data showed a hierarchy of murine BM EPCs based on their proliferative potentials.

The isolated EPCs can be passed for many generations in EPC culture medium. The cells from passages 1–3 exhibited a similar proliferative potential, but cells from the 4th to 6th passages showed declined proliferative capacity under this culture condition (Fig. 3). Incubation of 1×10^6 murine BMC with EPC culture medium yielded $11.2 \times 10^4 \pm 4.24 \times 10^4$ EPCs at primary culture, resulting in approximately 3.9×10^9 EC over a 90-day culture period (Fig. 3).

The Effect of EC-CM on the Formation of EC Colony

To understand how EC-CM enhanced the purification and outgrowth of EPCs and inhibited the growth of MSC/fibroblasts, EC-CM were fractioned as >10 kDa fraction, 3–10 kDa fraction, and <3 kDa fraction, and their effects on the formation of EPC- or MSC-colonies were analyzed. For endothelial colony (En-col) assay, EPC cells collected after BMC incubation with EC-CM for 5 days were seeded in 1×10^4 /24-well with 10% serum in the presence or absence of EC-CM or its fraction for another 15–18 days culture. For MSC colony (MSC-col) assay, 1×10^6 BMCs were seeded in 1×10^6/35-mm dish containing 20% FBS/DMEM (unselected serum) in the presence or absence of EC-CM or its fraction described above. EC-CM and the >10 kDa fraction showed significant stimulating effect on the growth of En-col (Fig. 4A). There was minimal endothelial cell colony formed when EC-CM was not

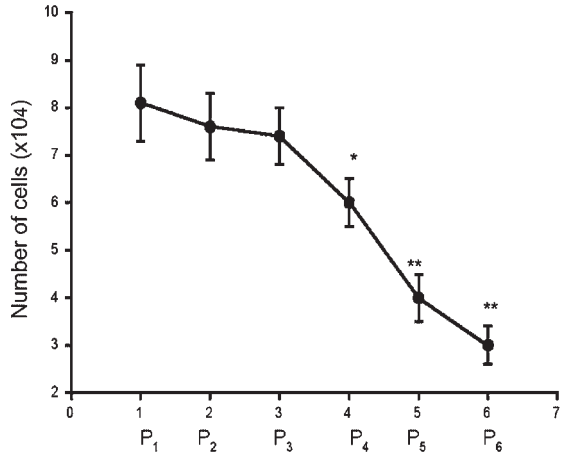

Fig. 3. Proliferative potential of EPCs in various passages in EPC culture medium over a 90-day culture period. BMC (1×10^6) were plated as described in Materials and Methods, and resulted in 11.2×10^4 EC at primary culture. Isolated EC at passage p1 (1×10^4) were seeded in a 24-well plate for triplicates. After 18 days in culture, the cell numbers in each well were counted. p1 cells (1×10^4) were replated in a 24-well plate for triplicates as p2 and total cells in each well were counted after 18 days incubation; and so on (only 1×10^4 EPCs at each passage were replated for the cell counts). The total number of EC after 90-day culture was 3.9×10^9. The mean ± SD of three wells were presented at each point in comparison to passage 1 (p1), $^*P < 0.05$, $^{**}P < 0.01$.

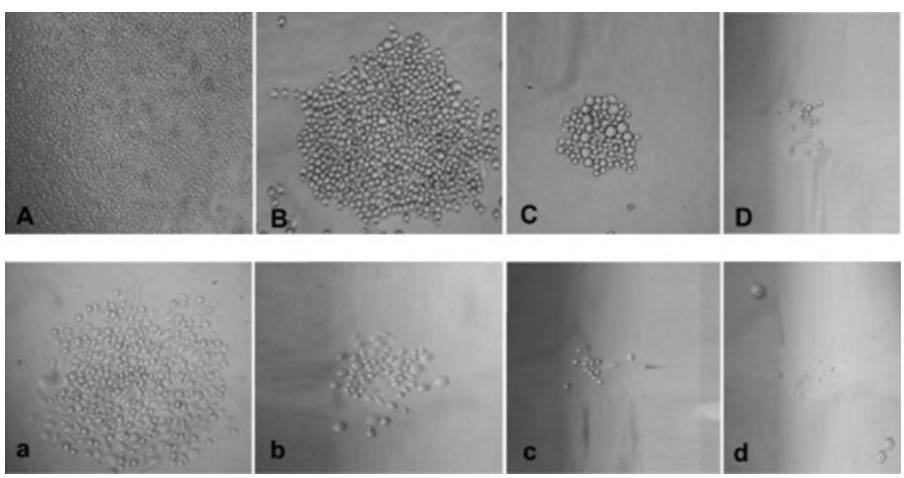

Fig. 2. The hierarchy of murine bone marrow EPCs. EPCs collected at day 5 culture were passed to 96-wells for a single cell colony assay. Representative photomicrographs demonstrate that EPC colonies (**A–C**) and cluster (**D**) gave rise to the secondary colonies (**a**,**b**) or cluster (**c**) or non-dividing cells (**d**). A,B: HPP-EPC colonies contained 5,808 cells and 1,012 cells, respectively. Cells from these colonies were replated onto another 96-well plate for a single cell colony assay, the secondary colonies formed after 18 days in culture with EPC medium (a,b). C: LPP-EPC colony contained 104 cells. Cells from this colony gave rise to clusters (c) after replating onto another well. D: Endothelial cluster-forming cells resulted in non-dividing endothelial cells only (d).

included in the En-col condition (Fig. 4A, control), or in the 3–10 kDa and <3 kDa fractions (Fig. 4A). There was a dose-dependent relationship between the concentration of EC-CM and the number of En-col (Fig. 4B). For MSC-col assay, EC-CM and the <3 kDa fraction showed a significant inhibitory effect on the growth of bone marrow MSC (Fig. 4C). The inhibitory effects of EC-CM or its <3 kDa fraction on MSC demonstrated a dose-dependent relationship (Fig. 4D).

Our previous study [Li et al., 2000] reported that many cytokines including VEGF, GM-CSF, SCF, MSP-1, endothelin-2, thymosin-10, connective tissue GF, PDGF-A Chain, MIP-2, PlGF, neutrophil activating protein ENA-78, INF-r, IL-1, IL-6, IL-13, IL-11, inhibin, and bFGF were expressed in murine bone marrow EC. Among them, the VEGF, GM-CSF, and bFGF are the cytokines with a MW >10 kDa. Thus, they were selected and tested in the En-col culture system individually. Each of them exerted stimulating effects on the growth of En-col (Fig. 5A). AcSDKP is a tetrapetide with a molecular weight of 487 and existed in <3 kDa fraction of EC-CM [Huang and Wang, 2001]. AcSDKP exhibited a significant inhibitory effect on MSC-col formation (Fig. 5B) but did not have any effect on EPC growth or the formation of En-col (Fig. 5A).

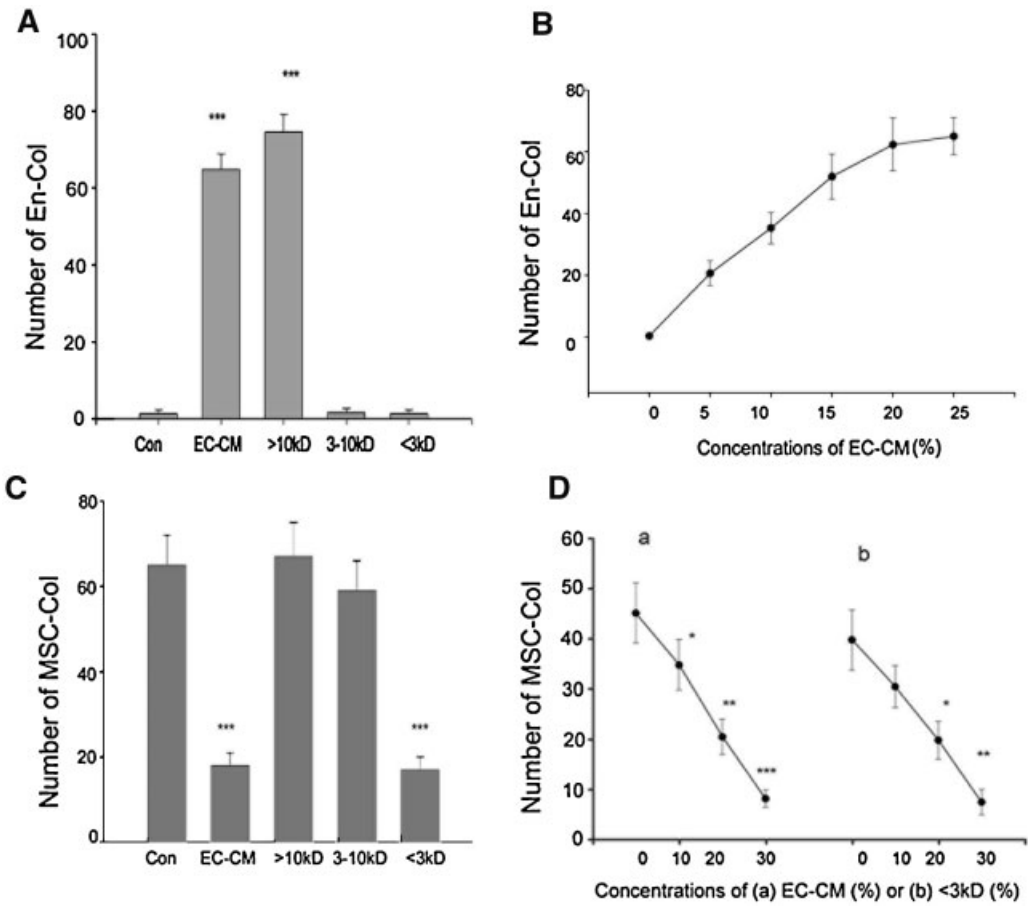

Fig. 4. The effects of EC-CM and its fractions on the growth of endothelial cell colonies (En-col) or mesenchymal stem cell colonies (MSC-col). **A**: EC-CM and its >10 kDa fraction promoted En-col formation. Purified EPCs (1×10^4) were plated in a 24-well plate with 10% serum/DMEM (control) or 10% serum/DMEM supplemented with EC-CM or one of the fractions. En-col were counted at each treatment. **B**: Dose-dependent relationship between the concentration of EC-CM and the number of En-col. EPCs (1×10^4) were plated in 24-well plate with an addition of a different concentration of EC-CM as indicated. Each point represents the mean ± SD of three wells. **C**: EC-CM and its <3 kDa fraction inhibited MSC-col formation. BMC (1×10^6) were seeded in a 35 mm-dish in triplicates with 20% serum/DMEM (control), or with an addition of EC-CM or one of its fractions to the dishes. Numbers of MSC-col were counted. ***$P < 0.001$. **D**: Dose-dependent relationship between the concentrations of EC-CM and the numbers of MSC-col. BMC (1×10^6) were plated in a 35 mm dish. Different concentrations of EC-CM (**a**) or the <3 kDa fraction (**b**) were tested individually. Each point represents the mean ± SD of three dishes, and in comparison with 0% group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

The <3 kDa fraction of EC-CM was pre-incubated with antibody against AcSDKP to better understand its biological activity in the EC-CM. After pre-incubating with antibody against AcSDKP, the inhibition of MSC-col formation was diminished significantly; in contrast, the<3 kDa fraction without pre-incubation of antibody continuously exhibited inhibitory activity on MSC-col formation ($P < 0.05$) (Fig. 5C).

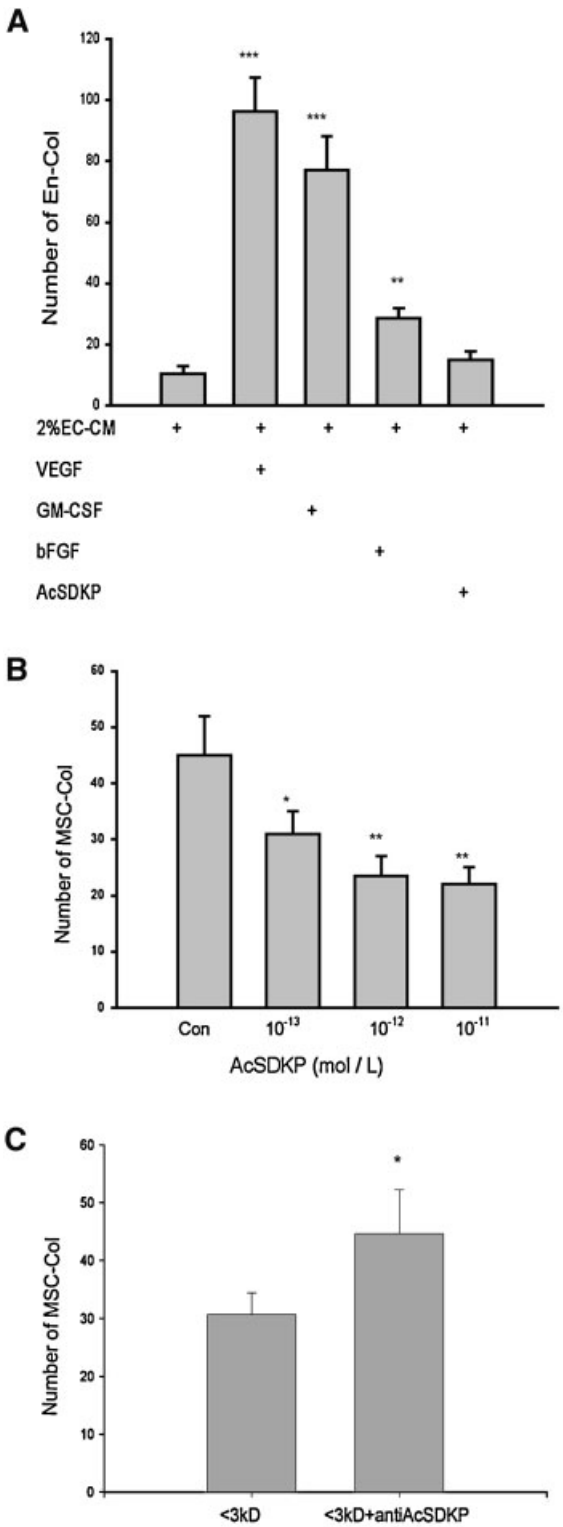

Fig. 5. The effects of cytokins on the formation of En-col or MSC-col. **A**: The effects of VEGF, GM-CSF, bFGF, and AcSDKP on the formation of En-col. EC-CM (2%) was included in the control and treated samples. **B**: The effect of different concentrations of AcSDKP on the formation of MSC-col. Controls did not include AcSDKP. **C**: AcSDKP antibody neutralization analysis. EC-CM fraction (<3 kDa) was pre-incubated with antibody against AcSDKP or with PBS, and were added individually to the dishes for MSC-col assay. MSC-col were counted. Each bar represents the mean ± SD of three experiments, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

DISCUSSION

In this study, we have purified and expanded the murine bone marrow EPCs in vitro after incubation of bone marrow mononuclear cells with EC-CM, a CM produced by an immortalized bone marrow endothelial cell line [Wang et al., 1998]. Bone marrow contains a small subset of stem cell precursors identified as functional EPCs, which express markers of CD133, CD34, and VEGFR2 [Hristov et al., 2003]. In cell culture on exposure to cytokines and on contact with Fn-coated dishes, these stem cell precursors can quickly lose CD133 and CD34 expression and gain a phenotype resembling endothelial cells [Hristov et al., 2003]. In our experiments, the bone marrow EPCs showed endothelial features after incubation in this novel EPC culture condition, for example, the precursor cells were positive for ac-LDL uptake and binding to *UEA-1*, expressed endothelial-specific markers, and formed tube-like structure (Fig. 1). The EPCs isolated from BMC demonstrated a high proliferative capacity: they were passed for six generations over a 90-day culture period, resulting in approximately 35,000-fold increase (Fig. 3) in cell number of endothelial progenies in this EPC culture condition without any additional growth factors. Based on the proliferative potential, Ingram et al. [2004] reported that there were HPP-EPCs and LPP-EPCs in human peripheral and umbilical cord blood; HPP-EPC formed secondary and tertiary colonies but LPP-EPC do not form secondary colony. The data in Figure 2 showed that some colonies were

highly proliferative cells, but some to clusters only with more senescent status. Therefore, a hierarchy of EPCs from murine BMCs were demonstrated in this study.

We summarize that the following conditions in the EPC culture system have contributed significantly to the enrichment and expansion of bone marrow EPCs in vitro: (1) EC-CM, the endothelial line was originally developed from bone marrow endothelial cells [Wang et al., 1998], the CM contains various angiogenic stimulators and MSC inhibitor(s) that have strong effects on EPC purification and outgrowth of EPCs; (2) selected serum, which had been screened to select the ones that favor the growth of endothelial cells; (3) cell density, with high density of BMC ($1-2 \times 10^6$/24-well) plated on the culture wells, EPCs were attached, grown and had autocrine signals to induce endothelial lineage and inhibited MSC. In contrast, with low density of BMC (1×10^6/35 mm dish), MSC were not been inhibited and started to grow (Fig. 4C,D); (4) Fn-coating, which also favors the attachment and growth of EPCs.

EC-CM appears to be the key component in the culture system for the purification and expansion of EPCs and EPC-derived EC. In combination with selected FBS, this culture condition is sufficient to support the isolation and growth of bone marrow EPCs and their progenies for many generations. We have demonstrated that cytokines in EC-CM played major roles in the growth of EPC colonies and in the inhibition of MSC. It has been well documented that endothelial cells secrete cytokines that stimulate the growth of endothelial cells [Li et al., 2000; Huang and Wang, 2001; Na and Wang, 2004; Zhong et al., 2004; Aparicio et al., 2005; Avasarala and Konduru, 2005; Shimizu et al., 2005]. The cytokines secreted by bone marrow EC were individually evaluated in En-col culture system, among them, the VEGF, GM-CSF, and bFGF were the strongest stimulating factors for endothelial cell proliferation (Fig. 5A). The molecular weight of VEGF, bFGF, or GM-CSF was higher than 10 kDa and existed in EC-CM of the $>$10 kDa fraction, we propose that the cocktail of these cytokines should result in a strong stimulation for EPC growth. More importantly, we have identified that AcSDKP in $<$3 kDa fraction of the CM [Huang and Wang, 2001] had significant inhibitory effect on the growth of MSC. Since the overgrowth of MSC has been a major contaminant in the EPC culture, inclusion of AcSDKP in EPC culture condition would significantly control the MSC growth in EPC culture. The inhibitory effect of AcSDKP on the growth of MSC is also consistent with the observations by Yang et al. [2004]. Taken together, we propose that AcSDKP is the primary inhibitory factor in EC-CM for the inhibition of MSC in the EPC culture.

We have identified that VEGF, bFGF, GM-CSF, and AcSDKP are important components of the EC-CM for the EPC culture, however, the effect of the combination of the four factors for the isolation of EPCs from murine BMC was about 80% efficiency of that of EC-CM (data not shown). Thus, the other components in the CM also contributed to the synergism and efficient selection and outgrowth of EPCs.

There are many examples of the autocrine/paracrine regulation in the proliferation of endothelial cells. For example, HUVEC synthesize PlGF and VEGF, and play a role in the autocrine and paracrine regulation of angiogenesis [Yonekura et al., 1999]. We have tested the HUVEC-CM in the isolation and growth of bone marrow EPCs. EGM2 (Cambrex, Walkersville, MD) was used to grow HUVEC. HUVEC-CM showed less inhibitory effects on the non-endothelial cells in comparison with EC-CM reported here; however, HUVEC-CM demonstrated a significant effect in support of growth of EPC-derived EC relative to the controls without HUVEC-CM (data not shown). We propose that other types of endothelial cells could also produce a panel of angiogenic factors and fibroblast inhibitors, similar to the ones identified in EC-CM in this report. The exogenously added VEGF or GM-CSF in the EC-CM culture condition substantially enlarged the size of En-col compared to the En-col formed in EC-CM alone (data not shown). Therefore, we predict that the combination of human endothelia cell-derived CM with AcSDKP, VEGF, GM-CSF, and bFGF, will also significantly improve the purification and growth of human EPCs or EPC-derived EC in vitro.

The EC-CM contains sufficient cytokines to stimulate endothelial cell growth, and also strong inhibitor(s) to suppress the growth of MSC or fibriblasts. This report not only provides an efficient approach to achieve the formation of murine bone marrow EPCs but also highlights the cytokine regulation in bone marrow stromal cells.

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