Murine Adherent CD34$^+$ Cell Population Expanded by Single Cell Cloning

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Abstract

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Introduction: While human endothelial progenitor cells (EPCs) have been a subject of somehow extensive investigation, EPCs from adult mouse hematopoietic system were poorly studied. Present investigation is focused on FVB mouse endothelial progenitor cells in terms of their isolation, purification, and expansion.

Material and Methods: Mononuclear cells collected from murine peripheral blood were cultured in fibronectin coated plate for two weeks, at which point, the adherent cell population were lifted and analyzed in terms of some surface markers. Using FACS Vantage equipped with one-cell deposition unit, single CD34 positive cells were plated per well already containing medium optimized for single cell growth. Several clones were then emerged, expanded, and examined in terms of some surface markers. Furthermore, the cells were investigated regarding ability to uptake DiI-ac-LDL and form capillary network on matrigel surfaces.

Results: Adherent population of mononuclear cells from mouse peripheral blood was appeared morphologically heterogeneous. About 5% of the adherent cells were CD34 positive. Having optimized their culture condition, several CD34 positive clones were expanded. The cells comprising the clones were DiI-ac-LDL$^+$ and formed capillary-like tube when being seeded on matrigel surfaces.

Conclusion: The primary culture of the mononuclear cells from murine peripheral blood contains a very limited number of cells positive for endothelial lineage markers. These cells (adherent CD34 positive) could be expanded by single cell cloning technique.

***Keywords*:** Murine adherent CD34 positive cells, endothelial cells

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Introduction

Peripheral blood of adults contains a unique subtype of circulating cells capable to differentiate into mature endothelial cells, and so called as endothelial progenitor cells (EPCs). The first detailed description of isolation of EPCs was published in 1997 by Asahara *et al.* They isolated a CD34$^+$ cell population from human peripheral blood using magnetic microbeads. The cells were plated on fibronectin-coated surfaces and grew into cells with endothelial characteristics. Furthermore, CD34$^+$ cell population was participated in vasculogenesis following transplantation in animal hind limb ischemic model (1). This finding was followed by identification of a variety of EPCs using the same methodology or others by several groups (2-6). Human EPCs share several markers, including CD34 antigen with hematopoietic stem cells (HSCs), and this has convinced some researchers that they would have derived from a common ancestor called as hemangioblast (7-10).

The location of EPCs is not limited to peripheral blood and there has been reports regarding isolation of EPCs from bone marrow as well (3, 11). Scientists believe that the bone marrow is the source of EPCs in peripheral blood, and these can mobilize into blood, especially following appropriate stimulus such as ischemic condition being occurred in the body (12-15). Previous experiments have demonstrated that some growth factors like vascular endothelial growth factor (VEGF),

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hematopoietic stimulators such as granulocyte colony-stimulating factor (G-CSF), angiopoietin-1, and stroma-derived factor-1 (SDF-1), and some recombinant pharmaceuticals can augment proliferation and mobilization process of bone marrow EPCs (2, 4, 16-19). EPCs have also been isolated from human cord blood and fetal liver (5, 20-21).

The therapeutic potential of EPCs in peripheral and coronary artery diseases have been shown in experiments with animal model of disease. Shatterman *et al.* conducted local injection of freshly isolated human $CD34^+$ mononuclear cells (MNCs) into diabetic nude mice with hind limb ischemia and showed an increase in the restoration of limb flow. Likewise, Kocher *et al.* (22) attempted to intravenously infuse the freshly isolated human $CD34^+$ MNCs into nude rats with myocardial ischemia and preserved left ventricle function associated with inhibition of cardiomyocyte apoptosis (21).

Despite promising potential for regenerative application, the fundamental scarcity of EPC populations in the hematopoietic system, even after *ex vivo* expansion, constitutes important limitation of EPC transplantation. *Ex vivo* expansion of EPCs cultured from the peripheral blood yield approximately 5.0×10^6 cells per 100 ml of human blood (23). Animal studies suggest that transplantation required 0.2-2×10 EPCs/gr weight to achieve satisfactory results (24).

Almost all available knowledge in the field of endothelial progenitor cells were produced by experiments carried out on human subject. Despite the fact that the mouse is appropriate model for human disease, very limited investigation has been conducted in this field. Human EPCs were usually obtained by culturing of mononuclear cells from peripheral blood on fibronectin coated dish, and resulting spindle-shaped cells were taken as progenitor cells that are positive for CD34 and Flk1 antigen. Accordingly, we cultured the cells from FVB mice and noticed that the primary culture of mononuclear cells become heterogeneous morphologically, as the cultivation period extended in order to expand the cells. Surprisingly, $CD34^+$ cell population was only comprised of a very small portion of adherent cell population. So, we decided to isolate and expand this cell type by single cell cloning method. However, current culture medium used for isolation and expansion of human EPCs failed to support their *in vitro* expansion. Therefore, we first attempted to provide appropriate culture condition in which single $CD34^+$ cells could be expanded, and then examined whether or not the expanded cells have EPC characteristic by some current EPC assays.

Material and Methods

Cell culture

Twelve FVB mice of 6-8 weeks were scarified and separate culture was established for each mouse. For this purpose the mice were anesthetized by intra-peritoneal injection of 90mg/kg of sodium phenobarbital (Sigma, USA), 20-guage needle was inserted into the heart, and approximately 1 ml blood was aspirated and used for study. To isolate mononuclear cells, the blood was mixed with 2 ml DPBS (Dulbecco's phosphate buffer solution; Sigma, USA), loaded on 1.5 ml histopaque (Sigma, USA), and centrifuged at 1100g for half an hour at room temperature (RT). The mononuclear cells were then collected and plated in fibronectin-coated 6-well plastic plate (Falcon, Germany) at 5×10^5 cell/ml in EBM-2 (endothelial basal medium). The cultures were kept in humidified incubator with atmosphere of 95% air and 5% CO_2 at 37 °C for 14 days. The adherent cells were removed with medium exchange on day 4. Subsequent changes in culture medium were performed every other day.

Flow cytometric analysis of primary culture

Since the cells in primary culture appear morphologically heterogeneous, the cultures were analyzed for the expression of some endothelial progenitor and hematopoietic lineage markers including CD34, Flk1, Sca1, Ckit, and Gr1. For cell labeling, the following procedure was performed. The culture of mononuclear cells was terminated at the end of day 14 by lifting the cells after 5 minute exposure to accutase enzyme (Sigma, USA). The remaining enzyme on the cells was removed by washing twice in HBSS (Hanks balanced salt solution) and subsequent centrifugation at 300 g for 5 minutes. About 200,000 cells were placed in 100 µl centrifuge tubes, incubated for 20 minutes at 4 °C with 1 ml of FC blocker, and then pelletted by centrifugation at 450 g for 10 minutes. The cells were resuspended in 1 ml PBS and double stained with FITC-conjugated CD34 and PE-conjugated Sca1, CD34 and Ckit, CD34 and Flk1, and CD34 and Gr1 (all purchased from Becton Dickenson) by adding antibody at a concentration of 2 µg/ml at 4 °C for 20 minutes. The cells were then washed and analyzed by flow cytometry (FACS calibur cytometer equipped with 488 nm argon laser). Isotype-identical antibodies were employed as

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negative control to detect non-specific binding of antibodies.

Single Cell cloning
Since CD34+ cells from human peripheral blood have first been shown as endothelial progenitor cells, we decided to isolate and expand the same cells from murine cultures by single cell cloning method. For this purpose, the cells from primary culture in day 14 were lifted by exposing to accutase enzyme, washed by HBSS and centrifugation, and counted by neubar chamber. About one million cells were then labeled by FITC-conjugated CD34 antibody as mentioned in previous section and kept on ice until sorting. Using FACS Vantage equipped with a one-cell deposition unit (OCDU), single cells were deposited as one cell per well in 96-well cell culture plastic dish already coated with fibronectin and containing 150 µl culture medium. Culture media including EBM-2 (endothelial basal medium), EGM-2 (endothelial growth medium), and IMDM (Iscoves modified Dulbecco's medium) each supplemented with a variety of growth factors (including hEGF, hPDGF, VEGF, IGF, and FGF at a concentration of 10 to 100 ng/ml) were all failed to support single cell growth and proliferation. Proliferation of the cells were initiated when DMEM-LG supplemented with 5% FCS, 20 ng/ml VEGF (vascular endothelial growth factor), 10 ng/ml hEGF (human epidermal growth factor), 20 ng/ml hPDGF (human platelet derived growth factor), 10^{-9} M dexamethazone, and 10^{-9} M ascorbic acid 2-phosphate were used. The culture was maintained in humidified atmosphere at 5% CO_2 and 37 °C temperature for a period of almost 10 days. The culture media, for the first time were replaced 5 days after culture initiation and the subsequent exchanges of the media were done every 2 days till day 10, when the cells were trypsinized and subcultured. The cells were increased by performing several rounds of subculture and finally the passage 4 cells were used for further analysis regarding the same set of surface marker being examined in primary culture.

Ac-LDL Assay
The clones prepared from single CD34+ cells were examined for Ac-LDL to clarify their EPC nature. For this purpose, monolayer of each clone was incubated in the presence of 10 mg/ml DiI-ac-LDL (1,1´-dioctadecyl-3,3,3´,3´-tetramethylindo-carbocyanine perchlorate-labeled acetylated LDL, Biomedical Technology) at 37 ˚C for about one hour and viewed with an inverted fluorescent microscope.

In vitro tube formation on matrigel
Capillary network formation on matrigel has also been indicated as a unique property of EPCs. To assay this capability, chamber slides were coated with matrigel (Sigma, USA) and after 1 hour of incubation at room temperature, 5×10^4 cells were added to chamber slide with 0.5 ml EGM media. Twelve hour later, the culture was observed under inverted microscope and a representative field was photographed.

Results

Primary cultures
Daily observation of the cultures showed that some mononuclear cells were adhered on fibronectin surfaces and survived, whereas the remaining failed to attach and then remained floating in culture medium and removed in the first medium replacement. During early days of cultivation, the cultures were predominantly consisted of almost spindle-shaped cells, but after 6 days of cultivation, morphologically heterogeneous cells including spindle, star-shaped, and round cells were appeared and the culture reached confluency by the end of day 14 (Fig 1, A and B).

Flow cytometric analysis of primary culture
According to fluorescence-activated cell sorting analysis (FACS) (Fig. 2), more than 90% of the cells were negative to all of the markers used in this investigation, about 5% were CD34+ (common marker of hematopoietic and endothelial progenitor lineages), 2.4% were Flk-1+, 2.6% were sca-1+, 0.06% were c-kit+, and were 0.06% Grl+. Blood sample from 12 mice were cultured individually for 14 days and analyzed by FACS. The results were almost the same and the representative FACS analysis was shown in Figure 2.

Cell cloning
The cells were seeded in DMEM-LG supplemented with mentioned growth factors and chemicals which allowed their survival and expansion. After about 3 days of cultivation, cell proliferation initiated in about 30% of the wells of 96-well plate. About 10% of cells continued their proliferation during the culture period, while the remaining stopped proliferation and detached from the culture surfaces. By the end of day 10, almost 20% of the available fibronectin surfaces were covered by the proliferating cells. At this time, the cells of each well were lifted, subcultured

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in 6-well plate, expanded by several passages (3-4 passages for 20 days), and eventually used for further study. Overall, 9 clones were obtained and studied. The morphology of the clones was almost the same.
Photomicrographs of the three clones are shown in Fig 1. Before confluency, the cells comprising the clones were mainly round in shape (Fig. 1, C, E and G), but they appeared as cobble stone monolayer in confluent culture (Fig 1 D, F, and H).

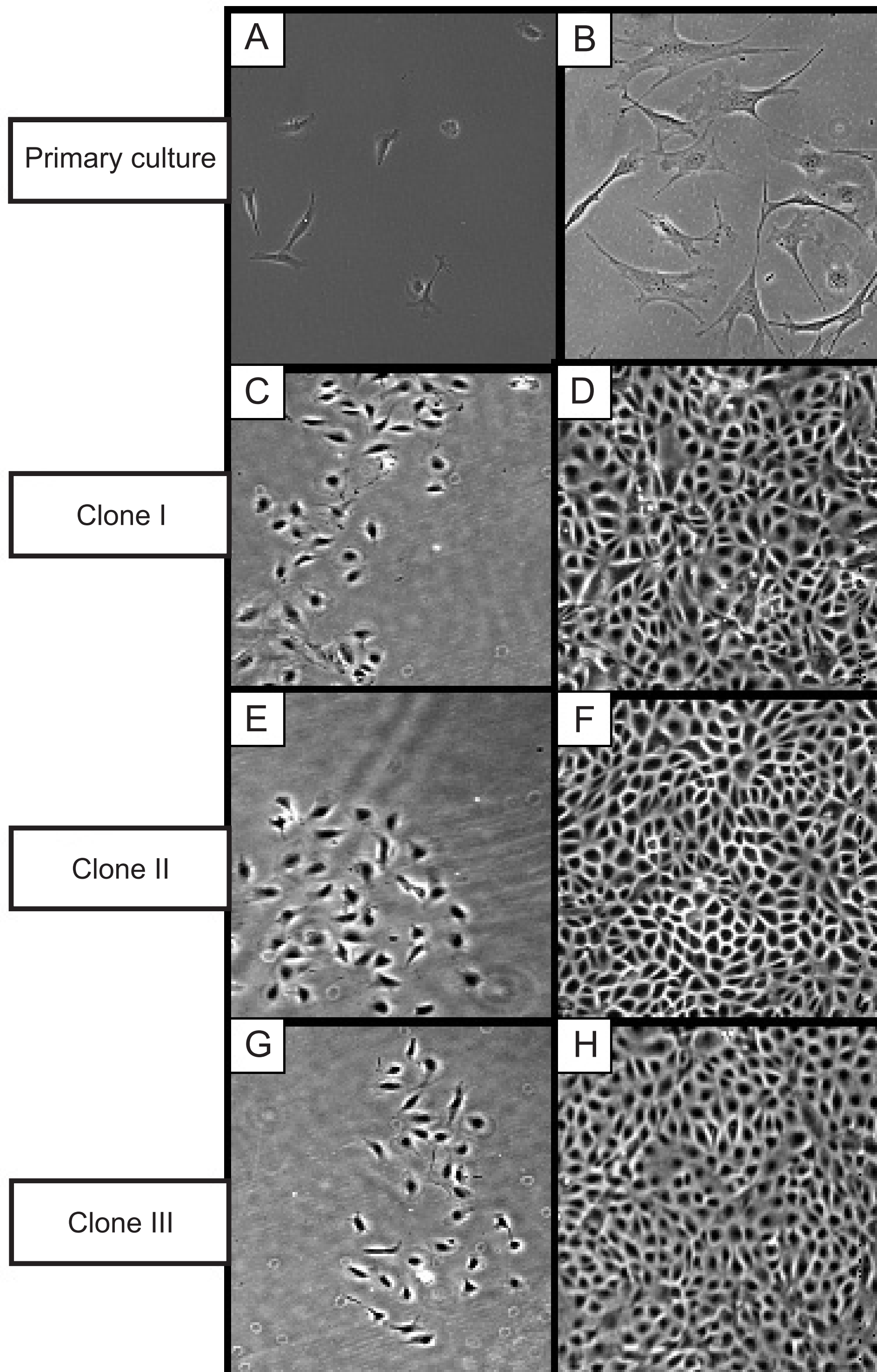

Fig 1: Representative photomicrograph indicating the mononuclear cells from mouse peripheral blood in primary culture. Day 5, the cells are spindle shape (A); day 12, cell population became morphologically heterogeneous (B); three different clones called as I, II, and III are shown before confluency (C, E, G) and during confluency (D, F, H); magnification ×100.

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FACS analysis of the clones

Cell population comprising each clone was analyzed using FACS to determine the cell purity. In this analysis, the same set of markers used in FACS analysis of the primary cultures was employed. The results of the positive clones (Fig. 1) are shown in Fig 2.

About 80-85% of the cells were CD34$^+$ and almost 15-20% had lost the antigen while expanding. Flk1, Ckit, and Gr1 markers were not expressed in the cells. About 20% of cells were double-stained with CD34 and Sca-1 markers, an interesting finding.

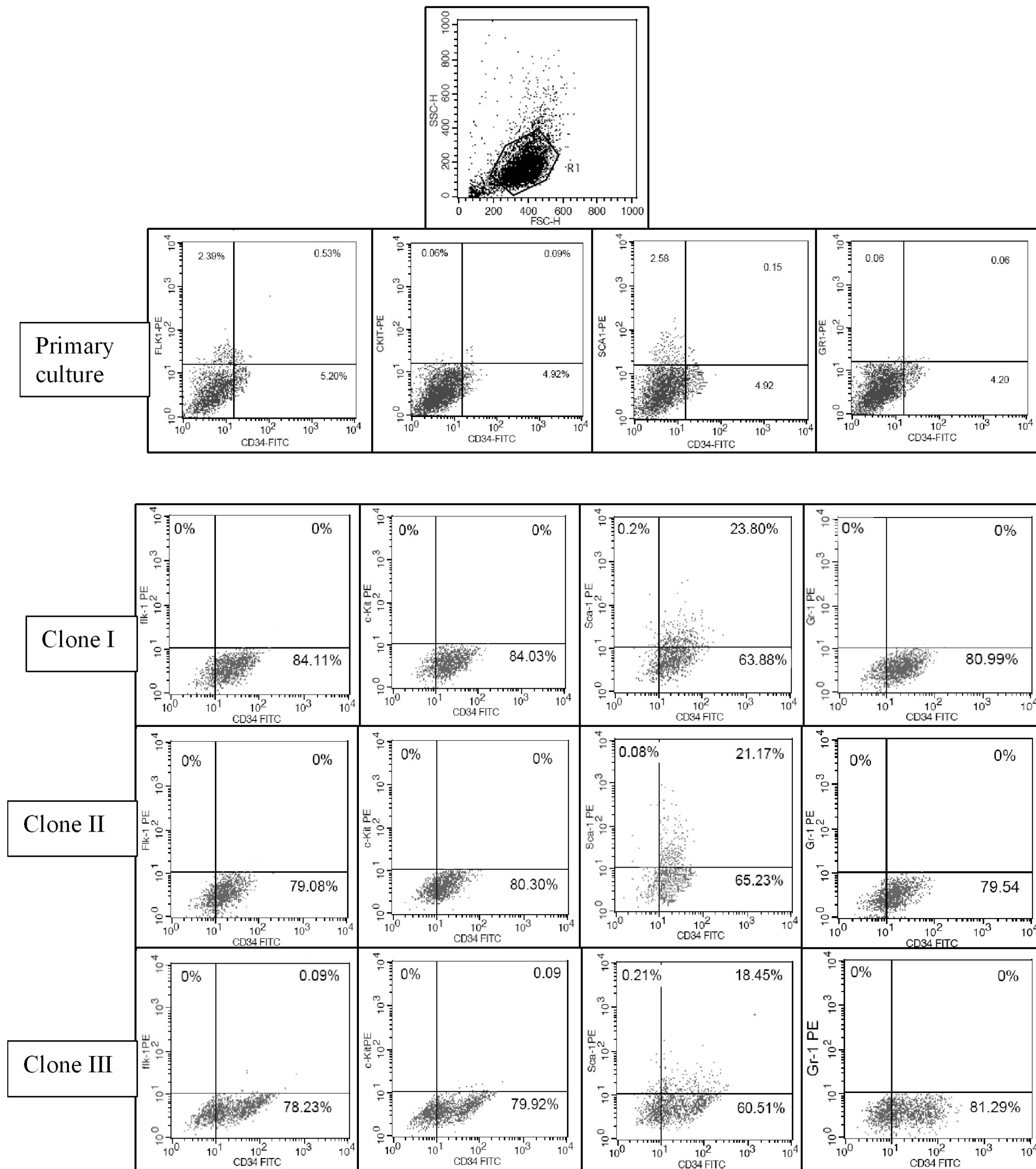

Fig. 2: Representative 4-quadrant FACS analysis of day 14 culture of mononuclear cells from FVB mouse peripheral blood (A), clone I (B), clone II (C), and clone III (D).

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EPC culture assay
Nearly all (>96%) clones expanded in this study were stained as red because of DiI-ac-LDL up-taking (Fig 3).

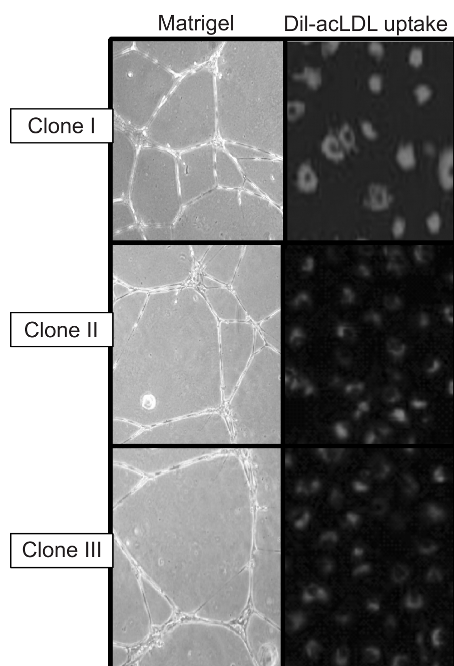

Fig. 3: Clones I, II, and III which was shown in Fig. 1 were cultivated on matrigel surface. The same clones were evaluated regarding DiI-ac-LDL uptake.

Tube formation Assay
The cells of all clones were differentiated into endothelial cells and arranged themselves into tube-like structures on matrigel surfaces (Fig 3).

Discussion

Despite of rather extensive investigation of human endothelial progenitor cells, those from mouse as an appropriate model for human disease have gained much less attention. In preliminary study to expand mononuclear cells from mouse peripheral blood on fibronectin-coated surfaces, the culture became morphologically heterogeneous and the cells other than spindle-shaped ones predominated the culture as the culture period was extended. This is in contrast to human cultures that consist of nearly homogenous spindle-shaped cells. Morphologically heterogeneous nature of the murine culture has also been reported in experiments which isolated mesenchymal stem cells from murine bone marrow cells.
Since it was established that endothelial progenitor cells are positive for CD34, Flk1, and Sca-1, we decided to analyze the primary culture for expression of these markers along with Ckit and Gr1 antigens to obtain a better picture from cell population of the cultures. According to our results, more than 90% of the cells were negative for all markers used in the study and this point needs more investigation. Ckit and Gr1 antigens were expressed negligibly as expected, since they are the specific markers for hematopoietic lineages. Endothelial progenitor cell markers including CD34, Flk1, and Sca1 were also expressed in a low proportion of the cells. It should be noted that the number of the cells obtained by cultivation of mononuclear cells is not sufficient for any experiments, and they should be multiplied before any *in vitro* and *in vivo* experiment.
Given the heterogeneous nature of primary culture of mouse mononuclear cells obtained from peripheral blood, development of an efficient isolation, purification, and expansion scheme for mouse EPCs seemed to be necessary. In present investigation, this has been achieved by single cell cloning method. The cell expansion using single cell cloning method is advantageous, as the purified cells can be used for experimental therapeutic neovascularization.
By far, there are no reports indicating the feasibility of single cell cloning for adherent $CD34^+$ cells in mice. In present study, this has been done by multiple experiments using different combinations of the media and growth factors in different quantities. EBM-2 and EGM-2 media, currently used for EPC isolation and proliferation from human peripheral blood, did not start the cell proliferation. Likewise, IMDM which routinely is used for proliferation of the lineage-committed hematopoietic progenitor cells (known as colony forming cells) failed to support cell proliferation. Single $CD34^+$ cells were only expanded when cultivating in a medium consisting of DMEM-LG supplemented with factors described in Materials and Methods.
To examine the homogeneity of the cloned cells, the cells were labeled with CD34 antibody for flow cytometric analysis. Our results showed that some cells had lost the CD34 antigen. So, we decided to perform FACS analysis using the same set of antibodies employed before cloning, and the results were interesting; Sca1 and CD34 antigens were co-expressed in some cells. These findings require rational explanation.
Lack of the CD34 antigen in about 20% of the cells obtained from single cell cloning can be explained in the light of data presented in previous experiment. According to some experiments, the human bone marrow EPCs are $CD34^+$, $CD133^+$, and $flkl^+$ (25). These cells

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lose CD133 marker while entering into peripheral blood and lose CD34 marker at the beginning of differentiation into endothelial cells (26). The profile of surface marker changes in mouse EPCs has not yet been reported. However, given the missing of CD34 expression during cell differentiation according to our results, lack of CD34 marker in about 15% of the cloned cells could be due to their spontaneous differentiation toward endothelial lineage.

Expression of Sca1 antigen in about 20% of the cloned cells also demands a rational explanation. The cells are expected to be devoid of Sca1 antigens, as they are the progeny of a single $CD34^+$ cell. A simple explanation would be that the antigen was expressed because of specific formulation of the cell expansion microenvironment. Alternatively, one can consider that only the cells positive for CD34 and Sca1 antigens survived during the cloning procedure, and then some of them lost due to specific formulation of culture medium, while others still maintained the Sca1.

The cells expanded in present study were not examined for their *in vivo* role in vasculogenesis, but according to some convincing evidence, they could be considered as an equivalent to endothelial progenitor cells (EPCs). The first line of evidence comes from the Ac-LDL up-taking assay.

It has been well established that EPCs could uptake acetylated low-density lipoprotein so that this property was used in several experiments to identify cells as EPC (16, 26-28).

Other evidence indicating endothelial progenitor nature of the cells comes from the tube formation assay. It has been shown that EPC could form a capillary network when seeded on matrigel surfaces (28-29). Ac-LDL up-taking capability of the cells combined with their tube formation property are both indicative of their endothelial progenitor identity.

Previous studies have shown a panel of surface markers for mouse EPC isolated from peripheral blood. Asahara *et al.* have demonstrated that the mononuclear cells of mouse peripheral blood positive for CD34, Flk1, and VE cadherin had EPC characteristics (16). In contrast, Iwakura *et al.* have considered Sca1 and Flk1 antigens as markers for EPC population existing among mononuclear cell of mouse peripheral blood (18). According to our results, $CD34^+$, $Flk1^+$-cells could be considered as EPCs. These data demonstrate the heterogeneous nature of the cells called as endothelial progenitor cells.

Conclusion

Taken together, the primary culture of the mononuclear cells from murine peripheral blood is heterogeneous regarding cell morphology and some markers. The number of the cells with endothelial lineage markers is very limited among the adherent population of primary culture. Adherent $CD34^+$ cells could be expanded by single cell cloning technique, and $CD34^+$ $Flk1^+$-cells have EPC characteristics.

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سلول‌های CD34 مثبت تکثیر یافته با روش Single Cell Cloning

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چکیده

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* **هدف**: جدا سازی و تکثیر سلول‌های $CD34^+$ از کشت اولیه سلول های تک هسته ای خون محیطی موش FVB با روش Cloning و ارزیابی آنها با سنجش های مربوط به سلول های پروژنیتور آندوتلیالی

* **مواد و روش‌ها**: سلول‌های تک هسته ای از خون محیطی موش جدا شده و در پلیت های شش خانه ای فالکون کشت شدند.۱۴ روز بعد از آغاز کشت، جمعیت سلولی چسبنده از لحاظ برخی مارکر های سطحی آنالیز شده و با استفاده از دستگاه فلوسایتومتری، سلول‌های $CD34^+$ جدا شدند و به صورت یک سلول در خانه، در پلیت‌های ۹۶ خانه کشت شد. چندین کلون تهیه و از لحاظ مارکر های سطحی و سنجش Dil-ac-LDL و تشکیل لوله عروقی مورد آزمایش قرار گرفت.

* **یافته‌ها**: جمعیت سلول های چسبنده در کشت اولیه از لحاظ مورفولوژی و برخی مارکرهای سطحی، هتروژن بود. حدود ۵درصد سلول‌ها $CD34^+$ بود. با استفاده از دستگاه فلوسایتومتری و مطلوب‌سازی شرایط کشت، چندین کلون از سلول‌های $CD34^+$ تهیه شد. این کلون‌ها $Dil\text{-}ac\text{-}LDL^+$ بوده و در زمان کشت بر روی ماتریژل، لوله عروقی تشکیل دادند.

* **نتیجه‌گیری**: کشت اولیه سلول‌های تک هسته‌ای خون محیطی موش FVB حاوی تعداد کمی سلول با مارکر رده عروقی است که این سلول‌ها (با مارکر $CD34^+$) قابل کلون کردن هستند.

کلیدواژگان: سلول‌های چسبنده $CD34^+$ موشی، سلول‌های آندوتلیال

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