論文の内容の要旨

**Real Angiogenic Potential of Early or Late Outgrowth Endothelial Progenitor Cells is Rigorously Dependent on the Time of Emergence**

（血管内皮前駆細胞の治療的血管新生能と培養開始からコロニー出現までの期間との関連についての検討）

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Endothelial progenitor cells (EPC) are still one of promising sources of regenerative medicine even though induced pluripotent stem (iPS) cells are becoming increasingly important in this field. The isolation of EPC was first reported by Dr. Asahara in 1997. They reported that the CD34 positive cells in the human adult peripheral mononuclear cell fraction differentiate into vascular endothelial cells, and demonstrated that these cells were derived from bone marrow and contribute to the postnatal vascular repair. Owing to this report, the interest in EPC as a potential source of cell therapy increased, and numerous basic and clinical studies of EPC have been conducted. However, their overall impact is still controversial, mainly because of the existence of some unresolved issues, including proper clinical trial designs, the optimal time point for cell transplantation, cell delivery methods for the target organs and the definition of EPC itself. In particular, there have been several definitions of ‘EPC’ in both pre-clinical and clinical studies from different researchers, and these were widely defined, ranging from bone marrow-derived MNCs to cells with specific surface marker expression. Thus, the exploration of the ‘best EPC’ for cell therapy and the appropriate classification of heterogeneous EPC are crucial for there to be steady progress in EPC studies for clinical applications. Recently, the definition of ‘Early EPC’ and ‘Late EPC’ was proposed according to the cell appearance and the timing of colony formation. Late EPC is defined as cells with a cobblestone shape, emerging relatively late in the MNC culture, producing more nitric oxide, and forming capillary-like tubes *in vitro*. Later, some researchers reported that the main mechanism by which Late EPC increased angiogenesis was via direct incorporation into pre-existing vascular networks. Therefore, Late EPC might be closer to the putative EPC.

Prompted by these reports, we focused on the Late EPC and tried to further

investigate their functional features. We noticed that the timing of colony emergence of the Late EPC was widely distributed, from 10 to 30 days after the seeding of MNCs on the culture plates. Thus, we hypothesized that Late EPC consist of some heterogeneous cells with different properties and angiogenic potentials. In this study, the period of colony emergence of Late EPC was divided into three groups. Then, the cells were redefined according to these periods, and their angiogenic properties were compared to clarify the best EPC to be used as a source of cell therapies.

In the present study, various types of EPCs were originally defined. First, human peripheral blood MNCs (hMNCs) were cultured on human fibronectin-coated wells with EBM-2 medium for 3 to 7 days, and adherent clustered cells were defined as Early outgrowth cells (EOC). Next, human MNCs were cultured on rat tail collagen I-coated wells, and the cells of the emergent colonies with a cobblestone appearance from days 10 to 30 were defined as the Late EPC. Then, these were sub-divided into three groups as follows: 10 to 16 days as MOC (mid-term outgrowth cells), 17 to 23 days as LOC (late-term outgrowth cells), and 24 to 30 days as VOC (very late-term outgrowth cells). According to this definition, the angiogenic properties of EPCs were investigated by following several methods *in vitro* and *in vivo*.

To investigate the differences in cell surface antigen expression among the various types of EPCs, the expression of some important markers were evaluated by flowcytometry. Most cells of all four types were strongly positive for CD31 and CD105, and negative for CD133. On the other hand, the positive rate of some markers varied among four types of EPCs, e.g., EOC: $CD34^{-}CD14^{+}CD45^{high}$, MOC: $CD34^{+}CD14^{low}CD45^{+}$, LOC: $CD34^{+}CD14^{-}CD45^{low}$ and VOC: $CD34^{+}CD14^{-}CD45^{-}$. The proliferative potential of EPCs was assessed by the commercially available kit (MTS assay). LOC demonstrated significantly high proliferative activity with approximately 1.3 fold of other EPCs. Next, the *in vitro* angiogenic potential of EPCs was evaluated using Matrigel tube formation assay. Only the LOC could form thicker and tighter tube-like structures on the Matrigel, whereas MOC and VOC could form only a thin cord-like network. The EOC never formed capillary-like tubes in Matrigel. The total length of these structures was significantly longer in the LOC. To compare the mRNA expression levels of crucial markers for angiogenesis among EPCs, a quantitative Polymerase Chain Reaction (qPCR) analysis was performed. The mRNA expression level of eNOS in LOC was significantly higher than those in the other EPCs. The expression levels of Flk-1, Flt-1 and Tie-2 in LOC were also highest among EPCs whereas no statistical difference was observed. From these in vitro analyses, the different properties of four types of EPCs were clarified, and the superiority of

proliferative activity and angiogenic potentials in LOC among four types of EPCs was suggested.

To demonstrate this superiority of LOC *in vivo*, several analyses using mouse hindlimb ischemic model were conducted. First, to confirm that the injected EPC subpopulation could directly incorporate into the mouse vasculature, GFP-labeled EPCs were intravenously injected into immunocompromised mice on days 1, 3, 5 and 7 after unilateral hind limb ischemia surgery ($1\times10^5$ cells per injection). Ten days after surgery, GFP signals were detected in some capillaries of the LOC-injected mouse (in approximately 4% of capillaries) but were hardly detected in EOC-injected mouse capillaries. Next, we investigated the continuous therapeutic efficacy of EPC transplantation using the same model. Four weeks after surgery, the mice in the LOC-injected group showed significantly enhanced blood flow recovery as measured by Laser Doppler scanning (blood flow ratios of the ischemic/nonischemic leg: $0.94 \pm 0.02$ [LOC group] versus $0.74 \pm 0.07$ and $0.78 \pm 0.09$ [EOC and MOC groups], $P<0.05$). In addition, capillary density measured by histological staining of both anti-mouse CD31 and anti-human CD31 was highest in LOC-injected group. These results suggested that the ability of both enhancing recipient cell-derived capillary formation and direct incorporation of injected cells into a part of recipient capillary was greatest in the transplantation of LOC among groups.

From these in vitro and in vivo analyses, we concluded that Late outgrowth EPC emerging on days 17–23 in *ex vivo* culture of human PBMNCs are superior to other EPC subpopulations with regard to the angiogenic potentials as the source of cell therapy.