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—【Original】——原　著—

PYRUVATE SUPPLEMENTATION ENHANCES VASCULAR ENDOTHELIAL GROWTH FACTOR PRODUCTION BY BONE MARROW-DERIVED MONONUCLEAR CELLS

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Bone marrow-derived mononuclear cells (BMMNC) include endothelial progenitor cells (EPC), which are characterized by their secretion of angiogenic factors such as vascular endothelial growth factor (VEGF) to recruit local endothelial cells, thereby enabling the establishment of new blood vessels. Implantation of BMMNC has been clinically used for therapeutic purposes in the treatment of critical limb ischemia (CLI); results showed that it was ineffective in a substantial number of cases. To evaluate the appropriate concentration of pyruvate to achieve the highest VEGF gene expression, cells were cultured with pyruvate at final concentrations up to 20 mM in 5% CO_2 for 2-4 days. The intracellular concentration of pyruvate was measured enzymatically and cell number and viability were determined. Expression levels of VEGF genes and numbers of $CD31^+/CD34^+$ cells were evaluated. Finally, VEGF levels in the conditioned medium were examined in each condition. Pyruvate supplementation in murine BMMNC cultures successfully increased intracellular pyruvate levels in a concentration-dependent manner, and 5 mM pyruvate was found to be the most appropriate to maintain viable cell number and up-regulate *VEGF* gene after 2-day culture. In addition, VEGF in the conditioned medium was significantly elevated by the use of 5 mM pyruvate after 4-day culture. From these results, we suggest that preconditioning of BMMNC with 5 mM pyruvate for 2 days may be a useful way to safely and inexpensively enhance the angiogenic properties of BMMNC and the therapeutic effectiveness of cellular therapy for CLI.

Keywords: critical limb ischemia, regenerative medicine, vascular endothelial growth factor, Endothelial progenitor cells

INTRODUCTION

Critical limb ischemia (CLI) is the end-stage status of arterial stenosis or obstruction in the lower extremities due to arteriosclerotic peripheral artery disease (PAD) or thromboangitis obliterans (TAO, Buerger's disease). CLI is characterized by symptoms such as leg pain, skin ulcers or uncontrollable infection, eventually resulting in limb amputation due to necrosis of the affected leg. For patients suffering from severely ischemic legs, surgical bypass grafting or endoatherectomy has been applied; however, approximately 20-30% of patients are not candidates for these procedures. As a result, about 100,000 and 120,000 CLI patients undergo leg amputation per year in the European Union and United States, respectively[1)].

Bone marrow-derived mononuclear cells (BMMNC) have been demonstrated to contain endothelial progenitor cells (EPC), which secrete angiogenic factors and recruit endothelial colony-forming cells with proliferative potential for neoangiogenesis[2)~5)]. Implantation of BMMNC or peripheral blood mononuclear cells (PBMNC) has been used for therapeutic neovascularization and is becoming an indispensable therapeutic option to rescue ischemic limbs. In Japan, the clinical feasibility of this approach has been analyzed in a randomized controlled study called TACT: Therapeutic Angiogenesis by Cell Transplantation[6)]. Three-year amputation-free rates were 91% in TAO and 60% in PAD, suggesting that cellular therapy is effective for most patients affected by TAO, but that several obstacles must be overcome to improve overall clinical effectiveness, especially for PAD. A Phase I/IIa clinical trial

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reported that granulocyte-colony stimulating factor (G-CSF)-mobilized $CD34^+$ cells were safely utilized and that improvement of efficacy was revealed both subjectively and objectively[7].

Normal cells can activate genes in the adaptation to a hypoxic environment, and hypoxia-inducible factor (HIF-1) is known to be responsible for transcriptional activation. HIF-1 acts as a heterodimer, and a subunit, HIF-1α, becomes unstable in normoxic conditions. An exception to this is in cancer cells, where it is stable under normoxic conditions[8]. Subsequently, hypoxia-inducible genes such as glucose transporters, glycolytic enzymes, erythropoietin, carriers for iron/copper, and vascular endothelial growth factor (VEGF) and its cognate receptor are activated, leading to neoangiogenesis accompanying tumor formation.

HIF-1α is post-translationally regulated by two oxygen sensors, HIF prolylhydroxylase (HIF-PH) and FIH (factor-inhibiting HIF). Under normoxia, HIF-PH hydroxylates both proline^{402} and proline^{564}, resulting in degradation by the ubiquitin-proteasome pathway. Similarly, FIH hydroxylates aspargine^{803}, and this hydroxylation interferes with the protein-protein interaction between HIF and CBP/p300[9]. 2-oxoglutarate is a crucial cofactor for HIF-PH and pyruvate binds to the 2-oxoglutarate site of HIF-PH, leading to stabilization of HIF-1 through inactivation of HIF-PH[10]. As a result, pyruvate enables HIF-mediated trans-activation of genes associated with the mobilization, migration and recruitment of endothelial cells to form new blood vessels.

VEGF is an angiogenic growth factor which is activated through HIF-1α stabilization. Previous studies attempted to enhance VEGF production of EPC using hypoxic preconditioning[11] or HIF-PH inhibitors[12]. In the present study, we show that preconditioning of BMMNC using an appropriate concentration of pyruvate successfully activates *VEGF* gene and augments VEGF production, and discuss the possible application of short-period conditioning to cellular therapy for CLI.

MATERIALS AND METHODS

Tissues and cells

Male C57BL/6 mice aged 7-8 weeks were obtained from Japan SLC (Shizuoka, Japan) and housed under pathogen-free conditions. Bone marrow cells were obtained from femurs and tibiae aseptically by flushing with RPMI1640 into 15-m*l* plastic tubes using a 21-gauge needle and a 1-m*l* syringe. Clumps of cells were dispersed by repeated passage through the 21-gauge needle. The bone marrow cells were the washed with RPMI1640 once, and mononuclear cells were obtained after Ficoll-Paque density gradient centrifugation. Aliquots of bone marrow cells were diluted in Turk's solution and nucleated cells were counted. Cells were maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), and a mixture of penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO).

Measurement of intracellular concentration of pyruvate

To evaluate the effects of pyruvate on hypoxia-inducible gene expression, BMMNC (8e5 cells/m*l*) were cultured in RPMI1640 supplemented with 10% FCS and pyruvate at final concentrations of 0, 1, 3, 5, 10 and 20 mM in 5% CO_2 at 37℃ for 2-4 days. Viability was calculated by the trypan blue dye exclusion method. A murine leukemic cell line, CBA2, was used as a control[13].

Cultured cells were collected by centrifugation at 800 g, 4℃, for 3 minutes, and washed with ice-cold PBS once. The cell pellet was resuspended with 2 m*l* of 0.3 N perchloride, and rigorously mixed by vortexing. After centrifugation at 5,000 g, 4℃, for 5 minutes, supernatants were used to measure the intracellular concentration of pyruvate as previously described[14]. Protein assays were performed by the method of Bradford using a commercial kit (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA).

We purchased cobalt chloride, L-mimosine and roscovitin from Sigma (USA). These reagents were used as HIF-PH inhibitors, and final concentrations in culture medium were 75 μM, 1 mM and 20 μM, respectively.

Flow cytometric analysis

Fluorescence-activated cell sorting (FACS) analysis was performed using the EPICS ALTRA analyzer XL-MCL (Beckman Coulter, USA), and the data were analyzed with EXPO™32 ADC software (Beckman Coulter, USA). Antibodies against human CD31 and CD34 labeled with FITC (fluorescein isothiocyanate) and PE (phycoerythrin), respectively, were obtained from Beckman Coulter.

Quantitative RT-PCR (Q-RTPCR)

Total cellular RNA of cultured BMMNC was extracted with an RNeasy purification kit (QIAGEN), and

Table 1 Oligonucleotide primers used in this study

VEGF-1	5'-GAGCTTCCTACAGCACAGCA-3'
VEGF-2	5'-TCTTTCCGGTGAGAGGTCTG-3'
GAPD-1	5'-TGCGACTTCAACAGCAACTC-3'
GAPD-2	5'-ATTGTGAGGGAGATGCTCAG-3'

500 ng of total RNA was reverse-transcribed at 42℃ for 90 minutes with 50 pmol oligo (dT) 17 primer, 0.5 U/μl cloned RNase inhibitor (Takara Bio, Shiga, Japan), 10 mM DTT, 1 mM dNTP and 50 units of Expand Reverse Transcriptase (Roche Diagnostics). Aliquots (1/100) were subjected to quantitative reverse-transcription PCR (Q-RTPCR) using primer pairs described in Table 1. Q-RTPCR was performed using Mx3000P (Stratagene, CA) with SYBR Premix ExTaq polymerase (Takara Bio). The reaction mixtures were subjected to 40 cycles of amplification consisting of 95℃ for 30 seconds, 63℃ for 60 seconds and 72℃ for 30 seconds. Primers are listed in Table 1.

Relative gene expression levels were calculated using the standard curve, which was made by Q-RTPCR using 0, 0.8, 4, 20 and 100 ng of total RNA extracted from untreated murine BMMNC. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA level was used as an internal control.

Measurement of VEGF excretion

After 4-day culture, conditioned medium was used to determine VEGF concentration using the Quantikine-Mouse VEGF Immunoassay (R&D Systems, USA). VEGF concentration is expressed as picograms per milliliter of conditioned medium, and calculated mean and standard error (SE) of three independent experiments were used for statistical analysis. Statistical comparisons between groups were performed using Student's t test. $P<0.05$ was considered statistically significant.

RESULTS

In order to evaluate whether BMMNC can efficiently uptake extracellular pyruvate in culture medium, we measured the intracellular concentration of pyruvate after 2-day culture. As shown in Fig. 1, supplementation of pyruvate resulted in accumulation of intracellular pyruvate in a concentration-dependent manner. Using 20 mM pyruvate, accumulation was increased 33-fold. In contrast, we were unable to observe the accumulation of intracellular pyruvate with a leukemic cell line (data not shown).

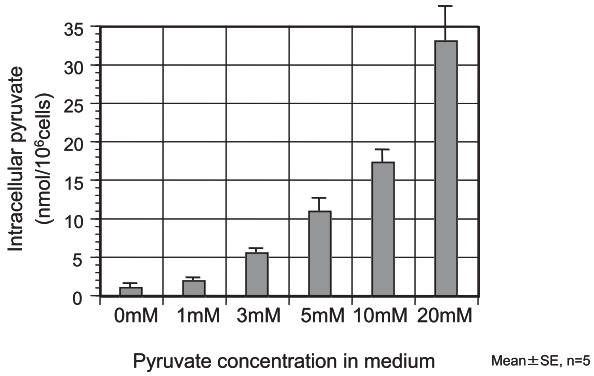

Fig. 1 Intracellular pyruvate concentration after a short period of culture supplemented with pyruvate.
Supplementation of pyruvate for 2 days resulted in accumulation of intracellular pyruvate in a concentration-dependent manner. Intracellular pyruvate concentrations were as follows: 0 mM, 1.11 ± 0.51; 1 mM, 2.00 ± 0.37; 3 mM, 5.61 ± 0.57; 5 mM, 11.04 ± 1.66; 10 mM, 17.39 ± 1.59; 20 mM, 33.15 ± 4.50 (n = 5, mean ± SE).

Fig. 2 depicts numbers of viable BMMNC after 2- or 4-day culture with various concentrations of pyruvate added to the culture medium. Cell number on day 0 was designated as 1. On day 2, 5 mM pyruvate most significantly increased the viable cell number compared with the others (paired t-test, two-tailed p-value< 0.01, n=3), to approximately two-fold that on day 0. In contrast, cell number on day 4 was decreased to less than half of that on day 2, and no significant differences were recognized between 3, 5 and 10 mM pyruvate.

Flow cytometry was used to evaluate circulating EPC level by the quantification of cells double-positive for EPC markers defined as $CD31^+/CD34^+$. A representative scatter graph is shown in Fig. 3. Although no statistical analysis was carried out, 5 mM pyruvate for 4 days seemed more effective for the increment of $CD31^+/CD34^+$ cells.

Next, we further studied the influence of other HIF-PH inhibitors, such as cobalt chloride, L-mimosine and roscovitin, on the induction of VEGF. After 2-day culture of BMMNC supplemented with 0, 1, 3 and 5 mM pyruvate, or other HIF-PH inhibitors, VEGF mRNA was measured by Q-RTPCR (Fig. 4). *VEGF* mRNA quantities included in 5 ng of RNA from each condition are shown with untreated murine BMNC as a control. Supplementation with 1, 3 and 5 mM pyruvate augmented *VEGF* mRNA up to approximately 8.4, 20.9 and 27.8 times, respectively. Among the HIF-PH inhibi-

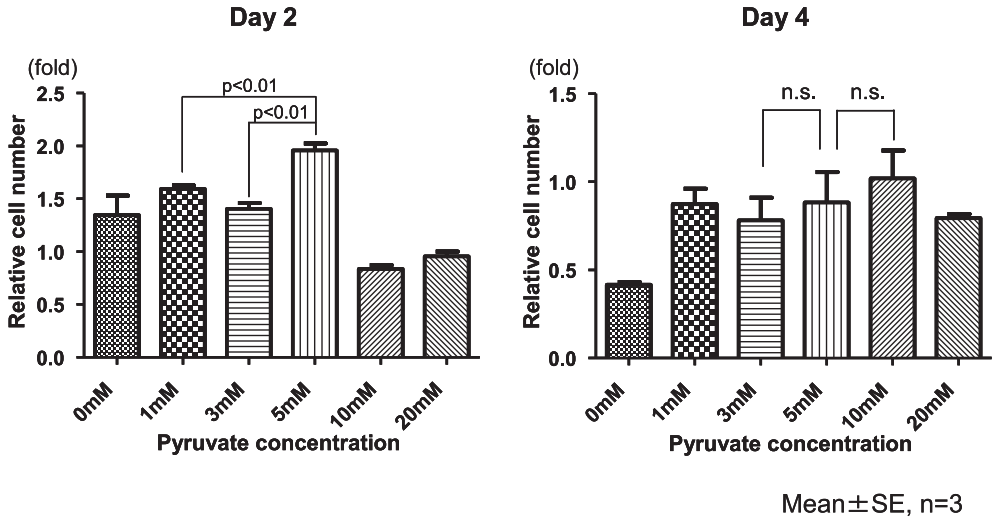

Fig. 2　Cell proliferation and viability after a short period of culture supplemented with pyruvate. Viable cell numbers after 2- or 4-day culture with various concentrations of pyruvate in culture medium are shown. On day 2, 5 mM pyruvate most significantly increased the viable cell number (1.96-fold) compared with 1 mM (1.59-fold), 3 mM (1.40-fold) and others (paired t-test, two-tailed p value＜0.01, n＝3). On day 4, no significant differences were recognized between 3, 5 and 10 mM pyruvate.

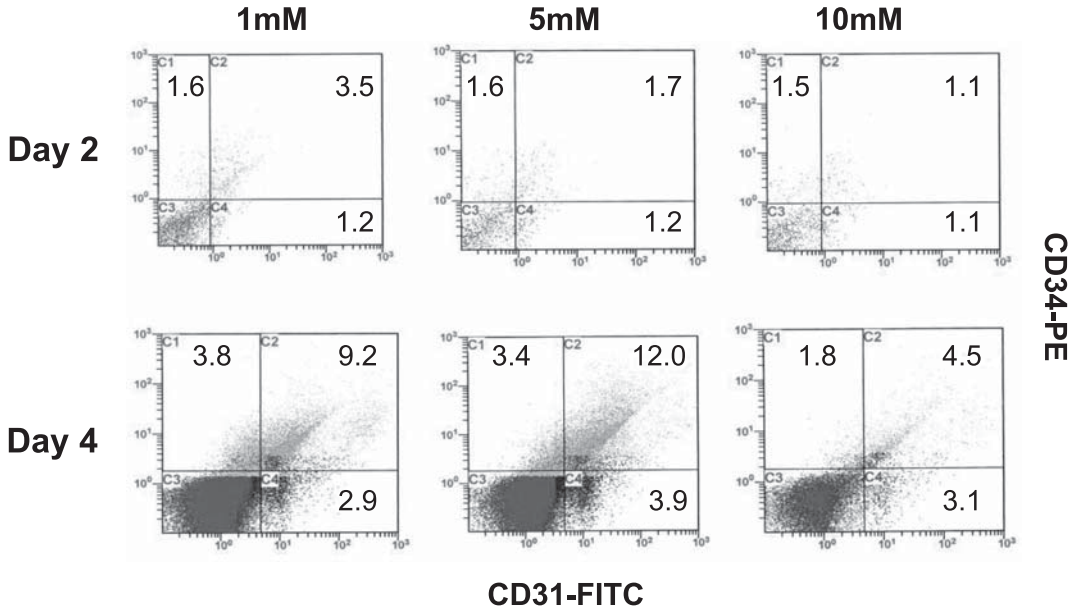

Fig. 3　$CD31^+/CD34^+$ cells after 4-day culture with various concentrations of pyruvate
As $CD31^+/CD34^+$ cell fraction contains EPC, we evaluated these cell surface markers by flow cytometry. Untreated BMMNC contain 1.0%, 4.3% and 1.6% of $CD31^+/CD34^-$, $CD34^-/CD34^+$ and $CD31^+/CD34^+$ cells, respectively (data not shown). Numbers in scatter graphs indicate % of the corresponding fractions. No obvious increment of $CD31^+/CD34^+$ cells was observed on day 2, while 4-day culture resulted in an increase in double-positive cells to 9.2% at 1 mM, 12.0% at 5 mM, and 4.5% at 10 mM.

tors tested, cobalt chloride showed the largest increase in *VEGF* mRNA, at approximately 109 times more than the control. Levels with L-mimosine and roscovitine were 28.3 and 42.1 times more than that of the control, respectively.

Finally, we examined VEGF concentration in conditioned medium (Fig. 5). In this experiment, only the 4-day culture with 5 mM pyruvate showed significant elevation of excreted VEGF level compared with 0 mM pyruvate (n=5, two-tailed p value<0.05).

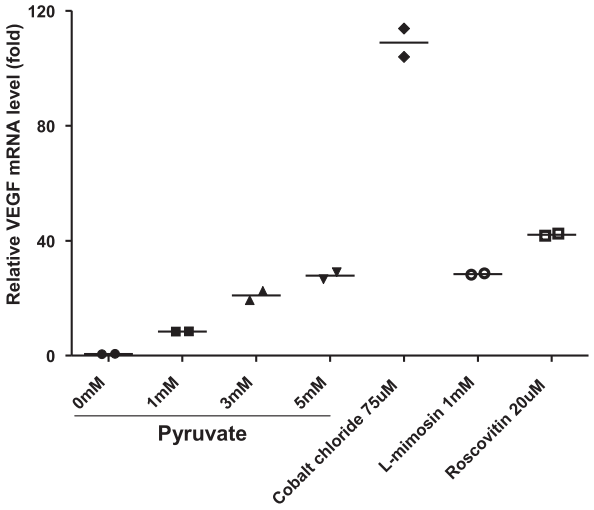

Fig. 4 Transcriptional activation of VEGF gene by pyruvate compared with activation by other HIF-PH inhibitors.
VEGF mRNA was titrated after 2-day treatment with pyruvate as well as other HIF-PH inhibitors. Vertical axis shows relative *VEGF* mRNA quantities compared with those of untreated murine BMMNC as a control. Data of two independent experiments are shown. *VEGF* mRNA increased 8.4 times at 1 mM, 20.9 times at 3 mM and 27.8 times at 5 mM, compared with that of untreated murine BMMNC. Levels with HIF-PH inhibitors such as cobalt chloride, L-mimosine and roscovitine were 109, 28.3 and 42.1 times more than control, respectively.

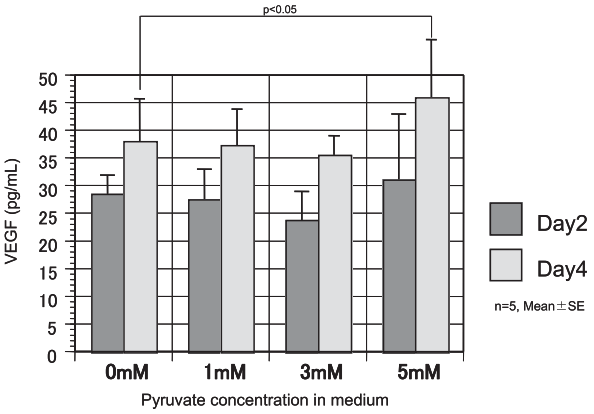

Fig. 5 VEGF levels in the conditioned medium
VEGF concentration in the medium was measured after 2- and 4-day culture, and statistical analysis was performed by paired t-test (n = 5). A significant increase was observed only for 5 mM pyruvate on day 4 (two-tailed p value < 0.05).

DISCUSSION

EPC can be recruited from bone marrow by several growth factors, such as VEGF, stromal cell-derived factor-1 (SDF-1), angiopoietin-1 or G-CSF[15]. EPC also secretes VEGF in autocrine and paracrine fashions, and acts to differentiate mature EPC into endothelial cells[16]. Thus, activation of *VEGF* gene in EPC is most likely to be effective in augmenting therapeutic efficacy for CLI patients. Previous studies have elucidated that circulating EPC express several endothelial cell-specific surface markers such as CD31, CD34, VEGF receptor-2, CD146 and von Willebrand factor[17].

In order to examine whether BMMNC can efficiently uptake extracellular pyruvate in a short-period culture, we measured the intracellular concentration of pyruvate after 2- and 4-day culture. As shown in Fig. 1, the intracellular concentration of pyruvate successfully increased in an extracellular pyruvate concentration-dependent manner. Carry-over of extracellular pyruvate in assay samples was unlikely, since intracellular pyruvate was unchanged in a leukemic cell line, which rapidly proliferates in culture. Viable cell counts were evaluated using 1-20 mM pyruvate in medium (Fig. 2), and 5 mM pyruvate was shown to be the most appropriate concentration to increase viable cells on day 2, whereas no significant differences were observed among 1-10 mM pyruvate on day 4. Numbers of $CD31^+/CD34^+$ double-positive cells were increased in 5 mM pyruvate on day 4 (Fig. 3), and 5 mM pyruvate upregulated *VEGF* gene to the same extent as L-mimosine on day 2 (Fig. 4). It should be noted that VEGF concentration in conditioned medium showed a significant increase at 5 mM on 4-day culture. Since viable cell numbers and *VEGF* mRNA became significantly enhanced on day 2, and secreted VEGF in conditioned medium must be washed out and resuspended in saline before administration, we conclude that 2-day culture in 5 mM pyruvate is the most appropriate condition for enhancing VEGF production of murine BMMNC.

Mechanisms attributable to the activation of HIF-inducible genes under normoxia have been extensively studied, and several chemicals and organic small molecules have been identified as HIF-PH inhibitors[12]. Roscovitine is a purine derivative, and inhibits cyclin-dependent kinase, inducing cell cycle arrest and apoptosis. This chemical is under consideration as a potential anti-tumor reagent. L-mimosine is a plant amino acid that has a similar structure to tyrosine, and also induces cell cycle arrest in mammalian cells and inhibits translation of cyclin-dependent kinase. Cobalt chloride and roscovitin showed greater effectiveness for *VEGF*

gene activation (Fig. 4); however, both are much more expensive and have cytotoxicity, which is a serious concern when cells are administered to patients.

Although a previous report demonstrated that pyruvate is effective for angiogenesis in chick embryo[18] as well as in *in vivo* mouse Matrigel plug assays, we confirmed that pyruvate supplementation raised the intracellular concentration in culture and that VEGF concentration was increased in conditioned medium. *Ex vivo* activation of the hypoxia-inducible genes and subsequent administration of cells are considered to not only enhance clinical effectiveness but to also provide a novel approach in cellular therapy for CLI, since a recent study[19] showed that the conditioned medium generated from *in vitro* culture of progenitor cells was effective for CLI.

Quite recently, a prospective, randomized, double-blinded clinical study revealed a significant increase in amputation-free survival in patients receiving cellular therapy for CLI[20]. In the study, autologous bone marrow cells were used, designated as tissue repair cells (TRC), which were generated by 12-day culture in long-term bone marrow culture medium[21]. TRC are derived from a 50-m*l* bone marrow aspirate, and reportedly contained more $CD14^+$ and $CD90^+$ cells than primary BMMNC[21].

In conclusion, the present study showed that this culture system successfully enhanced *VEGF* gene expression and secretion of VEGF into the medium. *Ex vivo* expansion of EPC may become an ideal strategy as a cellular source for therapeutic neoangiogenesis.

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ピルビン酸添加による骨髄単核球における血管内皮増殖因子の発現増強を目指した短期培養法の研究

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要旨：
血管内皮前駆細胞（EPC）を用いた血管再生療法は血管バイパス形成術や血管内膜切除術の適応とならない重症虚血肢に対する治療オプションとして近年脚光を浴びている. 骨髄からの EPC 動員や局所における EPC の血管内皮細胞への分化には血管内皮増殖因子（VEGF）が重要な役割を果たしていることから，今回我々はマウス骨髄単核球（BMMNC）を用いて *VEGF* 遺伝子発現の増強および VEGF 分泌量の増加を図るための新規培養法の開発を目指し，以下の実験を行なった.
VEGF は低酸素下で転写因子 HIF-1 が安定化することで誘導される. その背景にはプロリル水酸化酵素（HIF-PH）の活性がピルビン酸により阻害されることが明らかになっている. ピルビン酸が BMMNC における VEGF 発現を増強することが期待されたため, マウス BMMNC のピルビン酸添加培養を行なった. EPC 表面マーカーである $CD31^+$/$CD34^+$二重陽性細胞は 5mM ピルビン酸により最も高くなり，VEGF 遺伝子発現量は 2 日間の培養により培養前の 27.8 倍にまで達した．さらに培養液中 VEGF 濃度は 5mM ピルビン酸，4 日間培養にて有意な上昇を認めた.
ピルビン酸は細胞のエネルギー代謝上必須の有機酸であり，無害で安価のため，今回開発した EPC 体外増幅法は今後治療的血管新生への応用が期待出来る.

キーワード：
重症虚血肢，再生医療，血管内皮細胞増殖因子（VEGF），血管内皮前駆細胞

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