見られた。内、9匹においては心機能の低下・心室性不整脈の出現から心停止に陥った。1匹は生存し、経胸壁心エコー検査上、著明な前壁の運動の低下と、心拡大が見られた。

D 考察

(1)再生アソシエイト細胞の獲得免疫反応に対する免疫寛容効果の in vitro 研究
マウスよりヒトと同様の方法をもって作成した再生アソシエイト細胞は、ヒト再生アソシエイト細胞と類似した形態を呈しており、本作成手法の有用性が示唆された。今後、このマウス由来の再生アソシエイト細胞の表現型を同定し、ヒト再生アソシエイト細胞との相同性を検討しつつ、培養条件を最適化する必要がある。
(2)再生アソシエイト細胞（同種同系）移植実験
本 in vitro 研究の中心的な実験手技となる混合リンパ球培養試験の実施に成功した。今回の検討では、再生アソシエイト細胞の、他家細胞に対する免疫寛容効果は明かではなかった。この原因として、レシピエントマウスが有する他家抗原に対する獲得免疫が十分でないことが考えられ、今後の実験では脾臓を摘出する前に、レシピエントマウスに抗原刺激を加えることを考慮する必要がある。また手技的な問題も考えられ、実験を繰り返すことにより、実験技術に習熟する必要がある。

(3)再生アソシエイト細胞（同種同系）移植実験
マウスの急性心筋梗塞の作成に成功し、さらにマウスの心機能を経胸壁心エコー法にて評価することに成功した。今回の検討ではまだ死亡率が高く、心機能低下が一定でないことから、実験を繰り返し技術的に習熟することにより、一定の心機能低下を有するモデルを作成する必要がある。

E 結論

マウス由来の再生アソシエイト細胞の作成に成功した。再生アソシエイト細胞の獲得免疫反応に対する免疫寛容効果の in vitro 研究に必要な実験手技の実施に成功し、また再生アソシエイト細胞（同種同系）移植実験に必要なマウス急性心筋梗塞モデルの作成及び心機能の評価に成功した。今後、これらの基盤となる実験手技を用いて、本研究仮説を検証していく。

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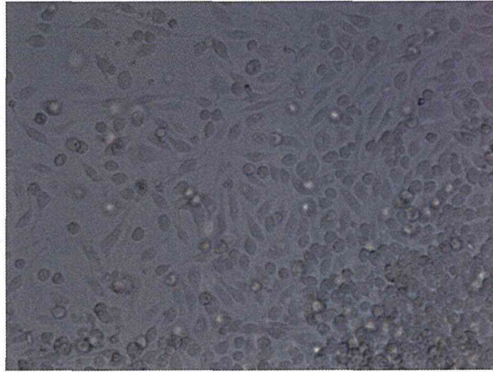
G　知的財産権の出願・登録状況

特記すべきことなし

別添図表一覧

図１：



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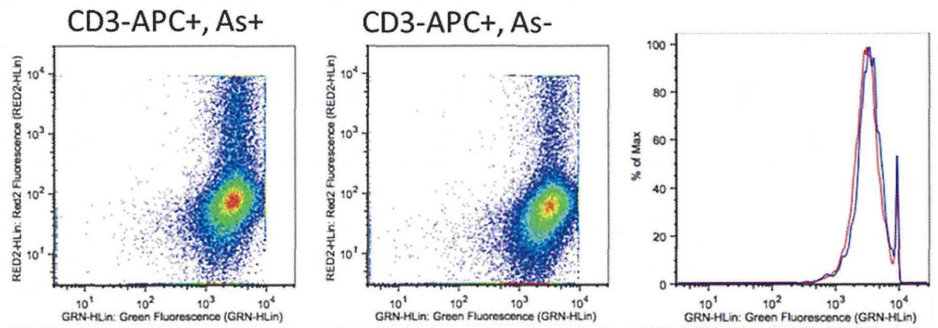

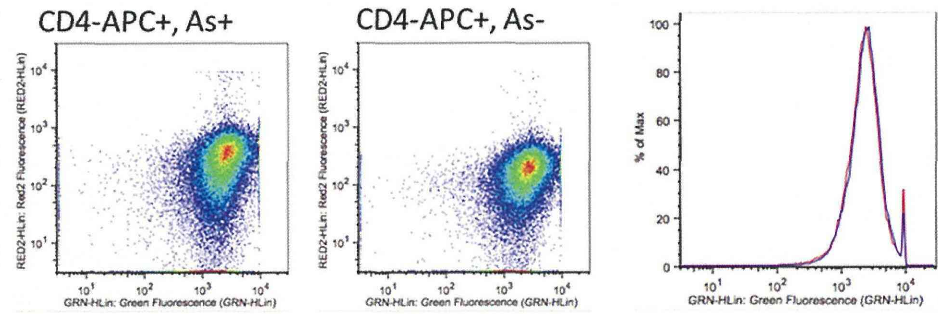

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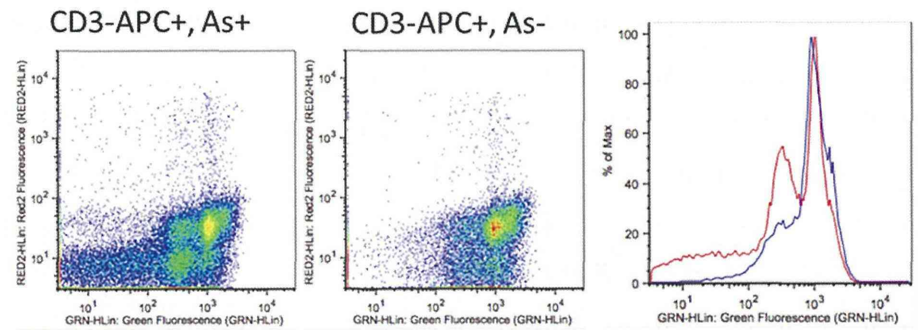

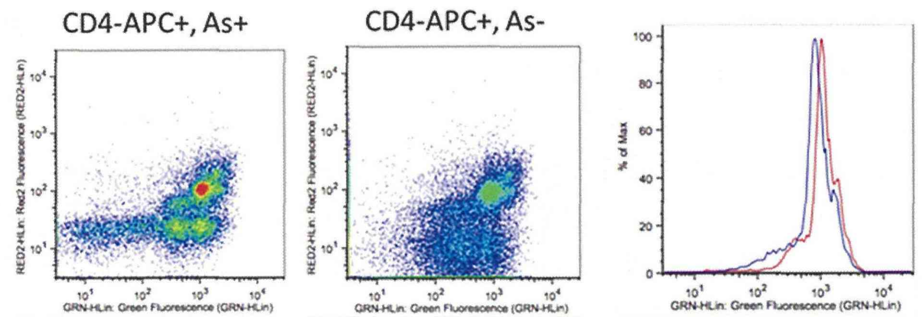

厚生労働科学研究費補助金　難病・がん等の疾患分野の医療の実用化研究事業（再生医療関係研究分野）

（総括・分担）研究報告書

「再生アソシエイト細胞による iPS 細胞移植時の免疫寛容治療研究」

研究分担者　田中里佳（順天堂大学医学部　形成外科学　准教授）

研究要旨：本提案課題では、血液単核球細胞を再生シグナル環境で培養することで、血管再生・抗炎症・免疫寛容作用を有する「再生アソシエイト細胞」に誘導する技術を研究し、iPS 組織の生着・機能化のための臨床応用技術として開発し、心筋細胞シート（拠点大阪大学：澤グループ）、網膜色素上皮シート（拠点理化学研究所：代表笹井：高橋グループ）産業化を前提とした臨床応用まで進めることを目的とする。田中里佳率いる順天堂大学グループにおいては、糖尿病患者の再生アソシエイト細胞有効性の確認、糖尿病疾患に対する再生アソシエイト細胞の血管再生・抗炎症・免疫寛容作用を確認、将来的に iPS 組織の生着・機能化のための臨床応用技術を開発する。平成 25 年度は、マウス再生アソシエイト異種移植実験を実施するための培養法と実験手技、評価確立を行い、糖尿病疾患における再生アソシエイト細胞の有効性を確認する研究計画を予定した。

A. 研究目的

順天堂大学において平成 25 年度は下記を目的に実験を実施した。

①マウス再生アソシエイト細胞培養法の確立

②糖尿病患者における再生アソシエイト細胞の血管新生、抗炎症効果

③マウス再生アソシエイト細胞の同種同系潰瘍移植実験による血管新生、抗炎症反応の組織評価指標確立。

B.研究方法

①マウス末梢血から単核球を採取し、単核球を再生アソシエイト細胞培養カクテルにて 5 日間培養を行い培養後に再生アソシエイト細胞の血管再生と抗炎症効果の指標となる細胞表面マーカーを FACS 解析にて、血管再生能を EPC-colony forming assay にて解析した。

②糖尿病患者末梢血単核球から再生アソシエイト細胞を作成し、血管再生と抗炎症効果の指標となる細胞表面マーカーを FACS 解析にて、血管再生能を EPC-colony forming assay, EPC Culture Assay にて解析をおこなった。糖尿病患者再生アソシエイト細胞をヌード潰瘍モデルマウスに移植し、組織における血管新生、抗炎症、創傷治癒効果を解析した。

③マウス再生アソシエイト細胞の同種同系潰瘍移植実験による血管新生、抗炎症反応の組織評価指標を確立するため、健常マウス(C57BL6J)と糖尿病マウス(db/db)に潰瘍を作成し潰瘍作成後 Day1,Day3, Day5, Day7, Day10 における局所の M1/M2 細胞活性を評価した。

（倫理面への配慮）

ヒトゲノム・遺伝子解析研究に関する倫理指針（平成２５年文部科学省・厚生労働省・経済産業省告示第１号)、臨床研究に関する倫理指針（平成２０年厚生労働省告示第４１５号）厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針（平成１８年６月１日付厚生労働省大臣官房厚生科学課長通知）及び順天堂大学でで定めた医学部倫理員会の規定を遵守した。動物実験は、順天堂大学実験動物委員会と学長の承認のもと動物愛護法を遵守、臨床研究については順天堂大学病院倫理委員会の承認のもとに実施している。

C.研究結果

①マウス末梢血再生アソシエイト細胞は培養前に比べ培養後に細胞数は減少する（5.0±0.0 vs. 1.0±0.2）が EPC の数は増加する傾向にある。EPC colony forming assay の結果、再生アソシ

エイト細胞は、primitive, definitive, および total-CFUs 全てにおいて Colony 数は優位に増加し高い血管再生能を有する細胞であることがわかった。
②DM 患者において再生アソシエイト細胞は EPC､M2 マクロファージ数を約 10 倍に増幅し、上記に示した血管再生能を検証するアッセイ法と RT-PCR においても DM 再生アソシエイト細胞は健常人以上の抗炎症効果と血管再生能を示した。ヌードマウス移植効果は In vitro の結果同様、DM 再生アソシエイト細胞植群において高い創傷治癒効果と組織内血管再生効果を示した。
③健常マウス(C57BL6J)と糖尿病マウス (db/db)に潰瘍を作成し潰瘍作成後 Day1,Day3, Day5, Day7, Day10 における局所の M1/M2 細胞活性を評価した。健常マウス潰瘍において M1 細胞は Day1 に上昇しその後減少する。M2 は Day 7 をピークに上昇し､その後 Day10 まで潰瘍内部に存在する。一方糖尿病マウス潰瘍において M2 細胞は Day 1 における上昇なすくなく､Day7 においても M1 細胞が遷延して潰瘍ないに存在する。M2 の発現は Day10 に軽度の発現が認められるにとどまる。

D.考察

今年度の研究結果においてマウスと糖尿病患者の末梢血再生アソシエイト細胞は高い抗炎症・免疫寛容・血管再生能を有することが示された。また、今後の動物実験において組織抗炎症効果・免疫反応を解析するための潰瘍モデルの有効性と解析プロトコールの作成準備が整ったため今年度の実験データをもとに来年度の実験をスムーズに進められると考える。

E.結論

我々は抗炎症効果、高い血管再生能を有するマウス末梢血再生アソシエイト細胞の確立に成功した。来年度は同種同系再生アソシエイト細胞移植実験、同種異種再生アソシエイト細胞移植実験を実施し、免疫寛容効果と抗炎症効果、血管・組織再生効果を検証する。末梢血再生アソシエイト細胞は少量の血液からを抗炎症・免疫寛容細胞の移植することが可能となり、iPS 組織の生着・機能化のための臨床応用技術として高い可能性を有する。

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①糖尿病患者に対する新しい血管再生の開発
Author：田中 里佳(順天堂大学 医学部形成外科学), 増田 治史, 有田 佳代, 実川 佐智恵, 平野 理恵, Dewi Sukmawati, 藤村 聡, 三田 智也, 綿田 裕孝, 浅原 孝之, 水野 博司
Source：糖尿病(0021-437X)56 巻 Suppl.1 PageS-253(2013.04)

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G　知的財産権の出願・登録状況

発明の名称：血管内皮前駆細胞を含む細胞群の生体外増幅方法
基礎出願の番号：特願２０１２－２１８２０６、ＰＣＴ出願番号：ＰＣＴ／ＪＰ２０１３／７６６１８、国際出願日：2013 年 9 月 30 日（基礎出願日：2012 年 9 月 28 日）発明者：浅原孝之、増田治史、田中里佳

Ⅲ．研究成果の刊行に関する一覧表

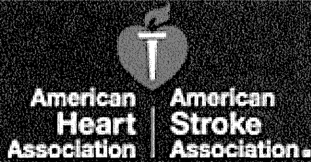
研究成果の刊行に関する一覧表

原著論文

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Masuda H, Asahara T et al.	Vasculogenic Conditioning of Peripheral Blood Mononuclear Cells Promotes Endothelial Progenitor Cell Expansion and Phenotype Transition of Anti-inflammatory Macrophage and T Lymphocyte to Cells with Regenerative Potential.	Journal of American Heart Association. (in press)			2014
Masuda H, Asahara T.	Clonogenic assay of endothelial progenitor cells.	Trends in cardiovascular medicine.	23	99-103	2013
Tanaka R, Masuda H, Asahara T. et al.	Quality-control culture system restores diabetic endothelial progenitor cell vasculogenesis and accelerates wound closure.	Diabetes.	62	3207-3217	2013
Kawamura M, Miyagawa S, Fukushima S, Saito A, Miki K, Ito E, Sougawa N, Kawamura T, Daimon T, Shimizu T,	Enhanced survival of transplanted human induced pluripotent stem cell-derived cardiomyocytes by the combination of cell sheets with the pedicled omental flap technique in a porcine heart.	Circulation.	128	87-94	2013

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
前頁続き Okano T, Toda K, Sawa Y.					
Tanaka R, Masuda H, Kato S, Imagawa K, Kanabuchi K, Nakashioya C, Yoshiba F, Fukui T, Ito R, Kobori M, Wada M, Asahara T, Miyasaka M.	Autologous G-CSF-mobilized peripheral blood CD34+ cell therapy for diabetic patients with chronic nonhealing ulcer.	Cell transplantation.	23	167-179	2014

Ⅳ．研究成果の刊行物・別刷



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Journal of the American Heart Association
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URL: http://jaha-submit.aha-journals.org

Title: Vasculogenic Conditioning of Peripheral Blood Mononuclear Cells Promotes Endothelial Progenitor Cell Expansion and Phenotype Transition of Anti-Inflammatory Macrophage and T Lymphocyte to Cells with Regenerative Potential

Manuscript number: JAHA/2013/000743-T2

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Vasculogenic Conditioning of Peripheral Blood Mononuclear Cells Promotes Endothelial Progenitor Cell Expansion and Phenotype Transition of Anti-Inflammatory Macrophage and T Lymphocyte to Cells with Regenerative Potential

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34 **ABSTRACT**
35
36 **Background:** Cell-based therapies involving mononuclear cells (MNCs) have been developed
37 for vascular regeneration to treat ischemic diseases; however, quality control of therapeutic
38 MNCs has not been evaluated. We investigated the therapeutic potential of peripheral blood (PB)
39 MNCs; operated by recently developed quality and quantity culture (QQc) of endothelial
40 progenitor cells (EPCs).
41 **Methods and Results:** PBs were collected from healthy volunteers; PBMNCs isolated from
42 these PBs were subjected to QQc for 7 days with medium containing SCF, TPO, Flt-3 ligand,
43 VEGF, and IL-6. The resulting cells (QQMNCs) in EPC-colony forming assay generated
44 significantly more definitive EPC colonies than PBMNCs. In flow cytometry, macrophages and
45 helper T lymphocytes of QQMNCs became phenotypically polarized into angiogenic,
46 anti-inflammatory, and regenerative subsets: classical M1 to alternative M2; Th1 to Th2;
47 angiogenic- or regulatory T cell expansion. Quantitative real-time PCR (qRT-PCR) assay
48 revealed the predominant pro-angiogenic gene expressions in QQMNCs versus PBMNCs. Using
49 murine ischemic hindlimb models, the efficacy of QQMNC intramuscular transplantation (Tx)
50 was compared to that of PBMNCTx, cultured 'early EPC' Tx (eEPCTx), and G-CSF mobilized
51 CD34+ cell Tx (GmCD34 Tx). Laser Doppler imaging revealed the blood perfusion recovery in
52 ischemic hindlimbs following QQMNCTx superior to following PBMNCTx and eEPCTx, but
53 also earlier than following GmCD34Tx. Histological evaluations and qRT-PCR assays in
54 ischemic hindlimbs demonstrated that QQMNCTx, similarly to GmCD34Tx, enhanced
55 angio-vasculogenesis and myogenesis, while preponderantly inhibited inflammation and fibrosis
56 versus PBMNCTx and eEPCTx.
57 **Conclusions:** QQc potentiates the ability of PBMNCs to promote regeneration of injured tissue;
58 considering the feasible cell preparation, QQc-treated PBMNCs may provide a promising
59 therapeutic option for ischemic diseases.
60
61 **Keywords:** Vascular regeneration, Cell-based therapy, Peripheral blood mononuclear cells,
62 Serum-free Culture, Anti-inflammation
63
64 **Clinical Trial Registration:**
65 URL; irb.med.u-tokai.ac.jp/d/2/monthly/2010.html, IRB No.; 10R-020
66 URL; irb.med.u-tokai.ac.jp/d/2/monthly/201312.html, IRB No.; 13R228

67 **NON-STANDARD ABBREVIATIONS AND ACRONYMS**

68

69	acetylated low density lipoprotein, labeled with 1,1'-dioctadecyl –	
70	3,3,3',3'-tetramethyl-indocarbocyanine perchlorate	acLDL-DiI
71	Angiopoietin-1	Ang-1
72	Angiopoietin-2	Ang-2
73	Anterior tibial muscle	ATM
74	Bone marrow	BM
75	Chemochine receptor2	CCR2
76	Colony forming unit	CFU
77	3,3'-Diaminobenzidine, tetrahydrochloride	DAB
78	Definitive EPC-CFU	dEPC-CFU
79	Early EPC	eEPC
80	Endothelial progenitor cell	EPC
81	Fms-like tyrosine kinase 3 ligand	Flt-3 ligand
82	Forkhead box P3	Foxp3
83	Gastrocunemius muscle	GCM
84	Granulocyte colony stimulating factor	G-CSF
85	G-CSF mobilized CD34+ cell	GmCD34
86	Human umbilical vein endothelial cell	HUVEC
87	Inducible nitric oxide synthase	iNOS
88	Insulin-like growth factor-1	IGF-1
89	Interferon-γ	□□□̃γ
90	Interleukin-1β	□□̃ıβ
91	Interleukin-6	□□-6
92	Metalloprotease	MMP
93	Mononuclear cell	MNC
94	Natural killer cell	NK cell
95	Peripheral blood	PB
96	Primitive EPC-CFU	pEPC-CFU
97	QQc cells of CD34+ cell depleted PBMNC	QQ-34–MNC
98	Quality and quantity culture	QQc
99	Quantitative real-time PCR	qRT-PCR

100	Region of interest	ROI
101	Smooth muscle α actin	SMα actin
102	Stem cell factor	SCF
103	Tumor necrosis factor- α	TNF-α
104	Transforming growth factor-β	TGF-β
105	Transplantation	Tx
106	Thrombopoietin	TPO
107	Vascular endothelial growth factor	VEGF
108	von Willebrand factor	vWF
109		

110 **INTRODUCTION**

111

112 Based on the isolation of bone marrow (BM)-derived endothelial progenitor cells (EPCs),[1-3]
113 autologous total mononuclear cells (MNCs) freshly isolated from BM or peripheral blood (PB)
114 have been applied to clinical vascular regenerative therapy in patients with severe ischemic heart
115 or limb diseases.
116 These initial clinical experiences indicate that cell-based therapy for vascular regenerations is
117 safe, feasible, and effective.[4-7] However, the translational and clinical trials have also
118 demonstrated insufficient or contradictory effectiveness on recovery from ischemic diseases.[8-11]
119 PB- and BMMNCs constitute hematopoietic lineage cells; most are lymphoid cells or myeloid
120 monocytes, and very few are stem/progenitor cell types, such as hematopoietic stem/progenitor
121 cells, EPCs, or other mesenchymal stem cells. The scarcity of EPCs in MNC populations is a
122 main reason for failure of constant and potent contributions in clinical cases.
123 Enriched EPCs, such as CD34+ or CD133+ cells, constitute less than 0.01% of PBMNCs and
124 0.1% of BMMNCs, and the frequency of colony forming EPCs is 0.005% in PBMNCs.[12]
125 Taking into account the majority in MNCs, we need to issue the critical functions of
126 monocyte/macrophages. Monocytes are precursors of macrophages; monocytes play key roles in
127 both pro-inflammatory and in regenerative processes by phenotype alterations following tissue
128 infiltrations.[13] Macrophages are a heterogeneous cell population that adapts and responds to a
129 large variety of micro-environmental signals. For example, the cytokines and growth factors in
130 some microenvironments induce macrophages to adopt regenerative phenotypes, while the
131 inflammatory deterioration and fibrosis in uncontrolled inflammatory environment in tissues
132 induce pro-inflammatory macrophage transitions and suppress regenerative processes.
133 Also the inflammatory environment is interacted by major lymphoid phenotypes simultaneously
134 stimulated by pro-inflammatory signals and controlled macrophage phenotype transition for
135 pro-inflammatory drive. [13, 14]
136 To suppress unproductive inflammatory process and enhance vasculogenic regeneration,
137 purified EPCs have been transplanted into patients with severe ischemic heart or limb diseases,
138 and these initial clinical experiences indicate that this cell-based therapy is safe and effective. [15-19]
139 Nevertheless, the problems caused by EPC scarcity must be overcome for therapeutic EPC
140 transplantation to become reproducible and effective. To this end, the following three stepwise
141 strategies have been used: 1] leukapheresis, 2] daily administration of granulocyte colony
142 stimulating factor (G-CSF), and 3] isolation of machinery CD34+ or CD133+ cells. Such a

143 isolation process of autologous EPC sources burdens the patients to be treated with 'EPC therapy',
144 in terms of medical invasiveness and costs.
145 More importantly, the number and functionality of EPCs decline as patients age[20] and in patients
146 with cardiovascular risk factors.[21, 22]
147 Here, we describe a method for MNC culture that enhances the vasculogenic potential of EPCs
148 and facilitates the preparation of monocytes for regenerative phenotype activation.
149 Our method for quality and quantity control culture (QQc) of MNCs (QQMNC) is based on an
150 established culture method to that increases the quality and quantity of EPCs derived from
151 enriched EPC populations, such as CD34+ and CD133+ cells. Notably, the therapeutic potential
152 of QQc is demonstrably greater than that of naïve EPCs for cardiovascular regeneration following
153 infarcted myocardia in rats.[23] Interestingly, we found that the vasculogenic signaling condition of
154 MNCs in QQc potentiates the vascular and tissue regeneration ability of naïve PBMNCs. The
155 regenerative function of QQMNC turned out to be operated via the activation of
156 anti-inflammatory and angiogenic monocytes/helper T lymphocytes as well as vasculogenic EPC
157 expansion.
158 Moreover, the present experimental study demonstrated that therapeutic efficacy of QQMNC
159 transplantation is equal to and greater than that of G-CSF mobilized CD34+ cell transplantation.
160 Therefore, in cell-based therapy for ischemic diseases, QQMNC provides a practical option of
161 cell sources including PBMNC and G-CSF mobilized CD34+ cell.
162
163

164 **METHODS**

165

166 **Collection of PBMNCs cells or CD34 cells from healthy volunteers.**

167 The experiments using human samples were performed with institutional approval and
168 guidelines from the Clinical Investigation Committee at Tokai University School of Medicine
169 (IRB No.; 10R-020, *URLweb site*; irb.med.u-tokai.ac.jp/d/2/monthly/2010.html, and IRB
170 No.;13R228, *URLweb site*; irb.med.u-tokai.ac.jp/d/2/monthly/201312.html). The whole healthy
171 human volunteers between the ages of 20 and 55 years gave informed consent.
172 PB (20 to 100 mL/subject) was drawn by heparinized venous puncture at the forearm. PBMNCs
173 were isolated by density gradient centrifugation using Lymphocyte Separation Solution (d=
174 1.077)(Nakalai Tesque), as previously reported.[24] CD34+ cells were purified by an autoMACS
175 separator (Miltenyi Biotec), using magnetic beads-coated mouse anti human CD34 antibody and
176 a CD34 Cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

177

178 **QQ culture and early EPC culture of PBMNCs.**

179 ***1. QQ culture condition for PBMNCs:*** QQc medium of Stem Line II (Sigma-Aldrich) contained
180 the five human recombinant proteins; SCF, TPO, Flt-3 ligand, VEGF and IL-6. Then, isolated
181 PBMNCs were cultured for 7 days at the cell density of 2 $x10^6$ cells/2mL QQc medium per well of
182 6 well Primaria tissue culture plate (BD Falcon). The cell density in QQc was corresponded to the
183 approximate density of 1 $x10^6$ MNCs per 1 mL of PB. The culture well plates and the contents of
184 QQc medium are listed in **TABLE 1, 2**.
185 ***2. Early EPC culture of PBMNCs:*** As previously reported,[24, 25] 'early EPCs' were acquired
186 following 7 days culture of isolated PBMNCs using EGM-2-MV SingleQuots kit (Lonza). In
187 brief, EGM-2-MV complete medium was adjusted by adding 5%FBS (SAFC Biosciences) and
188 supplemented growth factors except hydrocortisone to EBM-2 basal medium. PBMNCs were
189 adjusted to the similar cell density (1 x 10^6 cells/mL) with 5%FBS/ EGM-2-MV complete
190 medium to that in QQc of PBMNC. The cells were then plated on human fibronectin-coated
191 6-well Primaria tissue culture plate (2x 10^6 cells/2 mL/well) and cultured. The medium was
192 changed 4 days after seeding, then cultured for 3 more days. Seven days later, non attaching cells
193 were removed; the adherent cells were harvested with 2 mM EDTA/PBS after washing with PBS.
194 The harvested 'early EPCs' (eEPCs) were suspended in IMDM; adjusted the cell density ($1x10^4$
195 cells/40 μL or $2x10^5$ cells/40 μL), then transplanted into skeletal muscle of murine ischemic
196 hindlimb, as described below.

197 **EPC colony forming assay.**
198 To investigate the vasculogenic potential of PBMNCs or QQMNCs, we used semi-solid culture
199 medium and 35 mm Primaria dishes (BD Falcon), to grow and then counted the adhesive EPC
200 colonies by EPC colony forming assay (EPC-CFA) (MethoCult SF^{BIT}, Stem Cell Tec.) with
201 pro-angiogenic growth factors/cytokines, as previously reported (**TABLE 3**).[12] Aliquots of those
202 cells were seeded at 2 x10^5 cells/dish (3 dishes per volunteer) for EPC-CFA. Sixteen to eighteen
203 days after initiation of the culture, the number of adherent colonies per dish was measured using a
204 gridded scoring dish (Stem Cell Tec.) under phase-contrast light microscopy (Eclipse TE300,
205 Nikon). Primitive EPC-CFUs (pEPC-CFUs) and definitive EPC-CFUs (dEPC-CFUs) were
206 separately counted.
207
208 **Flow cytometry.**
209 ***1. Performance of flow cytometry for lineage cell populations:*** Freshly isolated PBMNCs and
210 the QQMNCs were subjected to flow cytometry to detect surface antigen positivities of
211 hematopoietic stem or lineage committed cells as well as endothelial lineage cells. The antibodies
212 were listed in **TABLE- 4**, **5**, **6**. The cells suspended in 2mM EDTA/0.2% BSA/PBS buffer (5
213 x10^5 cells/200 μL) were incubated after addition of 10 μL FcR blocking reagent at 4°C for 30 min
214 and then equally dispensed into reaction tubes for subsequent staining (100 μL/tube). Each
215 aliquot was incubated with 2 μL of each first antibody at 4°C for 20 min, and then washed twice
216 with 1mL of 2mM EDTA/0.2% BSA/PBS buffer. The cells were suspended in 2mM EDTA/0.2%
217 BSA/PBS buffer (2 x10^5 cells/200 μL). Flow cytometry analysis was performed using
218 LSRFortessa cell analyzer (BD) and FlowJo software (Tomy Digital Biology). When staining for
219 von Willebrand factor (vWF), after incubation with each first antibody, cells were incubated with
220 biotin conjugated rat anti-mouse IgG_1, and then conjugated to streptavidin-PE/Cy7.
221 ***2. Performance of flow cytometry for activated helper T lymphocyte subsets:*** PBMNCs or
222 QQMNCs in 10% FBS/RPMI 1640 medium (1 x10^6 cells/mL) were treated with 25 ng/mL
223 phorbol 12- Myristate 13- acetate (PMA) (Promega) and 1 μg/mL ionomycin (Wakos) for 12 h at
224 37°C. Subsequently, for the last 3 h, cells were incubated with 2 μM Monensin (BioLegend).
225 Thereafter, cells were washed and suspended with 2mM EDTA/PBS buffer and stained for cell
226 surface markers with CD4-PerCP/Cy5.5 and CD25-PE before fixation. Stained cells were washed,
227 resuspended with 2mM EDTA/PBS buffer, and distributed into aliquots for each staining. After
228 treatment with Fixation Buffer (BioLegend) and Permeabilization Buffer (BioLegend), cells
229 underwent intracellular staining with INF-γ-Pacific Blue and IL-4-APC. Alternatively, after