2. 学会発表

なし

H. 知的財産権の出願・登録状況 （予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

幹細胞分離デバイスの事業化に向けた研究

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大規模な設備を必要とせずにシンプルな器具と操作によって細胞治療を実施可能とする幹細胞分離デバイスは，製品化を伴って医療の均てん化に貢献するであろうと期待できる．このようなデバイスが臨床で普及するためには製品としての上市が効果的であり，特にアカデミアやクリニカルサイドで発出した技術シーズについては企業へ着実に移転することが不可欠である．このような技術移転では，国内はもとより海外における特許などの動向と，競合となる可能性がある製品についての動向が，新しい製品を上市するにあたって把握しておくべきところである．本研究分担者らは，特に海外の特許動向と製品化状況を探索し，幹細胞分離デバイスの事業化の推進を図った．

A. 研究目的

ヒトの細胞を安全に処理して様々な治療に応用することを目的とした施設が細胞調整施設 CPC (cell processing center) であるが，その設備投資や維持にかかるコストなどからCPCを具えることができる施設は限られているのが現状である．安価で使い易い細胞調整のためのデバイスは，これら現状の課題を解決へと導くものであり，CPCは国内だけでなく欧米など海外でも用いられていることからも，このようなニーズはグローバルに求められてゆくであろうと推測される．特に，医療機器の市場は国際的には米国が最も大きく，同国における製品化動向を把握しておくことは極めて重要である．さらに，このようなデバイスを製品化して臨床現場での実用に供するためには，日本国内のみならず広く海外をも視野に入れ，グローバルな展開を目指すことがビジネス化へのポテンシャルを育むことにつながる．そのプロセスにおいては，特許の出願動向を抑えておくことと，市場での製品動向を抑えておくことが必要である．

そこで本研究では，デバイスが取り扱う幹細胞を含む単核白血球など海外での特許の出願状況を把握するとともに，類似した製品など市場の動向について明らかにすることを目的とした．

B. 研究方法

本研究費補助金事業において開発を進めている幹細胞分離デバイスと同様に，遠心分離など密度差を利用して単核白血球を分離・収集する特許や活栓で成分の混合・分離を図る海外の特許出願状況について，公開情報などを参考に調査・整理した．

また，当該デバイスに関連すると考えられる製品化に類する情報について，文献・公開情報などとともに本研究の分担研究者・研究代表者が所属する国立循環器病研究センターと秘密保持契約が締結されている海外の専門家からの情報も参考として収集・分析した．

C. 研究結果

遠心分離など密度差を利用して単核白血球を分離・収集する特許や活栓で成分の混合・分離を図る海外の特許について検索した結果，既に企業から出願されているものが複数認められた（表 1）．

また，現在までのところ，遠心分離器を使用して骨髄単核細胞を分離する装置としては Res-Q™ 60 BMC System (ThermoGenesis Corp.), MarrowStim™ Concentration System (Biomet Biologics Inc.) など，市

場に供給されているものが確認できた．前者は分離器本体，シリンジ，チューブなど，後者は分離器本体，ニードル，シリンジなどをセットとしてキット化されたものである．これら二つのデバイスについては，分離に伴う作業時間は15分程度であって，本研究費補助金事業において開発を進めている幹細胞分離デバイスとほぼ同等とみなせるものであった．

表1．海外の特許出願状況

公告番号	出願日	出願人
US5840502A	1994/08/31	Activated Cell Therapy, Inc.
US6652475B1	2000/07/06	Mission Medical, Inc.
US7211191B2	2004/09/30	Thermogenesis Corp.
US7771590B2	2005/08/23	Biomet Manufacturing Corp.
US20080171951A1	2006/03/23	Claude Fell
US8167139B2	2007/07/06	Thermogenesis Corp.
WO2010138895A3	2010/05/28	Ecw Therapeutics, Inc.
WO2011116221A1	2011/03/17	Synergenesis, Inc.
US8163184B2	2011/03/25	Biomet Biologics, Llc

D. 考察

本研究では，遠心分離など密度差を利用して単核白血球を分離・収集する特許や活栓で成分の混合・分離を図る海外の特許についてのみを考慮して検索したが，実際には更に広い技術的概念のものであっても特許の請求項が重複することは考えられる．よって，関連する可能性がある先行技術は相応数あるものと推測される．また，既に企業から製品化された細胞分離デバイスの事例も存するため，本研究で開発の対象としているデバイスを事業化のために製造する際には，他にも現在のところ未探索の企業への権利侵害のリスクを念頭におき，特許調査はより精細に実施することが望まれる．

現在のところ，米国では幹細胞治療用に製品として臨床での使用が承認されているものはないため，Res-Q™ 60 BMC System (ThermoGenesis Corp.)，MarrowStim™ Concentration System (Biomet Biologics Inc.) などは，主に研究用として使用されていると想定される．一方で，将来的には各社とも臨床を見据えているものと考えられるため，それに向けた種々のハードルを想定しておく必要がある．本研究費補助金事業で取り扱っているデバイスについても，例えば手作業に伴うプロセスが多いため，使いやすさやそれに相応して作業に付随するリスクマネジメントも考慮しておかなければならない．さらに，米国における保険償還制度は複雑であるため，これらについても充分な配慮が必要である．

E. 結論

この幹細胞分離デバイスの製品化にあたっては，海外の特許出願状況を踏まえて，より詳細に権利化関係を確認するとともに，既に米国において製品化されているデバイスなどの現況を考慮した製品化が望まれる．

F. 健康危険情報

なし。

G. 研究発表

1. 論文発表

なし

（発表誌名巻号・頁・発行年等も記入）

2. 学会発表

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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

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tsuji M, Taguchi A, Ohshima M, Kasahara Y, Sato Y, Tsuda H, Otani K, Yamahara K, Ihara M, Harada-Shiba M, Ikeda T, Matsuyama T.	Effects of intravenous administration of umbilical cord blood CD34(+) cells in a mouse model of neonatal stroke.	Neuroscience.	263	148-58	2014
Kaneko M, Shintani Y, Narita T, Ikebe C, Tano N, Yamahara K, Fukushima S, Coppen SR, Suzuki K.	Extracellular high mobility group box 1 plays a role in the effect of bone marrow mononuclear cell transplantation for heart failure.	PLoS One.	8(10)	e76908	2013
Kasahara Y, Ihara M, Nakagomi T, Momota Y, Stern DM, Matsuyama T, Taguchi A.	A highly reproducible model of cerebral ischemia/reperfusion with extended survival in CB-17 mice.	Neurosci Res	76(3)	163-168	2013
Kasahara Y, Ihara M, Taguchi A.	Experimental evidence and early translational steps using bone marrow derived stem cells after human stroke.	Front NeurolNeurosci.	32	69-75	2013
Tsuji M, Ohshima M, Taguchi A, Kasahara Y, Ikeda T, Matsuyama T.	A novel reproducible model of neonatal stroke in mice: comparison with a hypoxia-ischemia model.	Experimental Neurology	247(9)	218-225	2013
Ihara M, Taguchi A, Maki T, Washida K, Tomimoto H	A Mouse Model of Chronic Cerebral Hypoperfusion Characterizing Features of Vascular Cognitive Impairment	Methods in Molecular Biology	1135	95-102	2014

Taguchi A, Kasahara Y, Matsuyama T.	Letter by Taguchi et al Regarding Article, "Granulocyte Colony-Stimulating Factor in Patients With Acute Ischemic Stroke: Results of the AX200 for Ischemic S	Stroke	45(1)	e8	2014

Ⅳ．研究成果の刊行物・別刷

Neuroscience 263 (2014) 148–158

EFFECTS OF INTRAVENOUS ADMINISTRATION OF UMBILICAL CORD BLOOD CD34$^+$ CELLS IN A MOUSE MODEL OF NEONATAL STROKE

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Abstract—Neonatal stroke occurs in approximately 1/4000 live births and results in life-long neurological impairments: e.g., cerebral palsy. Currently, there is no evidence-based specific treatment for neonates with stroke. Several studies have reported the benefits of umbilical cord blood (UCB) cell treatment in rodent models of neonatal brain injury. However, all of the studies examined the effects of administering either the UCB mononuclear cell fraction or UCB-derived mesenchymal stem cells in neonatal rat models. The objective of this study was to examine the effects of human UCB CD34$^+$ cells (hematopoietic stem cell/endothelial progenitor cells) in a mouse model of neonatal stroke, which we recently developed. On postnatal day 12, immunocompromized (SCID) mice underwent permanent occlusion of the left middle cerebral artery (MCAO). Forty-eight hours after MCAO, human UCB CD34$^+$ cells (1×10^5 cells) were injected intravenously into the mice. The area in which cerebral blood flow (CBF) was maintained was temporarily larger in the cell-treated group than in the phosphate-buffered saline (PBS)-treated group at 24 h after treatment. With cell treatment, the percent loss of ipsilateral hemispheric volume was significantly ameliorated (21.5 ± 1.9%) compared with the PBS group (25.6 ± 5.1%) when assessed at 7 weeks after MCAO. The cell-treated group did not exhibit significant differences from the PBS group in either rotarod (238 ± 46 s in the sham-surgery group, 175 ± 49 s in the PBS group, 203 ± 54 s in the cell-treated group) or open-field tests. The intravenous administration of human UCB CD34$^+$ cells modestly reduced histological ischemic brain damage after neonatal stroke in mice, with a transient augmentation of CBF in the peri-infarct area.

Key words: neonatal stroke, neonatal encephalopathy, brain injury, umbilical cord blood, CD34$^+$ cell, cell-based therapy.

INTRODUCTION

Perinatal/neonatal stroke occurs in 1/2800 to 1/5000 live births and results in life-long neurological impairments: e.g., cerebral palsy, mental retardation, and epilepsy (Nelson and Lynch, 2004; Chabrier et al., 2011). The current treatment for infants with stroke is predominantly supportive, as there is no evidence-based specific treatment available (Roach et al., 2008; Chabrier et al., 2011). The onset of neonatal stroke is antenatal in some cases and is unknown in others. Hence, treatments that have a narrow therapeutic window, such as tissue plasminogen activator, are not feasible for perinatal/neonatal stroke. Cell-based therapy has attracted much attention as a novel treatment for a number of neurological diseases, including neonatal encephalopathy (NE) (Bennet et al., 2012), which encompasses stroke and hypoxic–ischemic encephalopathy (HIE) (Dammann et al., 2011). Apart from its possible regenerative properties, its wide therapeutic time window, up to days after the insult (Yasuhara et al., 2010; Donega et al., 2013), is one of the most attractive features of this therapy. This is astonishing considering the fact that almost all candidate drugs examined in animal models exhibit neuroprotection only when administered before or within a few hours after the insult.

Human umbilical cord blood (hUCB) contains many stem cell types, i.e., hematopoietic stem cells, endothelial progenitor cells, and mesenchymal stem cells (MSCs) (Ingram et al., 2004; Lee et al., 2004). CD34 is widely used as a marker of hematopoietic stem

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Abbreviations: ANOVA, analysis of variance; CBF, cerebral blood flow; GDNF, glial cell line-derived neurotrophic factor; HIE, hypoxic–ischemic encephalopathy; hUCB, human umbilical cord blood; HuNu, anti-human nuclei antibody; MCAO, middle cerebral artery occlusion; MNC, mononuclear cell; MSC, mesenchymal stem cell; NE, neonatal encephalopathy; PBS, phosphate-buffered saline; ROI, region of interest; SCID, severe combined immunodeficiency; UCBC, umbilical cord blood cell; VEGF, vascular endothelial growth factor.

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and endothelial progenitor cells (Rafii and Lyden, 2003). hUCB contains 10-times more $CD34^+$ mononuclear cells (MNCs) than does adult peripheral blood (Murohara et al., 2000). The proportion of $CD34^+$ cells in hUCB ranges from 0.3% (Sun et al., 2010) to 2.4% (de Paula et al., 2012), which is comparable to bone marrow (Cox et al., 2011). Because of this feature, hUCB has been used for hematopoietic stem cell transplantation in patients with hematological diseases and inherited metabolic disorders/neurodegenerative diseases, i.e., Hurler's syndrome, adrenoleukodystrophy, and Krabbe disease (Prasad et al., 2008). Apart from their hematopoietic properties, hUCB cells (hUCBCs) have myriad effects. Human $CD34^+$ cells secrete numerous cytokines, chemokines, and growth factors, including vascular endothelial growth factor (VEGF) (Majka et al., 2001). $CD34^+$ cells are less prevalent in the neonatal peripheral blood immediately after birth than in UCB and tend to decrease within the first 48 h after delivery (Kim et al., 2007). The basic concept underlying the intravenous administration of autologous UCBCs for NE is to replenish the reduced stem cells in systemic circulation, which may contribute to neuroprotection and/or enhance cerebral plasticity.

There are several dozen reports in the literature that have examined the effects of cell therapies in animal models of NE. Several cell types have been investigated (Chicha et al., 2014), including neural stem cells (Comi et al., 2008; Sato et al., 2008), MSCs (van Velthoven et al., 2010), multipotent adult progenitor cells (Yasuhara et al., 2006), and dental pulp-derived stem cells (Yamagata et al., 2013). Several cell sources have been investigated as well, i.e., rodent embryo (Comi et al., 2008; Sato et al., 2008), rodent or human bone marrow (Yasuhara et al., 2006; van Velthoven et al., 2010), and hUCB (Meier et al., 2006). Furthermore, several administration routes have also been investigated, i.e., intracerebral (Xia et al., 2010), intraperitoneal (Meier et al., 2006), and intranasal delivery (van Velthoven et al., 2013). Many studies have shown the benefits of cell therapy. Among these different cell therapies, the intravenous administration of autologous UCB treatment may have the lowest risk for clinical use in NE (Bennet et al., 2012). A few clinical trials using an intravenous administration of autologous UCB for NE are currently in progress (http://www.clinicaltrials.gov/, NCT00593242, NCT01506258, NCT01649648). However, little is known about the optimal protocol and the mechanisms of action of UCBC treatment. To date, there have been 15 reports in the literature examining the effects of UCBC treatment in rodent models of NE. These studies used either whole of the MNC fraction (Meier et al., 2006; de Paula et al., 2009, 2012; Pimentel-Coelho et al., 2010; Rosenkranz et al., 2010, 2012, 2013; Yasuhara et al., 2010; Geißler et al., 2011; Bae et al., 2012; Dalous et al., 2012; Wasielewski et al., 2012; Wang et al., 2013) or MSCs derived from hUCB (Xia et al., 2010; Kim et al., 2012). The effects of other cell populations in UCB for NE remain unknown. In this study, we focused on the $CD34^+$ cell fraction of hUCB. We have previously reported the beneficial effects of the systemic administration of hUCB-$CD34^+$ cells in an adult mouse model of stroke (Taguchi et al., 2004a). The objective of this study was to examine the effects of the intravenous administration of hUCB-$CD34^+$ cells on post-stroke recovery in a mouse model of neonatal stroke.

EXPERIMENTAL PROCEDURES

Animals and surgery

All experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Experimental Animal Care and Use Committee of the National Cerebral and Cardiovascular Center.

Ninety-one postnatal day 12 (P12) male and female mouse pups with severe combined immunodeficiency (SCID) (CB-17/Icr-scid/scidJcl; CLEA Japan Inc., Tokyo, Japan) were prepared for the experiments. P8–12 mice are considered comparable to human full-term (P0) neonates with regard to brain maturation (Hagberg et al., 2002); some authors argue that P12 mice are more representative of human full-term neonates (Charriaut-Marlangue et al., 2013). The novel model of neonatal stroke that we recently reported (Tsuji et al., 2013) uses CB-17 (CB-17/Icr-+/+Jcl) mouse pups, which are immunocompetent. As human cells were administered to mice, in the present study, we used immunocompromized animals to minimize immunological reactions due to xenotransplantation. The SCID mice used in the present study were derived from the same strain with the same genetic background as CB-17 mice. All efforts were made to minimize the number of animals used and their suffering.

Permanent middle cerebral artery occlusion (MCAO) was produced according to previously reported methods (Tsuji et al., 2013). Under isoflurane anesthesia (4.0% for induction and 1.5–2.0% for maintenance), a hole was made in the left temporal bone. The left middle cerebral artery (MCA) was electrocauterized and disconnected distal to crossing the olfactory tract. Thirteen pups underwent open-skull surgery without MCA electrocoagulation and served as sham-surgery controls. Five pups were excluded from the experimental analysis owing to bleeding during surgery. All analyses were performed by investigators who were blinded to the experimental group.

Cerebral blood flow (CBF) measurements

The cortical surface CBF was measured by a laser speckle flowmetry imaging system (Omegazone, Omegawave Inc., Tokyo, Japan) 24 h after MCAO, 24 h after treatment (i.e., 72 h after MCAO), and 1 and 7 weeks after treatment, as described previously, with one minor modification (Ohshima et al., 2012). We measured the CBF in two regions of interest (ROIs) through the intact skull with an open-scalp: the Core (the ischemic core region of the MCA territory) and the MCA region (the broader region covering most of the MCA territory, including the Core). The same grid was

used to set the two matching regions on the contralateral side. To analyze the influence of the treatment on the CBF in the peri-infarct regions, we measured the area in which the CBF was not attenuated, which we defined as the "well-perfused area." By observing the contralateral CBF visually, the area in which the CBF appeared equal to the corresponding contralateral side was manually demarcated using NIH Image software (ImageJ, 1.43r, NIH, Bethesda, MD, USA). For analytical accuracy of ROIs between animals and serial imaging, we set the ROI based on a line drawn from bregma to lambda, rather than basing it on the actual MCA-perfused territory. The percent of the well-perfused area was calculated by the ratio of the well-perfused area out of an area of a square grid, which primarily covered the MCA-perfused territory (Fig. 1A). All pups exhibited CBF reduction in the MCA territory after the MCAO. However, three mice exhibiting a mild CBF reduction, which was defined as a CBF ratio (ipsilateral/contralateral MCA region) > 0.80 at 24 h after MCAO, were removed from the study.

Administration of hUCB-CD34$^+$ cells after stroke

Seventy mice with MCAO were randomly assigned to one of two groups that received hUCB-CD34$^+$ cells (UCBC group) or phosphate-buffered saline (PBS group). Human UCB-CD34$^+$ cells were purchased from Lonza Inc. (Walkersville, MD, USA). The purity was >95%, and the viability of the cells was >95%. Forty-eight hours after the stroke, a skin incision was made under isoflurane anesthesia, and the left femoral vein was exposed. hUCB-CD34$^+$ cells (1×10^5 cells), or the same volume (40 μl) of PBS, were carefully infused into the femoral vein over 3 min using a 35G needle. We selected the dose of 1×10^5 hUCB-CD34$^+$ cells in the present study as the dose of 5×10^5 cells was beneficial in our previous study in an adult mouse model (Taguchi et al., 2004a). We selected the timing of cell administration at 48 h after the insult on the basis of our previous studies (Taguchi et al., 2004a; Uemura et al., 2012).

Behavioral tests

Rotarod and open-field tests were performed as described previously (Tsuji et al., 2013). Sensorimotor skills were evaluated 9 days and 7 weeks after the insult in the rotarod test. The rotarod accelerated from 4 to 40 rpm over 5 min (Muromachi Kikai Co., Ltd., Tokyo, Japan). The time until the mouse fell off the rotating drum was recorded for five consecutive sessions, and the average time spent on the drum was used for statistical comparison.

Locomotor and exploratory behaviors were evaluated 5 and 7 weeks after the insult using the open-field test.

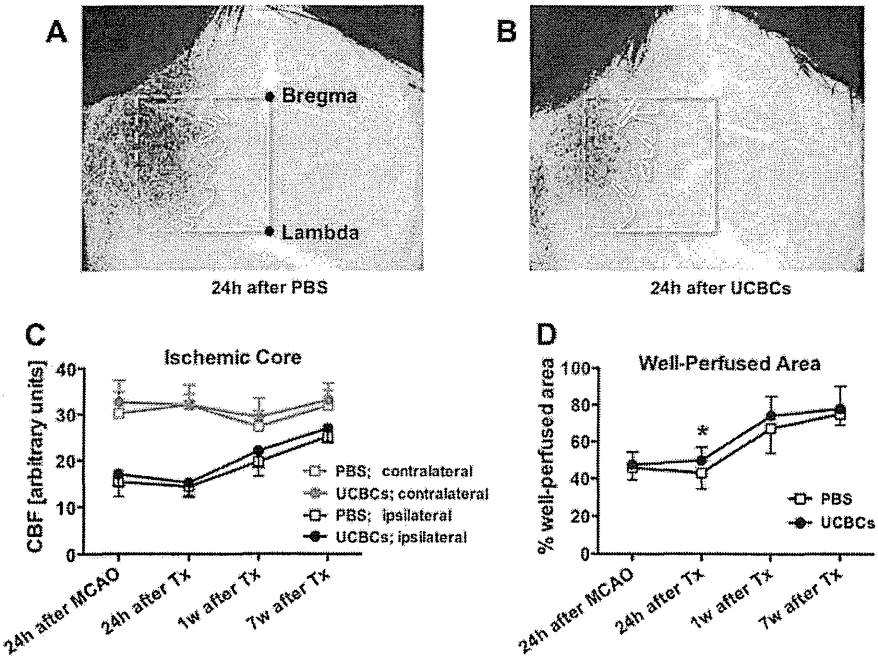

Fig. 1. Cerebral blood flow. (A, B) Representative images of the cerebral blood flow (CBF) 24 h after treatment (i.e., 72 h after the middle cerebral artery occlusion (MCAO)). The CBF was decreased in the MCA region on the ipsilateral side, which is indicated by the bluish color, after the MCAO insult. (C) There were no significant differences between the PBS-treated and human umbilical cord blood CD34$^+$ cell (UCBC)-treated groups with regard to CBF in the ischemic core. (D) However, with regard to the area of CBF reduction, there was a significant difference in the ratio of the area in which CBF was maintained (the area delineated by white lines in A, B) out of an area of a square (the square delineated by white dotted lines). The square was set based on a line drawn from bregma to lambda. We defined the ratio as the "% well-perfused area." Differences between groups were tested using a two-way repeated measures ANOVA. A post hoc test showed that the% well-perfused area was significantly larger in the UCBC group compared with the PBS group at 24 h after the treatment. *$P < 0.05$ (PBS-treated group n = 10–12 at each time point, except at 7 weeks after treatment (n = 5); UCBC-treated group n = 11–13 at each time point, except at 7 weeks after treatment (n = 4)). Tx; treatment.

Animals were allowed to search freely in a box (30 × 30 cm) for 30 min in a light environment and for the subsequent 30 min in a dark environment (Taiyo Electric Co., Ltd., Osaka, Japan). Infrared beams were mounted at specific intervals on the X-, Y-, and Z-banks of the open-field. The total number of beam crossings by the animal was counted and scored as "locomotion" for the horizontal movement and as "rearing" for the vertical movement.

Histological analyses

A morphological evaluation of the brain injury was performed as described previously (Tsuji et al., 2012, 2013). Animals were perfusion-fixed intracardially with 4% paraformaldehyde at 12 days or 7 weeks after the insult. The brain was removed and sectioned coronally in 1-mm-thick slices. The areas of the ipsilateral and contralateral hemispheres in each brain section were measured using ImageJ. The hemispheric volume was estimated by integrating the hemispheric areas. The % stroke volume was calculated as follows: ((contralateral volume – viable ipsilateral volume)/contralateral volume) × 100%. This model causes a pure cortical stroke with mild secondary injury in the thalamus and the corpus callosum, and the hemispheric volume effectively represents the histological injury (Tsuji et al., 2013).

Immunohistochemistry

Coronally sectioned brain slices were covered in tissue freezing medium (O.C.T. Compound, Sakura Finetek USA Inc., Torrance, CA, USA). Coronal sections (10 μm) were prepared using a cryostat (Leica Biosystems Inc., Wetzler, Germany). Sections were subjected to immunohistochemistry with anti-human nuclei antibody (HuNu) (Merck Millipore, Billerica, MA, USA, 1:30) and mouse-specific antibody to CD31 antigen expressed by endothelial cells (BD Biosciences, San Jose, CA, USA, 1:100); the secondary antibodies included anti-mouse Envision+ system-HRP Labeled Polymer and biotinylated anti-rat immunoglobulin (Dako Cytomation, Glostrup, Denmark), respectively. Nuclei were stained with hematoxylin after the HuNu staining. When analyzing the CD31-positive blood vessel, we defined the "peri-infarct area" as the external (non-ischemic) regions within 200 μm of the border of the post-stroke area, as described previously (Nakano-Doi et al., 2010). The lengths and diameters of blood vessels were measured using ImageJ.

Statistics

The mortality rate of the animals was analyzed using the Fisher's exact test. Differences in body weight were assessed using a one-way analysis of variance (ANOVA), followed by the Bonferroni test. The percent volume loss was assessed using a Student *t*-test. CBF, rotarod and open-field test outcomes were assessed using a two-way repeated measures ANOVA, followed by the Bonferroni test. Parameters in blood vessels were assessed using a two-way ANOVA, followed by the Bonferroni test. Differences were considered significant at $P < 0.05$. The results are expressed as the mean ± standard deviation (SD), unless otherwise noted.

RESULTS

Mortality and body weight

Mortality rates did not differ between the PBS and UCBC groups: 1 out of 35 mice in the PBS group and 2 out of 35 mice in the UCBC group. Body weights at the time of surgery (P12), and at 7 days (P21) and 7 weeks after the treatment (P63), did not differ among the three groups, including the sham-surgery control group (Table 1).

CBF

With regard to the degree of CBF reduction, there were no significant differences between the PBS and UCBC groups, either in the ischemic core (Fig. 1A–C) or in the MCA region (data not shown). However, with regard to the area of CBF reduction, a two-way repeated measures ANOVA showed that there was a significant group difference in the % well-perfused area: i.e., the areas where CBF was maintained were different (Fig. 1A, B, and D). A post hoc test showed that the % well-perfused area was significantly larger in the UCBC group compared with the PBS group at 24 h after the treatment, but not at the other time points measured.

Morphological brain injury

All pups subjected to MCAO exhibited cortical infarct and consistent hemispheric volume loss when assessed either 12 days or 7 weeks after the insult (Fig. 2A, B). Twelve days after MCAO, the mean % stroke volume did not differ between the PBS and UCBC groups, 20.7 ± 3.3% and 21.3 ± 2.4%, respectively (Fig. 2A). Seven weeks after MCAO, the mean% stroke volume in the UCBC group (21.5 ± 1.9%) was significantly ameliorated compared with the PBS group (25.6 ± 5.1%) (Fig. 2B–D). No sex differences in % stroke volume were observed in either of the groups (male 26.2 ± 3.9 vs. female 24.8 ± 6.5% in the PBS group, male 21.4 ± 1.2 vs. female 21.5 ± 2.3% in the UCBC group, at 7 weeks after MCAO).

Table 1. Body weights

	P12	P21	P63
Sham-surgery	6.9 ± 0.6	8.6 ± 0.5	20.0 ± 1.7
MCAO + PBS	6.7 ± 0.7	8.0 ± 0.7	18.2 ± 3.6
MCAO + UCBCs	6.7 ± 0.7	7.9 ± 0.7	18.2 ± 3.1

Body weights (g) (mean ± SD) at postnatal day 12 (P12, the day of surgery), P21 (7 days after the treatment), and P63 (7 weeks after the treatment) were not different between groups. MCAO, middle cerebral artery occlusion; PBS, phosphate-buffered saline; UCBCs, human umbilical cord blood $CD34^+$ cells.

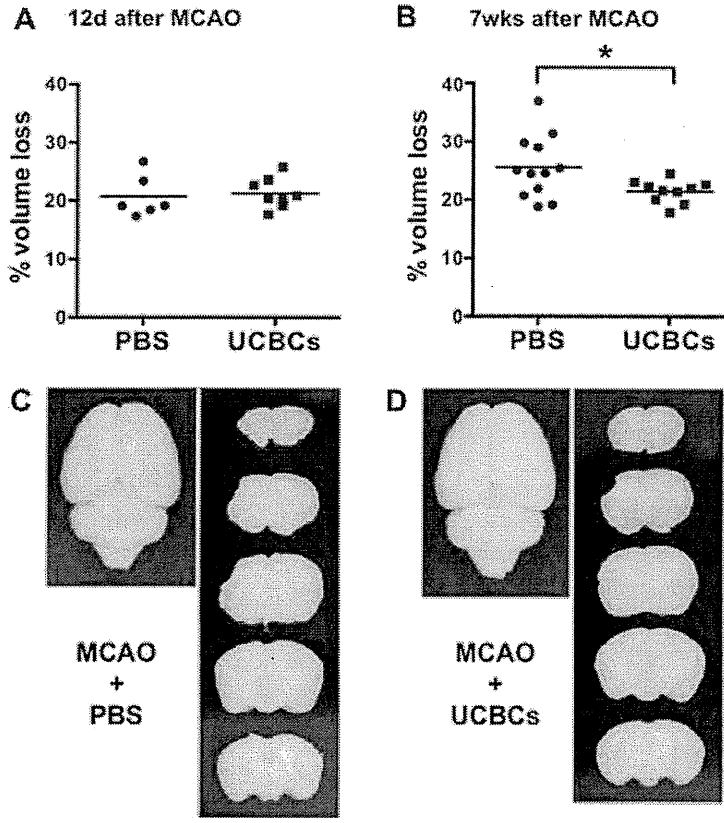

Fig. 2. Morphological brain injury. (A) Twelve days after middle cerebral artery occlusion (MCAO), the mean % stroke volume did not differ between the PBS-treated and human umbilical cord blood CD34$^+$ cell (UCBC)-treated groups. (B) Seven weeks after MCAO, the mean % stroke volume in the UCBC group was significantly ameliorated compared with the PBS group. (C) Representative images of brains 7 weeks after MCAO. *P < 0.05.

Localization of hUCB-CD34$^+$ cells

Very few donor cells stained with human antinuclear antibody were identified in the brain (a few stained cells per the entire coronal section) 24 h after the intravenous injection (n = 5), most of which were located around blood vessels (Fig. 3). The stained cells were hardly identified 10 days after the injection (n = 5) (data not shown).

Blood vessels

We analyzed blood vessels in the region bordering the cortical infarct at 7 weeks after MCAO (Fig. 4A). There were no significant differences in either the number of vessels or the total length of vessels between the PBS and UCBC groups (Fig. 4B–E). However, the mean diameter of vessels was significantly larger in the UCBC-treated mice compared with the PBS-treated mice (Fig. 4B, C, and F). These large vessels in the UCBC-treated mice were observed only in the region bordering the cortical infarct and not in the other regions of the ipsilateral side or in the contralateral side.

Rotarod performance

Sensorimotor performance, as assessed by a rotarod treadmill at 9 days and 6 weeks after the insult, was analyzed by a two-way repeated measures ANOVA. There were significant group, but not time, differences. Compared with the performance in the sham-surgery group (238 ± 46 s, at 6 weeks), the performance was significantly impaired in mice with MCAO + PBS (175 ± 49 s), while no significant impairment was

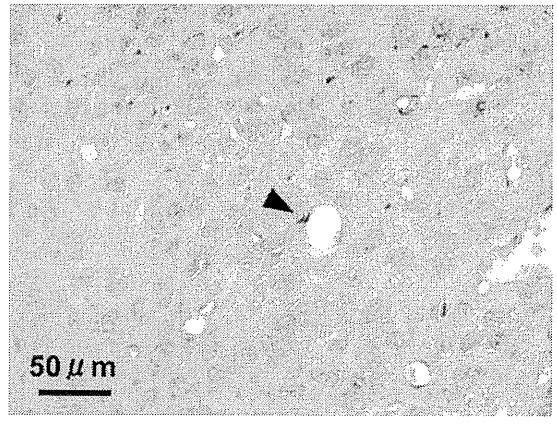

Fig. 3. Administered human umbilical cord blood CD34$^+$ cells. Very few donor cells, i.e., human umbilical cord blood CD34$^+$ cells stained with human antinuclear antibody, were identified in the brain 24 h after the intravenous injection. Donor cells that were identified were localized around blood vessels (arrowhead).

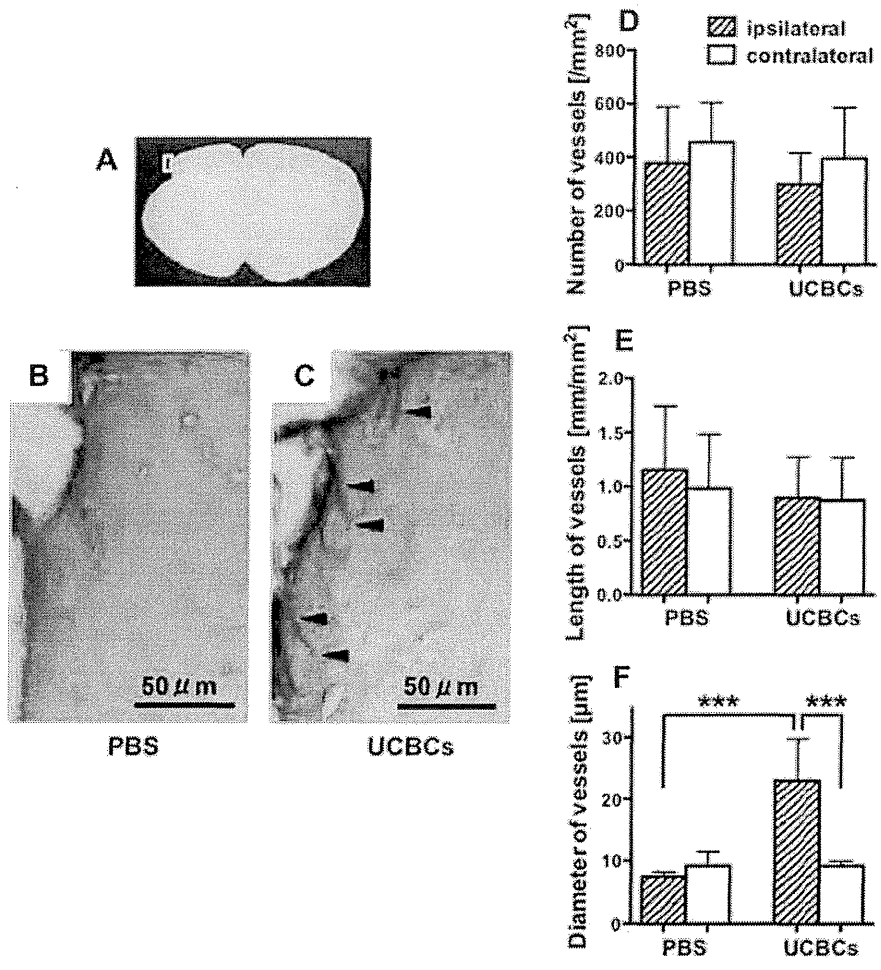

Fig. 4. Blood vessels. (A) Blood vessels in the peri-stroke region (small white square) were analyzed 7 weeks after MCAO. (B, C) Representative images of vessels stained with CD31 antibody (a marker of endothelial cells) in the peri-stroke regions. (D, E) There were no significant differences in either the number of vessels or the total length of vessels between the PBS-treated and human umbilical cord blood $CD34^+$ cell (UCBC)-treated groups. (F) However, the mean diameter of vessels in the peri-stroke region was significantly larger in the UCBC-treated mice compared with the PBS-treated mice. These large vessels in the UCBC-treated mice were only observed in the peri-stroke regions of the cortical infarct. ***$P < 0.001$.

observed in mice with MCAO + UCBCs (203 ± 54 s) (Fig. 5). However, there was no significant difference between the MCAO + UCBC group and the MCAO + PBS group. No sex differences in the performance were observed in either of the groups (male 188 ± 32 vs. female 155 ± 61 s in the PBS group, male 185 ± 43 vs. female 220 ± 57 s in the UCBC group, at 6 weeks).

Open-field activities

We initially analyzed the overall activities during 60-min sessions at 5 and 7 weeks after the insult using a two-way repeated measures ANOVA (data not shown). We then analyzed the temporal changes throughout a 60-min session in 5-min increments using a two-way repeated measures ANOVA (Fig. 6A, B). Compared with sham-surgery mice, mice with MCAO did not exhibit significant behavioral alterations in either locomotion or rearing at either time point; one exception

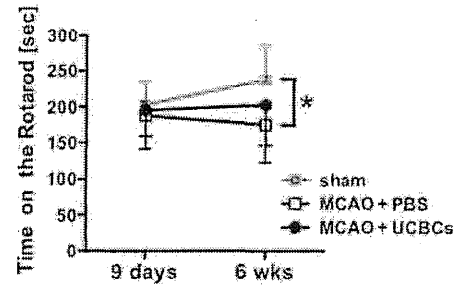

Fig. 5. Rotarod test. Repeated-measures two-way ANOVA showed significant group differences in sensorimotor performance. Performance was significantly impaired in mice with MCAO treated with PBS compared with sham-surgery mice. In contrast, performance was not impaired in mice with MCAO treated with human umbilical cord blood $CD34^+$ cells (UCBCs) compared with sham-surgery mice. However, there was no significant difference between the MCAO + UCBCs group and the MCAO + PBS group. *$P < 0.05$. (sham $n = 10$; MCAO + PBS $n = 12$; MCAO + UCBCs $n = 16$, 9 days after the insult, i.e., 1 week after the treatment. $n = 10$ in each group, 6 weeks after the insult).

was mice with MCAO treated with either PBS or UCBCs that exhibited significantly less prominent responses to the dark environment with respect to locomotion at both 5 and 7 weeks after the insult. Overall, UCBC treatment did not significantly alter the behaviors in the mice with neonatal stroke, as assessed with the open-field test.

DISCUSSION

Only two cell types, the whole MNC fraction and MSCs, in hUCB have been investigated as cell therapies in animal models of NE to date. In the present study, the intravenous administration of hUCB-CD34$^+$ cells, which are mostly hematopoietic stem cells and endothelial progenitor cells, modestly ameliorated histological brain injury after neonatal stroke in mice. The effects were, at least in part, due to the improved CBF in the ischemic penumbra during the subacute phase of stroke, which may be associated with the increased mean diameter of blood vessels observed in the peri-infarct area during the chronic phase.

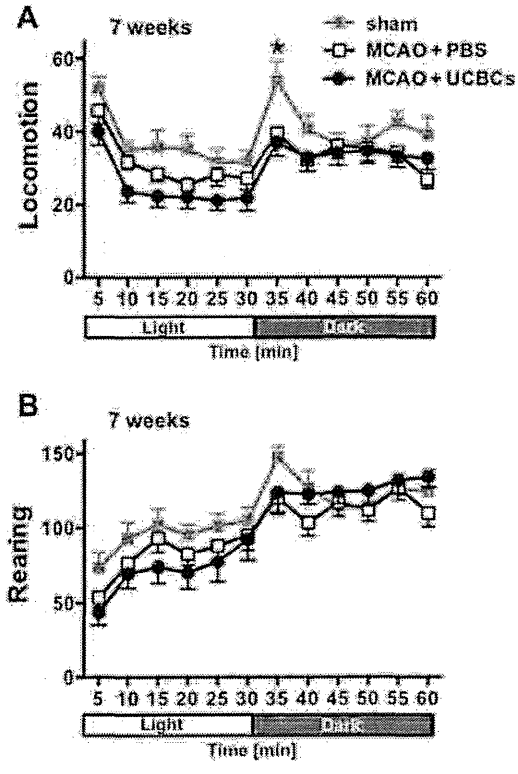

Fig. 6. Open-field test. Temporal changes in activities were analyzed in 5-min increments by a repeated-measures two-way ANOVA. (A) With respect to locomotion (horizontal movement), the mice in all three groups became hyperactive in response to the dark environment when assessed at 7 weeks after the insult. However, the response was significantly weaker in the mice with MCAO treated with either PBS or human umbilical cord blood CD34$^+$ cells (UCBCs) compared with the sham-surgery mice. (B) With respect to rearing (vertical movement), there were no significant group differences at 7 weeks after the insult. UCBC treatment did not significantly alter behavior in the mice with neonatal stroke. $^*P < 0.05$ compared with the MCAO + PBS and MCAO + UCBCs groups. Mean ± SEM. (sham $n = 13$; MCAO + PBS $n = 17$; MCAO + UCBCs $n = 13$).

The purpose of the present study was to examine the potential of the CD34$^+$ cell fraction in human UCB; SCID mice, which are deficient in functional B and T lymphocytes because of a single gene mutation, were transfused with hUCB-CD34$^+$ cells. Hence, the present study used xenotransplantation; thus, caution should be exercised when translating the data obtained in the present study into the clinic. We used immunocompromized mice to minimize the undesirable immunological and inflammatory reactions caused by xenotransplantation as these reactions are not induced in autologous transfusion, which is the expected paradigm in the clinical application. To date, no study has examined the effects of allogeneic transplantation with UCBCs in rodent models of brain injury. One reason is because the rodent UCB is different from the human UCB; unlike in humans, mouse CD34$^+$ cells are not hematopoietic cells (Osawa et al., 1996). In addition, collecting rodent UCBCs is technically difficult.

We chose the timing of the cell transfusion to be 48 h after the brain injury for numerous reasons. The optimal time window of hUCBC therapies in animal models of NE has not been examined. UCBCs were administered 24 h after the brain injury in most NE studies (Table 2). Recent data on UCBCs and other types of stem cells in adult rodent models suggest that a later timing of transfusion, i.e., 48 or 72 h after the insult, is more beneficial (Newcomb et al., 2006; Rosenblum et al., 2012; Uemura et al., 2012).

One of the key features of this study is that it was performed using a highly reproducible model of NE, in which the locations of the infarct and peri-infarct areas were easily distinguishable and consistent between animals. During the first hours after brain injury, a detrimental biological cascade begins, and cells are destined to be damaged. We assumed that the neuroprotective effects would be limited when animals were treated 48 h after the insult, even if the cell treatment was potent. In fact, the hUCB-CD34$^+$ cell treatment achieved a modest, but statistically significant, amelioration of the brain injury. Similarly, the cell treatment exhibited a statistically significant augmentation of the CBF in the peri-infarct region, but not in other regions. These results suggest that the cell therapy exerts neuroprotective effects only in the peri-infarct area and not in the ischemic core. Moreover, these results suggest that it is crucial to examine the effects of treatment in a region-specific manner in a highly reproducible model.

The amelioration rate of cerebral tissue loss by the UCBC therapy was relatively small, and none of the UCBC-treated mice exhibited outstanding improvement; there may be a limit of the improvement in % stroke volume, e.g., 17.5% in this study. This implies that the ischemic core cannot be rescued or restored by cell treatment. The result is well conceivable as the model used in the present study is a permanent MCAO model in mice with a CB-17 strain background, which has little anastomoses between MCA and other cerebral arteries (Taguchi et al., 2010). Even after removing the pup with

Table 2. *Reported studies with umbilical cord blood cells in rodent models of neonatal brain injury*

Research group		Model	Cell type	Cell dose	Timing	Delivery route	Follow-up	Improvement		Author and reference
								Morphology	Behavior	
A	1	P7 rat, HI	MNC	1×10^7	24 h	i.p.	2 weeks	NA	+	Meier et al. (2006)
	2	P7 rat, HI	MNC	1×10^7	24 h	i.p.	2 weeks	NA	NA	Rosenkranz et al. (2010)
	3	P7 rat, HI	MNC	1×10^7	24 h	i.p.	6 weeks	NA	+	Geißler et al. (2011)
	4	P7 rat, HI	MNC	1×10^7	24 h	i.p., intrathecal	6 weeks	+	+	Wasielewski et al. (2012)
	5	P7 rat, HI	MNC	1×10^7	24 h	i.p.	2 weeks	+	NA	Rosenkranz et al. (2012)
	6	P7 rat, HI	MNC	1×10^7	24 h	i.p.	2 weeks	NA	NA	Rosenkranz et al. (2013)
B	7	P7 rat, HI	MNC	1×10^7	24 h	i.v.	3 weeks	–	–	de Paula et al. (2009)
	8	P7 rat, HI	MNC	1×10^6, 10^7, 10^8	24 h	i.v.	8 weeks	+	+	de Paula et al. (2012)
C	9	P7 rat, HI	MNC	1.5×10^4	7 days	i.v.	3 weeks	+	+	Yasuhara et al. (2010)
D	10	P7 rat, HI	MNC	2×10^6	3 h	i.p.	7 days	+	+	Pimentel-Coelho et al. (2010)
E	11	P7 rat, HI	MNC	$1 \times 1 \times 10^7$	24 h	i.v.	10 weeks	+	+	Bae et al. (2012)
F	12	P5 rat, excitotoxicity	MNC	1, 3×10^6, 1×10^7	0, 24 h	i.p., i.v.	5 days	–	NA	Dalous et al. (2012)
G	13	P7 rat, HI	MNC	3×10^6	24 h	Intraventricular	2 weeks	+	NA	Wang et al. (2013)
H	14	P7 rat, HI	MSC	5×10^4	3 days	Intraparenchymal	4 weeks	+	+	Xia et al. (2010)
I	15	P10 rat, MCAO	MSC	1×10^5	6 h	Intraventricular	4 weeks	+	+	Kim et al. (2012)
Present study		P12 mouse, MCAO	$CD34^+$ cell	1×10^5	48 h	i.v.	7 weeks	+	–	

P, postnatal day; HI, hypoxia–ischemia; MCAO, middle cerebral artery occlusion; MNC, mononuclear cell; MSC, mesenchymal stem cell; i.p., intraperitoneal; i.v., intravenous; NA, not assessed.

the most severe brain damage in the PBS group, the ameliorating effects of the cell therapy were statistically significant, confirming the fact that the treatment effect is modest but significant.

$CD34^+$ cells as a neuroprotective treatment

The intravenous administration of hUCB-$CD34^+$, but not $CD34^-$ cells, ameliorates damage in adult mice with permanent MCAO (Taguchi et al., 2004a) and in a rat model of spinal cord injury (Kao et al., 2008). Boltze et al. (2012) compared the effects of the intravenous administration of hUCB-MNCs (which contains a variety of cells, including $CD34^+$ cells), $CD34^+$ cells, and $CD34^-$ cells in adult rats with permanent MCAO. The MNCs provided the most prominent neuroprotective effects, with $CD34^+$ cells appearing to be particularly involved in the protective action of MNCs. A study in a rat model of myocardial infarction showed that $CD34^+$ cell treatment elicited the greatest attenuation of the damage with the high-dose MNC group (which contained the same absolute $CD34^+$ cell dose as the $CD34^+$ cell group) exhibiting a moderate attenuation (Kawamoto et al., 2006). The beneficial effects of the intravenous administration of hUCB-$CD34^+$ cells have also been reported in adult rat models of transient MCAO (Chen et al., 2001; Ou et al., 2010), heatstroke (Chen et al., 2007) and traumatic brain injury (Chen et al., 2013). Among the variety of cell types in hUCB, $CD34^+$ cells play a crucial, if not absolute, role in the neuroprotection afforded by hUCBC treatment. In our clinical studies of adult patients with cerebral ischemic events, the number of circulating $CD34^+$ cells was inversely correlated with cerebral infarction and positively correlated with CBF (Taguchi et al., 2004b, 2009). These results suggest that circulating $CD34^+$ cells have a role in the maintenance of the cerebral circulation in ischemic stress.

The administration of whole nucleated cells or the MNC fraction isolated by a density gradient separation is a simple approach for clinical application. Of note, the "MNC fraction" does not necessarily indicate that the cells in the fraction are exclusively mononucleate cells. The hUCB-MNC fraction isolated by gradient separation using Ficoll-paque (GE Healthcare UK Ltd., Amersham Place, England) contains 1–20% granulocytes among the recovered cells. As much as 46% of the MNC fraction is composed of granulocytes after separation from child bone marrow (Cox et al., 2011). As some studies have shown that granulocytes are detrimental for NE (Palmer et al., 2004), the administration of only the beneficial cell fraction may be important to improve the clinical outcome.

Augmentation of CBF and modulation of blood vessels by UCBC treatment

The present study shows that augmentation of CBF is one of the beneficial effects of hUCB-$CD34^+$ cell treatment. Our previous study demonstrated that the degree of CBF reduction in the subacute phase following neonatal HI (24 h after the insult) correlated strongly with the

subsequent morphological development of brain damage in mice (Ohshima et al., 2012). This implies that augmentation of the CBF during this phase may lead to improvements in brain damage during the chronic phase. We have previously reported that the intravenous administration of hUCB-CD34$^+$ cells enhanced CBF just outside of the penumbra in an adult mouse model of permanent MCAO (Taguchi et al., 2004a). We have reported that the intravenous administration of murine bone marrow MNCs markedly augmented CBF in the early phase after treatment (6 h after administration) in an adult mouse model of ischemic white matter damage (Fujita et al., 2010). Augmentation of CBF induced by CD34$^+$ cell treatment has also been reported in adult rat models of transient three-vessel-occlusion (Shyu et al., 2006) and heatstroke (Chen et al., 2007).

In the present study, we found that the cell therapy can modulate the morphologies of blood vessels after an ischemic insult, i.e., an enlarged diameter of blood vessels. We have previously reported that cell therapies can modulate the morphologies of blood vessels after ischemic insults, leading to an increased density of blood vessels in the adult models (Taguchi et al., 2004a; Fujita et al., 2010). Angiogenesis facilitated by CD34$^+$ cell treatment has also been reported in other models of brain injury (Shyu et al., 2006; Chen et al., 2007, 2013) and myocardial infarction (Kawamoto et al., 2006). In the present study, there was no accumulation of hUCB-CD34$^+$ cells in the border area of the infarct; the number of donor cells in the brain was substantially lower at 24 h after administration, and the donor cells were virtually absent by 10 days after administration. Therefore, it is highly unlikely that the donor cells contributed physically to the enlargement of the blood vessels after their incorporation. Although we previously identified that a few donor cells reside in the vascular walls and express endothelial markers or features of pericytes, this is not a prevalent phenomenon in the ischemic brain (Taguchi et al., 2004a; Fujita et al., 2010). A body of evidence demonstrates the beneficial effects of cell therapies in animal models of brain injury in the absence (Borlongan et al., 2004; Boltze et al., 2012) or paucity (Yasuhara et al., 2010) of hUCBCs in brain tissue.

There have been no studies that directly examined angiogenesis after hUCBC treatment in animal models of NE. However, one study demonstrated a possible association between hUCBC treatment and angiogenesis in an animal model of NE. The study showed that an intraperitoneal application of hUCB-MNCs increased the expression of the proteins Tie-2, occludin, and VEGF in the brain, which are associated with angiogenesis (Rosenkranz et al., 2012). Increased levels of VEGF in the central nervous system following the intravenous administration of hUCB-CD34$^+$ cells have been reported in an adult rat model of spinal cord injury (Kao et al., 2008). Increases in endothelial nitric oxide synthase activation by bone marrow-MNC treatment have been observed in ischemic brains (Fujita et al., 2010). We suggest that the direct structural incorporation of donor cells within blood vessels may not be the main mechanism underlying the modulation of CBF and blood vessels but rather VEGF, nitric oxide, or unknown factors that are induced by cell treatment are responsible.

Other effects of UCBC treatment

Apart from its effects on CBF and blood vessels, the pluripotent nature of hUCBC treatment has been reported to be one of the mechanisms responsible for the beneficial effects of this treatment for NE (Verina et al., 2013). Although there are no reports of the use of hUCB-CD34$^+$ cells in NE, there are 15 reports of the use of other types of hUCBCs (Table 2). Systemic (i.e., intraperitoneal or intravenous) injection of hUCB-MNCs in a neonatal rat model of HIE reduced apoptosis (Pimentel-Coelho et al., 2010; Rosenkranz et al., 2012); increased the expression of brain-derived neurotrophic factor (BDNF) (Rosenkranz et al., 2012), nerve growth factor, and glial cell line-derived neurotrophic factor (GDNF) in the brain (Yasuhara et al., 2010); reduced the activation of astrocytes (Wasielewski et al., 2012) and microglia (Pimentel-Coelho et al., 2010; Rosenkranz et al., 2013); reduced the increase in serum levels of pro-inflammatory cytokines (Rosenkranz et al., 2013); and restored neural processing in the primary somatosensory cortex (Geißler et al., 2011). Studies in adult rodent models of CNS disorders have shown that hUCB-CD34$^+$ cell treatment increased brain levels of trophic factors, i.e., GDNF, and decreased serum levels of systemic inflammatory molecules, i.e., tumor necrosis factor-α and intercellular adhesion molecule-1 (Chen et al., 2007, 2013; Kao et al., 2008; Ou et al., 2010).

Taken together, the major mechanisms responsible for the beneficial effects of hUCB treatment for cerebral ischemia appear to be related to either immunomodulation/anti-inflammation and/or trophic factor/cytokine production, independently of CBF/blood vessel modulation. Our present study suggests that the modulation of these immuno-inflammatory responses is not the single mechanism of action of hUCBC treatment, as SCID mice (which lack both functional T and B lymphocytes) exhibited improvement after cell therapy.

CONCLUSIONS

This study shows that the intravenous administration of hUCB-CD34$^+$ cells 48 h after neonatal stroke modestly ameliorates brain injury in a mouse model.

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Extracellular High Mobility Group Box 1 Plays a Role in the Effect of Bone Marrow Mononuclear Cell Transplantation for Heart Failure

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Abstract

Transplantation of unfractionated bone marrow mononuclear cells (BMCs) repairs and/or regenerates the damaged myocardium allegedly due to secretion from surviving BMCs (paracrine effect). However, donor cell survival after transplantation is known to be markedly poor. This discrepancy led us to hypothesize that dead donor BMCs might also contribute to the therapeutic benefits from BMC transplantation. High mobility group box 1 (HMGB1) is a nuclear protein that stabilizes nucleosomes, and also acts as a multi-functional cytokine when released from damaged cells. We thus studied the role of extracellular HMGB1 in the effect of BMC transplantation for heart failure. Four weeks after coronary artery ligation in female rats, syngeneic male BMCs (or PBS only as control) were intramyocardially injected with/without anti-HMGB1 antibody or control IgG. One hour after injection, ELISA showed that circulating extracellular HMGB1 levels were elevated after BMC transplantation compared to the PBS injection. Quantitative donor cell survival assessed by PCR for male-specific *sry* gene at days 3 and 28 was similarly poor. Echocardiography and catheterization showed enhanced cardiac function after BMC transplantation compared to PBS injection at day 28, while this effect was abolished by antibody-neutralization of HMGB1. BMC transplantation reduced post-infarction fibrosis, improved neovascularization, and increased proliferation, while all these effects in repairing the failing myocardium were eliminated by HMGB1-inhibition. Furthermore, BMC transplantation drove the macrophage polarization towards alternatively-activated, anti-inflammatory M2 macrophages in the heart at day 3, while this was abolished by HMGB1-inhibition. Quantitative RT-PCR showed that BMC transplantation upregulated expression of an anti-inflammatory cytokine *IL-10* in the heart at day 3 compared to PBS injection. In contrast, neutralizing HMGB1 by antibody-treatment suppressed this anti-inflammatory expression. These data suggest that extracellular HMGB1 contributes to the effect of BMC transplantation to recover the damaged myocardium by favorably modulating innate immunity in heart failure.

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Introduction

Transplantation of stem or progenitor cells is an emerging approach to repair and/or regenerate damaged myocardium undergoing adverse ventricular remodeling. Unfractionated bone marrow mononuclear cells (BMCs) contain several kinds of stem/progenitor cells and are the most frequently used donor cell type in clinical cell therapy to the heart [1]. The therapeutic effect of BMC transplantation in not only acute myocardial infarction (MI) but also post-MI chronic heart failure (ischemic cardiomyopathy) has been confirmed in animal and human studies [1–3]. Because injected BMCs do not vigorously differentiate to functioning cardiomyocytes or vascular cells *in vivo*, the major mechanism of the therapeutic effects is proposed to be their secretion of cytokines, chemokines and growth factors that help repair of the damaged myocardium suffering post-MI adverse remodeling [1–3]. However, the precise mechanism of this "paracrine effect" remains uncertain.

Interestingly, cardiac function recovery by BMC transplantation occurs despite of markedly poor donor cell survival [1,3,4]. It has also been shown that active secretion from BMCs is less extensive compared to other donor cell types [5,6]. It was also reported that injection of extract of dead BMCs by freeze-thaw cycles induces the similar therapeutic effect to injection of living BMCs [7]. These findings led us to hypothesize that dead donor BMCs might be a supplementary or alternative source of the paracrine mediators, which could contribute to the repair of the failing myocardium.

High-mobility group box 1 (HMGB1) was initially identified as a nuclear protein that regulates transcriptional factors to stabilize the nucleosome [8]. This molecule is also known to be actively secreted from activated inflammatory cells and also passively

released from dead cells [9–11]. Extracellular HMGB1 induces and intensifies inflammation in most cases, while it can also operate to attenuate inflammation and enhance the healing of damaged tissues, according to the form/amount of HMGB1 and nature of the tissues [9–12]. In the heart, there is increasing evidence that extracellular HMGB1 attenuates myocardial damage and induces recovery/regeneration [13–18], though there are contradicting reports [19,20]. We have demonstrated that HMGB1 administration achieved the similar benefits to the BMC-mediated paracrine effects, including decreased fibrosis, increased vascular formation, attenuated cardiomyocyte hypertrophy, and attenuated inflammation in a rat ischemic cardiomyopathy model [17]. It has also been reported that extracellular HMGB1 augments tissue regeneration through activating endogenous progenitor cells [15,21].

Collectively, these data formed a hypothesis that extracellular HMGB1 released from dead donor cells contributes to the paracrine effect of BMC transplantation to repair the post-MI failing myocardium and to improve cardiac performance.

Materials and Methods

Ethics Statement

All studies were performed with the approval of the UK Home Office (Project Licence Number: 70/7254). The investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US NIH Publication, 1996). All animal surgery was performed under inhalation anesthesia of isoflurane and administration of buprenorphine hydrochloride was made just after surgery to reduce postoperative pain, and all efforts were made to minimize suffering. Surgical procedures, cardiac function measurement, and sample analyses were performed in a blinded manner.

BMC Collection

Bone marrow was isolated from both femurs and tibias of male Lewis rats (150–200 g; Charles River, UK), from which BMCs (mononuclear cells) were purified by Ficoll-Paque gradient centrifugation (GE Healthcare) as previously described [3]. Flow cytometry analysis (FACSAria, BD Biosciences) using monoclonal anti-rat CD34 (Santa-Cruz) and anti-rat CD45 (BD Pharmingen) antibodies showed that 4.6±1.7% of the BMCs were positive for CD34 and 75.5±4.3% were positive for CD45 (**Figure S1**). To trace the injected cells, BMCs were labeled with CM-DiI (Molecular Probes) before transplantation according to the company's protocol. The viability of donor BMCs just before injection measured by trypan blue staining was 97.1±0.6% (n = 11 animals).

Assessment of Cardiac Function

Cardiac function and dimensions pre and post treatment were measured by using echocardiography (Vevo−770, VisualSonics) as previously described [3,17]. Diastolic and systolic LV endocardial areas at the papillary muscle level were measured from parasternal short-axis views, from which LV fractional area change (LVFAC) was calculated. Post treatment hemodynamics parameters were measured by catheterization (SRP−320/PVAN3.2, Millar Instruments and Chart 5 software, ADInstruments) as described previously [22].

Generation of Ischemic Cardiomyopathy and Cell Transplantation in Rat

Female Lewis rats (150–200 g, Charles River) underwent left coronary artery ligation as described previously [3,17,23,24]. Four weeks later, the animals that showed appropriate cardiac dysfunction (LVFAC 22–32%; base line in intact rats = 61.6±1.7% [n = 5]) by echocardiography were chosen and randomly assigned to 4 treatment groups; intramyocardial injection of 1×10^7 syngeneic male BMCs (BMC group), injection of BMCs with 50 μg anti-HMGB1 neutralizing antibody (Medical & Biological Laboratories; AB group), injection of BMCs with 50 μg control IgG (Sigma-Aldrich; IgG group), and injection of PBS only (CON group). BMCs were suspended in 200 μl of PBS and intramyocardial injection was performed into 2 sites (100 μl each) of the LV free wall, targeting the border areas [3].

To optimize the antibody dose, 1×10^7 BMCs were injected with 0, 10, 50, or 100 μg of anti-HMGB1 antibody in the same model ($n\geq3$). At day 28, LVFAC was 31.5±1.2, 30.3±1.5, 26.6±1.3, and 26.4±1.8%, respectively. Then, 50 μg antibody was used in the main study.

Detection of Released HMGB1

Peripheral blood was collected, from which serum was obtained by centrifugation. HMGB1 levels in the serum were determined in duplicate using a commercial ELISA kit (IBL international GMBH) according to the manufacture's instruction.

Histological Analysis

The hearts were excised, fixed with 4% paraformaldehyde, embedded in OCT compound, and quickly frozen in liquid nitrogen. Cryosections were cut and incubated with biotin conjugated Griffonia simplicifolia lectin I-isolectin B4 (1:100, Vector), monoclonal anti-rat CD68 antibody (1:100, AbD Serotec), monoclonal anti-rat CD86 antibody (1:50, BD), monoclonal anti-rat CD163 antibody (1:100, AbD Serotec), monoclonal anti-rat Ki-67 antibody (1:50, DakoCytomation), and/or polyclonal anti-rat cardiac troponin-T (cTnT) antibody (1:200, HyTest) followed by visualization using appropriate fluorophore-conjugated secondary antibodies (Molecular Probes). Samples were observed by a fluorescence microscopy (BZ8000, Keyence) with or without nuclear counter-staining using 4′, 6-diamidino-2-phenylindole (DAPI). Ten different fields were randomly selected in each border area of the samples and assessed. Another set of sections were stained with 0.1% picrosirius red, which enabled calculation of extracellular collagen volume fraction in border area by using NIH image-analysis software [3,17,22].

Quantitative Analysis of Donor Cell Survival

Genomic DNA was extracted from the whole LV samples of female rats. To detect donor cell (male) survival, expression of the Y chromosome-specific *sry* gene in these samples was assessed by real-time polymerase chain reaction (PCR; Prism 7900HT, Applied Biosystems). The *sry* levels were normalized to the DNA amount using the autosomal single copy gene, oesteopontin. The number of surviving donor cells was estimated by correcting the relative *sry* expression using a standard curve as previously described [3,17,22].

Measurements of Myocardial Gene Expression

Total RNA was extracted from the whole LV samples and assessed for myocardial expression of *IL-1β*, *TNF-α*, and *IL-10* by quantitative RT-PCR (Prism 7900HT, Applied Biosystems) as previously described [22]. TaqMan primers and probes were