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Human umbilical cord blood cells improve cardiac function after myocardial infarction

Yasutaka Hirata[a,*], Masataka Sata[b], Noboru Motomura[a], Minoko Takanashi[c], Yoshihiro Suematsu[a], Minoru Ono[a], Shinichi Takamoto[a]

[a] *Department of Cardiac Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[b] *Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[c] *Tokyo Metropolitan Red Cross Blood Center, Tokyo, Japan*

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Abstract

Human umbilical cord blood (UCB) contains an abundance of immature stem/progenitor cells and has been clinically used as an alternative to bone marrow transplantation. In addition, cord blood can be obtained non-invasively, in contrast to invasive bone marrow aspiration. We investigated the potential of human UCB $CD34^+$ cells to improve cardiac function following myocardial infarction. Myocardial infarction was induced in Wistar rats by ligation of the left coronary artery. Either 2×10^5 human UCB $CD34^+$ cells or equivalent cell-free medium was injected into the injured myocardium of the rats following induction of myocardial infarction. $CD34^+$ cell transplantation significantly improved ventricular function as compared to the control group. Immunofluorescence staining for human CD34, CD45, and PECAM-1 revealed surviving cells in the myocardium. Our findings suggest that transplanted human cells survived and improved cardiac function following myocardial infarction. These results may show the usefulness of UCB $CD34^+$ cells for myocardial infarction.

Keywords: Human cord blood cell; Myocardial infarction; Stem cell therapy

Permanent loss of cardiomyocytes and non-functional fibrous scar formation following myocardial infarction (MI) result in irreversible damage to cardiac function. Although an efficient repair will allow the ventricle to function despite the loss of some of its cardiomyocytes, it has been well established that adult cardiac myocytes do not replicate, thus, these pump units are not actually replaced. The recent development of cellular cardiomyoplasty has offered a new approach to restore impaired heart function in which no parenchymal regeneration occurs [1]. Many recent studies have focused on the use of embryonic stem cells and bone marrow-derived cells, including adult hematopoietic stem cells, mesenchymal stem cells, and bone marrow side population cells [2–5]. Further, $CD34^+$ cells isolated from bone marrow and peripheral blood have been successfully used for regeneration of blood vessels following experimental MI [6,7] and a recent study described transdifferentiation of human peripheral blood-derived $CD34^+$ cells into cardiomyocytes in vivo [8].

Human UCB is rich in mesenchymal progenitor cells [9] and contains a large number of endothelial cell precursors [10], as well as many immature stem/progenitor cells with extensive in vitro proliferation capacity. Further, it has been used as a source of transplant stem and progenitor cells [11], and also of marrow-repopulating cells for the treatment of pediatric and adult disease [12,13]. Previously, it was shown that freshly isolated human UCB $CD34^+$cells injected into ischemic

* Corresponding author. Fax: +81 3 5684 3989.
E-mail address: h-yasu@umin.ac.jp (Y. Hirata).

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adductor muscles gave rise to endothelial and skeletal muscle cells in a mouse model of immunosuppression [14]. This high degree of stem cell plasticity prompted us to examine whether neovascularization of the infarcted myocardium could be prompted by transplanting human UCB $CD34^+$ cells into infarcted rats.

In the present study, we examined whether intramyocardially transplanted UCB $CD34^+$ cells could survive, enhance neovascularization, and improve left ventricular functional recovery following MI in rats.

Materials and methods

Isolation of human $CD34^+$ cells from umbilical cord blood. Human UCB was collected into a citric acid containing bag immediately after delivery, following written approval from each mother. After obtaining the UCB mononuclear fraction by Ficoll–Histopaque (Sigma Chemical, USA) gradient separation, isolation of $CD34^+$ cells was performed using a MINIMACS system with a direct CD34 isolation kit (Miltenyi Biotech, USA) according to the manufacturer's instructions to purity of about 85–98%. Suspensions of $CD34^+$ cells were washed three times in PBS and re-suspended in DMEM. The final density of $CD34^+$ cells was 2×10^6 cells/ml.

Animals, surgical procedures, and transplantation of human cells. Male Wistar rats (Saitama Experimental Animals, Japan), each weighing 270–320 g, were used. All procedures involving experimental animals were performed in accordance with protocols approved by the Institutional Committee for Animal Research of The University of Tokyo and complied with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996). To prevent rejection, the rats were treated with FK506 (Fujisawa, Japan), administered intraperitoneally at a dose of 0.3 mg/kg 6 h before, just after, and then daily after the surgical procedure for the entire experimental period. Following anesthesia with an intraperitoneal administration of pentobarbital at 50 mg/kg, the rats were intubated and mechanically ventilated with a rodent ventilator (SAR-830/P Ventilator, CWE, USA). A left thoracotomy was performed, during which the pericardium was opened and the left anterior descending (LAD) coronary artery was ligated 2 mm beneath the left atrial appendage. Within 20 min after MI induction, 2×10^5 $CD34^+$ cells in 100 μl DMEM were injected using a tuberculin syringe into the anterior and lateral aspects of the viable myocardium bordering the infarction ($n = 10$). Control rats with MI received cell-free medium at the same volume as that given to rats treated with $CD34^+$ cell transplantation ($n = 10$). Sham rats underwent placement of the suture without ligation ($n = 6$).

Echocardiographic study. Four weeks after cell transplantation, the rats were anesthetized with an intraperitoneal administration of pentobarbital at 30 mg/kg. Echocardiographic studies were performed with a high frequency linear array transducer (LOGIQ500, GE Yokokawa Medical Systems, Japan), using parasternal short- and long-axis views in B and M imaging modes. Two-dimensional short- and long-axis images were obtained and M-mode tracings were analyzed.

Measurement of hemodynamics. Following the echocardiographic study, the rats were intubated and ventilated. A midline sternotomy was performed, and then a 2.5 F Millar (Millar Instruments, USA) and 3F conductance (S-I Medico-tech, Japan) catheters were introduced into the left ventricle through the apex. LV pressure curve and pressure–volume loops were obtained, and E_{max} and $+dp/dt$ max were calculated using SIP-100 software (S-I Medico-tech, Japan). Afterload was changed by gentle clamping and releasing of the ascending aorta by forceps.

Tissue harvesting. Following the hemodynamic analyses, pentobarbital was administered intravenously at 100 mg/kg and the rats were humanely killed. Transverse sections of each heart were embedded in OCT compound (Sakura Finetek USA, USA), frozen in liquid nitrogen, and stored at −80 °C. Rat hearts in OCT blocks cut into 5 μm serial sections were placed on individual slides.

Measurement of capillary density. Capillary density was evaluated by histological examination of five randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD. Frozen sections were blocked with 0.5% goat serum. Endogenous biotin and biotin binding proteins were blocked with a blocking kit (Vector Laboratories, USA). The sections were incubated with anti-CD31 (BD Biosciences, USA), followed by the avidin–biotin complex technique and Vector Red substrate (Vector Laboratories). Sections were counterstained with hematoxylin. Capillaries were recognized as tubular structures positive for CD31.

Double-immunofluorescence study. Immunofluorescence double staining was performed as described elsewhere [15]. After blocking in 0.5% goat serum, frozen sections were incubated with first antibodies (Cy3 conjugated α-smooth muscle actin (α-SMA), FITC conjugated anti human PECAM-1, FITC conjugated anti human PECAM-1, and FITC conjugated anti human CD45 (Chemicon International, USA); FITC conjugated anti human CD34 class II (Dako, USA)). These monoclonal antibodies were all mouse IgG, and thus negative control slides were incubated with appropriate dilution of non-immune mouse IgG (Vector Laboratories, USA).

The nuclei were counterstained with Hoechst 33258 (Sigma Chemical, USA). The sections were mounted with Perma Fluor aqueous mounting medium (Immunotech, France) and observed under a confocal microscope (FLUOVIEW FV 300, Olympus, Japan).

Estimation of the number of human cells in the rat myocardium. Estimation of the number of human cells in the rat myocardium was performed by counting the number of cells positive for human CD34, CD45, and PECAM-1 in randomly selected five sections. Assuming a cell thickness of 5 μm and that injected cells existed within 3 mm width of the injection site, the total number of human cells per heart was estimated as 600 times the average number of the cells per section.

Data analyses. Data are expressed as means ± SEM. Student's t test was used for two-group comparisons and ANOVA followed by an unpaired Student's t test, with Bonferroni's correction for multiple group comparisons. A probability value of $P < 0.05$ was considered to denote statistical significance.

Results

Myocardial function improved by human UCB $CD34^+$ cells

Of the rats that underwent LAD ligation, 4 rats with medium injection and 3 rats with $CD34^+$ cells injection died within 4 weeks after the operation. Overall mortality was 40% for medium injection group and 30% for $CD34^+$ injection ($P = \text{NS}$). There was no mortality among the sham operated group. We investigated the effects of injected $CD34^+$ cells on myocardial function in rats following infarction. Fig. 1 shows the results of our echocardiographic assessment of cardiac function in rats 4 weeks after MI and $CD34^+$ cell implantation. LV function was improved in rats that received $CD34^+$ cells as compared to the controls (mean fractional shortening was $31 \pm 2\%$ versus $24 \pm 2\%$, $P < 0.05$). Similarly, LV dilatation was significantly suppressed by injection of $CD34^+$ cells (7.3 ± 0.3 mm versus 8.5 ± 0.4 mm, $P < 0.05$).

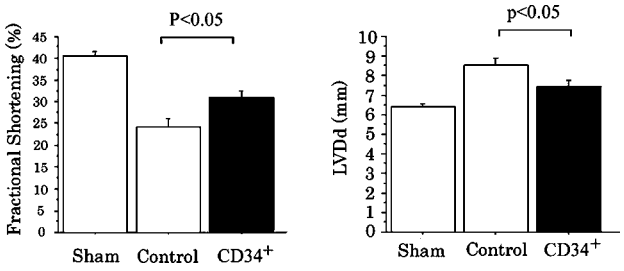

Fig. 1. Human UCB $CD34^+$ cell transplantation significantly improved LV function, as LV dilatation was suppressed in rats with cell transplantation. Sham, rats without MI ($n = 5$); control, rats with MI injected with cell-free medium ($n = 6$); and $CD34^+$, rats with MI transplanted with $CD34^+$ cells ($n = 7$).

Fig. 2 shows the results of hemodynamic analyses of the hearts 4 weeks after MI. E_{max} was significantly greater in infarcted hearts that received $CD34^+$ cells (778 ± 36 mmHg/ml versus 610 ± 20 mmHg/ml, $P < 0.01$). The maximum rising rate of pressure ($+dp/dt$ max) was also significantly recovered in rats that received $CD34^+$ cells (5160 ± 260 mmHg/s versus 4470 ± 130 mmHg/s, $P < 0.05$).

Enhancement of neovascularization by human UCB $CD34^+$ cells

Fig. 3 shows the immunohistochemistry for rat CD31. Capillary density in the segments of in the LV myocardium subserved by the occluded LAD was significantly greater in rats that received $CD34^+$ cells (223 ± 12 vessels/mm^2 versus 119 ± 12 vessels/mm^2, $P < 0.001$). This result indicates that engrafted $CD34^+$ cells cause or enhance neovascularization in injured myocardium.

Quantitative and qualitative analysis of surviving UCB cells

Immunofluorescence double staining by human antibodies (CD34, PECAM-1, and CD45) and α-SMA revealed surviving human cells around the vessels in the ischemic myocardium (Fig. 4). Among the cells that expressed CD34 and CD45, there were cells that also expressed α-SMA (Figs. 4A–F, arrows). Around these vessels, there were cells that did not express α-SMA (arrow heads). PECAM-1 positive cells were found both inside and outside the vessels but both of these cells did not express α-SMA (Figs. 4G–I, arrowheads). Non-immune mouse IgG gave rise to only background fluorescence staining (Figs. 4J–L). Anti-human CD34, CD45, and PECAM-1 also did not show reactivity with the control rat myocardium without human cell injection (Fig. 5). Table 1 shows the prevalence of the injected human cells in the myocardium of the rats. The estimated numbers of human cells that expressed CD34, CD45, and PECAM-1 at the time of sacrifice were about 220%, 330%, and 140% compared to the originally in-

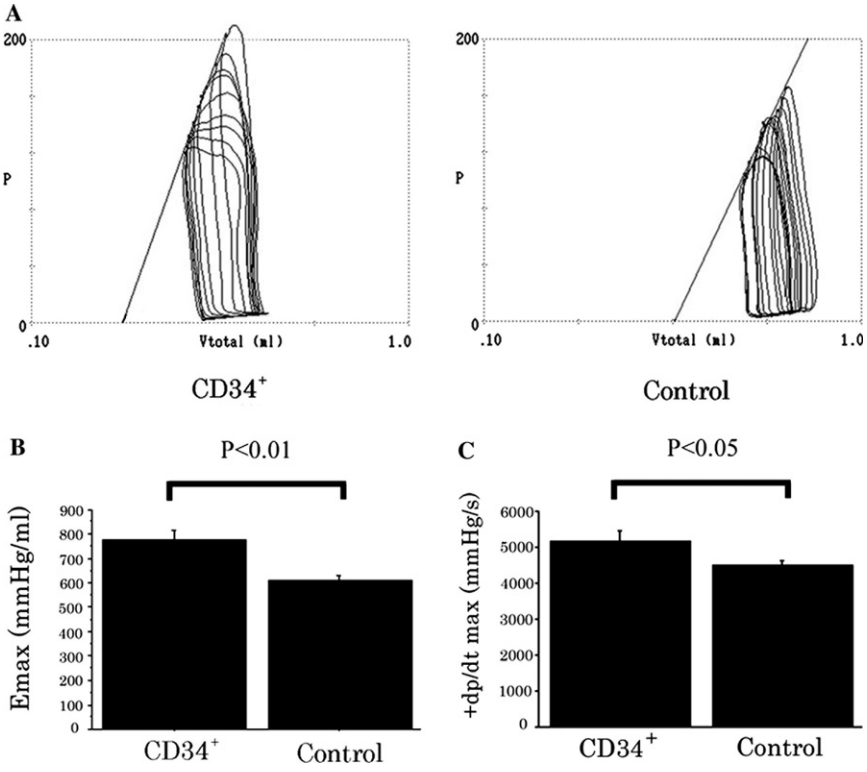

Fig. 2. Measurement of hemodynamics 4 weeks after MI. (A) Representative images of pressure-volume loop for each group; (B) E_{max}; (C) $+dp/dt$ max. $CD34^+$, rats with MI transplanted with $CD34^+$ cells ($n = 7$); control, rats with MI injected with cell-free medium ($n = 6$).

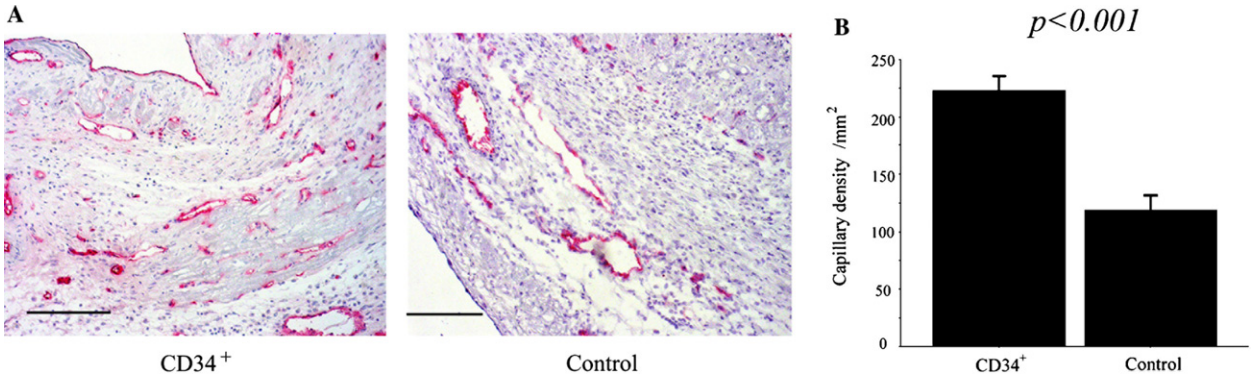

Fig. 3. Effect of transplantation of human UCB CD34$^+$ cells on neovascularization after MI. (A) Representative images of immunohistochemistry for rat CD31. Bar = 100 μm. (B) Capillary density evaluated by histological examination of five randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD. CD34$^+$, rats with MI transplanted with CD34$^+$ cells ($n = 7$); control, rats with MI injected with cell-free medium ($n = 6$).

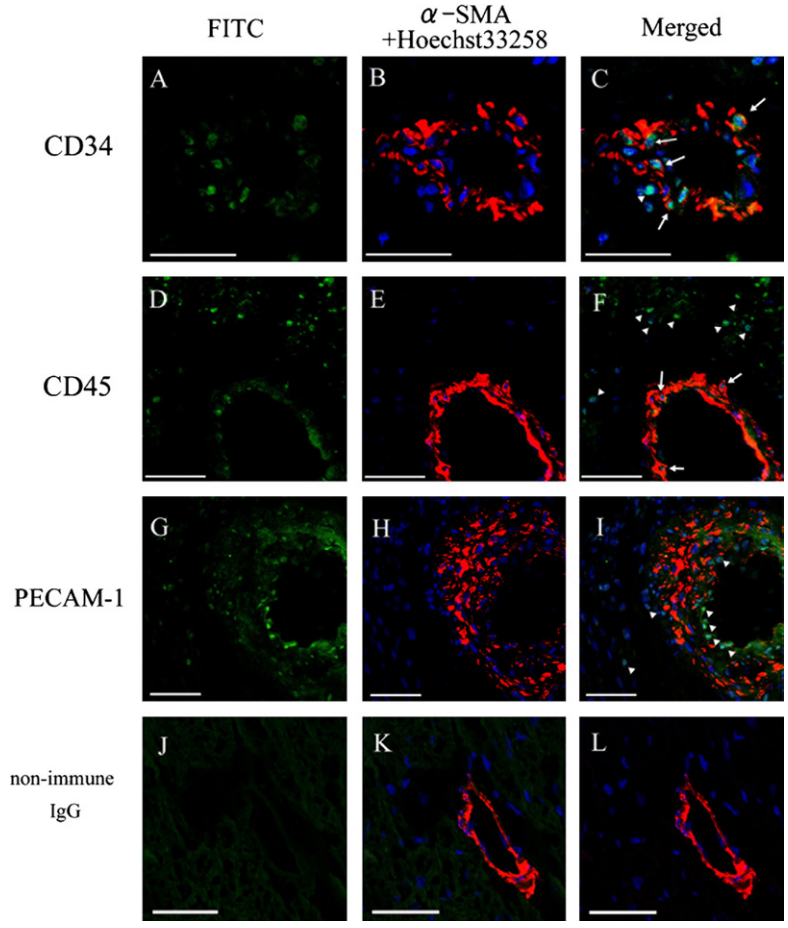

Fig. 4. Survival and incorporation of human UCB CD34$^+$ cells in rat myocardium. Immunofluorescence double staining by human antibodies (CD34 (A–C); CD45 (D–F); and PECAM-1 (G–I)) and α-SMA shows surviving human cells around the vessels in the ischemic rat myocardium. Non-immune mouse IgG gave rise to only background fluorescence staining (J–L). There were cells that express α-SMA (arrows) and do not express α-SMA (arrowheads). Bar = 50 μm.

jected CD34$^+$ cells, respectively. These data show that considerable number of human cells could survive in the rat myocardium under immunosuppression. The cells that were incorporated into the vessels of the rat myocardium comprised about 1% of the total surviving cells.

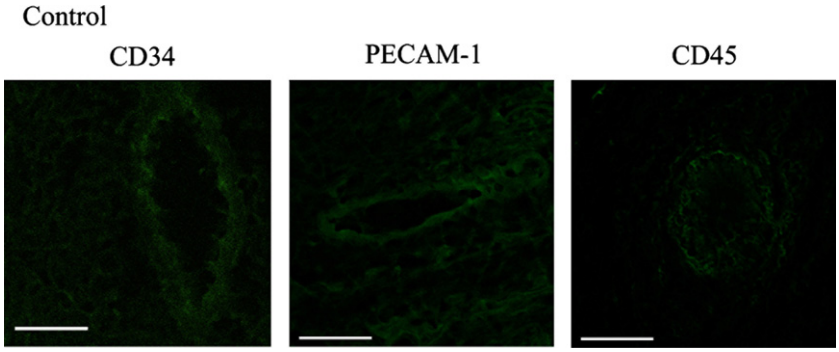

Fig. 5. Immunofluorescence staining human CD34, CD45, and PECAM-1 on control rat myocardium without human cell injection. These antibodies did not have reactivity with the rat myocardium. Bar = 50 μm.

Table 1
Estimate of human cells in the rat myocardium

	Number of cells/section	Estimated number of cells present at the time of sacrifice	Original number of human cells present at the time of sacrifice (%)	Vascular incorporation/section	Estimated number of cells present at the time of sacrifice	The ratio of vascular incorporation/existed cells (%)
CD34	730 ± 120	438,000	220	10 ± 2	5760	1.3
CD45	941 ± 195	668,000	330	13 ± 3	7560	1.1
PECAM-1	461 ± 119	276,000	140	6 ± 2	3600	1.3

The numbers of cells positive for human CD34, CD45, and PECAM-1 were counted in randomly selected five sections. Assuming a cell thickness of 5 μm and injected cells were within 3 mm width of the injection site, the total number of human cells per heart was estimated as 600 times the average number of the cells per section.

Discussion

Our present results demonstrated that $CD34^+$ cells isolated from human UCB are capable of enhancing neovascularization, incorporating into vasculature of the myocardium, and improving cardiac function.

We also found that human UCB $CD34^+$ cells can survive in rats with immunosuppression. Previously, freshly isolated human UCB $CD34^+$ cells injected into ischemic adductor muscles gave rise to endothelial and skeletal muscle cells in a mouse model of immunosuppression [14], while intravenously administered human UCB $CD34^+$ cells survived and improved functional recovery after stroke in Wistar rats without immunosuppression [16]. As previously suggested [14], there are two possible explanations for our finding that human cells can survive in rat ischemic myocardium tissue. First, transplanted cells are not mature and may represent suboptimal targets for the action of preformed anti-species antibodies. Second, the infarcted cardiac tissue likely consisted of mixed host/donor structures. The present finding may show a lower immunogenic potential of neo-formed tissues and longer tolerance by the host. In addition, our results showing the ability of $CD34^+$cells to incorporate into vasculature of rat myocardium may represent an advancement toward validation of the use of human UCB for allotransplantation in patients.

We used anti-human antibodies to track human cells along with immune fluorescence staining. This procedure does not require genetic modification or fluorescent labeling of the cells to track cell fate. We consider that this might be the reason that considerable number of our ''unmodified'' cells could survive in the rat myocardium.

Our findings show that most of the injected human cells were not incorporated into the rat vessels. This might indicate that injected cells contributed to the neovascularization mainly as hematopoietic cells to promote paracrine effect in ischemic tissues (e.g., secretion of angiogenic factors) as recent papers highlighted [17,18].

Neovascularization by human UCB $CD34^+$ cells seems to be superior to transplantation of fetal [19] or neonatal cardiac myocytes [20], and to that of embryonic stem cell-derived cardiac myocytes [21,22], all of which are difficult because of unresolved ethical issues. The problem caused by allogenic tissues might be avoided by HLA-matching, since cord blood banks have been already established as an alternative source of hematopoietic reconstituting cells for allogenic transplantation. Moreover, autologous UCB $CD34^+$ cell transplantation may be available for children with congenital heart disease.

In conclusion, we showed that transplanted UCB $CD34^+$ cells can survive, incorporate into vasculature of myocardium, and improve cardiac function following MI. Therefore, these cells may be an excellent source for the treatment of MI and congestive heart failure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2004.12.044.

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