Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (2): 6-12
Published online June 15, 2014 (http://www.cellmolbiol.com)
Received on March 23, 2014, Accepted on June 11, 2014.
doi : 10.14715/cmb/2014.60.2.2

Human umbilical cord blood cell transplantation improves cardiac function in a myoardial infarction rat model but induces intestinal graft versus host disease

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Abstract

Human umbilical cord blood cell (HUCBC) has low immunity. In the present study we investigated intestinal graft-versus-host disease (GVHD) induced by HUCBC transplantation in a myocardial infarction (MI) rat model. MI was established by using left anterior descending coronary artery (LAD) ligation. HUCBCs were injected into the animals 5 days post MI. Four weeks after the HUCBC transplantation, histology changes in small intestine were observed under an optical microscope. In addition, cardiac functions were tested. Further, factor VIII, vascular endothelial growth factor (VEGF) in the myocardium and small intestine were assayed. The HUCBC transplantation significantly induced intestinal GVHD in the MI rats. The HUCBC implantation remarkably improved ejection fraction (EF), fractional shortening (FS), and dp/dtmax in the MI rats ($P<0.05$). In the myocardium, the capillary density was larger in the small intestine of the HUCBC-transplanted rats compared to the controls. Real-time PCR and western blotting revealed that VEGF mRNA and protein levels in the myocardium and the small intestine dramatically significantly upregulated in the HUCBC-transplanted rats ($P<0.05$). The HUCBC transplantation significantly improves aggravated cardiac function of MI rats, but it induces intestinal GVHD.

Key words: Human umbilical cord blood, Graft versus host disease, Myocardial infarction, Vascular endothelial growth factor, Intestinal.

Introduction

Cell transplantation is a means to improve cardiac function in myocardial infarction. Generally, autologous skeletal muscle progenitor cells and marrow derived stem cells were selected to treat myocardial infarction (1). While human umbilical cord blood cells (HUCBCs) have pluripotent capabilities and rapid proliferation. Moreover, HUCBCs are very rich. Importantly, their immunity is very weak. Its lymphocytes are immature, producing fewer cytokines and immunoglobulins compared with the adult. Little or no cytotoxic activity is generated by cord blood cells after xenogeneic stimulation. Further, class II HLA antigen is seldom expressed in HUCBC (2,3).

Hu et al. (4) found that the transplanted HUCBCs survived and were involved in the repair process in host heart. Echocardiography result showed that significantly improved left ventricular function was present in HUCBC-transplanted myocardial infarction rats. In addition, a study regarded it as a safe strategy in the absence of immunosupression (5).

Occasionally, we found that many rats suffered from abdominal distention after the HUCBC transplantion. With the prolongation of the treatment time, the distention became more and more significant. To the best of our knowledge, it is the first time for us to observe this phenomenon. To clarify this, the grafted HUCBC number, capillary density, vascular endothelial growth factor (VEGF) in myocardium and small intestine were assayed.

Materials and methods

Preparation of HUCBC

Human cord blood samples taken from healthy mothers were provided by Beijing Umbilical Cord Blood Center (Beijing, China). The volunteers read and signed the inform consents for research and clinical use. Cord blood cells were separated within 6 h according to the following method. Briefly, the collected blood samples were separated by Ficoll lymphocyte separation medium, then the umbilical cord blood cells were isolated by gradient centrifugation at 2000rpm for 30 min. The nucleus cells including CD34-positive by flow cytometry (6) were used for the following experiments.

Establishment of a myocardial infarction rat model

Male Sprague-Dawley (SD) rats, weighing 280-300 g (Experimental Center, Academy of Military Medical Sciences, Beijing, China), were selected in the present study. The rats were anesthetized with 10% chloral hydrate (0.3 mg/kg), and mechanically ventilated. Left anterior descending coronary artery was ligated to produce myocardial infarction. The establishment of MI model was identified by anterior wall blanching, akinesis, and ST segment elevation on the electrocardiogram. After that, the animals were given free access to food and water. All the experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals. This study was approved by the Ethnic Committee of Friendship Hospital (Beijing, China).

The HUCBC transplantation and grouping

1 ml of HUCBC (1×10^7-2×10^7) or an equal volume of PBS was respectively injected into 10 MI rats once through caudal vein 5 days post the surgery. Any immunosuppressive drug was not given. Meanwhile, another 10 MI rats received no treatment served as controls.

Four weeks after the infarction, the rats experienced cardiac function and hemodynamics assays. Then the animals were sacrificed and the myocardium and small intestine tissues were collected for capillary density, immunohistochemistry, Real-time PCR, and Western blotting assays.

Cardiac function examination

Transthoracic echocardiography was performed on all the animals prior to the infusion (baseline echocardiogram) and 4 weeks after the HUCBC transplantation using a Vevo 707 Echocardiography System (Visual Sonics Inc., Toronto, Canada) with a 35-MHZ transducer. The animals were anesthetized with abdominal injections of 10% chloral hydrate (0.3 mg/kg). Parasternal long and short axis views were obtained by both M-mode and 2-dimensional echo images. Left ventricle end-diastolic dimension (LVEDD) and Left ventricle end-systolic dimension (LVESD) were measured perpendicularly according to the long axis of the ventricle at the mid-chordal level. Fractional shortening (FS) and left ventricular ejection fraction (LVEF) were calculated automatically by the echocardiography system. All the measurements were averaged for three consecutive cardiac cycles and performed by an experienced technician who was blind to the treatments.

Hemodynamics assay

At the end of the experiment (4 weeks after the HUCBC transplantation), the rats from each group were anesthetized with 10% chloral hydrate (0.3 mg/kg). A pressure tipped catheter transducer was electrically calibrated and then transthoracically inserted into the left ventricle for pressure measurements. The distal end of the transducer was connected to a U1604A writing direct oscillograph (Agilent Technologies, CA,USA). Each animal was allowed to stabilize for 10-20 min prior to LV pressure and heart rate recordings. The parameters including LV pressure, dp/dtmax, and heart rate were measured based on 10 consecutive waveforms.

Capillary density assay of the myocardium and the small intestine

At the end of the experiment, the animals were anesthetized and sacrificed. Then, the myocardium and small intestine tissues were collected and were cut into a size of approximately 3.0×1.0 mm and fixed in polyoxymethylene for 12 h followed by dehydration and embedding in paraffin. The samples were consecutively cut into 4 μm-thick sections.

To measure the capillary density in the ischemic myocardium and the small intestine, the sections were stained using anti-Factor VIII antibody (ab61390) (Abcam, Cambridge, UK). For quantification of positively stained vessels, the number of capillaries was counted in 10 randomly chosen high-power fields by two independent reviewers. The total blood vessel number per section were imaged and averaged. The capillary criteria: number of endothelial cells surround and form a lumen or a single cell cavity.

Real-time PCR assay of VEGF mRNA in the myocardium and the small intestine

About 100 mg of myocardium or small intestine was homogenized in a glass homogenizer and then 1.5 ml of Trizol (Invitrogen, USA) was added in. Total RNA was extracted from the myocardium or small intestine tissue using an RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions.

25 μl of standard reaction system included 12.5 μl of real-time PCR Master Mix SYBR Green I, 0.5 μl of forward primer (10 μmol/l), 0.5 μl of reverse primer (10 μmol/l), 1 μl of cDNA, and 10.5 μl of ddH_2O. The following reactions were performed for 40 cycles. The reaction condition included denaturation at 95°C for 4 min and annealing at 56°C for 40 s.

The following primers were used: VEGF (315-bp product), forward (5'-CAGGGTTTCGGGAACTAG-3') and reverse (5'-GTGTATGTGGGTGGGTGT-3'); β-actin (318-bp product), forward (5'-ATCATGTTTGAGACCTTCAACA-3') and reverse (5'-CATCTCTTGCTCGAAGTCCA-3'). All the primers were synthesized by Invitrogen Biological Technology Co., Ltd. (Shanghai, China).

The data were analyzed using IQ5 software of Gene express module (Bio-Rad, CA, USA). Three replicate reactions were performed and values were normalized to the housekeeping gene β-actin, C_T values were determined by using the 7500 System SDS Software (version.1.2.3; Applied Biosystems, USA).

Western blotting assay of VEGF protein in the myocardium and the small intestine

Approximately 100 mg of the myocardium or the small intestine tissue was homogenated and then lyzed with 1 ml of pre-cooled RIPA lysate for 30 s and followed by an ice bath for another 10 min. The suspensions were centrifuged at 12,000×*g* at 4°C for 10 min and then the supernatants were harvest. The concentration of the total protein was quantified by Bradford method. A same concentration of the total protein was subject to electrophoresis on 10% SDS PAGE and then transferred onto nitrocellulose membranes. After blocked by 10% defat milk, the membranes were incubated with a rabbit anti-rat VEGF antibody (1:500) (Abcam, USA) for 6 h at room temperature. The membranes were washed with TBST for three times and each 3 min. Then the membranes were incubated with goat anti-rabbit antibody (1:5000) (Zhongshan Golden Bridge, Beijing, China) for 2 h at room temperature. They were washed with TBST for three times and each 10 min and then detected using an ECL reagent. β-actin served as an internal control.

Histology assays of the myocardium and the small intestine

After completion of all the other experiments, the rats were sacrificed after the anaesthesia. The collected myocardium or the small intestine tissue was fixed in 4% paraform for 24 h. The samples were dehydrated and embedded in paraffin. The samples were cut into 4 μm-thick sections consecutively and then stained with hematoxylin and eosin and then observed using an opti-

cal microscope.

Statistical analysis

Data are presented as mean±standard error. Statistical analysis was performed using a SPSS11.0 software (SPSS Inc., Chicago, IL, USA). The difference(s) between or among groups was (were) compared by Student-Newman-Kenls (SNK) test or one-way analysis of variance (ANOVA). Comparisons of rate among groups were used by chi-square test. A p value of less than 0.05 is regarded as significant.

Results

General status of the MI rats after the HUCBC transplantation

At the end of the experiment (4 weeks post the transplantation), the body weight loss in the MI rats was not different from the PBS-treated MI rats. However, the body weight loss in the HUCBC transplantation group was significantly lower than that in the MI and MI plus PBS groups (P<0.05, P<0.05) (Table 1).

At the end of the experiment all the animals were sacrificed. It showed that no intestinal GVHD was observed in the single MI rats. However, PBS treatment significantly induced intestinal GVHD, leaving a GVHD positive rate of 20%. The HUCBC transplantation further remarkably aggravated the intestinal GVHD and the positive rate was 90% which was significantly higher than that in the MI alone or PBS-treated group, respectively (Table 2).

Table 1. Body weight loss in the MI rats 4 weeks post the HUCBC transplantation.

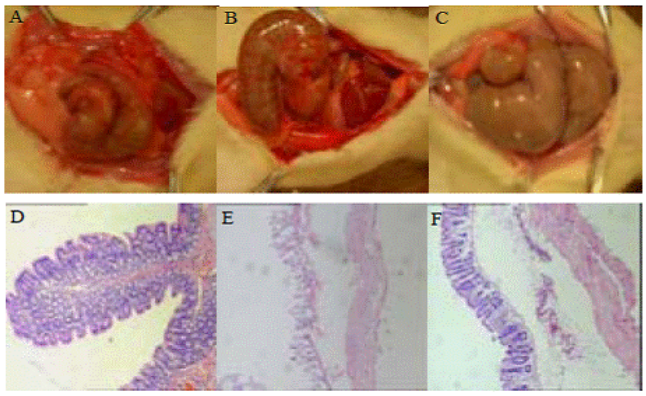
	Body weight loss (g)
MI	27.4±11.9
MI+PBS-treated	29.5±12.3
MI+HUCBC-transplanted	15.9±6.2 *, #

*P<0.05 vs. MI; #P<0.05 vs. MI+PBS-treated.

Histology changes in the small intestine after the HUCBC transplantation

In the control group, the small intestine was normal (Fig. 1A). In the PBS-treated group, the small intestine was a little bigger compare with the MI group (Fig. 1B). However, the small intestines of the MI rats received the HUCBC transplantation were significantly expanded (Fig. 1C). Briefly, the distent intestine almost overlapped ileum and went toward jejunum, but not in colon (Fig. 1C). Not any local obstruction and lump was found.

Histology results showed that the intestinal villi disappeared, leaving leukomonocyte infiltration and edematous submucosa (Fig. 1E and 1F). And it became more serious in the HUCBC-transplanted MI rats (Fig. 1F). All the evidences suggested occurrence of intestinal GVHD.



(H.E×40)

Figure 1. Histology changes of small intestine 4 week after the HUCBC transplantation ($\bar{x}$ ±s, n=10). A, small intestine of the MI rats; B, the PBS-treated small intestine of the MI rats. It is bigger than the control; C, the HUCBC-transplanted small intestine of the MI rats. It is inflated and much bigger than the control and the PBS-treated; D, mucous membrane of the small intestine from the MI rat; E, Lightly dropsical submucosa, leukomonocyte infiltration, and disappeared villus after the PBS treatment. F, Significantly dropsical submucosa, leukomonocyte infiltration, and disappeared villus after the HUCBC transplantation.

Cardiac and hemodynamic assays after the HUCBC transplantation

Echocardiography examinations were carried out in these animals 4 weeks prior to and after the HUCBC transplantation. Before the transplantation, there were no significant differences in cardiac function parameters such as LVEF and FS among these rats.

Four weeks after the transplantation, LVEF was significantly higher in the transplanted rats than the controls and the PBS-treated ones (P<0.05). FS in the HUCBC-transplanted rats were significantly elevated compared with the controls and the PBS-treated ones (P<0.05) (Fig. 2A).

The hemodynamic assay was performed four weeks after the HUCBC transplantation. It revealed that dp/dtmax in the HUCBC-transplanted group was significantly higher than that in the single MI group and PBS-treated group (P<0.01) (Fig. 2B). In addition,

Table 2. Small intestinal GVHD occurrence in the MI rats after the HUCBC transplantation.

	GVHD Negative	GVHD Positive	GVHD Positive Rate
MI	10	0	0
MI+PBS-treated	8	2	20% *
MI+HUCBC-transplanted	1	9	90% *, #

*P<0.01 vs. MI; #P<0.01 vs. MI+PBS-treated.

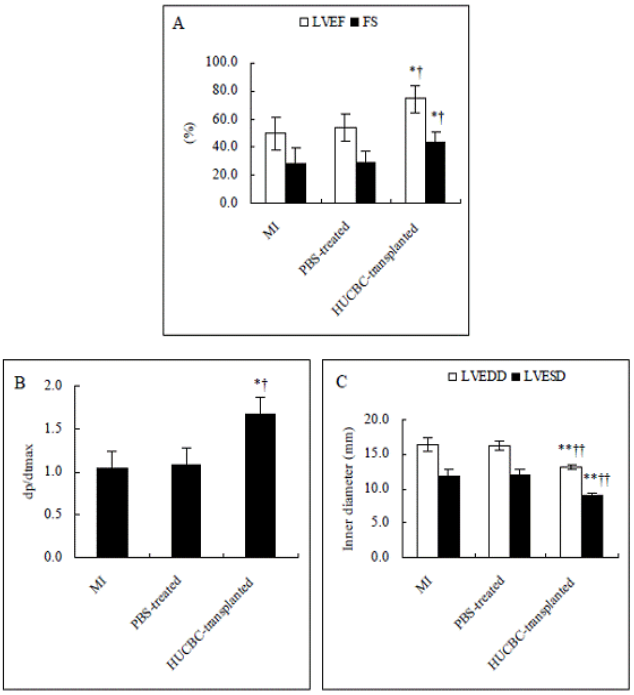


Figure 2. Cardiac functions 4 weeks after the HUCBC transplantation ($\bar{x}$ ±*s*, n=10). (A) LVEF and FS significantly increased in the HUCBC-transplanted group compared to the single MI and PBS-treated groups; (B) dp/dtmax in the HUCBC-transplanted group were significantly higher in the transplanted rats than that in the controls; (C) LVEDD and LVESD in the HUCBC-transplanted group significantly decreased compared to the single MI group and PBS-treated group. $^{*}P<0.05$, $^{**}P<0.01$ vs. MI; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. PBS-treated.

both LVEDD and LVESD significantly reduced in the HUCBC-transplanted group compared to the single MI group and PBS-treated group ($P<0.01$) (Fig. 2C).

All the results above suggested that the HUCBC transplantation significantly improved the impaired heart functions of the MI rats.

VEGF mRNA and protein levels are upregulated after the HUCBC transplantation

Both mRNA and protein levels of VEGF in the myocardium and the small intestine were significantly upregulated after the HUCBC transplantation compared with the single MI and the PBS-treated ones ($P<0.05$) (Fig. 3A). Actually, the PBS treatment also markedly upregulated the VEGF mRNA level compared to the control.

As expected, the HUCBC implantation markedly upregated the VEGF protein expression in both the myocardium and the small intestine. However, there was no significant difference in the myocardium between the PBS-treated group and the HUCBC implanted group. In addition, the VEGF protein expression in the small intestine was largest in the HUCBC-transplanted group, significantly higher than that in the single MI and PBS-treated groups. (Fig. 3B).

Capillary density assay of Factor VIII in the myocardium and the small intestine after the HUCBC transplantation

Factor VIII immunostaining was assayed to assess the capillary density in the myocardium border or the small intestine. The results showed that the HUCBC

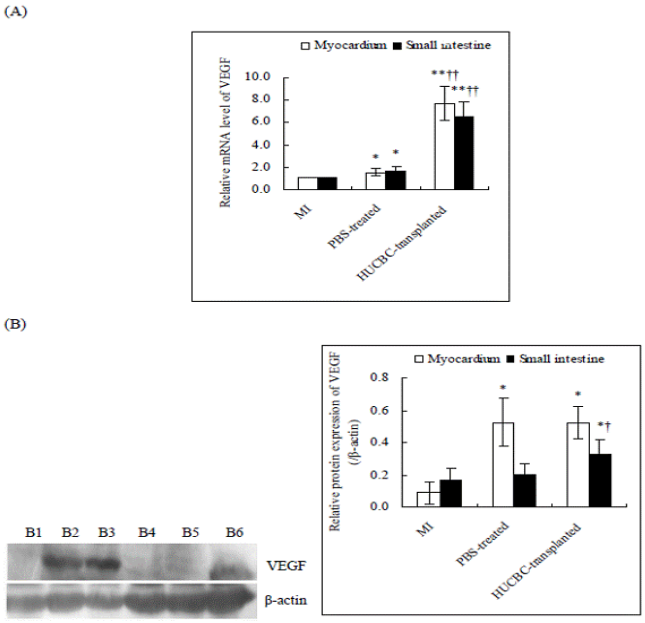


Fig 3. mRNA and protein expressions of VEGF in the myocardium and small intestine after the HUCBC transplantation ($\bar{x}$ ±*s*, n=10). (A) The VEGF mRNAs in the myocardium and small intestine significantly increased in the PBS-treated, and HUCBC-transplanted groups compared to the single MI group; **(B)** The VEGF protein expression in the myocardium was markedly upregulated in PBS-treated, and HUCBC-transplanted groups, while there was no significant difference between PBS-treated group and HUCBC-transplanted group. B1-B3: Myocardium collected from the single MI, PBS-treated, and HUCBC-transplanted rats, respectively; The VEGF protein expression in the small intestine was largest in the HUCBC-transplanted group and higher than that in the single MI and PBS-treated groups. B4-B6: Small intestine collected from the single MI, PBS-treated, and HUCBC-transplanted rats, respectively. $^{*}P<0.05$, $^{**}P<0.01$ vs. MI; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. PBS-treated.

transplantation greatly increased the total number of capillary vessels in the myocardium tissue. The positive factor VIII expression was significantly elevated after the HUCBC transplantation (Fig. 4A). There was a significant difference between the PBS-treated and the HUCBC-transplanted groups ($P<0.01$).

Similar phenomenon was also present in the small intestine. The positive factor VIII immunostaining was significantly enhanced in the HUCBC-transplanted group. And the positive expression of factor VIII in the small intestine of the HUCBC-transplanted MI rats was significantly higher than that in the controls and the PBS-treated ones (Fig. 4B) ($P<0.01$).

Discussion

The HUCBC transplantation is regarded as a good and potential therapeutic strategy in the treatment of acute myocardial infarction. HUCBC contains hematopoietic and mesenchymal progenitor cells that can potentially transdifferentiate into capillaries and myocardial cells. Moreover, HUCBC is less immunogenic (7-9).

Cellular immunity is mainly determined by the major histocompatibility complexes (MHC) including MHC class I molecule (HLA-ABC) and MHC class II molecules (HLA-DR and HLA-DQ). Only 5% of HUCBCs expressing MHC-I, and MHC-II was completely nega-

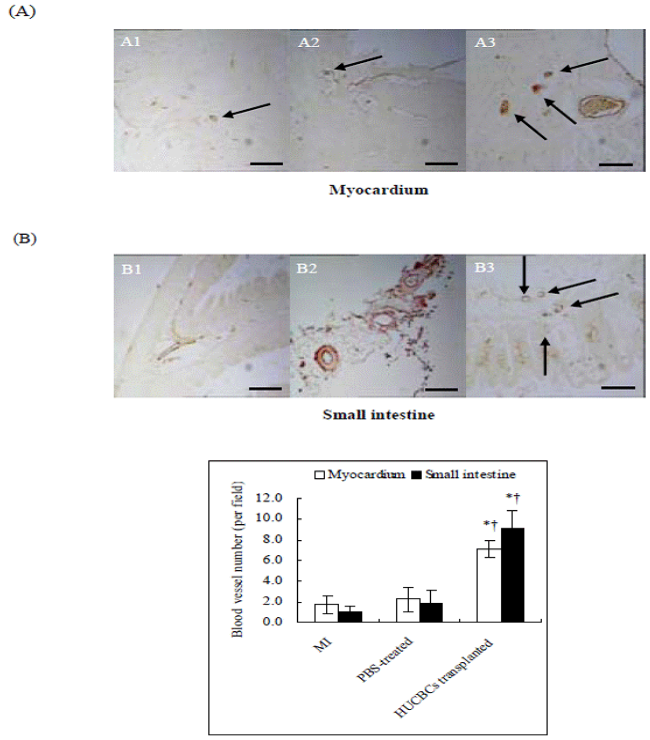


Figure 4. Immunohistochemistry assay of factor VIII **staining in the myocardium and small intestine after the HUCBC transplantation ($\bar{x}$ ±*s*, n=10). (A)** The immunohistochemical staining (gray) of factor VIII indicates the new-born blood vessel in the myocardium. The new-born blood vessel (Shown by arrow) in the myocardium in the HUCBC-transplanted group was more than that in the single MI group and the PBS-treated group. A1-A3: Myocardium collected from the MI, PBS-treated, or HUCBC-transplanted rats, respectively (Bar=150μm); **(B)** The immunohistochemical staining (gray) of factor VIII indicates the new-born blood vessel in the small intestine. The new-born blood vessel (Shown by arrow) in the myocardium in the HUCBC-transplanted group was remarkably increased compared to the single MI group or the PBS-treated group. B1-B3: Small intestine collected from the MI, PBS-treated, or HUCBC-transplanted rats, respectively (Bar=150μm). $^{*}P<0.01$ vs. MI, $^{\dagger}P<0.01$ vs. PBS-treated.

tive, which is similar to human embryonic stem cells. Meanwhile, HUCBC had no significant stimulation effect on proliferation of lymphocyte in autologous mixed leukocyte reaction. Therefore, it was not necessary to use immunosupression in the presence of transplantation of allogeneic HUCBC.

Previously, Henning et al. (5) had investigated possibility of application of HUCBC in the treatment of acute myocardial infarction rats. Further, they found that about 1×10^6 HUCBC directly localized in the infarct border one hour after myocardial infarction in the absence of immunodepressant. Four months after the HUCBC transplantation, left ventricular function of the transplanted rats was significantly improved. In addition, no obvious rejection reaction could be found. Based on these above, they concluded that the HUCBC transplantation was safe in the absence of immunosupression during myocardial infarction.

However, in our study we obtained different results. After the HUCBC transplantation, intestinal GVHD occurred in 90% of the MI rats. Thus, we designed a series of the related experiments to investigate the mechanism for the HUCBC transplantation-induced intestinal GVHD. Further, with the prolongation of the transplantation time, the symptom became more and more serious although the cardiac functions were greatly improved.

The autopsy examination suggested that the distent site was circumscribed around ileum. The histology result showed apoptosis of crypt epithelial cells, dropout, ultimately disappearance of crypts, patchy lymphocytic infiltration, and edematous submucosa, suggesting the occurrence of intestinal GVHD.

GVHD is one of the important causes of morbidity and mortality after allogeneic stem cell transplantation. Usually, intestinal GVHD appears at the third or more week after the transplantation accompanied by related symptoms such as anorexia, nausea, vomiting, early satiety, abdominal pain, and diarrhea. The severity of symptoms is depended on the damage degree of small intestinal mucosa. Some evidences have shown that small intestine, particularly ileum, is often inclined to be an insulted segment of gut during GVHD (10-13), which are consistent with our findings.

However, intestinal GVHD also appeared in the PBS-treated MI rats and it was wilder than the HUCBC-transplanted rats. Broadly speaking, PBS treatment is also a xenogeneic stimulation for the MI rats. Thus, the PBS treatment also can cause wide GVHD such as intestinal GVHD. However, PBS treatment has no specific action targets as well as HUCBCs. Therefore, the intestinal GVHD induced by PBS-treated was not worse in comparison with the HUCBC-transplanted.

Vascular endothelial growth factor (VEGF) is a dimeric glycoprotein that can improve vascular permeability and induce proliferation and migration of endothelial cells to form new blood vessels. VEGF plays a key role in improving cardiac function after myocardial infarction during the events of stem cell transplantation (14-16). VEGF is also involved in a variety of chronic inflammatory diseases (17-20). VEGF may be one of principal factors regulating the immune reactions associated with acute and/or chronic GHVD, which is not consistent with the study of Lunn et al. (21). They reported that serum VEGF was very high in the GVHD patients. It was thought that VEGF was an efficient predictor for GVHD. Min et al. (22) confirmed that there was a correlation between early serum VEGF (from day 7 to day 14) and outcome after allogeneic stem cell transplantation. Low VEGF level was closely associated with non-relapse mortality and aggravation of acute GVHD.

In the present study we found that the HUCBC transplantation significantly upregulated the mRNA and protein levels of VEGF and increased vessel density in the impaired myocardium and distent small intestine. Meanwhile, we observed that VEGF played different roles in the heart and small intestine. In the heart, the engrafted cells release vascular growth factors to increase regional perfusion by either induction of new vessel formation or growth (23-25). In the small intestine VEGF not only increased regional perfusion but also participated in immunological reaction.

VEGF induces macrophage and leukomonocyte recruitment, and releases a variety of cytokines and growth factors. Bachmann et al. (26) found that it was local VEGF but not serum VEGF might be the predictor for GVHD. They analyzed the effect of tyrosine kinase inhibitors blocking VEGF receptors on genesis of

hemangima and lymphangion. Consequently, tyrosine kinase inhibitors restrained the formation of the afferent and efferent arms of immune reflex arc and promote graft survival after corneal transplantation.

In conclusions, the HUCBC transplantation significantly improves aggravated cardiac function of the MI rats, but it induces intestinal GVHD. Further, it may be associated with the upregulation of local VEGF.

Acknowledgement

This study was supported by Basic and clinical research cooperation fund, capital medical university (13JK57).

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