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Effect of human umbilical cord blood cells on Ang-II-induced hypertrophy in mice

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Abstract

We have assessed the capacity of human umbilical cord blood (hUCB)-derived stem cells to differentiate into cardiomyocytes and repair Angiotensin II induced insult in culture and in mouse hearts when injected. hUCB were able to differentiate into cardiomyocyte-like cells, when induced with 5-Azacytidine or co-cultured with rat neonatal cardiomyocytes (NRCM). When co-cultured, hUCB reversed the pathological effects induced by angiotensin II (Ang-II) in NRCM and in mice injected after Ang-II infusion. As assessed by increased heart weight to body mass ratio and Ang-II-induced fibrosis, cardiac hypertrophy was also reduced after hUCB were injected. hUCB also reversed the pathological heart failure markers induced by Ang-II in mice. Further, we observed a shift from pathological hypertrophy towards physiological hypertrophy by hUCB in Ang-II-challenged mice. Our findings support hUCB as a feasible model for experimentation in stem cell therapy and emphasize the relevance of the hUCB in reversing heart failure conditions.

Keywords

human umbilical cord blood cells; hypertrophy; angiotensin II; apoptosis; alpha myosin heavy chain; neonatal rat cardiomyocytes

Introduction

Cell transplantation is being explored as an alternative therapy for treating patients with end-stage heart failure. Fetal cardiomyocytes, skeletal myoblasts, immortalized cell lines, fibroblasts, smooth muscle cells, and hematopoietic stem cells have been transplanted into host myocardium for improved cardiac function [1]. A number of studies using stem cell transplants report the differentiation of stem cells in the heart of murine and rodent models as well as the improvement of heart function deficits caused by cardiac injury [2]. The use of mesenchymal stem cells derived from cord blood has become increasingly important in light of ethical controversies surrounding the use of embryonic stem cells and, as such, may be highly suitable

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for transplantation [3–7]. Mesenchymal stem cells treated with 5-azacytidine transdifferentiate into a cardiac phenotype *in vitro* [8]. Moreover, these stem cells can also differentiate into cardiomyocytes when injected into normal or acutely injured myocardium [9]. However, there has been little use of hUCB to improve cardiac function

In this study, we explored hUCB treatment to facilitate the rehabilitation of α -MHC mRNA expression and induction of the shift from a pathological hypertrophy induced by Ang-II to a physiological hypertrophy, therefore representing a potential therapeutic strategy in treating or preventing cardiac dysfunction resulting from decompensatory cardiac hypertrophy.

Materials and methods

All animal procedures in the present study conformed to the principles outlined in the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH Publication No. 85-23, revised 1996) and also approved by the UICOM-P Institutional Animal Care and Use Committee. Current study also conforms with the principles outlined in the Declaration of Helsinki (see Cardiovascular Research 1997;35:2–3).

Culture and *in vitro* differentiation of hUCB

Human umbilical cord blood was collected from healthy volunteers with informed consent and according to a protocol approved by the Institutional Review Board. Cord blood was enriched by sequential Ficoll density gradient purification followed by selection of cells with CD44⁺ markers. The nucleated cells were suspended at a concentration of 1×10^7 /mL in Knockout DMEM basal medium supplemented with 10% FBS, 10% knockout serum and 1% Penicillin-Streptomycin and plated in 100 mm culture dishes. Three days later the non-adherent cells were removed with medium replacement. After the cultures reached confluency, the cells were lifted by incubation with 0.25% trypsin and 1 mM EDTA at 37°C for 3 to 4 min. They were diluted at a ratio of 1:2 or 1:3 and replated and cultured at 37°C in an incubator with a 5% CO₂ atmosphere.

For *in vitro* differentiation, a 24 h acclimatization step was carried out by replacing the growth medium with pre-induction medium consisting of Knockout DMEM medium supplemented with 10% FBS, 10% Knockout serum, 1% Penicillin-Streptomycin, bFGF (10 ng/mL, Invitrogen, Carlsbad, CA). Cardiomyocyte differentiation was then initiated by incubating the cells in cardiogenic medium or pre-induction medium with 10 μ M 5-Azacytidine (5-Aza) (Sigma, St. Louis, MO). The cells were then observed for differentiation from 10 to 30 days.

Differentiation of hUCB in co-culture

hUCB were trypsinized and washed with PBS. Approximately 100 thousands of re-suspended cells were added directly into the 10⁶ NRCM cells. The culture were double immuno stained with c-kit and sarcomeric alpha actinin to detect the hUCB that are differentiated into myocytes. A ratio of 1:100 of hUCB to NRCM was maintained during the Angiotensin II (Ang-II) treatments.

Neonatal rat myocyte culture and hypertrophic stimulation

Primary cultures of cardiomyocytes from 1- to 3-day-old Sprague–Dawley rats were prepared as described previously [10]. Briefly, ventricular tissue was enzymatically dissociated, and the resulting cell suspension was enriched for cardiomyocytes by differential plating. Cells were plated onto collagen-coated culture dishes or cover slips and cultured in cardiomyocyte culture medium (M199 supplemented with 5% FBS, 10% Horse Serum, 1% Penicillin + Streptomycin (Invitrogen, Carlsbad, CA). To induce hypertrophy, cardiomyocytes were cultured with 100ng/mL Ang-II for 60 to 72 h.

Immunocytochemistry and hypertrophy assay

Cells were fixed with 4% paraformaldehyde and blocked by incubation in 5% BSA solution for 1 h, followed by anti- α -actinin antibody (Sigma, St Louis, MO), TRITC-conjugated goat anti-mouse IgG (Sigma, St Louis, MO) for 1 h each, and mounted with Vectashield mounting medium containing DAPI (Vector Labs, Burlingame, CA). In double immunofluorescence experiments anti-rabbit c-kit antibody was used and visualized by FITC-conjugated secondary antibody. Immunofluorescence was analyzed under a fluorescence microscope (Olympus BX61).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the hUCB and hUCB co-cultured with NRCM with RNeasy kit (Qiagen, Valencia, CA) and was converted to cDNA with a first-strand cDNA synthesis kit (Biorad, Hercules, CA) according to the manufacturer's recommendations. Online supplement Table 1 contains sequences for PCR primers used in the study.

Western blotting

Whole cell lysates were subjected to immunoblotting analysis for Bax, Bcl2, cleaved caspase 3 and caspase 9 (Upstate Biotechnology, Charlottesville, VA). Protein samples from left ventricular homogenates were also analyzed by immunoblotting to detect the levels of thyroid hormone receptors (TR), TR- α and TR- β isoforms (Cell Signaling Technology, Beverly, MA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

NRCM were cultured with or without hUCB on eight-well chamber slides at a density of 5×10^3 per well. 24 h later, 100 nM Ang-II was added to the culture medium and phosphate-buffered saline (PBS) was added to the control cells. At 72 h after treatment, the cells were fixed in 10% phosphate-buffered formalin for 15 min. TUNEL staining for detection of apoptotic cells was carried out using the TUNEL Apoptotic Detection kit (Upstate Biotechnology, Charlottesville, VA) as per the manufacturer's instructions. Briefly, the fixed cells were permeabilized with 0.05% Tween 20 in PBS containing 0.2% bovine serum albumin then incubated with 50 μ L of terminal deoxynucleotidyl transferase end-labeling cocktail. The slides were incubated with 50 μ L of avidin-FITC, and mounted with anti-fading gel mount (Biomed, Foster City, CA). Slides were observed under a fluorescent microscope (Olympus BX61), and photographed. Fluorescent apoptotic cells were quantitatively evaluated (10 randomly selected microscopic fields per sample) using Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

Ang-II infusion in mice and hUCB injections

Eight-week-old female nude mice were infused with vehicle or Ang-II ($400 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) with a mini-osmotic pump (ALZET model 2004; DURECT Corp, Cupertino, CA) that was implanted subcutaneously. Two weeks later, 2×10^6 hUCB were intravenously injected into mice. All experimental procedures on animals were performed with protocols approved by the Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria.

Histological analysis of hearts

Animals were sacrificed 28 days after Ang-II infusion. Hearts were quickly excised and weighed. Paraffin-embedded hearts were cut into 4 μ m slices. The sections were stained with hematoxylin-eosin solution (H&E) to measure the cell areas or with Masson's trichrome (MT) for interstitial fibrosis evaluation. To measure the surface area of cardiomyocytes, suitable cross sections with nearly circular capillary profiles and nuclei were selected. Sections were

observed under a phase contrast microscope (Olympus) and photographed. Fibrosis was quantified by the blue coloration in MT-stained sections. Cellular areas and fibrosis amounts were determined using NIH Image J software. Eight to ten sections from each heart were examined, and the results obtained from 8 hearts in each group were averaged.

Statistical analysis

Values are shown as mean $\pm$ SEM of at least three independent experiments. The significance of difference was estimated by ANOVA. $P < 0.5$ was considered significant.

Results

In vitro differentiation of hUCB into cardiomyocyte-like cells expressing cardiac-specific genes

hUCB were induced to differentiate and express cardiac-specific antigens by 24 h exposure to bFGF/5-Aza and further continuation in cardiogenic medium, hUCB started to morphologically change after 20 days in culture. Immunocytochemistry showed that differentiated cells were strongly stained with cardiac sarcomeric α -actinin and cardiac troponin I at 1 month after 5-Aza treatment (Fig. 1A). Only hUCB stained positive for c-kit but not the NRCM. hUCB also stained positive to α -actinin only when co-cultured with NRCM (Fig. 1B). RT-PCR analysis revealed the expression of various cardiac genes such as BNP, cardiac actin, NKx2.5, MLC2a, Myosin Heavy Chain only in co-cultured hUCB, but not in hUCB grown alone. NRCM cDNA was included as a negative control to confirm the specificity of the primers used (Fig. 1C). Primers for 18s RNA were used as loading controls. cDNA from human was included as a positive control.

hUCB inhibit the Ang-II-induced hypertrophic response in cells

NRCM cultured with or without hUCB were stimulated with Ang-II for 48–72 h. Hypertrophic response of cardiomyocytes was assayed as a function of pronounced sarcomeric rearrangement and ANF expression. Unlike the control NRCM, hUCB co-cultured NRCM lacked prominent sarcomeric organization after Ang-II treatment (Fig. 2A). Treatment with Ang-II significantly increased ANF immunoreactivity in control cardiomyocytes but not in hUCB co-culture (Fig. 2B). Real-time quantitative RT-PCR analysis of Ang-II-treated NRCM showed a general induction of pathological hypertrophic markers such as β -MHC, ANF, BNP and α -SK actin by over 3.5 fold compared to controls and an inhibition of the physiological hypertrophic marker α -MHC by half fold. Upon hUCB treatment, the Ang-II induced pathological hypertrophic markers were inhibited significantly ($p < 0.01$). Interestingly, we observed increased α -MHC expression in NRCM treated with Ang-II and hUCB, indicating a shift of pathological hypertrophy to physiological hypertrophy by hUCB (Fig. 2C).

hUCB inhibits Ang-II-induced apoptosis in NRCM

NRCM were cultured with or without hUCB in 8-well chamber slides and treated with 10 nM Ang-II. Control cells were treated with PBS instead of Ang-II. Figure 3A shows Ang-II-induced apoptosis in NRCM (67%) as seen by TUNEL positive cells. Addition of hUCB resulted in only 21% apoptotic cells despite Ang-II addition to the culture. Online supplement figure 1 shows a representative photomicrograph of tunnel positive cells in Ang-II treated NRCM. Western blot analyses showed a relative decrease in Bcl-2 and an increase in Bax expression in Ang-II-stimulated cells, compared with controls (Fig. 3B). Addition of hUCB reversed the Ang-II-induced effect on expression of Bcl-2 and prevented the increase in the expression of Bax. Ang-II challenged NRCM showed an increase in cleaved caspase 3 (17kDa) and 9 (38kDa) compared to controls. hUCB prevented the cleavage of caspase 3 and 9 (Fig. 3B).

hUCB reverses Ang-II-induced pathological hypertrophy and up-regulates α -MHC

As shown in Figure 4A) infusion of Ang-II resulted in a 33% increase in the heart weight to body weight ratio in control mice after 28 days. In contrast, hypertrophic growth was blunted significantly ($P<0.05$) in hUCB-implanted mice, with approximately 15% increase in the heart weight to body weight ratio. Cellular cross-sectional area was reduced significantly ($P<0.05$) in hUCB-injected mice (a 15% increase in Ang-II versus control) compared with control mice (a 46% increase in Ang-II versus control). Mice treated with Ang-II revealed a 6.47% fibrosis whereas, Ang-II induced fibrosis was reduced to 2.87% (a 55% reduction) when injected with hUCB. Immuno peroxidase staining of heart sections revealed the presence of human specific nestin only in the Ang-II challenged mice but not in others (Online Figure 2D). The supporting evidence showing representative heart sections are shown in online supplement figure 2.

Quantitative RT-PCR analysis showed over 2.5-fold induction of the pathological hypertrophic markers in general and 50% reduction in α -MHC isoform by Ang-II treatment. The Ang-II-induced hypertrophic markers were inhibited by hUCB implantation. Importantly there was an increase in the physiological hypertrophic marker α -MHC by hUCB treatment (Fig. 4B). Western blotting results from control, Ang-II, Ang-II + hUCB, and hUCB alone treated mouse heart lysates (50 μ g) showed an increased expression of thyroid hormone receptors TR- α and TR- β levels in hUCB injected Ang-II challenged mice (Fig. 4C).

Discussion

Our study demonstrated successful usage of human umbilical cord blood (hUCB)-derived cells in AngII induced pathological hypertrophic effects on heart. Usefulness of hUCB in treating pathological conditions also takes out the ethical controversies raised against the use of embryonic stem cells. We were able to transdifferentiate hUCB into cardiomyocytes both by chemical treatment and co-culture with neonatal rat cardiomyocytes (NRCM). Of note, conditioned medium from NRCM did not induce myocyte differentiation in hUCB (data not shown). Our results are consistent with other studies showing the differentiation ability of stem cells derived from various origins into cardiac myocytes [11,12]. Several publications enlist the movement and tropism of stem cells towards site of injury implicating various growth and paracrine factors [13–17]. Similarly this study shows homing of hUCB in the Ang-II-treated mice hearts by vascular injections making this a less invasive method of delivery of hUCB.

Re-expression of fetal genes, increased cellular size and interstitial fibrosis are hallmarks of pathological hypertrophy leading to dilated cardiomyopathy (see for a review [18]). Here, we have shown the potential of hUCB to inhibit the pathological effects exerted by Ang-II on hypertrophy first by reduced sarcomeric organization, ANF expression, apoptosis and the cleavage of caspases 3 and 9 in isolated myocyte cultures. Later hUCB injected into mice inhibited the hypertrophy caused by Ang-II by reducing heart wt to body ratio, cellular cross sectional area and fibrosis. Further, we have shown that hUCB were capable of switching the pathological hypertrophy induced by Ang-II towards more useful physiological hypertrophy by inhibiting β -MHC and inducing α -MHC in the presence of Ang-II both in culture as well as in mice. Expression α MHC has been linked to physiological hypertrophy and shown to have beneficial effects on cardiac fitness [19–21]. In this context the encouraging increase of α -MHC by hUCB after Ang-II treatment indicates usefulness of hUCB to induce the shift from a pathological hypertrophy to physiological hypertrophy, to improve cardiac contractile function, increase the cardiac fitness and to prevent the progression of hypertrophy towards detrimental heart failure.

Limitations of the study

Although, we have successfully demonstrated the beneficial use of hUCB in reversing the pathological effects of cardiac injury, even when injected 2 weeks after Ang-II infusion in mice, this study lacks the mechanistic details of why and how the hUCB migrate towards only the Ang-II challenged hearts and how the molecular switch from α to β MHC occurs by hUCB addition. These questions were attempted to address by analyzing the ability of hUCB to reduce the Ang-II effects by inhibiting the pro-apoptotic Bax expression and inducing the anti-apoptotic Bcl 2 expression. Further, we have observed a decrease in the TR- α as well as TR- β levels in the Ang-II treated mice. This inhibition of TRs expression was reversed and resulted in higher level of TRs expression when injected with hUCBs in the Ang-II treated mice. The reversal of TRs inhibition might be the crucial juncture of induction of alpha MHC by hUCB and switch the pathological to physiological hypertrophy. Thyroid hormone receptors have been shown to induce the alpha myosin heavy chain isoform which is implicated in physiological hypertrophy [22]. Since, these results only indicate the reversal of hypertrophy form; we are further investigating the mechanisms involved in this switch.

Our results are consistent with the hypothesis that hUCB-derived stem cells migrate to and participate in the repair and recovery of stimuli-induced cardiac dysfunction. We continue to study the long-term survival and effects of hUCB on cardiac function and the underlying molecular events that allow hUCB to transform heart failure into cardiac fitness.

In conclusion, this study demonstrates for first time, the ability of hUCB to migrate and integrate with Ang-II challenged hearts as well as their potential in converting pathological hypertrophy into physiological hypertrophy, which may be extended to other types of stimuli that induce hypertrophy and subsequently lead to heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

hUCB	human umbilical cord blood stem cells
Ang-II	angiotensin II
5-Aza	5-Azacytidine
NRCM	neonatal rat cardiomyocytes

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Figure 1.

Human umbilical cord blood-derived cells differentiate into cardiomyocytes and express cardiac-specific markers. A) hUCB treated with 5-azacytidine (10 μ M) and grown in bFGF-supplemented medium were subjected to immunofluorescent staining with anti α -sarcomeric actinin and anti-troponin antibodies after 30 days in culture. NRCM are shown as positive controls. B) hUCB, NRCM and a co-culture of hUCB with NRCM were established in chamber slides. Three days later, co-cultures were immunostained for c-kit (green) and α -sarcomeric actinin (red). Top panel shows only hUCB (green), middle panel shows both NRCM and differentiated hUCB (red), and the bottom panel is the merge of both top and middle panels. C) Total RNA isolated from hUCB cultured with or without NRCM was subjected to RT-PCR analysis using human-specific primers for cardiac markers. Total RNA from human and rat heart were included as positive and negative controls respectively. Primers for 18s RNA were designed to amplify a 200 bp product from both human and rat RNA.

Figure 2.

hUCB reverse Ang-II-induced hypertrophy and apoptosis in NRCM. Cardiomyocytes isolated from neonatal rats treated with Ang-II (100 nM) were co-cultured with or without hUCB. A) Cells were immunostained for α -sarcomeric actinin. Induction of hypertrophy was determined as function of striated vs. punctate sarcomeric organization. B) Cells stained for ANF polypeptide. Hypertrophy was determined as function of perinuclear staining. C) Total RNA isolated from NRCM treated with Ang-II and cultured with or without hUCB was subjected to quantitative RT-PCR analysis using rat-specific gene primers for alpha and beta MHC, ANF, BNP and α -SK actin to measure the hypertrophic markers induced by Ang-II and its reversal by hUCB. Data represents average of triplicates normalized to β -actin, GAPDH, and 36B4 house keeping genes (* $p < 0.01$).

Figure 3.

hUCB reverse the apoptosis induced by Ang-II in NRCM A) Quantitative representation of average of ten microscopic fields of TUNEL positive cells. B) NRCM were stimulated with Ang-II for 24 h, followed by western blot analysis of 50 μ g proteins from each sample using anti-Bax, Bcl-2, caspase-3 and caspase-9 antibodies.

Figure 4.

hUCB reverse pathological effects induced by Ang-II in mice. Nude mice were infused with PBS (control) or Ang-II ($400 \text{ ng kg}^{-1} \text{ min}^{-1}$) for two weeks. Infusions were continued by injecting mice with hUCB (hUCB and Ang-II+hUCB) or PBS for two more weeks. A) Heart wt/body wt ratio expressed in mg/gm of whole heart and body mass measured at the termination of experiment (left), quantification of cellular areas measured from hematoxylin and eosin stained sections using Image J software (middle) and quantification of the % of interstitial fibrosis using Masson's trichrome-stained tissue sections four weeks after Ang-II infusion (right). * = $p < 0.05$ and # = $p < 0.01$. B) Quantitative RT-PCR analysis showing the expression of hypertrophic markers using mouse-specific primers in total RNA obtained from left ventricular tissue of mice treated with vehicle, Ang-II, Ang-II + hUCB, hUCB alone 28 days after Ang-II infusion. House keeping genes 36B4, GAPDH, and β -actin were used for normalization. These results are representative of three separate experiments. C) Western blot analysis of whole cell lysates from above treatments showing expression of TR- α and TR- β levels. GAPDH was used as loading control.