Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 325 (2004) 665–669
BBRC
www.elsevier.com/locate/ybbrc

Effect of human umbilical cord blood cells on glycemia and insulitis in type 1 diabetic mice

Norman Ende, Ruifeng Chen, Alluru S. Reddi*

Department of Pathology and Laboratory Medicine, UMDNJ-New Jersey Medical School, 185 South Orange Ave, Newark, NJ 07103, USA

Received 15 October 2004
Available online 5 November 2004

Abstract

Several studies have shown that transplantation of embryonic stem cells into diabetic animals either improved or normalized blood glucose levels. In this study, we examined the dose-dependent effect of early (prediabetic stage) intravenous administration of human umbilical cord blood (HUCB) mononuclear cells on blood glucose levels, survival, and insulitis in nonobese diabetic (NOD) mice with autoimmune type 1 diabetes. The results show that mice treated with HUCB cells significantly lowered their blood glucose levels and increased their lifespan, as compared with untreated mice. Also, a significant reduction in insulitis was observed in treated than in untreated mice. The mice that received the highest dosage (200×10^6) of cells had greater reduction in blood glucose levels and the degree of insulitis than the mice that received lower dosage ($100–150 \times 10^6$) of cells. Prolonged lifespan in the former group of mice seems to be related to better control of blood glucose levels. Thus, administration of HUCB cells in the prediabetic stage without any immunosuppression improves type 1 diabetes by protecting the islets from insulitis in NOD mice.

Keywords: Human umbilical cord blood cells; Stem cells; Blood glucose; Type 1 diabetes; Insulitis; NOD mice

Embryonic stem (ES) cell therapy for diabetes mellitus has received considerable attention in recent years [1–3]. These ES cells from multiple sources differentiate into insulin-producing cells [4–13] and improve glycemia in animal models of diabetes. Soria et al. [14] initially implanted one million mouse ES-derived insulin-secreting cells into the spleen of streptozotocin diabetic mice, and found normalization of blood glucose levels within a week. Subsequently, several other investigators [15–19] reported improvement or reversal of hyperglycemia by stem cell therapy. In addition, Stepanovic et al. [20] demonstrated wound healing in type 2 diabetic mice following mouse bone marrow cells injected under skin wounds. Thus, ES cells from different sources can improve glycemia as well as wound healing in diabetes.

In addition to ES cells, mononuclear cells derived from human umbilical cord blood (HUCB) were also found to differentiate into cells that improve a variety of disease conditions in animals [21]. In a preliminary study, transplantation of HUCB mononuclear cells into type 1 and type 2 diabetic mice improved not only their glycemia but also survival [22,23]. In these studies, we used a fixed dose of mononuclear cells. Furthermore, the mechanism by which these cells improve glycemia is unknown. We, therefore, extended our study to demonstrate that improvement in glycemia is dose-dependent and related to improvement in insulitis of the pancreatic islets in nonobese diabetic (NOD) mice. These mice develop type 1 diabetes later in life due to autoimmunity.

Materials and methods

Animals. Fifty-five female NOD mice, obtained from the Jackson laboratory, were used for the study. These mice are characterized by

* Corresponding author. Fax: +1 973 972 3578.
E-mail address: reddias@umdnj.edu (A.S. Reddi).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.10.091

insulitis and marked decrease in pancreatic insulin content, which occurs around 12 weeks of age in females. The onset of diabetes is marked by glucosuria and nonfasting glucose levels >250 mg/dl. The mice were divided into three groups. One group of 25 mice received $100–150 \times 10^6$ HUCB mononuclear cells retro-orbitally into the venous plexus. Another group of five mice received 200×10^6 cells in a similar fashion. The third group ($N = 25$ mice) did not receive any cells and served as controls (untreated group). The age of mice on injection varied from 7 to 9 weeks depending on the availability of the cord blood. Urine glucose was tested with Diastix reagent strips (Bayer, Elkhart, IN) weekly, and blood for glucose was obtained from the orbital plexus at the time of death and sacrifice. Glucose levels were determined by the glucose oxidase method using the reagents supplied by Sigma Chemical (St. Louis, MO). The mice were followed for a mean of 148 days. Treated mice did not receive any immunosuppression. All mice were fed Purina rodent chow 5001 ad libitum and allowed to drink tap water. Procedure for the collection and use of HUCB for this study was approved by the Institutional Review Board of New Jersey Medical School, Newark, NJ. The mice were housed in an AAALAC-1 approved animal facility, and the project was approved by the Institutional Animal Review Committee.

Collection and preparation of human cord blood cells. HUCB samples were obtained from placentas of healthy full-term neonates. Each cord blood sample was collected into a 50 ml sterile polypropylene test tube containing 5 ml of citrate phosphate dextrose as an anticoagulant. The volume collected varied from 20 to 40 ml, and the samples were kept at room temperature until they were sent to the blood bank for storage. The samples were then transferred into a polyolefin blood collection bag (Cryocyte Freezing Container, Baxter Healthcare, Deerfield, IL) that allows gaseous transfer and were stored at 4 °C in a blood bank refrigerator. Donor specimens were combined according to their blood type (ABO). After storage for 10–13 days, units were placed in a 15 ml disposable centrifuge tube and the mononuclear cells were separated from the whole cord blood by Ficoll–Hypaque (Sigma, St. Louis, MO) density gradient centrifugation. The cells were then washed twice with phosphate-buffered saline (PBS) and centrifuged for 10 min at 1000 rpm. One milliliter of PBS was added to the pellet for counting. After the viability and counting were determined, the mononuclear cells were centrifuged for 10 min at 1000 rpm, then 0.2 ml of PBS solution was added for final dilution and injection into the mouse (retro-orbital). This process was repeated the next day to bring the total number of mononuclear cells given to the mice up to the desired number of either $100–150 \times 10^6$ or 200×10^6.

Analysis of human growth hormone expression by RT-PCR. In order to identify the distribution of HUCB cells in transplanted mice, total RNA was isolated from blood, heart, lungs, brain, liver, spleen, pancreas, kidney, and bone marrow tissues of control and treated mice as well as from HUCB cells by guanidine isothiocyanate method [24]. The extracted RNAs were purified by RNAeasy columns (Qiagen, Valencia, CA). cDNA synthesis was performed by reverse transcription using Superscript (Invitrogen) or MMLV or AMV (Promega) reverse transcriptase enzyme. HGH gene was amplified by PCR (Perkin–Elmer, Foster city, CA) using 5′-TGT CTT CCC ACC AT TCC TCC CTT A-3′ and 5′-CCA CTC ACG GAT TTC TGT TGT GTT TC-3′ primers to produce 434 bp fragment. Similarly HPRT housekeeping gene was amplified using HPRT specific primers as positive control for PCR amplification. The PCRs were carried out in GeneAmp 9600 PCR system (Applied Biosystems) with initial denaturation at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 5 min. PCR products were run on 1.5% agarose gels on a horizontal electrophoresis apparatus and visualized with ethidium bromide on a flourimager (Molecular Dynamics, Sunnyvale, CA). Band densities were analyzed using Image Quant software.

Grading of insulitis. On death or sacrifice (under O_2/CO_2 anesthesia), portions of the pancreas were fixed from all animals in 10% buffered formalin, 3 μm thick sections were prepared and stained with hematoxylin and eosin for light microscopy. Since the number of islets varied in different portions of the pancreas and insulitis was not uniform in all the animals, we counted and graded all the islets (65 in untreated; 73 and 30 in low and high dose recipients, respectively) found on the histologic sections. The islets were graded from 1+ to 4+ according to the infiltration of mononuclear cells in and surrounding the islets. An islet graded 1+ was either normal (no infiltration) or had only rare infiltration with mononuclear cells. A grade of 4+ had a large cuff of mononuclear cells surrounding or obliterating the islet. Gradings of 2+ and 3+ were considered intermediate grades.

Statistical analysis. All data were analyzed by one-way analysis of variance. Results are expressed as means ± SD; a P value <0.05 was considered significant. Survival curves were plotted using the Kaplan–Meier method.

Results

Before injection of HUCB cells, the blood glucose levels were 112 ± 46 mg/dl in all mice. Fig. 1 shows the blood glucose levels in untreated and HUCB-treated groups at time of death and sacrifice. As evident, the glucose levels were significantly lower in treated than in untreated mice. Among treated mice, the glucose levels were significantly much lower in those mice that received the highest dosage of HUCB cells (untreated: 440 ± 68; treated ($100–150 \times 10^6$): 310 ± 82; and treated (200×10^6): 170 ± 42 mg/dl).

Fig. 2 shows Kaplan–Meier survival curves in untreated and treated mice. As shown, the survival of untreated mice was greatly reduced compared to that of treated mice. Among treated mice, only those that received the highest dose (200×10^6) of HUCB cells did not die prior to sacrifice.

Table 1 demonstrates the histologic grading of islet cells for the presence of insulitis in untreated and treated mice. About 58% of islets from untreated mice showed 3+ and 4+ insulitis compared to 32% and 3% in treated mice that received $100–150 \times 10^6$ and 200×10^6 HUCB cells, respectively. About 97% of the islets of mice that received 200×10^6 HUCB cells had either no or <1+ insulitis.

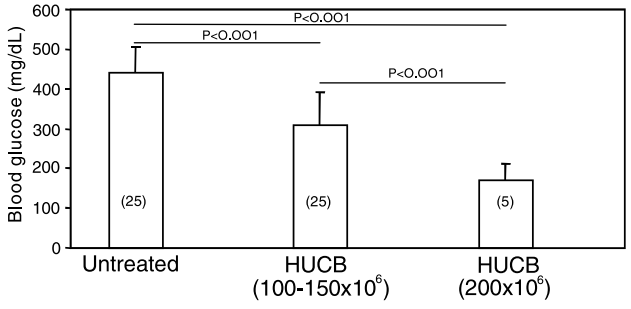

Fig. 1. Blood glucose levels (mg/dl) at time of death or sacrifice in untreated diabetic mice and diabetic mice that received various dosages of HUCB mononuclear cells. Data shown are means ± SD. Numbers in bars represent number of animals.

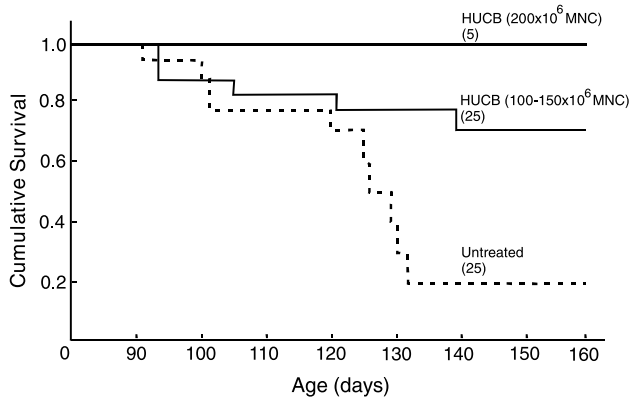

Fig. 2. Kaplan–Meier survival curves for untreated diabetic mice and diabetic mice that received various dosages of HUCB mononuclear cells. Numbers in parentheses represent number of animals. $P < 0.01$–0.001 between untreated and treated diabetic mice.

Table 1
Histologic grading of islets for insulitis in untreated and human umbilical cord blood (HUCB) cell-treated diabetic mice

Group	Insulitis grading (%)	
	1–2+	3–4+
Untreated[b]	42 (23)[a]	58 (42)
HUCB cells ($100–150 \times 10^6$)	68 (42)	32 (31)
HUCB cells (200×10^6)	97 (29)	3 (1)

[a] Number in parentheses represents number of islets examined.
[b] Untreated vs HUCB cells ($100–150 \times 10^6$): $P < 0.05$; untreated vs HUCB cells (200×10^6): $P < 0.01$.

No evidence of total RNA for HGH was found in any organ of the treated mice that died prior to the termination of the study. However, total RNA was detected in the spleen of two surviving mice that received 200×10^6 cells and another mouse that received $100–150 \times 10^6$ cells. The survival rate in these mice was much higher than those that did not demonstrate HGH mRNA.

Discussion

This study demonstrates for the first time a dose-dependent decrease in blood glucose levels in NOD mice that received HUCB mononuclear cells. This blood glucose-lowering effect of HUCB cells seems to be due to improvement in insulitis in these mice. Reversal of autoimmune diabetes has also been reported in NOD mice by stem cell therapy [15,16,19]. Thus, HUCB cells exert their glucose-lowering effect similar to those of other stem cells in streptozotocin-induced as well as autoimmune diabetes in animals [3,14,17,18].

Type 1 diabetes is an autoimmune disease [25], and the NOD mouse represents an animal model of this type of diabetes. Immunotherapy has been shown to prevent the development of diabetes if treatment is started in the prediabetic stage or early after the diagnosis of diabetes [26,27]. This prevention of diabetes seems to be due to preservation of β cell function via possible abolition of insulitis. In our study, the mice that received the highest dose (200×10^6) of HUCB cells prior to the development of hyperglycemia had better control of their blood glucose levels because of preservation of β cell function via prevention of insulitis. In these mice, the appearance of glucosuria was also significantly delayed compared to untreated mice (data not shown).

The survival of untreated mice was much shorter than those of both groups of treated mice. Among treated mice, the survival was dose-dependent. None of the mice receiving the highest dose died, which may be attributed to better control of glycemia. Although insulin levels were not measured in any of the mice, we assume recovery of β cell function as shown by less insulitis. Thus, transplantation of high doses of HUCB cells into NOD mice normalizes survival.

The mechanisms for improvement in both blood glucose levels and insulitis in NOD mice are not apparent from the current study. However, it is possible that administration of HUCB cells may have directly modulated the immune response causing insulitis. It is of interest to demonstrate whether or not some of the HUCB cells would transform into insulin-producing cells in addition to abolition of autoimmunity. Such demonstration will have a major impact in the management of type 1 diabetes.

Transplantation of HUCB mononuclear cells into mice was found to improve not only glycemia [22,23] but also the clinical condition of mice with lupus [28], amyotrophic lateral sclerosis [29,30], as well as stroke [31] and brain injury [32] in rats. In these studies, the pluripotential capacity of the HUCB cell or cells was not identified. However, a recent study showed that the CD45-negative population of the HUCB cells has the potential to differentiate into osteoblasts, chondroblasts, adipocytes, and hematopoietic and neural cells including astrocytes and neurons that express neurofilament, Na^+ channel protein, and various neurotransmitter phenotypes [33]. These CD45-negative cells were also found to differentiate into albumin-producing hepatocytes and cardiomyocytes when transplanted into fetal sheep [33]. Thus, HUCB mononuclear cells can be used as another modality of stem cell therapy to prevent a variety of disease states, including diabetes.

It is also interesting to note that the transplanted mice did not demonstrate any clinical or histologic evidence of either acute or chronic graft-vs-host disease. Also, no other evidence of adverse effects was noted in transplanted mice.

In conclusion, transplantation of HUCB mononuclear cells into NOD mice with autoimmune type 1diabetes improves blood glucose levels and survival in a

dose-dependent fashion without any evidence of graft-vs-host disease. This improvement in glycemia is related to improvement in insulitis. Further work is needed to examine the mechanisms by which insulitis is prevented by transplantation of HUCB mononuclear cells in NOD mice.

Acknowledgments

This study was supported by the Abraham S. Ende Research Foundation. We thank Dr. Milton Ende, Ms. Adaline Wood, and Ms. Yolanda Mohammed as well as the Obstetrics staff of the Southside Regional Medical Center, Petersburg, Virginia. The assistance of Mr. Sunil Kuppasani is greatly appreciated.

References

[1] B. Soria, A. Skoudy, E. Martin, From stem cells to beta cells: new strategies in cell therapy of diabetes mellitus, Diabetologia 44 (2001) 407–415.
[2] S. Bonner-Weir, Stem cells in diabetes: what has been achieved, Horm. Res. 60 (Suppl. 3) (2003) 10.
[3] M.A. Hussain, N. Theise, Stem-cell therapy for diabetes mellitus, Lancet 364 (2004) 203–205.
[4] A. Vinik, R. Rafaeloff, G. Pittenger, L. Rosenberg, W. Duguid, Induction of pancreatic islet neogenesis, Horm. Metab. Res. 29 (1997) 278–293.
[5] V.K. Ramiya, M. Maraist, K.E. Arfors, D.A. Schatz, A.B. Peck, J.G. Cornelius, Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells, Nat. Med. 6 (2000) 278–282.
[6] S. Assady, G. Maor, M. Amit, J. Itskovitz-Eldor, K.L. Skorecki, Insulin production by human embryonic stem cells, Diabetes 50 (2001) 1691–1697.
[7] N. Lumelsky, O. Blondel, P. Laeng, I. Velasco, R. Ravin, R. McKay, Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets, Science 292 (2001) 1389–1394.
[8] Y. Moritoh, E. Yamato, Y. Yasui, S. Miyazaki, J.-I. Miyazaki, Analysis of insulin-producing cells during in vitro differentiation from feeder-free embryonic stem cells, Diabetes 52 (2003) 1163–1168.
[9] A. Ianus, G.G. Holz, N.D. Theise, M.A. Hussain, In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion, J. Clin. Invest. 111 (2003) 843–850.
[10] D. Kim, Y. Gu, M. Ishii, M. Fujimiya, M. Qi, N. Nakamura, T. Yoshikawa, S. Sumi, K. Inoue, In vivo functioning and transplantable mature pancreatic islet-like cell clusters differentiated from embryonic stem cell, Pancreas 27 (2003) e34–e41.
[11] H. Kozima, M. Fuzimiya, K. Matsumura, T. Nakahara, M. Hara, L. Chan, Extrapancreatic insulin-producing cells in multiple organs in diabetes, Proc. Natl. Acad. Sci. USA 101 (2004) 2458–2463.
[12] S. Miyazaki, E. Yamamoto, J.-I. Miyazaki, Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells from embryonic stem cells, Diabetes 53 (2004) 1030–1037.
[13] H. Segev, B. Fishman, A. Ziskind, M. Shulman, J. Iskovitz-Eldor, Differentiation of human embryonic stem cells into insulin-producing clusters, Stem Cells 22 (2004) 265–274.
[14] B. Soria, E. Roche, G. Berná, T. León-Quinto, J.A. Reig, F. Martin, Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice, Diabetes 49 (2000) 157–162.
[15] S. Ryu, S. Kodama, K. Ryu, D.A. Schoenfeld, D.L. Faustman, Reversal of established autoimmune diabetes by restoration of endogenous β cell function, J. Clin. Invest. 108 (2001) 63–72.
[16] S. Kodama, W. Kühtreiber, S. Jujimura, E.A. Dale, D.L. Faustman, Islet regeneration during the reversal of autoimmune diabetes in NOD mice, Science 302 (2003) 1223–1227.
[17] R. Shah, R.M. Jindal, Reversal of diabetes in the rat by injection of hematopoietic stem cells infected with recombinant adeno-associated virus containing the preproinsulin II gene, Pancreatology 3 (2003) 422–428.
[18] M. Zalman, S. Gupta, R.K. Giri, I. Berkovich, B.S. Sappal, O. Karnieli, M.A. Zern, N. Fleisher, S. Efrat, Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells, Proc. Natl. Acad. Sci. USA 100 (2003) 7253–7258.
[19] T.D. Zorina, V.M. Subbotin, S. Bertera, A.M. Alexander, C. Haluszczak, B. Gambrell, R. Bottino, A.J. Styche, M. Trucco, Recovery of the endogenous β cell function in the NOD model of autoimmune diabetes, Stem Cells 21 (2003) 377–388.
[20] V. Stepanovic, O. Awad, C. Jiao, M. Dunnwald, G.C. Schatteman, Lepr^{db} diabetic mouse bone marrow cells inhibit wound vascularization but promote wound healing, Circ. Res. 92 (2003) 1247–1253.
[21] N. Ende, The Berashis cell: a review. Is it similar to the embryonic stem cell? J. Med. 31 (2000) 113–129.
[22] N. Ende, R. Chen, R. Mack, NOD/LtJ type 1 diabetes in mice and the effect of stem cells (Berashis) derived from human umbilical cord blood, J. Med. 33 (2002) 181–187.
[23] N. Ende, R. Chen, A.S. Reddi, Transplantation of human umbilical cord blood cells improves glycemia and glomerular hypertrophy in type 2 diabetic mice, Biochem. Biophys. Res. Commun. 321 (2004) 168–171.
[24] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[25] G.S. Eisenbarth, Type 1 diabetes mellitus: a chronic autoimmune disease, N. Engl. J. Med. 314 (1986) 1360–1368.
[26] K.C. Herold, W. Hagopian, J.A. Auger, E. Poumian-Ruiz, L. Taylor, D. Donaldson, S.E. Gitelman, D.M. Harlan, D. Xu, R.A. Zivin, J.A. Bluestone, Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus, N. Engl. J. Med. 346 (2002) 1692–1698.
[27] P.G. Stock, J.A. Bluestone, Beta-cell replacement for type 1 diabetes, Annu. Rev. Med. 55 (2004) 133–156.
[28] N. Ende, J. Czarneski, E. Raveche, Effect of human cord blood transfer on survival and disease activity in MRL-Lpr/Lpr mice, Clin. Immunol. Immunopath. 75 (1995) 190–195.
[29] R. Chen, N. Ende, The potential for the use of mononuclear cells from human umbilical cord blood in the treatment of amyotrophic lateral sclerosis in SOD1 mice, J. Med. 31 (2000) 21–30.
[30] S. Garbuzova-Davis, A.E. Willing, T. Zigova, S. Saporta, E.B. Justen, J.C. Lane, J.E. Hudson, N. Chen, C.D. Davis, P.R. Sanberg, Intravenous administration of human umbilical cord blood cells in a mouse model of amyotrophic lateral sclerosis: distribution, migration, and differentiation, J. Hematother. Stem Cell Res. 12 (2003) 255–270.
[31] J. Chen, P.R. Sanberg, Y. Li, L. Wang, M. Lu, A.E. Willing, R. Sanchez-Ramos, M. Chopp, Intravenous administration of human umbilical cord blood reduces behavioral deficit after stroke in rats, Stroke 32 (2001) 2682–2688.
[32] D. Lu, P.R. Sanberg, A. Mahmood, Y. Li, L. Wang, R. Sanchez-Ramos, M. Chopp, Intravenous administration of human

umbilical cord blood reduces neurological deficit in the rat after traumatic brain injury, Cell Transplant. 11 (2002) 275–285.

[33] G. Kögler, S. Sensken, J.A. Airey, T. Trapp, M. Müschen, N. Feldhahn, S. Liedtke, R.v. Sorg, J. Fisher, C. Rosenbaum, S. Greschat, A. Knipper, J. Bender, O. Degistirici, J. Gao, A.I. Caplan, E.J. Colletti, G. Almeida-Porada, H.W. Müller, E. Zanzani, P. Wernet, A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential, J. Exp. Med. 200 (2004) 123–135.