Administration of Human Umbilical Cord Blood Cells Produces Interleukin-10 (IL-10) in IL-10 Deficient Mice Without Immunosuppression

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Abstract: Recent studies from our laboratory have shown that intravenous administration of human umbilical cord blood (HUCB) mononuclear cells to mice improved blood glucose levels, atherosclerosis and prostate cancer. In this study, we examined the effect of HUCB cells on the production of IL-10 levels in IL-10 knockout mice. It has been proposed that administration of IL-10 may be beneficial in the treatment of inflammatory bowl disease. The results show that mice treated with HUCB cells ($100x10^6$) produce IL-10, as demonstrated by both qualitative and quantitative analyses, and that the levels of this cytokine persisted until the mice were sacrificed (5.5 months after administration). Immunohistochemical staining of the intestine using HuNu antibody cocktail demonstrated the presence of HUCB cells in the knockout mouse. Although the mice did not receive any immunosuppression, there was no evidence of graft-versus-host disease. Our data suggest that HUCB cells are capable of producing IL-10, and the use of these cells or HUCB may be indicated in the treatment of certain human diseases.

Keywords: Human umbilical cord blood, plasma IL-10 levels, IL-10 knockout mice, stem cells.

INTRODUCTION

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that downregulates tumor necrosis factor-α, and IL-6 production [1,2]. It also regulates growth and differentiation of several cells, including B cells, cytotoxic and helper T cells, NK cells, mast cells, dendritic cells, granulocytes as well as endothelial cells. IL-10 was originally isolated from T_H2 cells as a cytokine synthesis inhibitor factor. It was subsequently demonstrated that many other cell types produce IL-10 [1,2]. IL-10 knockout mice develop IBD [3,4], and it has been proposed that IL-10 is a useful agent in the treatment of this disease [5]. Recently, Srivastava *et al.* [6] demonstrated amelioration of piroxicam-induced colitis in IL-10 knockout mice by embryonic stem cell therapy. However, it has not been demonstrated whether or not HUCB cells produce IL-10 *in vivo*. The objective of this study was to determine the ability of HUCB mononuclear cells to generate IL-10 production in IL-10 deficient mice and without impairment in body growth.

MATERIALS AND METHODS

Animals

A total of 22 IL-10-/-knockout (C57BL/6 IL-10 ^{tm1Cgn} mice (Jackson Laboratory, Bar Harbor, ME) were used for the study. These mice usually develop advanced IBD by 260 days. These mice were divided into 2 groups. One group of 12 mice received an injection of $100x10^6$ HUCB mononuclear cells at 66 days of age retro-orbitally into the venous plexus. The other group of 10 mice did not receive HUCB cells, and served as controls. Treated mice did not receive any immunosuppressive agents. All mice were fed Purina rodent chow 5001 ad libitum and allowed to drink tap water until sacrifice. Blood from the orbital plexus was drawn at 1 and 5.5 months after injection of HUCB cells. The mice were housed in an AAALAC-1 approved animal facility, and the project was approved by the Institutional Animal Review Committee.

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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 4°C for 48-72 hours until they were transported overnight to the laboratory. The samples were then transferred into a polyolefin blood collection bag (Cryocyte Freezing Container, Baxter Healthcare, Deerfield, IL) that allows gaseous transfer. 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 minutes at 1000 rpm. One ml of PBS was added to the pellet for counting. After the viability and counting were determined, the mononuclear cells were centrifuged for 10 minutes at 1000 rpm, then 0.2 ml of PBS solution was added for final dilution and injection into the mouse (retro-orbital).

Measurement of Plasma IL-10

The levels of human IL-10 were determined using the commercially available Quantikine Human IL-10 enzyme-linked immunosorbent assays (ELISA), (R&D Systems, Minneapolis, MN). Upon sacrificing the mice, plasma was collected with heparin, spun immediately for 15 min at 1000x g and frozen at -20C. Samples were thawed, measured in duplicate, and the corresponding optical density was compared to a standard curve generated from each plate with human and mouse IL-10 standards provided by the manufacturer. A monoclonal antihuman IL-10 antibody was precoated on 96-well plates and developed with monoclonal antibody conjugated to horseradish peroxidase (R&D Systems, Minneapolis, MN). This ELISA is sensitive to detect 7.8 pg/ml IL-10. The absorbance (OD450) was measured on a Dynatech plate reader (Dynatech Laboratories, Chantilly, VA). IL-10 (pg/ml) levels were calculated after extrapolation from the standard curves using Excel (Microsoft, Redmond, WA). **Data for IL-10 levels were presented as difference between the treated and untreated mice**.

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DNA Isolation

In order to identify the distribution of HUCB cells in transplanted mice, genomic DNA was extracted from mouse bone marrow, lung, liver, kidney, spleen and gut using the DNeasy Blood & Tissue Kit (Qiagen, I – Valencia, CA). RT-PCR was used to detect human β-globin DNA in tissues.

Western Blot Analysis

Isolated cells from spleen of mice treated with HUCB cells for 5.5 months .were used for Western blot analysis. Cells were washed with cold PBS and were, then subsequently lysed in ice-cold 1% nonidet P-40 (NP-40) and lysis buffer. The lysis buffer was (10 mM Tris (tris [hydroxymethyl] aminomethane-HCl, pH 7.4). Next the following were added: 5 mM EDTA (ethylenediaminetetraacetic acid), 150 mM NaCl, 0.1% SDS (sodium dodecyl sulfate), 1% sodium deoxycholate containing 1:40 dilution of protease inhibitor cocktail for mammalian cells and 1:50 dilution of phosphatase inhibitor cocktail 2 (both from Sigma-Aldrich, St. Louis, MO). The protein concentration of each cell lysate was determined with the RC and DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on Immobilon-P polyvinylidene difluoride membranes (Millipore, Bradford, MA). The amount of protein that was loaded was 30 µg/lane. Membranes were blotted at 4^{o} C overnight with the following primary antibody (mouse anti human IL-10). Immunodetection was done with anti-mouse IgG horseradish peroxidase (HRP)-linked antibody (both from New England BioLabs) and the ECL Plus (Enhanced Chemiluminescence) detection system (Amersham Biosciences) with BioMax MR films (Eastman Kodak, Rochester, NY). Protein bands were quantified by laser densitometry and analyzed by ImageQuant software (Amersham Biosciences).

Histology

At time of sacrifice, various tissues were removed, fixed in formalin and stained with hemotoxylin & eosin for evaluation of IBD. For identification of the HUCB cells in the intestine, immunohistochemical staining was used with a HuNu antibody cocktail (courtesy of Dr. S.Garbuzova-Davis).

Statistical Analysis

All data are expressed as mean ± SEM. Student's t-test was used to calculate the significance between treated and untreated mice and a p-value <0.05 was considered significant.

RESULTS

Fig. (**1**) shows body weights of treated and untreated mice after 1 and 5.5 months of HUCB cell ($100x10^6$) administration. As evident, there was no difference in weights between treated and untreated mice which suggests that the HUCB mononuclear cells were well tolerated. The effect of HUCB cells on plasma IL-10 levels is shown in Fig. (**2**). As evident, HUCB cells ($100x10^6$) induced IL-10 levels 1 month and at 5.5 months after administration. IL-10 was not above the threshold of detection in mice treated with $50x10^6$ HUCB mononuclear cells (data not shown). As shown in Fig. (**3**), the western blot analysis indicates the presence of human IL-10 protein levels in the spleen with $100x10^6$ but not with $50x10^6$ HUCB mononuclear cells. Fig. (**4**) demonstrates the sagittal sections of the intestine from a mouse given HUCB cells ($100x10^6$) that show clear staining for the human nuclear antigen, indicating the engraftment of human cells in the IL-10 knockout mouse **after** 5.5 months of treatment. Only 2 out of 20 treated mice showed human DNA in the bone marrow after 5.5 months of treatment. The other tissues did not show any evidence of human DNA. It is of interest to note that none of the IL-10 knockout mice showed any clinical or pathologic evidence of IBD.

DISCUSSION

This study demonstrates that the production of IL-10 can be induced by HUCB mononuclear cells in IL-10 knockout mouse model without immunosuppression. IL-10 is considered to be an endogenous anti-inflammatory cytokine and has been successful in preventing inflammation and injury in several animal studies [5]. IL-10 appears to be well tolerated, and human recombinant IL-10 was shown to improve clinical parameters in IBD/Crohn's disease [7]. Unfortunately, clinical trials using IL-10 have not always been successful, probably because of its short half-life and the mode of its administration. Indeed high doses of subcutaneous recombinant IL-10 in human subjects may have pro-inflammatory effects through interferon-γ induction [8]. Lindsay *et al*. [9] employed an adenoviral vector in mice and removed this obstacle but the use of an adenoviral vector in the current clinical setting is controversial. In contrast, HUCB administration may allow for local need of essential regulatory components such as IL-10 which is not currently possible with injections or oral medications. In our study, the persistence of IL-10 levels even after 5.5 months of administration suggests the clinical usefulness of HUCB cells to induce IL-10 production in human subjects. Vendrame and colleagues [10] reported the production of IL-10 by splenocytes isolated from a rat model of stroke treated with HUCB cells. Also, the production of

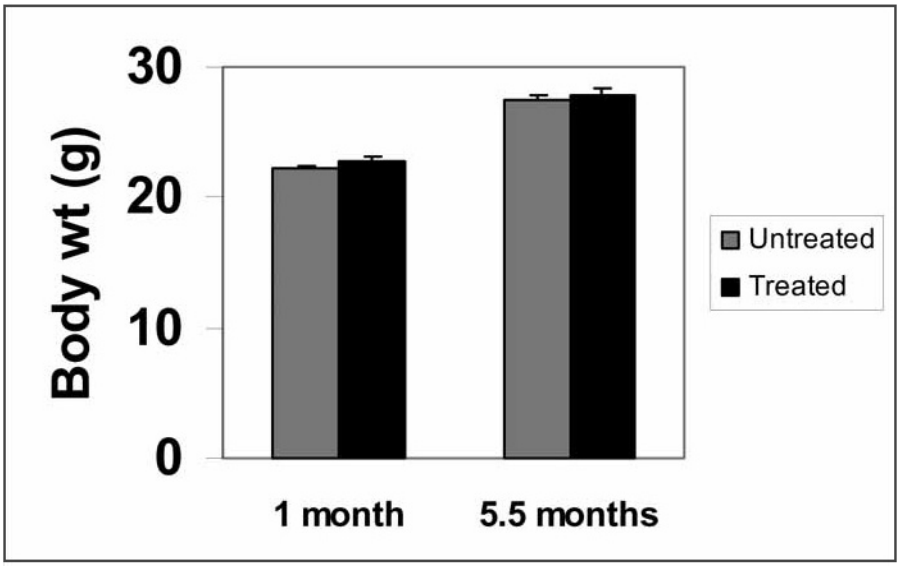

Fig. (1). Body weights of mice after 1 and 5.5 months of human umbilical cord blood cell administration.

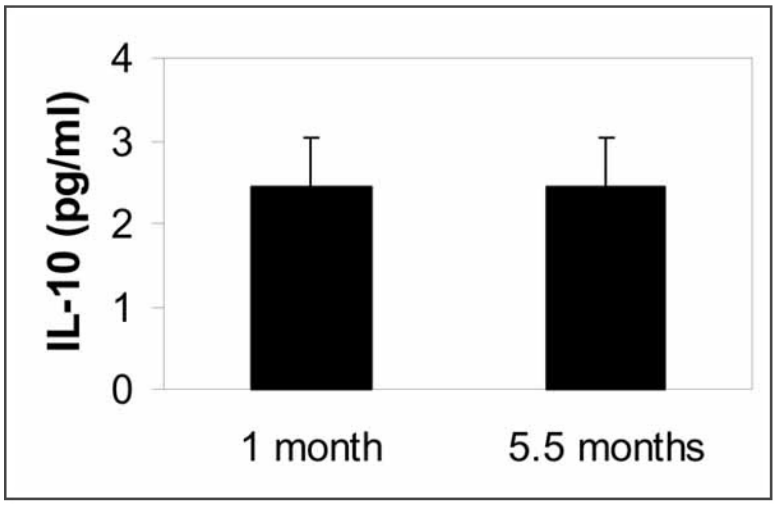

Fig. (2). Plasma IL-10 levels (pg/ml) in mice after 1 (N= 12) and 5.5 months (N= 6) of human umbilical cord blood cell ($100x10^6$) administration.

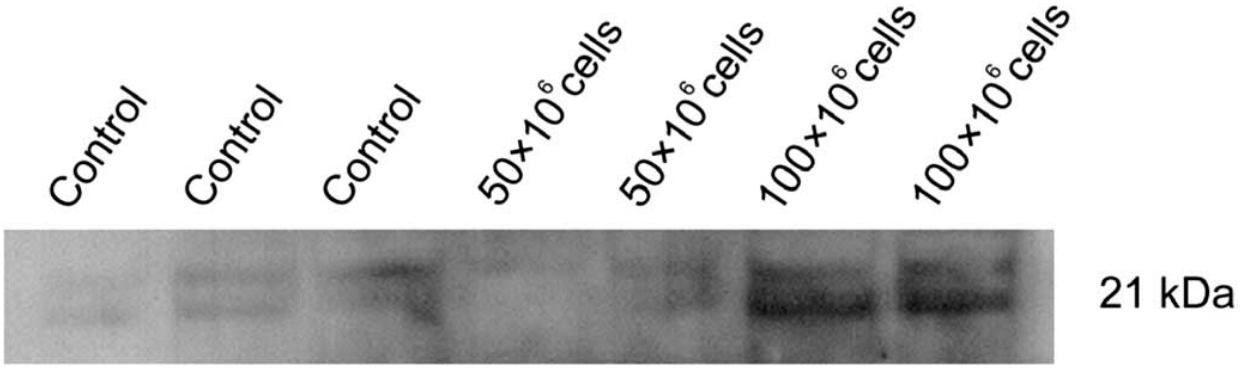

Fig. (3). Western blot analysis of IL-10 protein levels in splenocytes of IL-10 knockout (control) and human umbilical cord blood cell-treated mice.

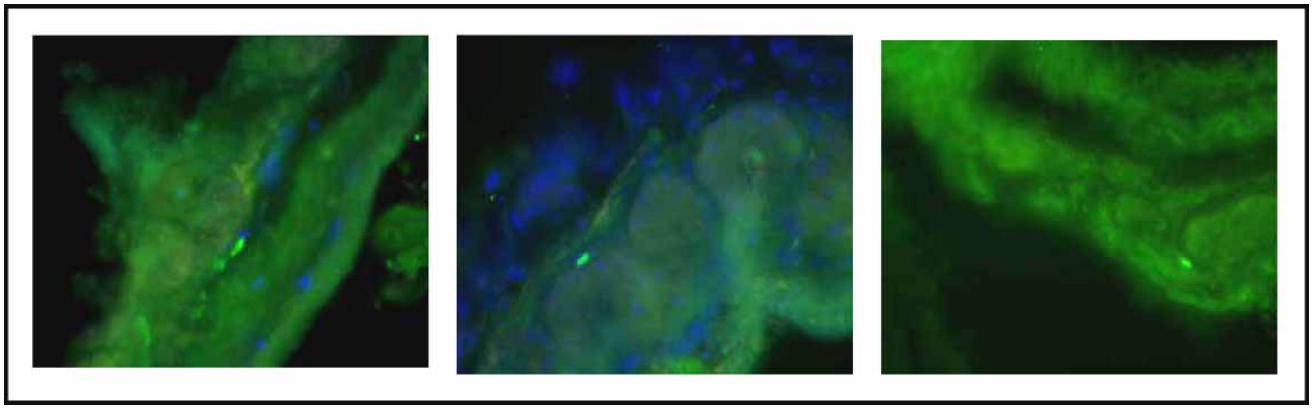

Fig. (4). Sagittal sections of intestine from a mouse given human umbilical cord blood cells showing immunohistochemical staining for human nuclear antigen (bright cells), indicating the engraftment of human umbilical cord blood cells (after 5.5 months of treatment).

interferon-γ was simultaneously reduced. Thus, the data suggest that HUCB cells are protective of brain injury in this rat model of stroke. In addition, HUCB mononuclear cells have been shown to produce a variety of cytokines that may have therapeutic potential in human disease [11,12]. The finding of human cells in the intestinal tract suggests that these cells may promote the production of needed cytokines in prevention of the disease. Besides the intestine, spleen also appears to generate IL-10. Thus, HUCB mononuclear cells are similar to other stem cells in the generation of several cytokines that are clinically useful.

There was no graft-versus-host disease in any one of the HUCB cell-treated mice. This lack of observation of graft-versus-host disease is consistent with our previous studies [13-15].

In summary, our data suggest that administration of HUCB cells to IL-10 knockout mice generates IL-10 production, which circulates in blood for long period of time that may be clinically significant in disease conditions that respond to IL-10 administration. Also, HUCB cell treatment did not cause any weight changes in these mice.

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