

Review

Bone marrow stem cell transplant into intra-bone cavity prevents type 2 diabetes: Role of heme oxygenase-adiponectin

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Abstract

Increase in endothelial cell sloughing and diminished function of endothelial stem cell progenitors in diabetic subjects are well known phenomena. We hypothesized that transplantation of bone marrow stem cells (BMSCs) including mesenchymal stem cells but not limited to CD34⁺ stem cells into type 2 diabetic ob mice would restore insulin sensitivity and glucose tolerance. This approach, when combined with induction of HO-1 (a cytoprotective antioxidant system) in the recipient, would further improve bone marrow function. Sublethally irradiated ob mice received BMSC or CD34⁺ stem cells from B129SF2/J mice (genetically related) via i.v. or intra bone marrow-bone marrow transplantation (IBM-BMT) at a dose of 5×10^6 cells. CD34⁺ i.v. administration to ob mice modestly improved glucose tolerance, whereas BMSC administered by the IBM-BMT significantly increased BMSC function, serum adiponectin and glucose tolerance. Induction of HO-1 in the recipients greatly enhanced the ability of BMSC to prevent diabetes. These findings suggest that transplantation of BMSC-mesenchymal stem cells via IBM-BMT in conjunction with induction of HO-1 can eradicate type 2 diabetes. The beneficial effect of HO-1 induction further suggests that the abnormality in endothelial progenitor cells is due to mesenchymal stem cell-stromal cell disorder exacerbated by oxidative stress and decreases in adiponectin. Thus, transplantation of BMSC using the IBM-BMT strategy in conjunction with HO-1 induction offers a novel approach for the treatment of type 2 diabetes.

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Keywords: Type 2 diabetes mellitus; Heme oxygenase; Obese; Transplant; Stem cells; Adiponectin

1. Introduction

Bone marrow stem cells are presently being considered for therapeutic use to prevent hematological, autoimmune disease and also cardiovascular disease. Bone marrow stem cells differentiate and contribute to vascular repair and improved renal function. Since individuals with diabetes suffer many cardiovascular complications they will benefit greatly from the use of bone marrow stem cells. We have reported that patients with type 2 diabetes have more endothelial cell death [40] and reduced endothelial cell progenitor function [37]. In

addition, bone marrow stem cells from diabetic patients exhibit a decreased ability to generate endothelial progenitor cell in culture [20,37,53]. However, these abnormalities may not be due to a defect in bone marrow stem cell function but to a disorder in stromal-mesenchymal stem cells and their microenvironment which may result in the release of immature progenitors. To this end, Ikehara's group has published a series of remarkable papers describing the use of stem cells for the treatment of type 1 and type 2 diabetes [22–26] and autoimmune disease [32,33]. They also have shown that the development of hyperglycemia and hyperinsulinemia in diabetic mice can be improved by bone marrow stem cell transplant [54].

Bone marrow mesenchymal and fat cells produce adiponectin. The serum levels of adiponectin are significantly decreased

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in type 1 and type 2 diabetes [8,18,19,21,27,28,31,34]. The decrease in adiponectin may be responsible for the observed defect in hematopoietic function in vivo and in vitro. Adiponectin has been identified as a hematopoietic stem cell growth factor. This finding suggests that a decrease in one of the cytokines such as adiponectin production may lead to a defect in the stromal microenvironment that is required to provide the optimum conditions for hematopoiesis and pluripotent stem cell differentiation to proper cell type [4,11,39]. We have previously shown that stromal stem cell and clonogenic capacity were severely reduced in HIV and in irradiated subjects which can be prevented by supplementation of cytokines and heme [4,11]. Cytokines, heme and heme oxygenase (HO) activity levels play a regulatory role in mesenchymal stem cell microenvironment and hematopoiesis [4,11].

HO is composed of two isozymes, HO-1 and HO-2. HO-1 is a stress response gene and has been shown to be critical for bone marrow cell proliferation and maturation [3,7]. The levels of HO-1 expression and the products of its catalytic activity, CO and bilirubin, are decreased in humans or animals with type 2 diabetes [9,14,30]. The decrease in HO-1 expression is frequently associated with increased superoxide production and oxidative stress leading to an impairment in the mesenchymal-stromal stem cell production of adiponectin and increased adipogenesis [2]. Oxidative stress has been implicated in the pathogenicity of insulin resistance in type 2 diabetes and of cardiovascular complications [48,50,57]. Induction of HO-1 has been shown to ameliorate diabetes-mediated cardiovascular complications including mitochondrial function [29,30,46]. Additionally, Li et al. have shown that upregulation of HO-1 in pre-diabetes preserved β -cell function and prevented the diabetic state of non-obese diabetic mice [35,36].

The present report examines whether purified bone marrow stem cells alone or bone marrow stem cells including stromal stem cells in combination with an HO-1 inducer can prevent type 2 diabetes and restore insulin sensitivity and glucose tolerance. The efficacy of bone marrow stem cell populations that included mesenchymal stem cells transplanted by IBM-BMT was compared to purified stem cells transplanted via the tail vein, and the capacity of HO-1 induction to improve type 2 diabetes was assessed. These studies used sublethally irradiated mice and long-term marrow culture (LTBMC) with the generation of hematopoiesis for comparison. We considered it critical to study the influence of IBM-BMT, CD34⁺ in the presence of HO-1 inducers on the bone marrow microenvironment and on pluripotent stem cell survival and hematopoiesis.

2. Materials and methods

2.1. Mice

Obese mice (ob/ob) mice and the wild type mice, B129SF2/J pathogen-free mice, weighing 24–30 g and 8–12 weeks old were used. Mice were maintained under specific pathogen-free conditions. All experiments were approved by the Animal

Care and Use Committee of the New York Medical College Approval Committee, Valhalla, NY.

2.2. Irradiation

Mice were irradiated with 8.5 Gy (two fractions with 3–4 h intervals) from a ¹³⁷Cs source, at a dose rate of 8.5 Gy/min.

2.3. Hematopoietic colony assays

Spleen-derived EPCs were determined and the methylcellulose technique for myeloid granulocyte-macrophage (CFU-GM) colony assays has been described in detail previously [4,5]. Non-adherent bone marrow cells were obtained after two cycles of plastic adherence, at a final concentration of 4×10^5 /ml CFU-GM. The cells were plated in 4-well multidishes using 0.5 ml of semisolid culture medium containing Iscove's modified Dulbecco's medium (IMDM), 1.12% methylcellulose, 30% heat-inactive FCS, 1% deionized BSA, and 5×10^{-5} M monothioglycerol, and growth factors. CFU-GM was grown in a medium supplemented with 10% pokeweed mitogen spleen cell conditioned medium. CFU-GM colonies were detected and scored on day 12 of seeding.

2.4. Long-term bone marrow culture

Details of the LT BMC technique used in our laboratory have been previously described [4,5,12,39]. Briefly, femoral bone marrow was flushed out into 25 cm² flasks with 10 ml of complete culture medium which consisted of Fisher's medium supplemented with L-glutamine, antibiotics, 10^{-6} M of hydrocortisone 21-hemisuccinate, 20% of heat-inactive sera [(2:1) horse:fetal bovine serum], respectively. Flasks were incubated at 33 °C with weekly replacement of 50% of the complete medium. The cellularity of LT BMC and the committed clonogenic progenitor content was then quantitated during 6–14 weeks.

2.5. Experimental design

Five groups of mice, 10–12 mice in each, were studied: (1) normal; (2) sublethally irradiated (8.5 Gy) mice received ob bone marrow; (3) ob mice received B129SF2 CD34-CoPP; (4) ob mice received CD34⁺ IBM-CoPP and (5) ob mice received B129SF2 IBM-BMT-CoPP. Mice were sacrificed 30 days after transplant and bone marrow cells removed for culture in LT BMCs, CFU-GM and CFU-S were determined.

2.6. Glucose tolerance test

Glucose clearance was determined using an intraperitoneal glucose tolerance test. Mice were made to fast for 8 h after which a glucose solution (2 g/kg body weight, injected as a 10% solution) was injected into the peritoneal cavity. Samples were taken from the tail vein at 0, 30, 60 and 120 min after glucose injection. Blood glucose was measured using the Accutrend Sensor glucometer.

2.7. Bone marrow preparation for HO-1, HO-2 and eNOS measurement

Bone marrow cells were placed in a homogenization buffer (10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF and 0.1% tergitol, pH 7.5). Homogenates were centrifuged at $27,000 \times g$ for 10 min at 4°C , the supernatant was isolated, and protein levels were assayed. The supernatant was used for measurement of HO-1, HO-2 and eNOS expression, and determination of HO activity [6,7]. Protein levels were visualized by immunoblotting with antibodies against mouse HO-1, HO-2 and eNOS. Briefly, 20 μg of cell lysate supernatant was separated by 12% SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were incubated with 10% milk in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20 buffer at 4°C overnight. After washing with TBST, the membranes were incubated with anti-HO-1, anti-HO-2 and anti-eNOS antibodies for 1 h at room temperature with constant shaking. The filters were washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG [6,29]. HO activity was assayed as previously described [7].

2.8. Adiponectin measurements

Adiponectin was determined using an ELISA assay (Pierce Biotechnology Inc., Woburn, MA).

2.9. Statistical analyses

The data are presented as mean \pm standard error of the mean (SEM) for the number of experiments. Statistical significance ($p < 0.05$) between the experimental groups was determined by the Fisher's method of analysis of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by a single factor analysis of variance (ANOVA) for multiple groups or unpaired t-test for two groups.

3. Results

3.1. Effect of IBM-BMT on glucose tolerance

We compared the efficacy of purified CD34^+ cells vs whole bone marrow cells treated with the HO-1 inducer CoPP in improving glucose tolerance in ob mice. As seen in Fig. 1, ob mice that received B129SF2/J- CD34^+ cells by IBM-BMT showed improvement in the glucose tolerance test compared to control ob mice. However, ob mice that received ob bone marrow did not show any improvement. In contrast, ob mice that received bone marrow from B129SF2/J maintained normal glucose levels.

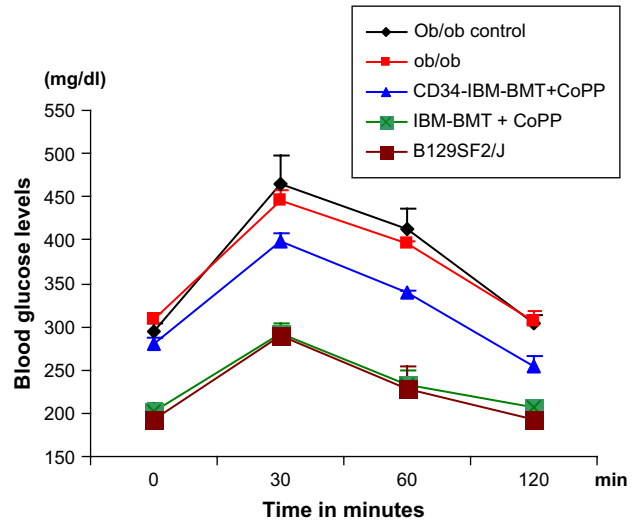


Fig. 1. Glucose tolerance in diabetic and transplant mice. Glucose tolerance tests were performed in control, ob transplanted with ob marrow (ob/ob), ob transplanted with B129SF2/J- CD34^+ treated with CoPP (CD34-IBM-BMT+CoPP), ob transplanted with B129SF2/J bone marrow cells treated with CoPP (IBM-BMT + CoPP).

3.2. Effect of BMT transplantations using tail vein or IBM-BMT with HO-1 inducers

In a series of in vitro experiments, the capacity of bone marrow cells to generate hematopoietic progenitors in LTBMCS was examined. Bone marrow cells were removed from mice 2 weeks after irradiation and cultured in LTBMCS. LTBMCS were then maintained for more than 10 weeks, during which time weekly determination of cellularity and CFU-GM clonogenic capacity were quantitated (Fig. 2). In Fig. 2, it can be seen that both the total cellularity and clonogenic capacity by LTBMCS derived from both ob mice and ob mice transplanted with ob marrow were severely reduced. In

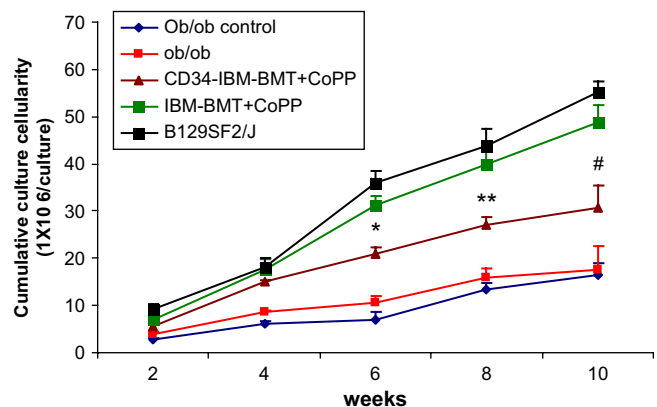


Fig. 2. Effect of bone marrow transplant-mesenchymal stem cells on hematopoiesis. Hematopoiesis in long-term bone marrow cultures established 2 weeks after transplantation for 10 weeks. Cumulative culture cellularity (1×10^6) in non-ob mice transplanted with ob bone marrow, ob transplanted with CD34 and the recipient injected with CoPP, ob mice transplanted with CD34-IBM-BMT-CoPP and IBM-BMT-CoPP. Results are expressed as mean \pm SEM; $N = 6$; * $p < 0.002$ vs ob/ob, ** $p < 0.0001$ vs ob/ob and # $p < 0.03$ vs CD34-IBM-BMT-CoPP.

addition, the cellularity generated from mesenchymal-stromal cell formation was markedly decreased in these cultures (ob transplanted to ob or ob transplanted with CD34 via tail vein). Although transplantation of CD34⁺ improved the cellularity, it did not reach the same levels as seen in IBM-BMT from B129SF2/J mice. Cultures from ob mice transplanted with normal bone marrow stem cells including normal mesenchymal stem cells i.e. IBM-BMT, display a marked improvement in cellularity compared to any type of transplant used. It is clear that IBM-BMT i.e. adherent cell-mesenchymal stem cell formation, the clonogenicity generated by IBM-BMT when used in combination with CoPP, gives the highest value of cellularity.

3.3. Effect of IBM-BMT on CFU-GM clonogenic potential

In order to further evaluate the time effect on the recovery of ob from transplantation, we measured the total cellularity of cultures during the 8–10 weeks under study as well as the CFU-GM clonogenic potential by the same group at 4 and 8 weeks. As seen in Fig. 3, the number of CFU-GM is increased in either cell transplanted by normal donor but not by ob bone marrow (Fig. 3). Ob mice transplanted with CD34 alone without adherent stromal cells (mesenchymal stem cells) display more than ob alone. However, mice receiving CD34-IBM-BMT and treated with the HO-1 inducer, CoPP, displayed a marked increase in CFU-GM. Recipients of the bone marrow by IBM-BMT and CoPP have expressed the highest number of CFU-GM suggesting that the cellularity and clonogenicity are normal compared to ob marrow or to the donors (Fig. 3).

3.4. Effect of BMT on HO-1 and HO-2 expression

Since the administration of CD34⁺ cells via tail vein did not improve glucose tolerance or increase bone marrow

cellularity or the number of CFU-GM, in the following set of experiments we used only the conditions whereas the recipient received HO-1 inducers. We determined the effect of CD34-IBM and IBM-BMT on HO-1 and HO-2 protein levels. Fig. 4 compares the HO-1 and HO-2 protein levels in bone marrow cells obtained from various treatments. As seen, CD34-CoPP-IBM increased HO-1 proteins compared to transplanted ob mice with their own type of marrow. Similarly, IBM-BMT increased the levels of HO-1 proteins to the donor levels. Optical density scanning for the ratio of HO-1 to actin confirmed the western blot analysis. Conversely, there was a significant decrease in the amount of HO-1 protein in ob transplanted with ob bone marrow compared to non-diabetic mice. Transplantation by either ob or CD34 or IBM-BMT did not change the HO-2 protein levels.

3.5. Effect of HO-1 expression on eNOS levels

HO-1 overexpression has been shown to increase eNOS levels in type 2 diabetes as a result of increases in EC-SOD and superoxide levels and to produce an increase in reduced glutathione levels [30,47,56]. The level of eNOS was measured to assess oxidative stress after BMT and was found to

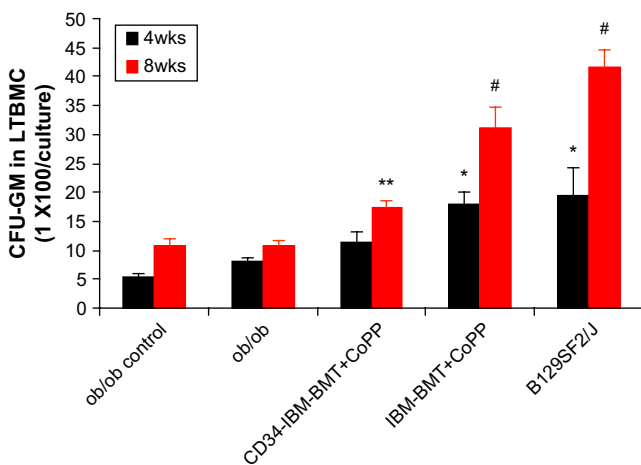


Fig. 3. Effect of transplantation on the interaction of adherent stromal-mesenchymal stem cells on the generation of CFU-GM. CFU-GM clonogenic potential was assessed at 4 and 8 weeks in cultures from the supernatant of LTBM cells. Results are expressed as mean ± SEM; N = 6; *p < 0.01 vs ob/ob, **p < 0.03 vs ob/ob and #p < 0.05 vs CD34-IBM-BMT-CoPP.

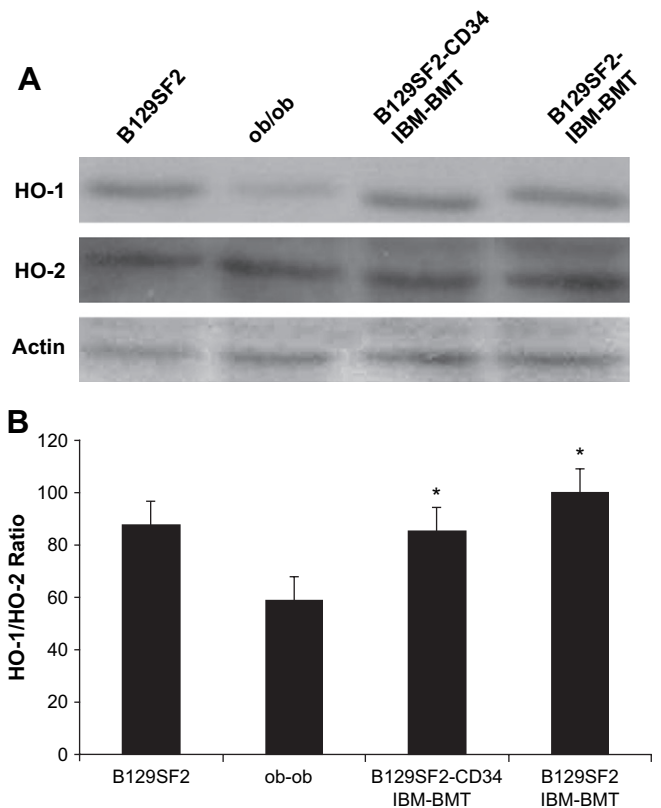


Fig. 4. Effect of transplantation on HO-1 protein levels. Western blot and densitometry analysis of HO-1 and HO-2 proteins in bone marrow cells from various transplanted mice. Immunoblots were performed using antibodies against mouse HO-1 and HO-2. Data are representative of six separate experiments. (B) Quantitative densitometry evaluation of HO-1/actin ratio. Mean band density normalized relative to α-actin. Results are expressed as mean ± SEM; N = 4 (*p < 0.005 vs ob mice).

be significantly reduced in ob transplanted with ob bone marrow compared to controls ($p < 0.05$, Fig. 5). Although CD34 BMT increased eNOS, this increase may be due to the increases in HO-1 proteins, even though the glucose levels were not dramatically decreased. IBM-BMT not only increased the levels of eNOS but also normalizes glucose levels compared to those found in controls ($p < 0.01$ vs ob or ob transplanted with CD34-IBM, Fig. 5).

3.6. Effect of IBM-BMT on serum adiponectin levels

There was a significant decrease in adiponectin between obese mice and wild type. IBM-BMT-HO-1 significantly increased adiponectin compared to ob/ob transplanted mice, $p < 0.01$ (Fig. 6).

4. Discussion

The present study demonstrates that transplantation of obese mice with bone marrow stem cells along with adherent cells (mesenchymal stem) from wild type (B129SF2/J) restores glucose tolerance in type 2 diabetes. Transplantation of purified stem cell from the wild type, B129SF2/J, CD34⁺, via the tail vein was ineffective in the restoration of glucose tolerance when compared to the transplantation of the whole bone marrow with mesenchymal stem cells. We also report that transplantation in conjunction with upregulation of HO-1 in the recipient animal using cobalt protoporphyrin enhances the function of the transplanted stem cells and increased serum adiponectin. IBM-BMT caused robust increases in serum adiponectin in ob mice. These observations support the conclusion that in type 2 diabetes there is a defect in the mesenchymal stem cell and its microenvironment which appears to lead to the release of immature hematopoietic stem cells. Mesenchymal stem cells exposed to high glucose

levels cause significant inhibition of cell growth and increase the number of adipocytes (manuscript in preparation).

Hematopoiesis is a carefully regulated system requiring complex interplay between different cell types, the extra cellular matrix and growth factors. The bone marrow stromal-mesenchymal stem cell provides the necessary microenvironment for blood cell development, and the interaction between mesenchymal stem cell-stromal elements and non-adherent progenitor cells is critical for the optimal functioning of regulated hematopoiesis. Bone marrow mesenchymal stem cells-adherent cells have been shown to generate cytokines which in turn recruit elements required to produce the multi-lineage growth factors that are considered necessary for the microenvironment [3]. Studies in our laboratory have revealed that heme and HO-1 are both necessary for the bone marrow to produce substance(s) which promote hematopoiesis and that inhibition of heme synthesis negatively influences the control of hematopoietic stem cell production [3,4,12,13,38,44,51,55].

Others have shown that heme and heme analogues stimulate production of growth factors by peripheral blood mononuclear cells and that the addition of exogenous heme in in vitro or in vivo experimental models has proven to be useful in both enhancing and/or correcting abnormal hematopoiesis brought about by toxic agents. Furthermore, heme has been shown to affect the hematopoietic microenvironment in both short and long-term bone marrow culture (LTBMC).

Upregulation of HO-1 in the bone marrow leads to an increase in the levels of bilirubin, antioxidants, and CO and anti-apoptotic molecules via an increase in heme degradation. Therefore, transplantation of bone marrow cell by IBM-BMT, in combination with HO-1 inducers, produces a definite advantage leading to the restoration of normal hematopoiesis and, in particular, mesenchymal stem cell elements. As seen in Section 3 the upregulation of HO-1 and increased HO activity in the recipient enhance transplantation with CD34⁺ and result in a better outcome for CFU-GM when compared to

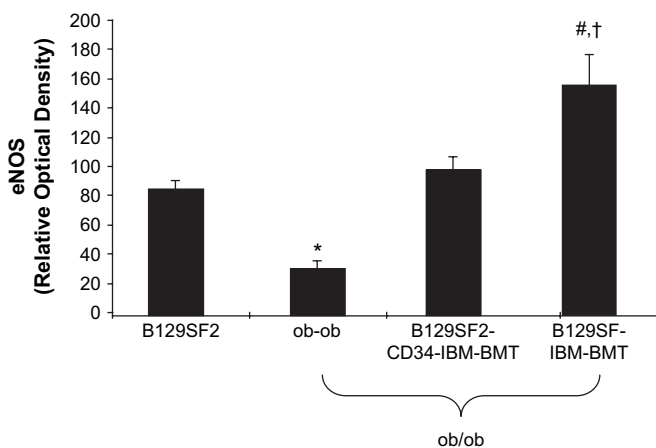


Fig. 5. Effect of bone marrow transplant on eNOS levels. Quantitative densitometry evaluation of eNOS/actin ratio is shown. Mean band density normalized relative to β -actin. Results are expressed as mean \pm SEM; $N = 4$ (* $p < 0.005$ vs ob/ob, # $p < 0.05$ vs B129SF2-CD34-IBM-BMT and † $p < 0.001$ vs ob/ob).

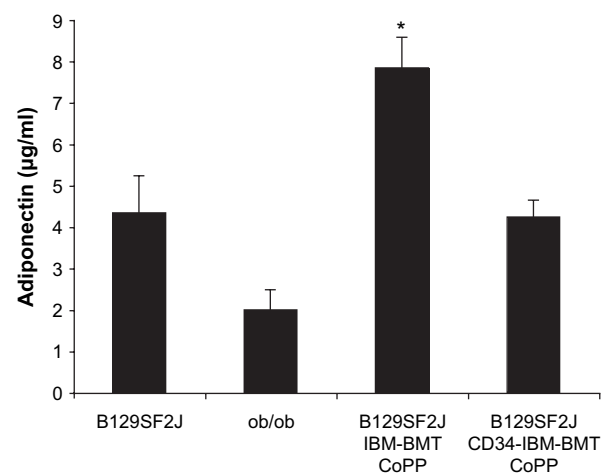


Fig. 6. Effect of IBM-BMT on serum adiponectin levels. There was a significant decrease between obese mice and wild type. IBM-BMT-CoPP significantly increased adiponectin compared to ob/ob transplanted mice or to CD34-IBM-BMT-CoPP ($p < 0.01$).

transplantation with HO-1 induction in the recipient. In addition, the ability of stromal-mesenchymal stem cell in LTBMCS was greatly improved when cultured from the recipient receiving IBM-BMT and increased HO-1 expression. In addition, IBM-BMT increased adiponectin at significant levels compared to CD34-IBM-HO-1. This may be due to the lack of mesenchymal stem cells in the transplantation of purified CD34⁺ cells, suggesting that IBM-BMT which encloses mesenchymal stem cells is of great value to the recovery from type 2 diabetes. Although the combined use of HO-1 induction, i.e. decrease in oxidative stress plus IBM-BMT, offers great improvement in diabetes and glucose tolerance, further studies remain to be done to clarify the mechanism(s).

The mechanism by which HO-1 improves the effectiveness of IBM-BMT is due to a decrease in superoxide, a major contributor to oxidative stress. Increase in oxidative stress may cause impairment in mesenchymal stem cell function and the release of crucial cytokines such as adiponectin. Secretion of adiponectin into circulation is derived from mesenchymal-adipocyte cells and this process is impaired in type 2 diabetes. Recently, L'abbatle et al. and others [41–43,52] showed that adiponectin plays an important role in diabetes. Adiponectin has been identified as being a novel hematopoietic stem cell growth factor. Adiponectin has been shown to stimulate production of NO in the vascular system [10,34,45]. Increases in NO or HO-1-derived CO increase blood flow and vascular function and prevent diabetes-mediated cell death [16,30,46,49]. Increase in blood flow will facilitate the delivery of various vasoactive substances, nutrients and oxygen to stromal-mesenchymal stem cells, which subsequently modulates the negative and positive crucial cytokines needed to regulate hematopoiesis.

Upregulation of HO-1 also enhances the release of adiponectin and its increase enables diabetic hearts to sustain oxidative stress [34]. In other studies, it has been shown that upregulation of HO-1 increases antioxidant molecules, glutathione, superoxide dismutase and mitochondrial function [15,30,46,56]. Additionally, hyperglycemia causes suppression of HO-1 expression, which is considered to be a major contributor to the increase in superoxide and cell death [6]. Therefore, upregulation of HO-1 in combination with IBM-BMT is ideal to alleviate diabetes-mediated cardiovascular disease. Over 7% of the USA population is estimated to live with type 2 diabetes with more than 70,000 deaths each year [1]. Therefore, bone marrow transplantation via IBM-BMT along with an increase in antioxidants could avoid numerous complications seen in diabetes. Control of hyperglycemia in diabetes patients is not easily achieved by most patients and in certain incidences the tight range control of glucose levels may increase the risk of hypoglycemia, stroke and the need for a better treatment is needed.

In conclusion, we have shown that bone marrow stem cell transplant using IBM-BMT along with an increase in antioxidant levels, via an increase in HO-1-adiponectin gene expression, is effective in the treatment and prevention of type 2 diabetes. Whether the combined use of HO-1 inducers offers the best approach to prevent type 2 diabetes remains to be further evaluated. Stem cells are being used in clinical trials for

other pathological conditions such as ischemic tissue repair, vascular regeneration, liver disease, neuroregenerative disease, and cancer and the use of BMT in diabetes should be considered as well. Finally, we note that this paper is part of a series of papers published in this special symposium [58–68]. For additional reading published in the Journal of Autoimmunity on the immunobiology of diabetes, including both type 1 and type 2, we refer to recent publications [69–80].

Acknowledgements

Research described in this article was supported by NIH grants HL55601, R01DK068134 and HL34300.

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