Antioxidant Therapy Attenuates Diabetes-Related Impairment of Bone Marrow Stem Cells

Mako Ohshima, MS; Tao-Sheng Li, MD; Masayuki Kubo, PhD; Shu-Lan Qin, MD; Kimikazu Hamano, MD

Background  Bone marrow cells from humans and animals with diabetes exhibit decreased angiogenic potency, thought to be related to oxidative stress, so the present study investigated if antioxidant therapy would attenuate the diabetes-related impairment.

Methods and Results  Diabetic mice were given antioxidant therapy, as a daily subcutaneous injection of superoxide dismutase-mimic (10 mg · kg⁻¹ · day⁻¹). Diabetic and healthy mice given a vehicle treatment were used as the control. After 4 weeks of treatment, bone marrow mononuclear cells (BM-MNCs) were collected for analysis and the endothelial progenitor cells in BM-MNCs were evaluated by flow cytometry. The intracellular reactive oxygen species (ROS) levels in BM-MNCs were measured using 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate. Endothelial differentiation from the BM-MNCs was estimated by immunostaining with VE-cadherin 7 days after culture. BM-MNCs from the control diabetic mice had fewer Flk-1/CD34 double-positive progenitor cells and higher intracellular ROS levels, with lower potency of endothelial differentiation than BM-MNCs from the healthy mice. Antioxidant therapy decreased the intracellular ROS level in BM-MNCs from that in the diabetic mice significantly (P<0.05), but increased significantly the percentage of endothelial progenitor cells (P<0.05) and their potency of differentiation into endothelial cells (P<0.05).

Conclusions  Antioxidant therapy attenuated the diabetes-related impairment of BM-MNCs by reducing oxidative stress. (Circ J 2009; 73: 162–166)

Key Words: Angiogenesis; Oxidative stress; Reactive oxygen species

In various experimental ischemic models, the implantation of autologous bone marrow-derived cells has been an effective method of inducing therapeutic angiogenesis and several clinical trials of this treatment for ischemic heart disease and peripheral arterial disease have been conducted safely; however, the implantation of autologous bone marrow-derived cells did not improve clinical symptoms or regional perfusion of ischemia in some patients. Some investigators speculate that this poor angiogenic potency is related to the complications of diabetes mellitus (DM), hyperlipidemia, and aging, because these risk factors contribute to the diabetes-related functional impairment of bone marrow cells for the induction of angiogenesis. Therefore, the impaired function of bone marrow cells in patients complicated by DM must be restored before they are used to induce therapeutic angiogenesis.

Reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radical, are biologically active species that have been increasingly recognized as playing a major role in vascular biology through redox signaling. It has been also suggested that they are important mediators of angiogenesis. A link between oxidative stress and DM has been suggested by many experimental models. Furthermore, oxidative stress has been found to induce the dysfunction of endothelial cells, and generate vascular complications in patients with type II DM. Thus, we evaluated the hypothesis that oxidative stress also contributes to the diabetes-related functional impairment of bone marrow stem cells, which would be attenuated by antioxidant therapy.

Methods

Animals  We used 18-week-old male C57BLKS/J Iar-+Lepdb/+Lepdb (db/db) mice (SLC, Shizuoka, Japan), which are a strain characterized by spontaneous type II DM with both hyperglycemia and hyperlipidemia. C57BLKS/J Iar-m+/+Lepdb (db/m+) healthy mice were used as a control. Mice were bred in clean conditions and all experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University.

Antioxidant Therapy  Antioxidant therapy was given to the db/db mice (n=8) by daily subcutaneous injection of superoxide dismutase-mimic, as 10 mg/kg Mn(III) tetrakis 4-benzoic acid porphyrin chloride (MnTBAP; BIOMOL International, L.P., Plymouth Meeting, PA, USA). We decided on the dose of 10 mg/kg MnTBAP based on the findings of another recent report. For a control, db/db diabetic mice (n=8) and db/m+ healthy mice (n=8) were given vehicle treatment as a daily subcutaneous injection of phosphate buffered saline (PBS). Treatments were continued for 4 weeks.

(Received January 31, 2008; revised manuscript received August 3, 2008; accepted August 17, 2008; released online November 19, 2008) Department of Surgery and Clinical Science, Yamaguchi University Graduate School of Medicine, Ube, Japan Mailing address: Kimikazu Hamano, MD, Department of Surgery and Clinical Science, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-kogushi, Ube 755-8505, Japan. E-mail: kimikazu@yamaguchi-u.ac.jp All rights are reserved to the Japanese Circulation Society. For permissions, please e-mail: cj@j-circ.or.jp
Monitoring Body Weight, Blood Glucose, and Urinary 8-OHdG Levels

We measured body weight and blood glucose levels in all mice before treatment, and then at 2 and 4 weeks after treatment, respectively. We also collected urine from the mice before treatment, and then at 2 and 4 weeks after treatment, and stored it at −80°C. We measured the concentration of 8-oxo-2'-deoxyguanosine (8-OHdG) in the urine using an ELISA kit (Nikkon SEIL Corporation, Shizuoka, Japan), according to the manufacturer’s instructions. Creatinine levels in urine were also measured using a creatinine assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The corrected values of urinary 8-OHdG levels were calculated as 8-OHdG ng/mg creatinine, and the mean value of triplicate assays of each experiment were used for statistical analysis.

Collection and Cultivation of Bone Marrow Cells

Bone marrow cells were collected from the femurs and tibias of mice at 4 weeks after antioxidant and vehicle treatment. Bone marrow mononuclear cells (BM-MNCs) were isolated by density gradient centrifugation as described previously. Freshly collected BM-MNCs were suspended at a density of 2×10^6 cells/ml in RPMI 1,640 medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, and cultured at 37°C in a humidified environment with 5% CO₂.

Flow Cytometry

We quantitatively evaluated the endothelial progenitor cells in the BM-MNCs of diabetic and healthy mice after treatment. Freshly collected BM-MNCs were stained with rat anti-mouse Fk-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody at 4°C for 30 min, followed by staining with FITC-labeled goat-anti rat IgG antibody at 4°C for 15 min. After washing, cells were stained with phycoerythrin-conjugated rat anti-mouse vascular endothelial (VE)-CD34 monoclonal antibody (BD Bioscience, San Jose, CA, USA) at 4°C for 30 min. Respective isotype controls were used as a negative control. Quantitative flow cytometry analysis was done using a FACS Calibur (BD Bioscience). We analyzed the data with Cell Quest software (BD Bioscience). Flk-1/CD34 double-positive stained cells were determined to be endothelial progenitor cells.

Cell Viability Analysis

To estimate cell viability, BM-MNCs were seeded on 96-well plates (2×10^3 cells/100 μl/well). After 3 days of culture, dead or dying cells were stained with trypan blue, and the surviving cells in each well were counted. The survival rate was calculated as the percentage of surviving cells among all of the seeded cells.

DCF Assay

The oxidative stress of bone marrow cells was evaluated by measuring the intracellular ROS levels, as described previously. Briefly, BM-MNCs (2×10^5 cells/ml) were plated on 96-well plates. After 3 days of cultivation, cells were washed and then incubated with 20 μmol/L 6-carboxy-2'7'-dichlorodihydrofluorescein diacetate (DCF; Lambda Fluorescence Technology, Graz, Austria) in the dark for 30 min at 37°C. After washing 3 times, fluorescence was measured immediately in a microplate reader with an excitation/emission wave length of 485/535 nm.

Immunocytochemistry

To evaluate endothelial differentiation, BM-MNCs were cultured on 4-well chamber culture slides (Nalge Nunc International, Naperville, IL, USA), which were precoated with 10 μg/ml fibronectin (Invitrogen, Carlsbad, CA, USA). After 1 week of culture, cells were fixed in acetone, blocked with Protein Block Serum-free (Dako Cytomation, Carpinteria, CA, USA), and then reacted with phycoerythrin-conjugated anti-mouse vascular endothelial (VE)-
The urinary 8-OHdG levels in the diabetic mice were much higher than those in the healthy mice. However, the blood glucose levels in the diabetic mice that received antioxidant therapy did not differ significantly from those in the diabetic mice that received vehicle treatment.

Similarly, the blood glucose levels in the diabetic mice that received antioxidant therapy or vehicle treatment were much higher than those in the healthy control mice, over the entire period (P<0.01, Fig 1B). However, the blood glucose levels in the diabetic mice that received antioxidant therapy did not differ significantly from those in the diabetic mice that received vehicle treatment.

Fig 2. Measurement of endothelial progenitor cells in bone marrow mononuclear cells (BM-MNCs) by flow cytometry after 4 weeks of treatment. The endothelial progenitor cells in BM-MNCs were identified by the double-positive expression of Flk-1 and CD34. The percentage of Flk-1/CD34 double-positive cells in the BM-MNCs of diabetic mice given antioxidant therapy (Diabetic-SOD) was significantly higher than that in the diabetic mice given a phosphate-buffered saline injection (Diabetic-PBS), but lower than that in the healthy mice (Healthy-PBS).

Statistical Analysis
All results are expressed as mean±SD. Statistical significance was evaluated using the unpaired t-test for comparisons between 2 means. A probability value of P<0.05 was considered significant.

Results
Changes in Body Weight, Blood Glucose, and Urinary 8-OHdG Levels
The body weight of the db/db diabetic mice that received either antioxidant therapy or vehicle treatment was significantly lower than that of the healthy mice after 28 days of treatment (P<0.05, Fig 1A). This was attributed to weight loss in the final stage of DM. Conversely, the body weight of the diabetic mice that received antioxidant therapy did not differ significantly from that of the diabetic mice that received vehicle treatment.

Similarly, the blood glucose levels in the diabetic mice that received antioxidant therapy or vehicle treatment were much higher than those in the healthy control mice, over the entire period (P<0.01, Fig 1B). However, the blood glucose levels in the diabetic mice that received antioxidant therapy did not differ significantly from those in the diabetic mice that received vehicle treatment.

The urinary 8-OHdG levels in the diabetic mice were much higher than those in the healthy mice, over the entire period (P<0.01, Fig 1C). The baseline levels of urinary 8-OHdG did not differ between the diabetic groups. Compared with the baseline, the 8-OHdG levels decreased gradually in the diabetic mice given antioxidant therapy, but it did not change in those given vehicle treatment. After 4 weeks of treatment, the urinary 8-OHdG levels in the diabetic mice given antioxidant therapy was significantly lower than those in the diabetic mice given vehicle treatment (P<0.05, Fig 1C).

Antioxidant Therapy and the Number of Endothelial Progenitor Cells in BM-MNCs
Endothelial progenitor cells in BM-MNCs were identified by Flk-1/CD34 double-positive stained cells, and the percentages of endothelial progenitor cells in BM-MNCs were measured quantitatively by flow cytometry analysis after 4 weeks of treatment. The percentage of Flk-1/CD34 double-positive cells in the BM-MNCs was much lower in the diabetic mice than in the healthy mice given vehicle treatment (0.68±0.12% vs 0.24±0.08%, P<0.01; Fig 2). Although the percentage of Flk-1/CD34 double-positive cells in the BM-MNCs of diabetic mice that received antioxidant therapy (0.46±0.12%) was also significantly lower than that in the healthy mice (P<0.05), it was significantly higher than that in the same diabetic mice given vehicle treatment (P<0.05, Fig 2).

Survival Rate of BM-MNCs
We counted the number of surviving cells 3 days after cultivation. Although the BM-MNCs of diabetic mice given a phosphate-buffered saline injection (Diabetic-PBS) had the lowest survival rate, the difference between that of the diabetic mice given antioxidant therapy (Diabetic-SOD) and that of the healthy mice (Healthy-PBS) was not significant.

Antioxidant Therapy and the Intracellular ROS Levels of BM-MNCs
We found significantly higher intracellular ROS levels in the BM-MNCs from the diabetic mice given antioxidant therapy (P<0.05) or vehicle treatment (P<0.01, Fig 4) than in those from the healthy mice. However, the intracellular ROS levels in the BM-MNCs from the diabetic mice given
Antioxidant Therapy and Endothelial Differentiation of BM-MNCs

After 7 days of culture, the endothelial differentiation of BM-MNCs was confirmed by staining with VE-cadherin, a specific marker expressed in early differentiated endothelial cells. There were significantly fewer VE-cadherin-positive cells in the BM-MNCs from the diabetic mice given antioxidant therapy (P<0.05) or vehicle treatment (P<0.01, Fig 5). However, significantly more VE-cadherin-positive cells were observed in BM-MNCs from the diabetic mice that received antioxidant therapy than in those from the diabetic mice given vehicle treatment (57.3±16.1 vs 176.6±29.5 cells/field, P<0.05, Fig 5).

Discussion

Bone marrow stem cells are among the most studied cell sources for repairing injured heart tissue and vessels because of their potential for myogenic differentiation and inducing therapeutic angiogenesis. Unfortunately, bone marrow stem cells from patients and animals with DM exhibit decreased potency for inducing therapeutic angiogenesis although the relative mechanisms are not fully understood. To increase the therapeutic potency of autologous bone marrow cells for inducing therapeutic angiogenesis in patients whose condition is complicated by diabetes, it is important to understand the mechanisms of diabetes-related impairment of bone marrow stem cells and then to restore the impaired function of the bone marrow stem cells before therapeutic usage.

Recent investigations have found that oxidative stress in type II diabetes contributes to endothelial (progenitor) cell dysfunction. Using mice with type II diabetes, we investigated if diabetes-related functional impairment of bone marrow stem cells was also related to oxidative stress. Increased urinary levels of 8-OHdG, an oxidative marker, confirmed enhanced systemic oxidative stress in these diabetic mice. We also found that bone marrow cells from diabetic mice contained less endothelial progenitors and showed poor endothelial differentiation. Furthermore, the bone marrow cells from diabetic mice had higher intracellular ROS levels than those from healthy mice. These findings support our hypothesis that oxidative stress is a critical factor contributing to the diabetes-related impairment of bone marrow stem cells.

We also investigated whether antioxidant therapy would attenuate the diabetes-related impairment of bone marrow stem cells. As expected, antioxidant therapy delivered by the administration of MnTBAP decreased the urinary level of 8-OHdG gradually in the diabetic mice. After 4 weeks of antioxidant therapy, the intracellular ROS level also diminished significantly in the bone marrow cells. Interestingly, the number of endothelial progenitor cells in the BM-MNCs and their endothelial differentiation increased in these diabetic mice after 4 weeks of antioxidant therapy. These results demonstrated clearly that antioxidant therapy could improve the diabetes-related functional impairment of bone marrow stem cells. Furthermore, we found that giving MnTBAP for 4 weeks did not change the levels of blood glucose in the diabetic mice, although a previous study reported that antioxidant therapy could improve the insulin resistance of type II diabetes. Our finding also suggests that the functional improvement of bone marrow stem cells achieved by antioxidant therapy is not dependent on the improvement in blood glucose levels.

Circulation Journal Vol.73, January 2009
Antioxidant therapy significantly attenuated the diabetes-related impairment of bone marrow stem cells. However, the intracellular ROS level, the number of endothelial progenitor cells, and the potency of endothelial differentiation of BM-MNCs were all slightly worse in the diabetic mice given antioxidant therapy than in the healthy mice. This finding indicated that 4 weeks of MnTBAP treatment resulted in partial, but not full recovery of the diabetes-related functional impairment of bone marrow stem cells. The precise reasons for the partial improvement achieved by antioxidant therapy remain unclear. A reasonable explanation is that, beyond oxidative stress, many other factors, including hyperglycemia and hyperlipidemia, also contribute to the functional impairment of bone marrow stem cells in type II diabetic mice. On the other hand, 4 weeks of antioxidant therapy may not be enough for full recovery of impaired bone marrow stem cells, and longer term therapy might be necessary.

In conclusion, oxidative stress contributes to the diabetes-related functional impairment of bone marrow cells, which can be attenuated significantly by antioxidant therapy. Further studies are required to investigate if the diabetes-related functional impairment of bone marrow stem cells can be improved further for inducing therapeutic angiogenesis in vivo.

Acknowledgment
This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References