ORIGINAL ARTICLE

Diabetes Decreases the Osteogenic Potential of Bone Marrow Progenitor Cells. Effects of Treatment with Metformin

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ABSTRACT

Diabetes mellitus is associated with an increased incidence of skeletal abnormalities, resulting in lower bone formation and/or remodeling. Osteopenia, osteoporosis and an increased incidence of non-traumatic fractures has been particularly observed in patients with type 2 diabetes. Recently, we have demonstrated that metformin has in vitro and in vivo osteogenic effects: (a) it stimulates the proliferation, differentiation and mineralization of osteoblasts in culture, and (b) in non-diabetic rats, it increases the repair of minimal bone lesions and improves femoral trabecular bone microarchitecture. In this study, we evaluated in rats the effect of diabetes induction by a partial destruction of pancreatic beta cells, on the osteogenic commitment of bone marrow progenitor cells (BMPC), and the modulation of this effect by orally administered metformin. We used young male Sprague Dawley rats (200 g), divided into four groups: untreated non-diabetic controls [C], non-diabetic rats treated for 2 weeks with metformin administered in drinking water (100 mg/kg/day) [M], untreated diabetic rats [D], and diabetic rats treated for 2 weeks with metformin (100 mg/kg/day) [DM]. Induction of Diabetes was performed one week prior to treatment with metformin, by successive intraperitoneal injections with 75 mg/kg body weight of nicotinic acid and 60 mg/kg body weight of streptozotocin. At the end of all treatments, blood samples were obtained to confirm the development of Diabetes, after which the animals were sacrificed by cervical dislocation under anesthesia. Femora and/or tibiae were dissected, and bone marrow cells were collected by flushing the bone diaphysal canal with Dulbecco’s modified essential medium (DMEM) under sterile conditions. Adherent cells were grown to confluence in DMEM-10 % fetal bovine serum (FBS), after which we assessed alkaline phosphatase specific activity (ALP) by an enzymatic kinetic method, and type 1 collagen production (Col-1) by a Sirius Red colorimetric method (basal osteoblastic differentiation of BMPC). Subsequently, BMPC were submitted to an osteogenic induction for 15 days with an osteogenic medium (DMEM-10 % FBS containing ascorbic acid and sodium beta-glycerophosphate), after which ALP and Col-1 were evaluated. Basal ALP activity and type 1 collagen production (BMPC without osteogenic differentiation) showed no significant differences between the four experimental groups. After 15 days of culture in osteogenic medium, BMPC from control rats increased their expression of ALP (5 times compared to baseline) and collagen production (11 times compared to baseline). BMPC from diabetic rats after 15 days culture in osteogenic medium also showed a significant (although smaller) increase in ALP (2-3 fold over basal activity) and collagen production (4-fold compared to baseline). BMPC obtained from rats treated with metformin (groups M and DM) and submitted to osteogenic induction for 15 days, showed an approximately 2-4-fold increase in both ALP and Col-1 (when compared with groups C and D, respectively). After 21 days of osteogenic induction, a decrease was observed in the mineralization of BMPC obtained from group D (65 % of that for group C). Treatment with metformin increased the mineralizing capacity of BMPC in all cases, including a reversal of the inhibitory effect of Diabetes on this parameter. In conclusion, we have found that our model of Diabetes reduces the osteogenic potential of bone marrow progenitor cells, and that this effect is partially reverted by orally administered metformin. These findings could explain, at least in part, the bone alterations that have been associated with Diabetes mellitus. Rev Argent Endocrinol Metab 49:70-76, 2012

No financial conflicts of interest exist.

Key words: diabetes mellitus, bone marrow progenitor cells, bone, metformin
INTRODUCTION

Diabetes Mellitus is a disease that affects 5% of the population. It is characterized by chronic hyperglycemia, which is caused by defects in insulin production or action. In the long term, diabetes is associated with a high incidence of macrovascular complications (acute myocardial infarction, stroke and peripheral vascular disease), microvascular complications (nephropathy, retinopathy, polyneuropathy) and mixed complications (diabetic foot, bone and joint alterations) \(^1\). In particular, alterations in bone metabolism are commonly observed with imbalances in calcium and phosphate levels, reductions in bone mineral content and bone mineral density (BMD), osteopenia, increased fracture risk and delayed bone injury healing \(^2\).

The occurrence and progression of chronic complications of diabetes may be partly explained by the irreversible accumulation of advanced glycation endproducts (AGEs) on long half-life proteins such as collagen \(^3\). We have previously demonstrated that the accumulation of AGEs on type I collagen (the major component of the bone matrix) reduces the bone-forming activity of osteoblasts in culture \(^4\). If this occurred \textit{in vivo}, it might result in a reduction of both bone formation and bone turnover, with a consequent reduction in bone quality.

Metformin is one of the most commonly used agents for the treatment of insulin resistance syndromes such as type 2 diabetes, metabolic syndrome and polycystic ovary syndrome. It is an insulin sensitizer biguanide that reduces blood glucose levels without directly affecting insulin secretion \(^5\).

Our group has previously reported that metformin has direct osteogenic effects on osteoblasts in culture, promoting their proliferation, differentiation and mineralization \(^6\). In other in vitro experiments, we demonstrated that metformin prevents the deleterious effects of AGEs on osteoblasts \(^7\). We have recently found that this orally administered biguanide promotes osteoblastic differentiation of bone marrow progenitor cells in non-diabetic rats; it improves femoral microarchitecture and cellularity \(^6, 9\) in diabetic and non-diabetic rats and it increases the repair of induced bone lesions \(^8\).

Bone is a dynamic tissue that constantly undergoes modeling and remodeling by the precise and localized coupling of bone formation and resorption processes. This requires the interaction of different cell types such as osteoblasts, osteocytes, bone lining cells, bone marrow progenitor cells (BMPC), macrophages and osteoclasts, in a process that is regulated by a variety of biochemical and mechanical factors \(^10\).

Progenitor cells present in the bone marrow micro-environment present the ability to differentiate into various cell types such as osteoblasts, chondrocytes and adipocytes \(^11, 13\). In this context, bone marrow metabolic conditions are determinants of the biologic balance between osteoblast-mediated bone formation and adipogenesis. Diverse endogenous factors such as hormones and cytokines, or exogenous treatments with certain drugs can affect this delicate balance, modifying the osteoblast-adipocyte ratio in the bone marrow \(^8\).

In this study, our aim was to investigate in an animal model the effect of diabetes induction by a partial destruction of pancreatic beta cells, on the osteogenic commitment of bone marrow progenitor cells (BMPC), and the potential modulation of this effect by orally administered metformin.

MATERIAL AND METHODS

Animal model: we used young male Sprague-Dawley rats (200-220 g) maintained in a temperature-controlled room at 23°C with a 12 hour light:12 hour darkness cycle, and fed standard rat laboratory chow and water \textit{ad libitum}. All experiments on animals were done in conformity with the Guidelines on Handling and Training of Laboratory Animals \(^14\), under the conditions established in the national (ANMAT Regulation 5330/97) and...
Animals were divided into four groups: untreated non-diabetic controls [C], non-diabetic rats treated for 2 weeks with metformin administered in drinking water (100 mg/kg/day) [M] (Química Montpellier S.A); rats with diabetes induced by partial destruction of pancreatic beta cells, not treated with metformin [D], and diabetic rats treated for 2 weeks with metformin (100 mg/kg/day) [DM]. Induction of Diabetes was performed one week prior to treatment with metformin, by successive intraperitoneal injections with 75 mg/kg body weight of nicotinic acid and 60 mg/kg body weight of streptozotocin (15). At the end of all treatments, blood samples were obtained to confirm the development of Diabetes, after which the animals were sacrificed by cervical dislocation under anesthesia.

Blood parameters: prior to sacrifice, blood samples were collected under anesthesia by cardiac puncture with no previous fasting, and serum was separated by centrifugation. The following parameters were evaluated in serum by commercial kits: glucose (Wiener Lab, Argentina), triglycerides (Wiener Lab, Argentina), cholesterol (Wiener Lab, Argentina), insulin (ELISA for rat insulin, ALPCO, USA) and fructosamine (BioSystelms, Spain).

Isolation and culture of BMPC: BMPC were collected by flushing the medullary canal of femurs and tibiae of C, M, D and DM rats with culture medium (8). Adherent cells were cultured to confluence in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Natocor; Córdoba, Argentina) and penicillin/streptomycin at 37°C in an atmosphere with 5% CO₂. When cells reached confluence, they were plated on culture plates for differentiation into osteoblasts.

Osteogenic differentiation of BMPCs: after cells reached confluence, they were induced to differentiate into osteoblasts using an differentiation medium containing 25 mg/ml ascorbic acid and 5 mM of β-glycerol-phosphate for 0, 15 or 21 days. At 0 and 15 days, Alkaline Phosphatase (ALP) activity and type I collagen (Col-1) production were evaluated; after 21 days, formation of mineralization nodules was quantified.

Alkaline Phosphatase Activity: The cell monolayer was washed with PBS and solubilised in 0.1% Triton-X100. An aliquot of the cell extract was used for protein determination by Bradford’s technique (16). Alkaline Phosphatase activity was measured spectrophotometrically using p-nitropheny phosphate (p-NPP) as substrate. The absorbance of p-nitrophenol (a colored product of hydrolysis) was recorded at 405 nm after incubation of the reaction mixture at 37°C in buffer pH 10.4 for a predetermined period (17). Results were expressed as nmoles p-NPP/mg of Protein/minute.

Production of type I collagen: production of type I collagen was used using a colorimetric microassay by Sirius Red technique (18); cells were fixed with Bouin’s solution (picric acid: formaldehyde 35%; glacial acetic acid - 15:5:1) for 1 h. The cell monolayer was washed with 0.1 N hydrochloric acid and stained with Sirius red for 1 hour. The stained material was dissolved in 1 ml of 0.1N sodium hydroxide, and the absorbance was recorded at 550 nm. Results were expressed as µg of collagen / 100µg of protein.

Mineralization: Extracellular calcium deposition (mineralization nodules) was evaluated after 21 days of culture using an osteoblastic differentiation medium. After fixation with formalin and staining with alizarin S red (19), stained calcium deposition were extracted with 1 mL of 0.1N sodium hydroxide, recording the optical density at 548 nm. Results are expressed as percentage (%) of baseline.

Statistical analysis: results are expressed as mean ± standard error of the mean (SEM). Differences between the groups were assessed by one-way analysis of variance.
ANOVA using the Turkey test. The GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used. Differences were considered statistically significant when p<0.05.

RESULTS

Effect of diabetes and metformin on biochemical parameters.

Treatment with nicotinic acid and streptozotocin produced a metabolic status consistent with diabetes with partial destruction of pancreatic beta cells. Table I shows the results of mean postprandial blood parameters (blood levels of glucose, triglycerides, cholesterol, insulin and fructosamine) for each of the four groups of rats.

Blood glucose levels showed a significant increase in the diabetic group (D) (270%) in relation to the control group. The group that only received metformin (M) did not show significant changes from the control group; while in the diabetic group treated with metformin (DM) a partial reversal of diabetes-induced changes was observed (185% vs control group), which correlates with the effects of this blood glucose normalizing agent and with the existence of a pancreatic insulin reserve (and possibly with peripheral resistance to its action) in this model of diabetes.

The same pattern is observed in blood triglycerides concentrations: group D showed a significant increase (200% vs. control group), while group DM presented a complete normalization of blood triglyceride levels.

Blood insulin levels show that diabetic rats had a marked reduction of insulin secretion, although with measurable levels. Treatment with metformin did not significantly change blood insulin levels, neither in diabetic nor in non-diabetic animals (Table 1).

The fructosamine test, a measurement of non-enzymatic glycation of serum proteins, showed a significant increase in group D (160% vs. C), which was not reverted by oral treatment with metformin in the group of diabetic rats (Table 1).

As regards cholesterol, there were no significant differences in any of the four groups.

Ex vivo effects on BMCPs of diabetes and treatment with metformin.

At baseline (no differentiated cells), osteoblastic markers expressed in BMCPs (type I collagen production (Graph 1) and alkaline phosphatase enzymatic activity (Graph 2) did not show significant differences between the different experimental groups in culture.

However, after 15 days of osteoblastic differentiation, group D showed a significant decrease in relation to group C in type I collagen production (30% of group C). In addition, oral treatment with metformin resulted in a significant increase in collagen expression in group M (122% of group C), while in group DM, there was a partial reversal of the effect of diabetes (68% of group C) (Graph 1).

When quantifying the alkaline phosphatase enzymatic activity, a pattern similar to that of collagen was observed: group D showed a significant decrease in this parameter (34% vs. group C) and group M showed a notable increase in this osteoblastic marker (171% vs. group C). Finally, in group DM metformin showed a significant reversal of the effects of Diabetes on ALP (125% vs. group C) (Graph 2).

After 21 days of osteoblastic differentiation, deposition of mineralization nodules was quantified (Graph 3; Figure 1). A decrease in the number of mineral deposits was observed in group D (65% of group C) and an increase in the number of deposits of mineralization nodules was observed in group M (130% vs. group C). Finally, in group DM, treatment with metformin reverted the effects of diabetes on this parameter (140% vs. the control group). (Graph 3).
TABLE I. Serum (non-fasting) biochemical parameters in the various experimental groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/ml)</th>
<th>Blood insulin (ng/ml)</th>
<th>Blood triglycerides (mg/ml)</th>
<th>Fructosamine (µmol/l)</th>
<th>Blood cholesterol (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>168 ± 8</td>
<td>1.26 ± 0.17</td>
<td>62 ± 7</td>
<td>147 ± 26</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>Metformin</td>
<td>176 ± 10</td>
<td>1.09 ± 0.3</td>
<td>52 ± 7 c</td>
<td>190 ± 13</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>453 ± 40 a</td>
<td>0.20 ± 0.09 e</td>
<td>252 ± 34 c,d</td>
<td>239 ± 12 e</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>Diabetic + metformin</td>
<td>309 ± 19 a</td>
<td>0.37 ± 0.12 c</td>
<td>58 ± 15</td>
<td>236 ± 16 e</td>
<td>56 ± 7</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of measurements.

\(^{a}p < 0.001\) vs. Control

\(^{b}p < 0.05\) vs. Metformin

\(^{c}p < 0.05\) vs. Control

\(^{d}p < 0.05\) vs. Diabetic

\(^{e}p < 0.01\) vs. Control

**DISCUSSION**

Our previous and present observations suggest that metformin can induce commitment of osteoprogenitor cells to the osteoblastic lineage and bone formation, exerting direct effects on bone cells. It has been previously reported that the mechanism of action of metformin may be mediated by MAPK, e/iNOS and AMPK signaling pathways, as well as by increasing the expression of the osteoblast-specific transcription factor Runx2/Cbfa1 (7,8).

In this study, diabetes was induced in rats by administration of nicotinamide and streptozotocin. In this model of partial destruction of pancreatic beta cells, nicotinamide partially prevents streptozotocin-induced NAD depletion in beta cells, reducing the toxic effect of streptozotocin (15). Thus, a partial decrease in insulin secretion was observed. Biochemical parameters: blood levels of glucose, triglycerides, insulin and fructosamine were consistent with the previously described diabetic status. In the case of fructosamine, no return to normal levels was observed after treatment with metformin, probably due to the half-life of albumin, in relation to the duration of the assay.

According to Owen (20), because of the differences in the metabolism of this drug between species, high concentrations of metformin are required in rats to achieve plasma concentrations similar to those found in human subjects and thus obtain its blood glucose normalizing effect in diabetic rats. Based on this concept, the dose of metformin to be used in in vivo studies was selected.

In our diabetes model, a decrease in the osteogenic potential of BMPCs was observed, an effect that was partially or fully reverted by oral treatment with metformin, as evidenced by type I collagen production, alkaline phosphatase specific activity and mineral nodules production.

Advanced glycation end-products (AGEs) are formed by non-enzymatic glycosylation reactions between amine groups of proteins, lipids or nucleic acids, with carbonyl intermediates or reducing sugars. These reactions occur in an accelerated manner in situations such as decompensated diabetes, metabolic syndrome and physiological
aging. Our group and other investigators have previously reported that the interaction between AGEs and their specific receptor RAGE induces apoptosis of osteoblasts and BMPC (4,21). Such observations, as well as our current results regarding the effects of experimental diabetes on the osteogenic potential of BMPC might explain, at least in part, bone alterations commonly observed in patients with Diabetes Mellitus.

Significantly, in our model of diabetes with decreased pancreatic insulin reserve, we have been able to verify that oral treatment with metformin may fully or partly prevent various anti-osteogenic effects of this metabolic pathology. This drug may be probably promoting osteoblastic differentiation of BMPCs via phosphorylation/activation of AMPK and subsequent induction of the osteogenic transcription factor Runx2, as we have previously reported in non-diabetic rats orally treated with metformin (8).

If our present results could be extrapolated to a clinical setting, they would be suggesting a plausible mechanism to account for the bone protective effect (reduced fracture rate) reported by other authors in cohort studies of patients treated with metformin (22).

Graph 1. Type I collagen production. Effect of diabetes and oral treatment with metformin on type I collagen production potential of BMPC. BMPCs isolated from each group of rats were cultured for different periods of time in osteogenic medium as indicated under Material and Methods. Results are expressed as: µg of collagen / 100µg of protein, and shown as mean ± SEM, n = 12. * p<0.01 vs. C; # p<0.001 vs. C; & p<0.001 vs. D.

Referencias de la figura:
Colágeno tipo I = type I collagen
(µg colágeno / 100 µ protein) = µg of collagen / 100µ of protein
Tiempo [Días] = Time [Days]
Graph 2. *Alkaline Phosphatase Activity.* Effect of diabetes and oral treatment with metformin on Alkaline Phosphatase specific Activity (ALP). BMPCs isolated from each group of rats were cultured for different periods of time in osteogenic medium. Specific ALP activity is expressed as: nmol p-NPP / mg of protein/min, and shown as mean ± SEM, * p<0.001 vs. C; # p<0.01 vs. C; & p<0.001 vs. D.

Referencias de la figura:
Actividad de fosfatasa alcalina = Alkaline Phosphatase Activity
(nmol p-NPP / mg de proteína/min) = nmol p-NPP / mg of protein/min)
Tiempo [Días] = Time [Days]

Graph 3. Effect of diabetes and oral treatment with metformin on BMPCs potential for extracellular matrix mineralization (mineral depositions). BMPCs isolated from each group of rats were cultured for 21 days in osteogenic medium. Results are expressed as % of baseline and shown as mean ± SEM, * p<0.05 vs. C; # p<0.01 vs. D.
Referencias de la figura:

Depósitos de mineral (% del basal): mineral deposition (% of baseline)

**Figure 1.** Mineral nodules: The cell monolayer was fixed with formalin and stained with alizarin red, after 21 days of culture in osteogenic medium. Objective lense: x10.

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