Metformin Enhances Osteogenesis and Suppresses Adipogenesis of Human Chorionic Villous Mesenchymal Stem Cells

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Metformin is the first-line anti-hyperglycemic drugs commonly used to treat type 2 diabetes. Recent studies have shown that metformin can enhance bone formation through induction of endothelial nitric oxide synthase (eNOS). Human chorionic villous mesenchymal stem cells (CV-MSCs) are promising candidates for regenerative medicine. The present study aimed to investigate the effects of metformin on the osteogenic and adipocytic differentiation of human CV-MSCs, and to elucidate the underlying mechanism.

CV-MSCs, prepared from human term placentae, were cultured with different concentrations of metformin. Treatment for 72 hours with 0.05 mM metformin had no noticeable effect on the proliferation of CV-MSCs. Consequently, CV-MSCs were cultured for seven or 14 days in the osteogenic medium supplemented with 0.05 mM metformin. Treatment for seven days with metformin increased the expression levels of osteogenic protein mRNAs, including alkaline phosphatase, runt-related transcription factor 2, and osteopontin. Metformin also enhanced the mineralization of CV-MSCs. Furthermore, metformin induced the expression of eNOS in CV-MSCs during osteogenic differentiation. By contrast, when CV-MSCs were cultured for 14 days in the adipogenic medium, 0.05 mM metformin inhibited the expression of adipogenic protein mRNAs, including proliferators-activated receptor-γ and CCAAT/enhancer binding protein-α. The lipid droplet accumulation was also reduced on 28 days after metformin treatment. These findings indicate that metformin can enhance osteogenic differentiation of CV-MSCs and reduce adipocyte formation. The effect of metformin on osteogenic differentiation of CV-MSCs may be associated with eNOS expression. Our findings will highlight the therapeutic potential of metformin in osteoporosis and bone fracture.

Keywords: adipocyte differentiation; chorionic villous mesenchymal stem cells; eNOS; metformin; osteogenic differentiation


Introduction

Metformin is the first-line anti-hyperglycemic drugs commonly used to treat type 2 diabetes (Kirpichnikov et al. 2002). It is considered to be an insulin-sensitizing drug that can inhibit the production of hepatic glucose and increase peripheral glucose uptake. Apart from diabetes, metformin also possesses positive effects on cardiovascular disease and nerve regeneration (Hettich et al. 2014; Fung et al. 2015). In recent years, the effects of metformin on bone metabolism have received considerable attention; there is a close relationship between glucose and bone metabolism (Meier et al. 2016; Starup-Linde and Vestergaard 2016). Bone metabolism includes two steps: osteoclastic bone resorption and osteoblastic bone formation. This process is important for bone repair and mineral homeostasis (Raggett and Partridge 2010). Diabetes can induce osteoporosis and increase the risk of bone fractures (Mai et al. 2011; Tsentidis et al. 2016; Wang et al. 2016). Mesenchymal stem cells (MSCs) possess multiple differentiation abilities and have great therapeutic potentials for bone repair (Doulatov and Daley 2013). Osteogenic genes including runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and osteopontin (OPN) are critical for osteogenic differentiation of MSCs (Ryoo et al. 2006; Vimalraj et al. 2015). Recent studies have shown that metformin can decrease fracture risk in patients with diabetes (Vestergaard et al. 2005). In addition, metformin can induce the osteogenic differentiation of MC3T3E1 cells (clonal osteoblast-like cells derived from mouse calvaria) and bone marrow (BM) progenitor cells (Gao et al. 2010; Jang et al. 2011). However, the effect of metformin on the differentiation of human chorionic villous mesenchymal stem cells (CV-MSCs) is still unknown.
The beneficial effects of metformin are associated with phosphorylation of AMP-activated protein kinase (AMPK) (Musi et al. 2002; Duca et al. 2015). AMPK is important in regulating the glucose and fatty acid metabolism. Metformin can augment the differentiation of endothelial progenitor cells through AMPK/endothelial nitric oxide synthase (eNOS)/nitric oxide pathway (Li et al. 2015). Takahashi et al. (2015) reported that metformin can treat ischemic peripheral artery diseases by increasing the activation of AMPK and eNOS.

In the present study, we investigate the effects of metformin on the differentiation of human CV-MSCs and explored the underlying mechanisms. The results showed that metformin can promote osteogenic differentiation of human CV-MSCs and inhibit adipocyte formation. The effect of metformin on osteogenic differentiation of CV-MSCs may be associated with metformin-induced eNOS expression.

Materials and Methods

Human CV-MSC isolation
MSCs were isolated from the chorionic villi of human term placentas and characterized as we and others previously described (Park et al. 2011; Pilz et al. 2011; Gu et al. 2016). Chorionic villi were minced and digested. Cells were filtered by a nylon tissue mesh to obtain a single cell suspension. Cells were centrifuged with a Percoll (Sigma Aldrich, St. Louis, USA) discontinuous gradient and then seeded in a 10-cm Petri dish using Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, South Logan, USA) containing 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY). After 4 days, media were changed, and nonadherent cells were washed. This study was approved by the ethics committee of the First Affiliated Hospital of Soochow University.

Cell proliferation assay
Cell proliferation was detected using a CCK8 kit (Dojindo, Tokyo, Japan) according to the manufacturer’s instructions. Briefly, cells were cultured in 96-well plates. Metformin (0, 0.05, 0.1, 0.5, and 1.0 mM) (Sigma Aldrich) was added to the cells 24 h after plating and incubated for 72 h. 10 μl CCK-8 solution was added to the wells and the cells were incubated for 2.5 h in the incubator. The Multi-Volume Spectrophotometer System (BioTek Epoch, Vermont, USA) was used to measure the absorbance at 450 nm.

Osteogenic differentiation of human CV-MSCs
Human CV-MSCs were cultured in DMEM medium supplemented with 10% fetal calf serum, 10 mM β-glycerol phosphate (Sigma Aldrich), 0.1 mM L-ascorbic acid (Sigma Aldrich), and 10 mM dexamethasone (Sigma Aldrich), 2 mM glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). In some experiments, metformin (0.05 mM) was added once every 3 days during osteogenic differentiation. Cells were collected at days 7 and 14 during osteogenic differentiation.

Alizarin red staining
Calcium deposits were detected by alizarin red S staining after 28 days of osteogenic induction. Cells were incubated with 2% alizarin red (pH 4.2) (Sigma Aldrich) for 10 min and washed with distilled water. Cells were detected by phase-contrast microscopy at day 28 to verify the presence of mineralized nodules.

Adipocyte differentiation of human CV-MSCs
Human CV-MSCs were cultured in adipogenic medium (Cyagen Biosciences Inc., USA) with or without metformin. Cells were detected by phase-contrast microscopy at day 28 to verify the presence of lipid droplets.

Oil red staining
At day 28 after adipocyte differentiation, cells were fixed in 1% formaldehyde, incubated with 0.5% Oil red solution (Sigma Aldrich) for 20 min, rinsed with 8% propylene glycol (Sigma Aldrich) for 3 min, washed in distilled water and mounted with aqueous mounting medium.

Real-time quantitative PCR
Total RNA was extracted from cells using TRIzol (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer’s instructions and reverse transcribed. Real-time PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) and conducted with the Biorad CFX96 (Bio-Rad). Primer sequences for real-time PCR were listed in Table 1. Glyceraldehyde-3-phosphate dehy-

Table 1. Oligonucleotides used in real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>OPN</td>
<td>GAGGCCCAAGCAGGAAGACATC</td>
<td>CAGATACGTCATCGACAAC</td>
</tr>
<tr>
<td>OCN</td>
<td>CACTCTCGCCCTATTGGC</td>
<td>CCCCTCCTGCTTGGACACAAAG</td>
</tr>
<tr>
<td>RUNX2</td>
<td>TGTTACTGTCATGCGGGA</td>
<td>TCTCAGATCGTTGAAACTTGCTA</td>
</tr>
<tr>
<td>ALP</td>
<td>GTGAACCGCAACTGGTACTC</td>
<td>GAGCTGCTAGCGATGTCC</td>
</tr>
<tr>
<td>eNOS</td>
<td>TGATGGCGAGCCAGTGAAG</td>
<td>ACTCTATTACACACAGG</td>
</tr>
<tr>
<td>FABP4</td>
<td>ACTGCGCAGAATTTGACG</td>
<td>CTGTTAGGAGAGTGGCCTT</td>
</tr>
<tr>
<td>PPARγ</td>
<td>GGATCGCTCCCTGAGTATCT</td>
<td>TGCACTTTGATCTTTGAAGT</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>GTGGAGACGCAGCAGAAG</td>
<td>TTCCAAGGGCAAGGTTATC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAGCGAGATCCCTCATGAGAAT</td>
<td>GCCTGTGTTCATACCTTCATG</td>
</tr>
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Metformin increases the osteogenic differentiation of CV-MSCs, metformin (0.05 mM) was added into the osteogenic medium. Real-time PCR was performed at days 7 and 14. Compared to the untreated control, metformin significantly increased the expression of ALP, RUNX2, and OPN at days 7 and 14 (p < 0.05; p < 0.01; Fig. 2A-C). However, metformin had no significant effect on the expression of osteocalcin (OCN) (Fig. 2D). To further confirm the effect of metformin on osteogenic differentiation of human CV-MSCs, Alizarin red staining was performed after 28 days of osteogenic induction. The results showed metformin increased the mineralization of human CV-MSCs at day 28 (Fig. 2E).

Metformin inhibits the adipocyte differentiation of human CV-MSCs

To investigate the effects of metformin on adipocyte differentiation of human CV-MSCs, metformin was added into the adipogenic medium. Real-time PCR showed that metformin did not affect the expression of adipogenic genes at day 7 compared to untreated control. However, the expression of proliferators-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα) was significantly reduced by metformin at day 14 (Fig. 3A, B). Moreover, metformin almost had no effect on the expression of fatty acid binding protein 4 (FABP4) at day 14 (Fig. 3C). Oil red O staining showed that metformin significantly inhibited the lipid droplet accumulation of CV-MSCs at day 28 (Fig. 3D).

Metformin induces eNOS expression

Nitric oxide is known to be involved in bone metabolism. To investigate the mechanism by which metformin affects osteogenic differentiation, we analyzed the expression of eNOS. Real-time PCR was performed at day 3. The results showed that metformin increased the mRNA expression of eNOS compared to untreated control (Fig. 4A). Moreover, western blot showed that metformin treatment increased the protein expression of eNOS at day 3 (Fig. 4B).

Discussion

Diabetes is considered to be a global health problem. Most diabetes patients have impaired bone formation and remodeling. The role of anti-diabetes drugs in the bone metabolism has received extensive attention in recent years (see Introduction). In the present study, we investigated the role of metformin in the differentiation of human CV-MSCs. Our results showed that metformin treatment could promote the osteogenesis but suppress adipogenesis of human CV-MSCs.

Metformin is an insulin sensitizing drug commonly used to treat type 2 diabetes. It has been reported that metformin can improve insulin resistance and lipid profiles (Kirpichnikov et al. 2002). Recently, in vivo and in vitro studies have suggested a role for metformin in regulating cell proliferation. High dose metformin can inhibit the
Fig. 2. The effects of metformin on the osteogenic differentiation of human CV-MSCs. Human CV-MSCs were cultured in osteogenic medium supplemented with 0.05 mM metformin. (A-D) The mRNA levels of RUNX2, OPN, OCN, and ALP were analyzed by real-time PCR at days 7 and 14. Data were shown as means ± SEM (n = 3). *p < 0.05, **p < 0.01. (E) Alizarin Red staining was performed at day 28.

Fig. 3. The effects of metformin on the adipogenic differentiation of human CV-MSCs. Human CV-MSCs were cultured in adipogenic medium supplemented with 0.05 mM metformin. (A-C) The mRNA levels of PPARγ, C/EBPα, and FABP4 were analyzed by real-time PCR at days 7 and 14. Data were shown as means ± SEM (n = 3). *p < 0.05, **p < 0.01. (D) Lipid droplets were stained with oil red O at day 28.
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growth of tumor cells (He et al. 2015). In contrast, low
dose metformin (0.64 μM) can increase the proliferation of
rat primary osteoblasts (Zhen et al. 2010). In the present
study, our data clearly showed that low dose (0.05 mM)
metformin exerted no noticeable effects on the proliferation
of human CV-MSCs. At the concentration range of 0.1-1
mM, metformin significantly reduced the proliferation of
human CV-MSCs. Interestingly, Li et al. (2015) reported
that 0.5-1 mM metformin did not affect the proliferation of
endothelial progenitor cells. Wu et al. (2011) found that 1
mM metformin did not affect the proliferation of human
CV-MSCs. Bone morphogenetic protein 2 (BMP2) is
another regulator that can stimulate osteoblast differentia-
tion of human CV-MSCs. Bone morphogenetic protein 2 (BMP2) is
another regulator that can stimulate osteoblast differentia-
tion of human CV-MSCs. BMP2 can promote the osteogenic dif-
ferentiation of human CV-MSCs. Molinuevo et al. (2010)
found that BMP2 can increase ALP activity and
mineralization in rat BM progenitor cells. Cortizo et al.
(2006) showed that metformin (25-100 μM) can increase
type I collagen production and ALP activity in MC3T3E1
cells. Moreover, metformin can reverse rosiglitazone-
induced bone loss in rats (Sedlinsky et al. 2011). Our
results were consistent with these reports and confirmed
that metformin possessed beneficial effects on bone forma-
tion. In contrast, others reported that metformin (1 mM)
had no effect on the osteogenic differentiation of MSCs
when cultured in β-glycerol phosphate and ascorbic2-phos-
phate medium (Wu et al. 2011). Cortizo et al. (2006) also
found that metformin had no effect on ALP activity in
UMR106 rat osteosarcoma cells. At the concentration of 2
mM, metformin reduced mineralization of osteoblasts
(Kasai et al. 2009). These conflicting results may be attribu-
ted to the different culture conditions and cell sources.

We also found that metformin reduced the expression of
C/EBPα and PPARγ in human CV-MSCs at day 14 after
dipogenic induction. However, metformin did not affect
the expression of C/EBPα and PPARγ at day 7 after adipogenic
induction. C/EBPα and PPARγ are critical factors in adipogenic
differentiation. Our results suggested that met-
formin did not affect adipogenesis at an early stage of dif-
ferentiation. Metformin only inhibited the adipogenesis of
human CV-MSCs at a later stage of differentiation.
Previous studies have shown that metformin can suppress
the adipogenic differentiation of murine MC3T3E1 cells in
combination with atorvastatin (Kim et al. 2015). In addi-
tion, metformin can decrease adipogenic gene expression in
subcutaneous adipose tissue explants (Moreno-Navarrete et
al. 2011). Our findings were in agreement with their
results. Interestingly, we found that the expression of
FABP4 was just slightly reduced after metformin treatment.
FABP4 is a target gene of PPARγ. PPARγ can induce the
expression of FABP4 in adipocytes. However, Garin-
shkolnik et al. (2014) have shown that FABP4 can nega-
tively regulate PPARγ expression and adipocyte differen-
tiation. We believe that there may be a balance between
FABP4 and PPARγ. Metformin can maintain the expres-
sion of FABP4. FABP4 then reduced the expression of
PPARγ, which may help to suppress adipogenesis in human
CV-MSCs.

We next investigated the possible factors which con-
tribute to metformin-induced osteogenic differentiation of
human CV-MSCs. It has been accepted that eNOS partici-
pates in the regulation of bone homeostasis (Wimalawansa
2010). eNOS knockout mouse showed impaired osteogenic
differentiation (Afzal et al. 2004). Bezafibrate can promote
the osteogenic differentiation of MC3T3E1 cells through
regulating eNOS (Zhong et al. 2011). In the present study,
we found that treatment of human CV-MSCs with metfor-
min led to an increase in the expression of eNOS. These
data suggest that eNOS may play an important role in met-
formin-induced osteogenic differentiation of human
CV-MSCs. Bone morphogenetic protein 2 (BMP2) is
another regulator that can stimulate osteoblast differencing.
tion (Salazar et al. 2016). BMP2 overexpression can augment osteogenic differentiation of human periodontal liga-
ment stem cells in vivo (Yi et al. 2016). Metformin can stimulate the expression of BMP2 in MC3T3E1 cells (Kanazawa et al. 2008). However, Wang et al. (2012) reported that metformin can suppress the expression of BMP2 in insulin-resistant mice. In the present study, we did not observe an obvious change in the expression of BMP2 in metformin-treated CV-MSCs (data not shown). Previous studies have shown that AMPK can stimulate the osteogenic differentiation of MC3T3E1 cells (Kanazawa et al. 2007). AMPK is an energy regulator that regulates glu-
cose metabolism. Molinuevo et al. (2010) have reported that metformin can activate AMPK signaling pathway in BM progenitor cells, followed by increased expression of eNOS and BMP2. It is possible that metformin can pro-

tome osteogenic differentiation through activation of AMPK/eNOS pathway in human CV-MSCs.

There are some limitations in this study. The effect of metformin on AMPK activation was not evaluated. Additional experiments need to be done to demonstrate the relationship between metformin-induced eNOS expression and osteogenic differentiation in human CV-MSCs.

In the present study, we investigated the effects of metformin on the differentiation of human placental CV-MSCs. Our results demonstrated that metformin can promote the osteogenic differentiation of human CV-MSCs and inhibit adipocyte formation. Moreover, metformin can induce eNOS expression in human CV-MSCs, suggesting a possible involvement of eNOS in metformin-induced osteogene-
sis of CV-MSCs in vitro.

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Conflict of Interest

The authors declare no conflict of interest.

References

Gao, Y., Li, Y., Xue, J., Jia, Y. & Hu, J. (2010) Effect of the anti-


