Effect of 6-bromoindirubin-3’-oxime on human deciduous tooth dental pulp cells

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Abstract: Stem cells have been a recent focus of regenerative medicine research. They originate from various tissues including human dental pulp containing human deciduous tooth dental pulp cells (hDPCs) that feature in the field of dentistry. However, only a very small number of stem cells are present in dental pulp tissue. It is known that 6-bromoindirubin-3’-oxime (BIO), a specific glycogen synthase kinase-3 (GSK-3) inhibitor, maintains the undifferentiated state of human and mouse embryonic stem cells. However, there are few reports that have applied BIO to hDPCs. Thus, we hypothesized that BIO may be necessary to maintain the undifferentiated state of hDPCs for clinical application. The aim of this study was to evaluate the effect of BIO on the undifferentiated state of hDPCs. hDPCs were extracted from the non-caries deciduous teeth of healthy children. BIO treatment at 0.5–1.5 μM slightly affected the proliferation of hDPCs, and 1–1.5 μM BIO induced the expression of Oct3/4 and Sox2 genes. Conversely, 0.5–1.5 μM BIO significantly reduced the gene expression of c-Myc as determined by real-time RT-PCR. Moreover, 1 μM BIO induced the expression of CD44 and CD90 as determined by flow cytometry. In summary, we found that 1 μM BIO was optimal for maintaining the undifferentiated state of hDPCs.

Key words: deciduous tooth, dental pulp, undifferentiated state, 6-bromoindirubin-3’-oxime

Introduction

In recent years, much attention has been focused on regenerative medicine, and stem cells are known to differentiate into various tissues including odontoblastic, chondrocytic, adipocytic and osteoblastic cell lineages1-3). To date, stem cells have been isolated from various tissues including human dental pulp4). Using stem cells from human dental pulp tissue, in vivo studies have demonstrated the stem cell differentiation into odontoblast-like cells lining the existing dentin surface5), and the formation of a continuous layer of dentin-like tissue on existing canal dentinal walls and on mineral trioxide aggregate cement surfaces6). In addition, transplantation of stromal stem cells into immunocompromised rats, which were obtained from human dental pulp or bone fragments in vitro, results in generation of a tissue structure with an integral blood supply similar to that of human adult bone5). Compared with adult bone marrow stromal stem cells, the stem cells from human exfoliated deciduous teeth (SHED) show a higher proliferation rate and number of population doublings8), and human deciduous tooth
dental pulp cells (hDPCs) have a higher proliferation rate than those from permanent teeth\(^9\). Therefore, it is thought that hDPCs in particular may be useful for tissue regeneration. However, the stem cells in dental pulp tissue are present in very small quantities. Some studies have reported that stem cells comprise 0.8% and 0.4% of human and mouse dental pulp, respectively\(^{10, 11}\). Thus, it is necessary to maintain the undifferentiated state of hDPCs for clinical application.

Some reports have described the use of 6-bromoindirubin-3′-oxime (BIO), a specific glycogen synthase kinase 3 (GSK-3) inhibitor that activates the Wnt signaling pathway and sustains the pluripotency of human and mouse embryonic stem cells (HESCs and MESCs, respectively). Specific GSK-3 inhibitors, such as BIO, may have practical applications in regenerative medicine\(^{12}\). In addition, immortalized pancreatic mesenchymal stem cells maintain their undifferentiated state in BIO medium\(^{13}\), and the addition of BIO to cultures significantly promotes the establishment of multipotent male germ line stem cell (mGSCs) colonies derived from goats and maintains their undifferentiated state\(^{14}\).

Moreover, BIO has demonstrated other effects such as enhancement of cardiomyocyte proliferation\(^{15, 16}\), neural differentiation\(^{17}\), alleviation of methylprednisolone-mediated bone microstructure damage \textit{in vivo}\(^{18}\), and suppression of tumor growth \textit{in vivo} with low toxicity in a mouse xenograft model of melanoma\(^{19}\). Based on these reports, BIO may have pharmacological potentials in various clinical fields.

However, very little is known about the effect of BIO on the undifferentiated state of hDPCs. Therefore, in this study, we examined cell proliferation, the expression of Oct3/4, Sox2 and c-Myc genes, which are related to the undifferentiated state, and the expression of mesenchymal stem cell markers CD44, CD90 and STRO-1 to evaluate the effect of BIO on the undifferentiated state of hDPCs.

**Materials and methods**

**Cell culture**

hDPCs were extracted from the non-caries deciduous teeth of healthy children for orthodontic reasons or prolonged retention of the deciduous tooth. Immediately after extraction, the teeth were placed in sterile 0.01 M phosphate-buffered saline (PBS) and cut horizontally under sterile conditions. The dental pulp tissue was gently removed from the teeth, minced and placed in 35-mm tissue culture dishes. Then, the dental pulp tissue was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin at 37\(^\circ\)C in a humidified atmosphere containing 5% CO\(_2\). Culture medium was exchanged every 3 days. hDPC cultures were established from cells growing out of the dental pulp tissue. At confluency, cells were subcultured by trypsinization. Passage 3–7 hDPCs were used in experiments.

Informed consent was obtained prior to procedures that were approved by the Ethical Committee of Osaka Dental University (No. 110713).

**BIO**

BIO (Calbiochem® EMD Chemicals Inc., San Diego, CA) was diluted to a concentration of 2.8 mM (1 mg/mL) in dimethyl sulfoxide. After hDPCs were incubated for 3 days, BIO was added to cultures, followed by another 48 h of incubation. The cells were then used in the following experiments.

**Cell proliferation assay**

Cell proliferation was evaluated by a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA).
Passage 3–5 cells were used in experiments. hDPCs were cultured at \(3 \times 10^5\) cells/mL in 96-well plates for 3 days. Then, BIO was added to cultures at 0.5, 1, 1.5, 2, and 2.5 \(\mu\)M. After incubation for 48 h, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and phenazine ethosulfate were added to the cultures. Absorbances at 490 nm and 690 nm were then measured using a microplate reader (Spectra Max M5; Molecular Devices Inc. Downingtown, PA, USA).

**Real-time reverse transcription polymerase chain reaction (RT-PCR)**

After incubation of hDPCs at \(8 \times 10^5\) cells/mL in a 12-well plate for 3 days, 0.5, 1.0 and 1.5 \(\mu\)M BIO was added to cultures, followed by incubation for 48 h. Total RNA was isolated and purified by a Mag Extractor (Toyobo, Osaka, Japan). Then, reverse transcription was performed with a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Briefly, reactions were carried out in a total volume of 20 \(\mu\)L consisting of 16 \(\mu\)L total RNA and 4 \(\mu\)L Master Mix for 5 min at 25\(^\circ\)C, 30 min at 42\(^\circ\)C, and then 5 min at 85\(^\circ\)C to terminate the reaction.

Real-time RT-PCR was performed in a Step One Plus (Applied Biosystems) using Taqman® Fast Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. RT-PCR was performed in a total volume of 10 \(\mu\)L consisting of 5 \(\mu\)L Master Mix, 3 \(\mu\)L RNase-free water, 0.5 \(\mu\)L primers (Oct3/4, Sox2, or c-Myc), 0.5\(\mu\)L GAPDH primer and the cDNA sample. Cycling conditions for Oct3/4 (Assay ID: Hs01654807_s1), Sox2 (Assay ID: Hs01053049_s1), c-Myc (Assay ID: Hs00905030_m1) and GAPDH (Assay ID: Hs02758991_g1) genes using Taqman® Gene Expression Assays (Applied Biosystems) were 2 min at 50\(^\circ\)C, 10 min at 94\(^\circ\)C, and then 40 cycles of 15 s at 94\(^\circ\)C, 30 s at 54\(^\circ\)C and 1 min at 72\(^\circ\)C. To correlate the cycle threshold values from the amplification plots with the copy number, a standard curve was generated and a no template control was included in every assay. The primers used are listed in Table 1.

**Flow cytometry**

hDPCs cultured at \(3 \times 10^5\) cells/mL in 6-cm dishes for 3 days were treated with 1 \(\mu\)M BIO for 48 h. Then, cells were collected and placed in 96-well plates (Corning Incorporated, NY, USA), washed with 1% BSA-PBS (Pierce Biotechnology Inc., Rockford, IL, USA) three times, and blocked with 100 \(\mu\)L/well Super Block® (Thermo Scientific, Rockford, IL, USA) diluted in PBS for 15 min at 4\(^\circ\)C. The cells were then incubated with primary antibodies (100 \(\mu\)L/well) at 4\(^\circ\)C for 30 min. Primary antibodies against the following mesenchymal stem cell markers were used: STRO-1 (1:100) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), CD44

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**Table 1 Primers used in real-time RT-PCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Assay ID</th>
<th>NCBI Gene Reference</th>
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<tbody>
<tr>
<td>Oct3/4</td>
<td>Hs01654807_s1</td>
<td></td>
</tr>
<tr>
<td>Sox2</td>
<td>Hs01053049_s1</td>
<td>NM_003106.3</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Hs00905030_m1</td>
<td>NM_002467.4</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Hs02758991_g1</td>
<td>NM_002046.3</td>
</tr>
</tbody>
</table>

TaqMan® Gene Expression Assays (Applied Biosystems) were used.
 Cells were washed twice, and then incubated with the secondary antibody (1:500) (Santa Cruz Biotechnology) at 100 μL/well for 30 min at 4°C. After two washes, Through Path (Onchip Biotechnologies, Tokyo, Japan) and propidium iodide (Sigma-Aldrich, St. Louis, MO) were added to wells at 100 μL/well. Flow cytometric analysis was performed with a microfluidic flow cytometer (Fishman R Full; Onchip Biotechnologies).

Statistical analysis

Results were expressed as the mean ± SE. Statistical analysis was performed by one-way ANOVA for non-repeated measures to detect differences between multiple groups. Differences between groups were determined by the Student-Newman-Keuls test. A value of $P<0.05$ was considered to be significant.

Results

Effect of BIO on the proliferation of hDPCs

We first assessed the toxicity of BIO by its effect on the proliferation of hDPCs. At 48 h after treatment, BIO slightly affected cell proliferation at 0.5–1.5 μM and significantly reduced cell proliferation at 2.0–2.5 μM (Fig. 1).

BIO induces the expression of Oct3/4 and Sox2 genes in hDPCs

The expression of Oct3/4, Sox2 and c-Myc genes in hDPCs treated with BIO is shown in Figure 2. Exposure of hDPCs to 1 and 1.5 μM BIO resulted in significantly increased gene expression.
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BIO treatments at 0.5, 1 and 1.5 μM resulted in a significant decrease of c-Myc gene expression.

**BIO induces the expression of CD44 and CD90 in hDPCs**

We detected the expression of CD44, CD90 and STRO-1 by flow cytometry. Representative histograms of hDPCs treated with 1 μM BIO, compared with those of untreated cells, are shown in Figure 3. BIO did not affect the expression of STRO-1 at 48 h after treatment. Conversely, BIO resulted in a significant increase of CD44 and CD90 expression.

**Discussion**

Indirubins have been known for over a century as a component of plant-derived indigo dye. They can be obtained from plants, mollusks, bacteria, and mammalian urine. BIO is a 6-brominated indirubin extracted from natural sources such as the marine mollusk *Hexaplex trunculus* L, and its pharmacological action is selective for GSK-3. A previous study showed that BIO is an effective and specific inhibitor of GSK-3 activity *in vivo*.20

GSK-3 is a ubiquitously expressed protein
kinase that exists in two isoforms, α and β\(^2\). GSK-3β is involved in the regulation of a wide range of cellular functions including differentiation, growth, proliferation, motility, and cell cycle progression\(^2\). In addition, it has been shown to induce apoptosis under various conditions\(^2\)\(^2\)-\(^5\).

GSK-3 is a component of the Wnt signal transduction pathway\(^2\). The cell-cell Wnt signaling pathway plays a critical and evolutionarily conserved role in directing cell fates during embryogenesis. Regulation of β-catenin levels is critical for Wnt signaling\(^2\), because Wnt signaling is regulated by the presence or absence of intracellular β-catenin\(^2\). Phosphorylation of β-catenin inhibits signaling in the absence of Wnt ligand\(^2\). β-catenin is phosphorylated by GSK-3\(^2\),\(^2\)\(^8\) for indirect regulation of the Wnt signaling pathway. It is known that BIO can activate Wnt signaling in HESCs\(^1\). Conversely, Wnt signaling is endogenously active in undifferentiated MESCs and is downregulated upon their differentiation\(^1\). It is a common pathway for the maintenance of the undifferentiated state of both MESCs and HESCs, and the activation of the canonical Wnt pathway by BIO facilitates maintenance of the undifferentiated phenotype of MESCs and HESCs\(^1\). Taken together, BIO suppresses phosphorylation of β-catenin, which is induced by GSK-3β, and activates Wnt signaling, leading to maintenance of the undifferentiated state.

For clinical application, it is necessary to maintain the undifferentiated state of hDPCs. In this study, we found that 0.5–1.5 \(\mu\)M BIO was effective in hDPCs, because 2–2.5 \(\mu\)M BIO significantly reduced hDPC proliferation. Moreover, 1–1.5 \(\mu\)M BIO significantly induced the expression of Oct3/4 and Sox2 genes. It has been reported that Oct3/4 is expressed in the majority of BIO-treated HESCs\(^1\) and mGSCs\(^4\). In addition, it has been reported that Sox2 expression is increased in mGSCs by BIO\(^4\). In hDPCs, our results were similar to those in these reports. Furthermore, a report has shown that the expression of pluripotency markers in undifferentiated cells, particularly Oct4 and Sox2, tends to decrease after adipogenic differentiation of dental pulp-derived cells\(^9\). Based on this report, it is thought that Oct3/4 and Sox2 genes are highly expressed in an undifferentiated state, and an increase of Oct3/4 and Sox2 gene expression indicates potentiation of the undifferentiated state.

C-Myc expression was reduced by 0.5–1.5 \(\mu\)M BIO in this study. Activation of Wnt signaling (and therefore GSK-3 inhibition) prevents c-Myc-induced apoptosis of Rat-1 cells\(^9\), and it has been confirmed that the BIO-mediated effect is caused by Wnt activation\(^1\). Therefore, these reports support our results, in which BIO reduced the gene expression of c-Myc in hDPCs.

Mesenchymal stem cell markers CD44 and CD90 were induced by 1 \(\mu\)M BIO. Many mesenchymal stem cell markers are used for investigation of dental pulp cells. Other studies have reported that CD44 and CD90 expression is increased in hDPCs\(^1\),\(^2\). Based on these studies, we used antibodies to analyze CD44 and CD90 expression, and our results were the same as those in previous studies. In contrast, BIO treatment did not significantly increase STRO-1 expression. Human dental pulp stem cells and human deciduous tooth stem cells include STRO-1-positive cells\(^3\). Compared with human bone marrow mesenchymal stem cells, SHED express significantly higher levels of STRO-1\(^3\). Therefore, we assumed that STRO-1 expression in hDPCs is induced by BIO. However, our results showed that BIO had no effect on the expression of STRO-1 in hDPCs.

The increase of Oct3/4 and Sox2 gene expression as well as CD44 and CD90 expression suggest that the undifferentiated state is maintained by BIO treatment of hDPCs. In addition, it has
been reported that BIO-mediated Wnt activation is functionally reversible, because withdrawal of BIO leads to normal multi-differentiation programs in both HESCs and MESCs\(^1\). Therefore, we believe that it is necessary to examine whether Wnt signaling is reversibly activated in hDPCs treated with BIO.

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**References**

乳歯歯髄由来細胞における 6-bromoindirubin-3’-oxime の影響

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大浦 清 2, 有田 憲司 1

近年、幹細胞は再生医療の研究において注目されており、歯科領域においてはヒト歯髄、特にヒト乳歯髄などの組織に存在している。しかし、ヒト歯髄組織に含まれる幹細胞の量は極めて少ない。glycogen synthase kinase-3 (GSK-3) に特異的な阻害剤である 6-bromoindirubin-3’-oxime (BIO) は、ヒトやマウス ES 細胞において未分化能を維持できる薬剤として知られている。しかし、この BIO をヒト乳歯歯髄由来細胞に用いた報告はない。乳歯歯髄細胞を臨床応用するためには、未分化能を維持することが必要であると考える。そこで今回、BIO の乳歯歯髄由来細胞における未分化能への影響について検討を行った。乳歯歯髄由来細胞は、う蝕のない乳歯から組織を採取した。0.5〜1.5 μM BIO では乳歯歯髄由来細胞に与える影響がわずかであった。リアルタイム RT-PCR による検索によると、1.0〜1.5 μM BIO で Oct3/4, Sox2 遺伝子発現が有意に増強し、一方、c-Myc 遺伝子発現はすべての濃度で有意に減少した。さらにフローサイトメトリーでは、1.0 μM BIO において CD44, CD90 発現が有意に増加した。以上より、乳歯歯髄由来細胞の未分化能の維持には 1.0 μM BIO が最適であることが明らかとなった。

キーワード：乳歯，歯髄，未分化能，6-bromoindirubin-3’-oxime