The Immunologic Properties of Undifferentiated Stem Cells from Human Exfoliated Deciduous Teeth (SHED) and Its Potential Application in Bone Regeneration

Nurul AA, Tan SJ, Asiah AB, Norliana G, Nor Shamsuria O, Nurul AS

School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

ABSTRACT

Introduction: Stem cells from human exfoliated deciduous teeth (SHED) are highly proliferative, clonogenic cells capable of differentiating into osteoblasts and inducing bone formation. It is a potential alternative for stem cell bone regeneration therapy. However, stem cell therapy carries the risk of immune rejection mediated by inflammatory cytokines of the human defense system.

Objective: This preliminary research studies the interaction between SHED and the immune system by determining the inflammatory cytokines profile and osteogenic potential of SHED.

Methods: Human fetal osteoblasts (hFOb) cell line and isolated SHED were cultured and total RNA was extracted, followed by reverse transcription cDNA synthesis. Semi-quantitative reverse transcription PCR and Multiplex PCR were performed to detect the expression levels of OPG/RANKL and TNF-α, IL-1β, IL-6, IL-8 and TGF-β in both cell types.

Results: Analysis showed that SHED expressed significantly lower amounts of IL-1β, IL-6, and IL-8 compared to hFOB. IL-1β is a potent bone-resorbing factor, while IL-6 and IL-8 induce osteoclastogenesis and osteolysis respectively. SHED did not express TNF-α which stimulates osteoclastic activity. SHED demonstrated high OPG/RANKL ratio, in contrast with that of marrow stem cells described in previous studies. Our findings suggest that SHED may have improved immunomodulatory profile in terms of promoting relatively lower inflammatory reaction during transplant and enhancing bone regeneration.

Conclusion: SHED has a potential to be a good source of osteoblasts for bone regeneration therapy. Further studies on the immunomodulatory properties of SHED-derived osteoblasts are necessary to enable stem cell therapy in immunocompetent hosts.

KEYWORDS: Stem cells, deciduous teeth, stem cells from human exfoliated deciduous teeth (SHED), osteoblast, cytokines.

INTRODUCTION

Stem cells from human exfoliated deciduous teeth (SHED) which was discovered in 2003 have been demonstrated to have a significant potential in renewal and regeneration of a variety of cells, including neural cells, odontoblasts and osteoblasts. Studies have provided evidence that SHED are postnatal cells, which are capable to proliferate and differentiate into several cytotypes, mainly osteoblasts, and also able to form lamellar bone after transplantation into immunosuppressed rats. SHED is a promising source of osteoblasts with significant potential for bone regeneration and stem cell therapy in humans. A balance between bone formation and resorption during stem cell therapy for bone regeneration is necessary for tissue integration and success of the transplant. Inflammatory cytokines, including interleukin-1β (IL-1B), interleukin-6 (IL-6) and tumour necrosis factors (TNF), as well as proteins like osteoprotegerin (OPG) and receptor activator of NF-kB ligand (RANKL), are mediators that regulate cell proliferation and differentiation in bone. The expression levels of these osteogenic and osteoclastogenic genes are important in determining the outcome of the bone remodeling process. In vitro studies have provided evidence that cytokines released by osteoblasts may directly stimulate bone resorption by osteoclasts.

RANKL and OPG are expressed by osteoblasts, and together with the protein RANK which is found on osteoclastic progenitors; this triad from the tumour necrosis factor family plays a significant role in bone formation, remodelling and resorption. RANKL provides an osteoclastic signal through RANK, which culminates in attachment, activation and survival of osteoclasts. On the other hand, OPG protects bone from excessive resorption by competitively inhibiting the action of RANKL and promoting apoptosis and reduction in the number of osteoclasts.

Corresponding author:

Dr. Nurul Asma Abdullah
School of Dental sciences,
16150 Kubang Kerian, Kelantan,
Malaysia.
Phone: +609 767 5841
Fax: +609 764 2026
E-mail: nurulasma@kk.usm.my
Inflammatory cytokines TNF-α, IL-1β and IL-6 are synergistic osteostrogenic factors that are produced by osteoblasts. Secretion of these cytokines will enhance osteoclastic differentiation and bone resorption, which is the main mechanism of immune rejection in cellular transplants. IL-1β directly stimulates osteoclastic resorption, increasing the proliferation and differentiation of the preosteoblasts as well as the osteoclastic activity, and inhibiting the apoptosis of osteoclasts. IL-6 stimulate bone resorption and play an important role in the initial stages of osteoclastogenesis. TNF-α, IL-1β and IL-6 also reduce the OPG/RANKL ratio, which promotes pathological bone loss. Interleukin-8 (IL-8) is a cytokine that similarly alters the OPG/RANKL ratio in favour of osteoclastic formation. On the other hand, transforming growth factor-β (TGF-β) is a potent stimulator of bone formation, promoting osteoblastic differentiation and the synthesis of the osteoid matrix. In essence, all these inflammatory cytokines modulate bone formation and resorption, and hence influence the immunological response towards cellular transplant.

The roles played by inflammatory cytokines in bone metabolism and the effect on osteoblasts have been extensively studied; however, the interaction between the human immune system and SHED still remains unclear. Research has demonstrated that SHED has potential capabilities for stem cell therapy, but the transplant tolerance for this premature cell is yet undefined. It is unknown whether SHED possess immunomodulatory functions as seen in osteoblasts, which will influence the success of transplants in an immunocompetent human. The present study will first evaluate the gene expressions of inflammatory cytokines, and the proteins factors OPG and RANKL in both osteoblasts and SHED to gauge the potential improvement in immunomodulatory profile in SHED.

MATERIALS AND METHODS

Cell line and cell culture

Human fetal osteoblasts (hFOB) cell line (Accession no: CRL-11372) was obtained from American Type Culture Collection (ATCC). The hFOB was cultured in commercially prepared Dulbecco’s Modified Eagles medium (DMEM): Nutrient Mixture F-12 (GIBCO, UK) supplemented with 10% fetal bovine serum (Sigma, USA) and 1% penicillin/streptomycin (Sigma, USA). The hFOB cell line was incubated in a CO₂ incubator at 37°C until confluence.

Stem cells were obtained from a previous study as described by Mohd et al. (2008). The cells were thawed and cultured in DMEM: Low Glucose 1X (GIBCO, UK) supplemented with 10% fetal bovine serum (Sigma, USA), 1% penicillin/streptomycin (Sigma, USA) as well as 1μM/ml of L-ascorbic acid (Sigma, Japan). The SHED was similarly incubated in a CO₂ incubator at 37°C until confluence.

Total RNA extraction

Total RNA was harvested from 1 X 10⁷ cells of both hFOB and SHED, using RNeasy Mini Kit (QIAGEN, USA) according to manufacturer’s instructions. Cells were lysed in RLT and applied onto the Qiashredder column and centrifuged. The lysate was homogenized with 70% ethanol and transferred into RNeasy mini spin columns for centrifugation. After that, 700μl of RW1 buffer was added to the column and again centrifuged. Finally, 500μl of RPE buffer was added to dissolve the total RNA. The final eluted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was assessed by the 260/280 nm absorbance ratio. The total RNA was stored at -70°C until the assays.

cDNA synthesis for hFOB and SHED

cDNA synthesis was carried out using revertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, USA). The synthesis was performed by adding the cDNA into 0.5μg oligo (dT)18 primer in nuclease-free deionized water, 1X reaction buffer, Ribolock™ Ribonuclease inhibitor (20 u/μl) and 10 mM dNTP mix. The mixture was mixed gently and centrifuged briefly before incubation for 5 minutes at 37°C. One μl of RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 u/μl) was added into the reaction mixture and incubated at 42°C for 60 minutes. These cDNA samples were then used for semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and multiplex polymerase chain reaction (MPCR).

Multiplex polymerase chain reaction (MPCR) of Human Inflammatory Cytokines Genes

Multiplex PCR was carried out on the cDNA samples using the MPCR Kit for Human Inflammatory Cytokines Genes Set-1 (Maxim Biotech Inc., USA) to detect the expression levels of the cytokines GM-CSF, TNF-α, IL-1β, IL-6, IL-8 and TGF-β genes in hFOB and SHED. The final reaction volume containing 2.5U of Taq DNA polymerase, 1X MPCR buffer mixture and 1X MPCR primers solution was added to 200ng of cDNA from hFOB and SHED, as well as to 1X control cDNA from the MPCR kit.

Amplification of cDNA samples were carried out according to the following parameters: denaturation and amplification steps at 96°C (1 min) and 66°C (4 min) for 2 cycles followed by 94°C (1 min), 66°C (2 min) for 35 cycles and elongation step at 70°C for 10 min.

The PCR products were analyzed on a 1.5% agarose gel and subjected to electrophoresis for 120 minutes at 60 V and the bands were observed. The intensity of each band was quantified with Molecular Imager Gel Doc XR System (Bio-Rad, USA).

Semi-quantitative reverse transcriptase-polymer-
Reverse transcriptase polymerase chain reaction (RT-PCR) amplification was performed in triplicate using a C-100™ thermal cycler (Bio-Rad, USA). A master mix containing 0.25 um primer solutions, 1X PCR buffer and 0.5U Taq DNA polymerase was added into 200ng of cDNA samples. The amplification of OPG and RANKL genes was performed according to the parameters as described in Figure 1.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>PCR fragment lengths</th>
<th>Sequence</th>
<th>Cycling conditions</th>
</tr>
</thead>
</table>
| GAPDH bp    | 313                  | F: GAAGTGGAAGGTCGGAGTC  
R: GAAGATGGTGATGGGATTTC | 94°C 1 min  
57°C 1 min  
72°C 1 min  
29 cycles |
| OPG bp GenBank  
Acc. no:  
NM_002546 | 469                  | F: AGACTTTTCAGCTGCTGA  
R: GGATCTCGCCAATTGTGA | 94°C 1 min  
57°C 1 min  
72°C 1 min  
29 cycles |
| RANKL bp GenBank  
Acc.no:  
AF019047  | 381                  | F: CAGGAGACCTATGCTACAGA  
R: CAAGGTCAGAGACATGGA | 94°C 1 min  
57°C 1 min  
72°C 1 min  
29 cycles |

Fig. 1. Primer sequence, PCR fragment length and cycling conditions for RT-PCR

Expression levels of OPG and RANKL were assessed for both hFOB and SHED. The house keeping gene GAPDH was used as the internal control.

The PCR products were analyzed on 1.5% agarose gel and subjected to electrophoresis for 120 minutes at 60 V. The GAPDH, OPG and RANKL amplicons were visualized and intensity of each band was quantified using the Molecular Imager Gel Doc XR System (Bio-Rad, USA).

Statistical analyses

Statistical analyses were carried out using the Mann-Whitney U test in SPSS software for Windows version 18.0. The p value < 0.05 was considered statistically significant.

RESULTS

Detection of expression levels of inflammatory cytokines by Multiplex PCR assay

The expression levels of inflammatory cytokines TNF-α, IL-1β, GM-CSF, IL-6, IL-8 and TGF-β were detected using Multiplex PCR. The TNF-α, IL-1β, GM-CSF, IL-6, IL-8 and TGF-β amplicons were visualized as 680, 555, 424, 360, 300 and 161 bp nucleotides in length, respectively (Fig. 2a).

The cytokine IL-1β was expressed in both hFOB and SHED, with SHED showing significantly lower levels of the IL-1β gene expression compared to hFOB. In addition, SHED also expressed significantly lower levels of IL-6 and IL-8 genes compared to hFOB. There was no significant difference in the expression of TGF-β gene between hFOB and SHED (Fig. 2b). It was also found that no TNF-α and GM-CSF was observed in both hFOB and SHED (Fig. 2a).
Fig. 2a. Pictures of gel analysis of MPCR products of inflammatory cytokines genes. All cytokines were less prominent in SHED when compared to hFOB, while the cytokines TNF-α and GM-CSF was not observed in both cell types.

Fig. 2b. SHED demonstrated significantly lower expression levels of inflammatory cytokines genes IL-1β, IL-6 and IL-8. The graphs were plotted with relative expression of genes with respect to GAPDH, the internal control. (□ hFOB, ■ SHED, * shows significance at P < 0.05).
Analysis of expression levels of OPG and RANKL by semi-quantitative RT-PCR assay

RT-PCR assay showed that both SHED and hFOB expressed the OPG and RANKL genes (Fig. 3a), with higher OPG levels compared to RANKL. SHED expressed high OPG/RANKL ratio in a same pattern as hFOB (Fig. 3b).

**Fig. 3a.** Pictures of gel analysis of RT-PCR products of a) GAPDH, b) OPG and c) RANKL. GAPDH was used as the internal control.

**DISCUSSION**

Stem cells have the capacity for unlimited self-renewal and retain the potential to differentiate into a variety of specialized cell types. These pluripotent cells are commonly used in regeneration therapy and transplants, which replace effete cells of damaged cartilage, bone, neural cells and others. However, the normal process of immune rejection of mismatched allogeneic tissue, mediated by inflammatory cytokines, would prevent the success of transplant therapy. In the present research, we studied the potential of SHED, in terms of its inflammatory cytokines profile and its relationship with bone regeneration. Inflammatory cytokines have pronounced modulatory effect on the mechanism of bone formation, remodeling, resorption and regeneration. Interactions between these cytokines and bone cells control their proliferation, differentiation and function, therefore, dictating normal bone metabolism. The interaction between SHED and the inflammatory cytokines in the immune system can influence the success of the stem-cell transplant.

**Fig. 3b.** Expression of OPG and RANKL were detected in both SHED and hFOB. SHED showed high OPG/RANKL ratio in the same pattern as hFOB. The graphs were plotted with relative expression of genes with respect to GAPDH. (□ OPG, ■ RANKL)
IL-1β is a potent inducer of bone resorption. It is now considered to be the principal component of osteoclast activating factor (OAF), a mixture of factors that stimulate bone resorption in organ cultures. IL-1β inhibits the formation of specific proteins in osteoblasts, i.e. osteocalcin and alkaline phosphatase, and has therefore been suggested to cause the inhibition of osteoblastic activity seen in myeloma. Since IL-1β has been documented to mediate inflammation and osteolysis, the differences in the expression levels of IL-1β in SHED and normal “self” hFOB cells is of considerable importance. The results of RT-PCR showed that SHED expressed significantly lower expression of IL-1β gene when compared to hFOB, therefore, indicating that SHED may only cause minimal stimulation of inflammatory bone resorption and insignificant inhibition of osteoblastic function.

Previous studies have demonstrated that IL-1 enhances the formation of IL-6 in osteoblasts. The expression of IL-6 mRNA and the production of biologically active IL-6 are induced in osteoblasts preferentially in response to local bone-resorbing agents such as IL-1α and IL-1β. We found this to be consistent with the result from our study, where SHED expressed low levels of IL-6 gene in accordance with similarly low levels of IL-1β. On the other hand, hFOB was shown to express high levels of IL-6 gene in line with similarly high levels of IL-1α. Studies have demonstrated that IL-1 enhances the formation of IL-6 in osteoblasts. The expression of IL-6 mRNA and the production of biologically active IL-6 are induced in osteoblasts preferentially in response to local bone-resorbing agents such as IL-1α and IL-1β. We found this to be consistent with the result of our study, where SHED expressed low levels of IL-6 gene in accordance with similarly low levels of IL-1β. On the other hand, hFOB was shown to express high levels of IL-6 gene in line with similarly high levels of IL-1β.

Induction of IL-6 production by osteoblasts represents one possible mechanism by which IL-1 augments bone resorption. Following that, IL-6 is produced by osteoblastic cells and appears to induce bone resorption by enhancing osteoclast formation. Other reports have shown an inhibitory effect of IL-6-type cytokines on bone formation and marker expression in vitro, as well as potent pro-apoptotic effects through enhancement of the Bax/Bcl2 ratio in the mitochondrial apoptotic pathway. Our research showed that SHED expressed significantly lower levels of IL-6 genes compared to hFOB. This result indicates that SHED may potentially promote relatively lower inflammatory bone resorption due to low levels of osteoclast formation and reduced osteoblastic apoptosis. Therefore, our result suggested that the low expression level of IL-1β and IL-6 genes in SHED should be further confirmed through in vitro studies to ascertain the significance in bone regeneration.

IL-8 is a member of the chemokine family of cytokines that are monocyte-derived factors capable of attracting and activating neutrophils, as well as inducing osteolysis. A study in University of Arkansas, USA demonstrated that the addition of recombinant human (rh) IL-8 to osteoblastic cells stimulated both RANKL mRNA expression and protein production, with no effect on the expression of osteoprotegerin (OPG); therefore stimulates osteoclast formation. Significantly lower level of IL-8 gene expression by SHED in the present study proves that stem cells may encourage comparatively reduced osteoclastic formation through the increase of OPG/RANKL ratio, thus decreasing inflammatory bone resorption and improving the stability and integration of cellular transplant and host.

TGF-β is one secreted factor that modulates the differentiation of osteoblasts and proliferation of osteoprogenitor cells. However, osteoblastic over expression of TGF-β in transgenic mice deregulates bone remodeling and leads to an age-dependent loss of bone mass. PCR results showed that SHED expressed slightly lower expression levels of TGF-β compared to hFOB. Hence, we speculate that SHED seems to modulate osteoblast differentiation and balancing between bone resorption and matrix formation in response to TGF-β secretion.

TNF-α is one of the most potent osteoclastogenic cytokines produced in inflammation. It stimulates osteoclastic bone resorption and mediates RANKL stimulation of osteoclast differentiation through an autocrine mechanism. GM-CSF promotes the fusion of prefusion osteoclasts into multinucleated osteoclasts into multinucleated cells which function in bone resorption. In this study, we demonstrated that the TNF-α and GM-CSF was not detected in both SHED and hFOB. This implies that SHED possess some similar properties with hFOB, which is an attractive feature for cell therapy because this reduces the probability of the human immune system recognizing transplanted SHED as foreign cells. However, future studies must be done on SHED-derived osteoblasts in comparison with hFOB, to determine the similarities or differences in gene expressions and regulation of these cells. The signals regulating osteoclast differentiation and development is mediated via proteins from the family of tumor necrosis factors, named osteoblast-like receptor activator of nuclear factor kβ (RANK), osteoprotegerin (OPG) and a ligand, receptor activator of NF-kβ ligand (RANKL) respectively. RANKL is expressed on osteoblasts and its interaction with the receptor RANK, present on osteoclast precursors, induces their differentiation and activation. This signaling pathway can be competitively inhibited by OPG (also produced by osteoblasts), which blocks the interaction between RANKL and the RANK receptor, thus resulting in increased apoptosis of mature osteoclasts, prevention of osteoclastogenesis and inhibition of activation of existing osteoclasts. In this study, we evaluated the levels of gene expression of OPG and RANKL genes in hFOB and SHED to lay the groundwork for further investigations on the mechanisms of OPG/RANKL regulation by SHED in...
relation with pro-inflammatory cytokines during stem cell therapy. The expression of OPG and RANKL is developmentally regulated by osteoblasts. The coordinated sequence (coupling) of osteoclastogenesis and osteoblastogenesis during the bone remodeling sequence has been attributed to osteoclast-to-osteoblast signaling. It has been shown that undifferentiated marrow stromal cells express a low OPG/RANKL ratio which initiates and supports osteoclastogenesis. In contrast with our study, we demonstrated that SHED expresses a high level of OPG/RANKL ratio. This implies that SHED may potentially initiate osteogenesis and prevent osteoclastogenesis. Therefore, we suggest that further studies should be carried out on the complete developmental sequence of SHED-derived osteoblasts, passage-by-passage, to clearly elucidate the expression levels of OPG and RANKL and the support of osteoclast formation. It is necessary to understand the contribution of the OPG/RANKL interaction in SHED and its derivative cells throughout the sequence of osteoclast and osteoblast differentiation during the bone remodeling.

CONCLUSION

This preliminary study shows that SHED has potentially improved immunomodulatory and osteogenic properties in terms of generally low expression levels of IL-1β, IL-6, IL-8 and TGF-β transcription genes, as well as high OPG/RANKL ratio. SHED is capable of differentiating into osteoblasts and forming lamellar bone after implantation into immunosuppressed rats, therefore, strongly implying that SHED is an important source of osteoblasts for stem cell and tissue-based clinical therapies. This study provides a description of the inflammatory cytokines profile of SHED, which largely favours the positive attributes required for stem cell transplants. However, we suggest that further research on the properties of SHED and its derivative cells (especially SHED-derived osteoblasts) in terms of immunomodulatory properties and interaction with human immune cells like PBMCs and T-lymphocytes, which are the main mediators in immune rejection of transplants. This is necessary to determine the efficacy of eventually using SHED in cellular-based therapies in immunocompetent hosts.

ACKNOWLEDGEMENTS

The authors are grateful to USM Incentive Grant for funding of this research, and also to the School of Dental Sciences, USM for providing the facilities and instruments.

REFERENCES

17. Dewhirst FE, Stashenko PP, Mole JE, Tsurumachi T. Purification and partial sequence of human osteoclast activating factor identity with interleukin 1 beta. J
19. Lacey DL, Grosso LE, Moser SA, et al. IL-1-induced murine osteoblast IL-6 production is mediated by the type 1 IL-1 receptor and is increased by 1,25 dihydroxyvitamin D3. J Clin Invest 1993; 91:1731-42.