INSULIN ACTIVITY ON DENTAL PULP STEM CELL DIFFERENTIATION: AN *IN VITRO* STUDY

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Insulin is a powerful and important hormone involved in the proliferation and differentiation of osteoblasts. Dental pulp stem cells (DPSCs) have the ability to self-expand and differentiate in pre-osteoblast, producing *in vitro* autologous bone tissue. The aim of our study is to investigate whether insulin can influence differentiation of DPSCs in osteoblast and bone tissue. Dental germ pulp was extracted from third molars of healthy subjects, following informed consent. DPSCs were treated with insulin at the concentration of 100 ng/μl for 24 and 48 h. Gene expression in treated DPSCs was compared with untreated cells (control) in order to check the effect of insulin on stem cell differentiation. After 24 h, significant up-regulated genes (Fold change > 2) in DPSCs were the Bone Morphogenetic Proteins BMP3, BMP4 and their receptor BMPR1A. BMP1 results over-expressed after 48 h of treatment. Significantly down-regulated genes were BMP4, BMP7 and TGFBR2 after 24 h of treatment and BMP5 and BMP7 after 48 h. Insulin was demonstrated to influence proliferation of DPSC, differentiation and expansion in osteoblasts. Further studies are needed to explore this new way of creating bone tissue.

Insulin may stimulate osteoblast differentiation, which means production of osteocalcin, the peptide that can stimulate pancreatic β cell proliferation and skeletal muscle insulin sensitivity (1).

Pre-osteoblasts show the isoform of the insulin receptor (IR) called IRA, while mature osteoblast the insulin receptor called IRB (1). This feature makes sense that insulin is a fundamental molecule in osteoblast differentiation from stem cells. This process may have importance in the production of the osteoblast peptide osteocalcin, a very important peptide for glucose metabolism. However, the direct effect of insulin on osteoblast differentiation is not completely clear.

In 2003, Miura et al. (2) isolated stem cells from human exfoliated deciduous teeth (SHED). After isolation from deciduous teeth, cells showed the ability to form *in vivo* a substantial amount of bone. Dental pulp from exfoliated teeth could be a good source of postnatal stem cells (2).

Dental pulp stem cells (DPSCs) can form complexes like pulp dentin consisting of mineralized matrix with odontoblasts very similar to human dentin (3, 4). The regeneration of a complete tooth

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is the objective of the use of stem cells in dentistry to restore the loss of natural teeth. Some studies have indicated that cell-based strategies show promising potential for regenerating the whole tooth structure in rodents (5). Moreover, stem cell-based regeneration of human tooth structures has been achieved in imunocompromised mouse models (6).

The transition from the deciduous tooth to a permanent one is a unique process in humans. The development and eruption of the permanent tooth is contemporary to the root resorption of the deciduous tooth. This process starts at about 6 years of age and stops after 12 years of age. In this time period all of the 20 deciduous teeth are normally replaced.

The dental pulp presents similar features to the gelatinous tissue of the umbilical cord and share characteristics of primitive stem cells (7). Ttherefore, the ability to isolate a population of totipotent stem cells from the dental pulp of naturally exfoliated deciduous teeth, could provide a unique source of stem cells for future clinical applications.

It is well known that dental pulp is one of the sources of stem cells. In fact, in adults stem cells are present in dental pulp and they are able to produce new dentin after mechanical or inflammatory stimuli (8). When dental pulp is damaged, usually as a consequence of dental decay, quiescent dental pulp stem cells (DPSCs) are activated to produce reparative dentin. The new dentin, called tertiary dentine, presents the same components of primary and secondary dentine, but a different cytoarchitecture. In fact, problems in mineralization are common in oral pathologies and the role of stem cells for hard tissue formation has increased interest among researchers and dentists. The factors and signalling molecules that control the quiescence/activation and commitment to differentiation of human DPSCs are not known.

Dental pulp stem cells (DPSCs) may be a source of differentiated cells inducing bone formation and controlled hydrossyapatite crystal growth. DPSCs have been isolated from teeth of subjects up to 30 years of age, and form sporadic dense nodules *in vitro*, but undergo mineralization and bone or dentin-like formation only when grafted *in vivo* (9). These DPSCs have the ability to self-expand and differentiate in pre-osteoblasts, producing *in vitro* autologous bone tissue.

The aim of our study is to investigate whether insulin can influence differentiation of DPSCs in osteoblast and bone tissue. We propose a working model that explains the possible interactions between insulin and DPSCs at the molecular level and describes the cellular consequences of these interactions. This model may be used to stimulate research on the clinical applications of DPSC in cellular therapy and tissue regeneration

MATERIALS AND METHODS

DPSCs isolation

Dental germ pulp was extracted from third molars of healthy young subjects with mean age of 18 years, following informed consent. Pulp was digested for 1 h at 37°C in a solution containing 3 mg/ml type I collagenase and 4 mg/ml dispase in 4 ml phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 500 µg/ml claritromycin. The solution was then filtered with 70 µm Falcon strainers (Sigma Aldrich, Inc., St Louis, MO, USA). Filtered cells were cultivated in α-MEM culture medium (Sigma Aldrich, Inc., St Louis, MO, USA) supplemented with 20% FCS, 100 µM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and placed in 75 ml flasks. Flasks were incubated at 37°C and 5% CO_2 and the medium changed twice a week.

Flow cytometric analyses

The purity of cell cultures was determined by analysis of different antigens after staining with fluorochrome (FITC- or PE-) conjugated mAbs anti-human CD14-FITC, CD14-PE, CD34-FITC, CD45-FITC, CD90-PE, CD105-PE (Immunotech, Marseille, France) and analyzed by FACScan. Nonspecific mouse IgG was used as isotype control (Immunotech). To avoid nonspecific fluorescence from dead cells, live cells were gated tightly using forward and side scatter.

Cell culture

For the assay, DPSCs were collected and seeded at a density of $1x10^5$ cells/ml into 9 cm² (3ml) wells by using 0.1% trypsin, 0.02% EDTA in Ca++ - and Mg–free Eagle's buffer for cell release.

One set of wells were added with Insulin (Sigma Aldrich, Inc., St Louis, MO, USA) at the concentration of 100 ng/ml. Another set of wells containing untreated cells was used as control. Cells were treated for 24 and 48 hours.

RNA processing

Total RNA was extracted from stem cells using the

GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich, Inc., St Louis, MO, USA). Cells were lysed and homogenized in a lysis buffer. Then ethanol was added to the lysate to ensure the binding of RNA to the silica membrane of the GenElute Binding Column. After washing with specific buffer to remove contaminants, RNA was eluted in 50 µl of Elution solution. Total RNA was measured with a NanoDrop spectrophotometer (Thermo Scientific Inc., MA, USA). cDNA was synthetized starting from 500 ng of each RNA sample (treated and control, at 24 and 48 h) using SuperScript® VILO™ cDNA Syntesis Kit (Invitrogen, Carlsbad, CA, USA). The reaction mix contained 5X VILO Reaction Mix, 10X SuperScript Enzyme Mix, RNA (500 ng) and DEPC water in a final volume of 20 µl. The reaction was incubated at 42°C for 60 min and then inactivated at 85°C for 5 min.

Real time PCR

cDNA was amplified by real-time PCR using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the KiCqStartTM Primers (Sigma Aldrich, Inc., St Louis, MO, USA), specific pre-designed primer pairs chosen for the investigated genes. Reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer, and cDNA.

All experiments were performed including non-template controls to exclude reagent contamination. PCRs were performed with two biological replicates.

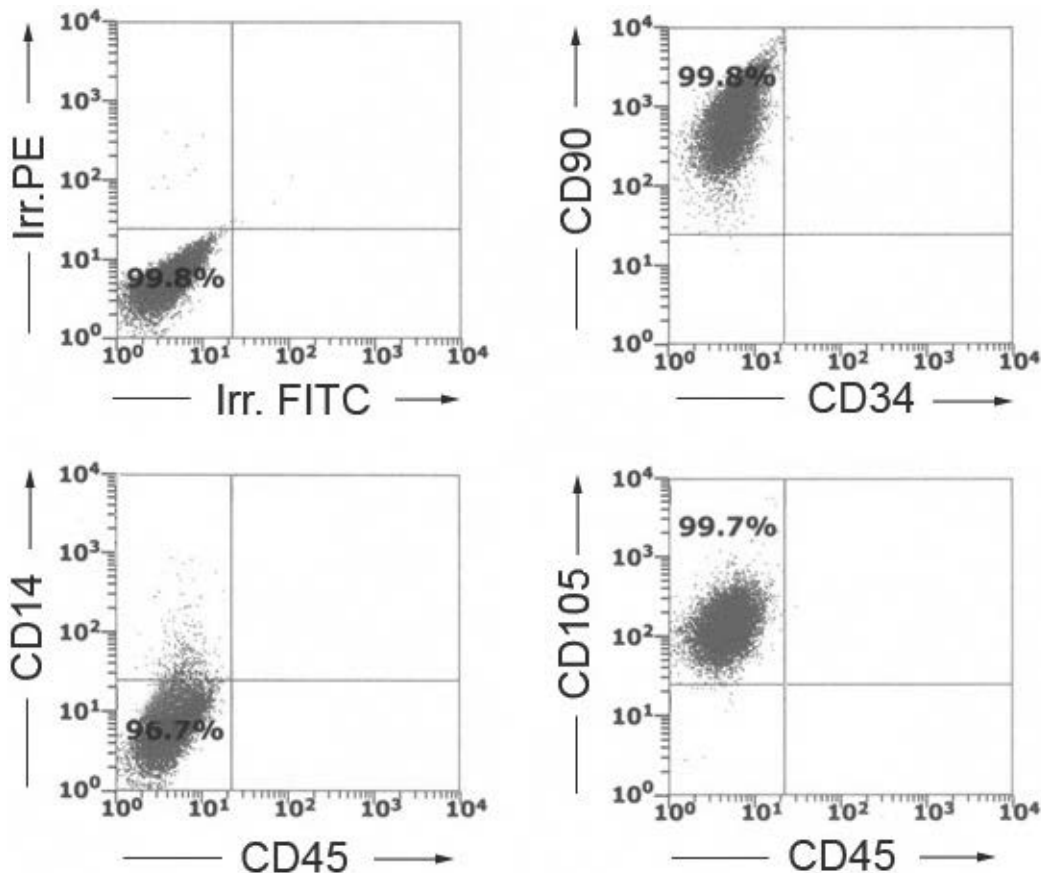The gene expression levels were normalized to

Fig. 1. *Surface antigen profile of dental pulp stem cells. Phenotypic characterization by flow cytometry of cell cultures derived from dental pulp, by staining with the indicated mAb. Representative dot plots documenting the purity of cell preparations and the homogenously CD105+, CD90+, CD34-, CD45-, CD14- surface antigen profile, are shown. Irr., irrelevant, isotype control Ab.*
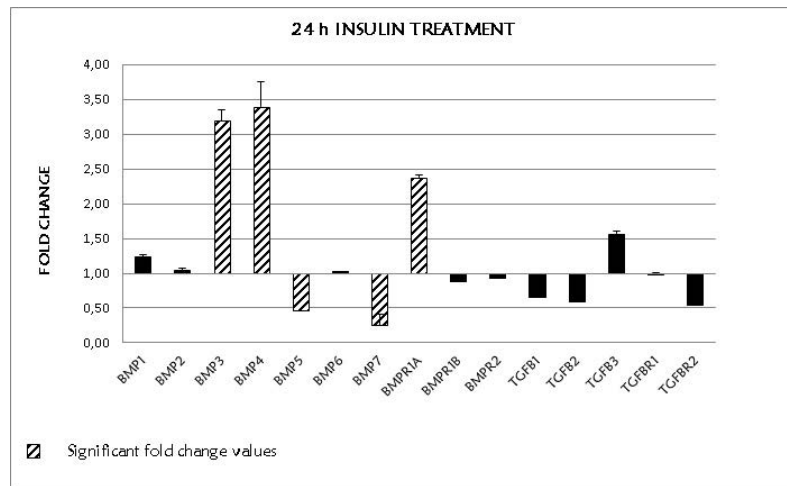
Fig. 2. *Gene expression profile of DPSCs after 24 h of treatment with insulin.*
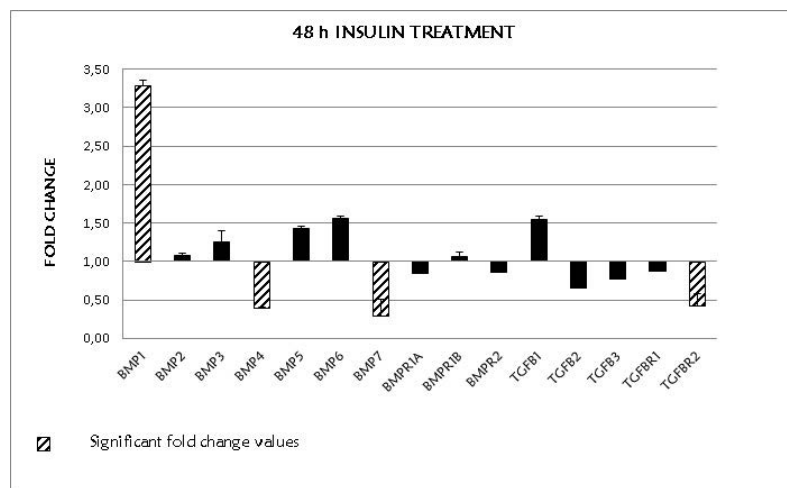

Fig. 3. *Gene expression profile of DPSCs after 48 h of treatment with insulin.*

the expression of the housekeeping gene GUSB and quantified as the fold changes relative to the expression of the untreated DPSCs. Quantification was made with the delta/delta calculation method (10).

RESULTS

Cell cultures were phenotypically characterized by flow cytometric analyses. Cell preparations derived from dental pulp were homogenously CD105⁺, CD90⁺, CD34⁻, CD45⁻, CD14⁻, which is

a typical mesenchymal stem cell surface antigen profile (Fig. 1).

DPSCs were treated with insulin at the concentration of 100 ng/ml for 24 and 48 h. Gene expression in treated DPSCs was compared with untreated cells (control) in order to check the effect of insulin on stem cell differentiation.

Genes related to ossification (BMP1, BMP2 BMP3, BMP4, BMP5 BMP6), osteoblast differentiation (BMPR1A, BMPR1B, BMPR2), bone mineralization (TGFβ1, TGFβ2, TGFβ3) and

skeletal development (TGFβR1 and TGFβR2) were investigated to study the potential effect of insulin in osteoblast differentiation and proliferation.

After 24 h of treatment many of the genes investigated were down-regulated in treated DPSCs *vs* untreated DPSCs. Significantly up-regulated genes (Fold change > 2) were the Bone Morphogenetic Proteins BMP3 and BMP4 (Fig. 2). Significantly up-regulated genes were Bone Morphogenetic Protein receptor BMPR1A. After 48 h, insulin induced the over-expression of bone related gene BMP1 (Fig. 3).

DISCUSSION

In our study, BMP3 and BMP4 after 24 h and BMP1 genes after 48 h were up-regulated, inducing proliferation and differentiation of human MSCs. Our study confirms the assumption that insulin promotes markers of bone formation, providing support of the view that insulin influences differentiation of DPSCs in an osteoblastic sense. Bone morphogenetic proteins (BMPs) have been shown to induce DPSCs to proliferate and then differentiate into osteoblastic cells. Insulin can modulate the expression of various osteogenic markers. Thus, the effects of insulin on DPSCs must be examined in light of their apparent maturity in terms of osteogenic potential. Previous studies showing that insulin promotes bone growth, suggest that insulin facilitates differentiation as the cell population reaches a more mature, post-proliferative stage.

DPSCs showed differentiation profiles similar to those of bone differentiation (11) and this event makes them very interesting as a model to study the osteogenesis (12). Dental pulp is a good source of stem cells that differentiate into osteoblasts, as well as other phenotypes, for hard tissue engineering. During life, bone tissue needs renewal, and dental pulp represents an additional source of mesenchymal cells to bone marrow. Selection and expansion of stem cells are complex procedures commonly used to obtain cells for potential therapy (12); thus, being able to obtain sorted cells quickly after collection and to transplant them directly is an advantage that can lead to easier application of bone-engineering protocols (13).

There is a considerable body of work showing osteogenic actions of insulin in cortical bone in several species. In our study we demonstrated that insulin can increase DPSCs and influence differentiation and proliferation of DPSCs in osteoblasts.

The extreme feasibility of managing dental pulp stem cells makes them ready to use in clinical trials on human patients: autologous stem cells collected from dental pulp of extracted wisdom teeth or from pulpectomy of teeth left *in situ* are a possibility that should be improved in clinical trials. Although dental pulp could play a fundamental role in all human tissues regeneration in the future (14), the main commitment is bone, and bone regeneration is the goal for these cells to find an immediate application (15).

Our study provides evidence that stem cells selected from human permanent dental pulp represent a population of postnatal stem cells able to extensively proliferate and differentiate into mesenchymal cells and mainly osteoblasts (16). Therefore, these cells constitute a large, ideal source of cells for tissue regeneration, tissue-based clinical therapies, and transplantation.

Insulin has been demonstrated to influence this process in production of DPSCs and differentiation and expansion in osteoblasts. Further studies are needed to explore this new way of creating bone tissue.

REFERENCES

1. Avnet S, Perut F, Salerno M, Sciacca L, Baldini N. Insulin receptor isoforms are differently expressed during human osteoblastogenesis. Differentiation 2012; 83(5):242-248.

2. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 2003; 100(10):5807-5812.

3. Gotlieb EL, Murray PE, Namerow KN, Kuttler S, Garcia-Godoy F. An ultrastructural investigation of tissue-engineered pulp constructs implanted within endodontically treated teeth. J Am Dent Assoc 2008; 139(4):457-465.

4. Yu J, Deng Z, Shi J, Zhai H, Nie X, Zhuang H, Li Y, Jin Y. Differentiation of dental pulp stem cells into regular-shaped dentin-pulp complex induced by tooth germ cell conditioned medium. Tissue Eng 2006; 12(11):3097-3105.

5. Chai Y, Slavkin HC. Prospects for tooth regeneration in the 21st century: a perspective. Microsc Res Tech 2003; 60(5):469-479.

6. Yen AH, Sharpe PT. Regeneration of teeth using stem cell-based tissue engineering. Expert Opin Biol Ther 2006; 6(1):9-16.

7. Hofmeister CC, Zhang J, Knight KL, Le P, Stiff PJ. Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. Bone Marrow Transplant 2007; 39(1):11-23.

8. Narayanan K, Srinivas R, Ramachandran A, Hao J, Quinn B, George A. Differentiation of embryonic mesenchymal cells to odontoblast-like cells by overexpression of dentin matrix protein 1. Proc Natl Acad Sci U S A 2001; 98(8):4516-4521.

9. Batouli S, Miura M, Brahim J, et al. Comparison of stem-cell-mediated osteogenesis and dentinogenesis. J Dent Res 2003; 82(12):976-981.

10. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4):402-408.

11. Hwang YC, Hwang IN, Oh WM, Park JC, Lee DS, Son HH. Influence of TGF-beta1 on the expression of BSP, DSP, TGF-beta1 receptor I and Smad proteins during reparative dentinogenesis. J Mol Histol 2008; 39(2):153-160.

12. Liu J, Jin T, Chang S, Ritchie HH, Smith AJ, Clarkson BH. Matrix and TGF-beta-related gene expression during human dental pulp stem cell (DPSC) mineralization. In Vitro Cell Dev Biol Anim 2007; 43(3-4):120-128.

13. Gronthos S, Brahim J, Li W, et al. Stem cell properties of human dental pulp stem cells. J Dent Res 2002; 81(8):531-535.

14. Laino G, d'Aquino R, Graziano A, et al. A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J Bone Miner Res 2005; 20(8):1394-1402.

15. Laino G, Carinci F, Graziano A, et al. In vitro bone production using stem cells derived from human dental pulp. J Craniofac Surg 2006; 17(3):511-515.

16. Papaccio G, Graziano A, d'Aquino R, et al. Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. J Cell Physiol 2006; 208(2):319-325.