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Expressions of pathologic markers in PRP based chondrogenic differentiation of human adipose derived stem cells

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ABSTRACT

Background: Optimization of the differentiation medium through using autologous factors such as PRP is of great consideration, but due to the complex, variable and undefined composition of PRP on one hand and lack of control over the absolute regulatory mechanisms in *in vitro* conditions or disrupted and different mechanisms in diseased tissue microenvironments in *in vivo* conditions on the other hand, it is complicated and rather unpredictable to get the desired effects of PRP making it inevitable to monitor the possible pathologic or undesired differentiation pathways and therapeutic effects of PRP. Therefore, in this study the probable potential of PRP on inducing calcification, inflammation and angiogenesis in chondrogenically-differentiated cells was investigated.

Methods: The expressions of chondrogenic, inflammatory, osteogenic and angiogenic markers from TGFβ or PRP-treated cells during chondrogenic differentiation of human adipose-derived stem cells (ADSCs) was evaluated. Expressions of Collagen II (Col II), Aggrecan, Sox9 and Runx2 were quantified using q-RT PCR. Expression of Col II and X was investigated by immunocytochemistry as well. Glycosaminoglycans (GAGs) production was also determined by GAG assay. Possible angiogenic/inflammatory potential was determined by quantitatively measuring the secreted VEGF, TNFα and phosphorylated VEGFR2 via ELISA. In addition, the calcification of the construct was monitored by measuring ALP activity and calcium deposition.

Results: Our data showed that PRP positively induced chondrogenesis; meanwhile the secretion of angiogenic and inflammatory markers was decreased. VEGFR2 phosphorylation and ALP activity had a decreasing trend, but tissue mineralization was enhanced upon treating with PRP.

Conclusions: Although reduction in inflammatory/angiogenic potential of the chondrogenically differentiated constructs highlights the superior effectiveness of PRP in comparison to TGFβ for chondrogenic differentiation, yet further improvement of the PRP-based chondrogenic differentiation media is required to inhibit the production of angiogenic/inflammatory markers, calcification and the release of synthesized GAG out of the construct.

1. Background

In the recent decade, PRP has been widely used as a therapeutic for OA patients with pain killing effects, although its overall effects depend on the stage of the disease, or in the other words on the micro-environmental conditions. The application of activated Platelet-Rich Plasma (PRP), with its vast range of cytokines and growth factors has achieved a considerable attention for chondrogenic differentiation in tissue engineering fields.

PRP is rich in growth factors such as TGF-β, Insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). As reported the overall effect of PRP depends on its composition since different preparation protocols might alter the content of its growth factors (Nagata et al., 2010; Bendinelli et al., 2010). Given that each factor or group of factors has inductive/promotive effects on individual or overlapped differentiation pathways, eval-

Abbreviations: ADSCs, adipose-derived stem cells; Col II, Collagen II; GAG, glycosaminoglycan; PRP, Platelet-Rich Plasma; IGF, Insulin-like growth factor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; BGN, biglycan; PPP, plasma poor platelet; DDMB, 1,9-dimethylmethylene blue; MMPs, Matrix metalloproteinases; OA, osteoarthritic.

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uation of the overall effect and monitoring possible undesired outcomes is of high importance.

It has been reported that PRP stimulates porcine chondrocyte proliferation and matrix biosynthesis (Akeda et al., 2006). Furthermore, some *in vivo* studies have shown that chondrocyte/PRP composites can enhance the regeneration of articular cartilage defects (Wu et al., 2007).

PRP has confirmed effects on extracellular matrix (ECM) production and as reported expressions of ECM related genes were enhanced upon treating with PRP, while expressions of ECM degrading enzymes were suppressed (Kim et al., 2014). In addition, it could up-regulate the expression of biglycan (BGN) and decorin (Miguez et al., 2011). BGN has numerous roles in differentiation processes such as collagen assembly, regulation of inflammation, binding and modulation of TGF-β and BMP-2,4 functions (Miguez et al., 2011). On the other hand, BGN also promotes osteoblast differentiation and increases matrix mineralization *in vitro* (Wang et al., 2010).

PRP potential for inducing angiogenesis, due to having potent angiogenic factors such as VEGF, is of great concern. Due to the presence of other factors which might have direct or indirect anti-angiogenic effects (Amable et al., 2013), and because ADSCs are intrinsically VEGF secreting cells (Lee et al., 2012), the overall angiogenic potential should be monitored when PRP is used to induce chondrogenesis. Again, the presence of IGF in PRP composition and its confirmed up-regulatory and mediatory effect on VEGF secretion (Slomiany and Rosenzweig, 2004) and VEGF alternative splicing (Nowak et al., 2010) highlights the importance of tracking the effects of PRP on differentiated cells from the VEGF secretion towards the activation of VEGFR2, which triggers the angiogenesis cascade.

There have been adequate supporting reports which confirm anti-inflammatory effects of PRP on different cell types including chondrocytes (Bendinelli et al., 2010). The molecular mechanism of anti-inflammatory effects of PRP as reported is through reduction of NF-κB transactivation and down regulation of the COX-2 and CXCR4 target genes (Bendinelli et al., 2010). Although the anti-inflammatory effects of PRP have been reported, its overall effect on chondrogenic differentiation of human ADSCs – which secret both VEGF and TNFα- to our knowledge has not been yet reported.

Therefore, the aim of this study was to investigate the effects of PRP on expression of tissue specific markers and for the first time the possible induction of angiogenesis, inflammatory cytokine secretion and calcification during chondrogenesis of ADSCs. To fulfill that, cells were differentiated using different PRP concentrations. Changes in gene expression levels for cartilage specific markers were appraised by real time PCR. Also chondrogenesis was assessed by measuring secreted glycosaminoglycan in the medium or that kept in cell ECM. The angiogenesis/inflammation induction potential was evaluated by measuring the VEGF and TNFα secretions from differentiated cells using the ELISA technique. Further, the secreted VEGF effect on VEGFR2 activation was determined.

2. Methods

2.1. Adipose stem cells isolation, expansion and characterization

Liposuctioned samples were collected from healthy patients after obtaining their written informed consent according to the ethics of Tehran University of Medical Sciences. For ADSCs isolation briefly, the liposuctioned sample was washed several times to remove the blood cells, 150 μg/ml collagenase I (Sigma) was added and the sample was incubated for 60 min at 37 °C while on shaker at 250 rpm. Collagenase was removed by centrifugation at 1200 rpm for 5 min. The pellet was then incubated with RBC lysis buffer three times each for 5 min, neutralized with PBS, centrifuged and cultured in DMEM supplemented with 10% FBS, 1% pen/strep and 1% amphotericin (all from sigma) in incubator and expanded accordingly. For differentiation experiments, cells between passages 3 to 9 were used.

Table 1
Sequences of human primers used for q-PCR.

H-beta Actin-F	CTT CCT TCC TGG GCA TG
H-beta Actin-R	GTC TTT GCG GAT GTC CAC
H – SOX9 – F	ATC TGA AGA AGG AGA GCG AG
H – SOX9 –R	TCA GAA GTC TCC AGA GCT TG
H – COL2A1 – F	GGT CTT GGT GGA AAC TTT GCT
H – COL2A1 – R	AAG ACG GCT TCC ACC AGT G
H-Aggrecan-F	AAA GAC CTC ACC CTC CAT CTC
H-Aggrecan-R	GGC TCC CAG CCA TTG ATA CAG
H-RUNX2-F	GCC TTC AAG GTG GTA GCC C
H-RUNX2-R	CGT TAC CCG CCA TGA CAG TA

2.2. Preparation and activation of PRP

Blood samples were collected in anti-agglutination tubes and then centrifuged at 3200 rpm for 10 min. The isolated plasma was centrifuged at 4000 rpm for 15 min to get platelet sediment. The sediment was dissolved in plasma poor platelet (PPP), activated using 10% $CaCl_2$ in 4° C overnight and finally centrifuged at 3000*g* for 15 min.

2.3. Chondrogenic differentiation in different PRP concentrations

For chondrogenic differentiation 2.5×10^5 ADSCs were centrifuged (1200 rpm, 5 min) to form a pellet. After pellet formation, expansion medium was replaced with standard differentiation medium (TGFβ1 10 ng/ml, Dexamethasone (10^{-7} M), proline (400 μg/ml), pyrovate (1 mg/ml), ascorbic acid (50 μg/ml) and ITS (1%) all from sigma). For evaluating the effects of PRP on chondrogenesis, two different PRP conditions (5%: T1, 15%: T2 group) were established by adding appropriate volume of PRP to DMEM medium. The differentiation medium containing TGFβ was used as control (Dif group).

2.4. Quantitative real time PCR

To evaluate gene expression, RNA samples were collected after 21 days of differentiation and extraction was carried out using RNA extraction kit (CinnaGen, Iran). cDNA was synthesized using a cDNA kit according to manufacturer instructions (Takara, Japan). Changes in gene expression were quantified by a RT PCR analyzer (Corbett). Result analysis was done by Rotor Gene software according to ΔΔCT formula. Specificity of the signals was confirmed by examining the melting curve of each gene. The primers used in this study are presented in Table 1.

2.5. Alcian blue staining and immunocytochemistry analyses

The samples were fixed in cold 4% paraformaldehyde and embedded in paraffin, then sectioned (5 μm thick) and used for immunocytochemistry and Alcian blue staining. After deparaffinization and rehydration, antigen retrieval was carried out using sodium citrate. The immunostaining was carried out using Col II (1:20 diluted, 4 °C O/N, rabbit anti-human, Abcam) or ColX (1:80 diluted, 4 °C O/N, rabbit anti-human, Abcam) antibodies. PE-conjugated secondary antibody (1;100 diluted, 1 h at 37 °C, goat anti-rabbit, eBioscience) was used for fluorescence visualization. For GAG staining, the sections were stained with Alcian blue 8GX (Roth).