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Study the effect of Urokinase plasminogen activator on the osteogenic differentiation capacity of mesenchymal stem cell

Nasrin Asadi[1], Nikoo Nasoohi[1], Mahshid Hodjat[2]*

[1]Department of biochemistry and biophysics, Faculty of advanced science and technology, Tehran medical sciences, Islamic Azad University, Tehran, Iran

[2]Dental Research Center, Dentistry Research Institute, Tehran University of Medical Sciences (TUMS), Tehran, Iran

mhodjat@TUMS.Ac.Ir; Mahshid.hodjat@DAAD-alumni.de

Abstract

Background and Objective: Human mesenchymal stem cells (MSC) have great application in tissue engineering, specifically bone tissue engineering**.** There is an evidence that the receptor of urokinase plasminogen activator (uPA) involves in osteogenic differentiation of mesenchymal stem cells**.** The aim of this study was to identify the effect of uPA and its inhibitor on osteogenic differentiation of mesenchymal stem cells (MScs).

Methods: The human dental pulp stem cells were treated with different concentrations of uPA and its inhibitor (amiloride) in osteogenic medium. MTT assay was used to study the cell viability. The expression of osteogenic genes was assessed by quantitative real-time PCR. Alizarin red staining and alkaline phosphatase activity (Alp) were used to evaluate the osteogenic potential of MScs

Results: Amiloride at concentrations more than 0.125 mM has a toxic effect while uPA showed a significant increase in cell proliferation at 1, 2, 4, 8 nM. The alkaline phosphatase activity increased in the presence of uPA while decreased in the presence of amiloride. The data showed that cells treated with osteogenic medium in the presence of urokinase did not have a positive effect on osteogenic differentiation while amiloride decreased mineralization. Urokinase did not have a significant effect on osteopontin and osteocalcin gene expression while amiloride significantly reduced the expressions of osteogenic related genes.

Conclusion: urokinase is an essential factor in osteogenic differentiation of mesenchymal stem cells and its inhibition leads to disruption of this process, however treatment of cells with urokinase has no effect on osteogenic potential.

Key words: urokinase plasminogen activator, Human mesenchymal stem cells**,** osteogenic differentiation, amiloride

Introduction

Bone, the component of the skeletal system is a dynamic, and highly vascular tissue. It is responsible for protecting organs such as the brain, lungs and heart, as well as providing mechanical strength and facilitating the movement of living organisms. They are also a source of minerals such as calcium, magnesium and phosphorus (1). Bone tissue comprises of different cells including osteoblasts, osteocytes, and osteoclasts. Osteoblasts that makeup about 4-6% of all bone cells are differentiated from mesenchymal stem cells and their primary function is to produce and secrete an extracellular matrix. Osteoblasts have two destinies: either they are actively in their bone matrix, or differentiate to the cells called osteocytes. Osteocytes makeup 95-90% of bone cells and have a lifespan of over 25 years (2). In turn, osteoclasts are giant multinucleated cells that are irregularly formed from a combination of monocyte progenitor cells and are responsible for reabsorption and bone formation, and their location is at the bone surface in damaged bones.

Human mesenchymal stem cells (MSC) have great application in tissue engineering, specifically bone tissue engineering. Various biochemical, biological, and biophysical factors affect the fate and lineage differentiation of mesenchymal cells make them suitable for therapeutic approaches. Indeed, MSc Priming has attracted massive attention in recent years. In vitro treatment of MScs with inflammatory mediators, pharmacological drugs or chemicals as well as hypoxia were used to improve their therapeutic efficacy, regenerative properties, and their function before clinical application.

Plasminogen activating system is an extracellular proteolytic enzyme system that is associated with various physiological and pathophysiological processes. It consists of several components, including: urokinase plasminogen activator (uPA), its receptor (uPAR) and plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2)(3). uPA is a key serine proteinase involved in the conversion of inactive plasminogen to active plasmin (3). There is an evidence that the receptor uPAR involve in osteogenic differentiation of mesenchymal stem cells via NFκB transcriptional pathway (4). It was shown that in knockout uPA animal models the calcification of vascular system is impaired (4). However still not much is known about the effect of extracellular treatment of uPA on MScs osteogenic differentiation.

The aim of this study was to identify the effect of urokinase plasminogen activator and its inhibitor on osteogenic differentiation of mesenchymal stem cells.

Material and Methods:

Urokinase plasminogen activator (CAS no. 9039-53-6) and amiloride were purchases from Sigma, Germany. Formaldehyde and Triton X-100 were from Merck, Germany. Dexamethasone, β-glycerolphosphate and l-ascorbic acid-2-phosphate were from Sigma.

Cells treatment

The human dental pulp stem cells were obtained from the cell bank of Dental Research Center of Tehran University of Medical Sciences. Cells were cultured in MEM-alfa Medium (Biosera, France) containing 10% FBS (Cegrogen, Germany) and streptomycin/ Penicillin that were incubated at 37 °C and 5% carbon dioxide. The cells were treated with selected concentrations of uPA and amiloride and were incubated for 7 days in osteogenic medium containing 100 nM dexamethasone, 10 mM β-glycerolphosphate and 50 µM l-ascorbic acid-2-phosphate. The plate's medium was changed every 72 hours for seven days.

MTT assay

The suitable doses of uPA and amiloride were obtained using viability test MTT assay. After 7 days incubation in culture medium, the cells supernatants were removed and 50µL of MTT solution (0.05 mg/10ml PBS) was added to each well. After 4 hours of incubation at 37 ° C, MTT solution was removed. At this stage, formazan crystals were visible in living cells under microscope. In the next step, 50µL DMSO was added to each well. After shaking several times to dissolve the formazan crystals using orbit shaker, the absorption was measured at 570 nm by ELISA reader (BioTeK, Germany).

Alkaline phosphatase test

To perform alkaline phosphatase test, pulp mesenchymal stem cells were cultured in a flask and incubated for 72 hours at 37 ° C and 5% CO2. The cells were then cultured on 6-cell plates 3 cm in diameter. Then, 5 ml of MEM medium containing 10% FBS and 300,000 cells of cell suspension were added to each well. The plates were then incubated at 37 ° C and 5% CO2 for 24 hours. Cells were treated with different concentrations of urokinase and amiloride. The plate's medium was changed every 72 hours for seven days. Cells were washed with PBS solution, followed by 2 min treatment with solution (10 mM mgcl2 + 0.2% Triton X-100, pH 7.2). In the next step, the cells were remove using scraper and the lysate were transferred to the microtube to be sonicated for 1min. Therefore the cells membranes were disrupted and the enzyme alkaline phosphatase were released. The microtubes were centrifuged at 7000 rpm for 5 min. The supernatant were collected to perform the alkaline phosphatase test. The alkaline phosphatase activity were measured according to the manufacturer protocol kit (Pars Azmon, Iran). Finally, the absorbance were measured at 405nm. Bradford protein assay was used to normalize protein concentration of each sample.

Alizarin Red staining

Culture medium was removed from wells of 24 well plate and the cells were washed with PBS. After 5 min fixation using formaldehyde 4%, the Alizarin red solution (2gr/100ml in H2O, pH to 4.1-4.3) (Sigma, Germany) was added to each well and incubated for 5 min while rotating gently. The staining solution was removed and the culture wells were washed with PBS. Calcified nodule formation was observed under light microscopy and the intensity of red color was detected in the macroscopic observation.

Gene expression analysis

The total RNA was extracted using RNA extraction kit (Biofact, Korea) according to the manufacturer protocol. The purity of RNA was estimated using 260/280 absorbance proportion values between1.8-2. The RNA quality was also evaluated on agarose gel. 1000ng RNA was transferred to cDNA using cDNA synthesis BioFact™ RT-Kit (BioFACT, Korea). The mixture including, 1 µL random hexamer primer, 9 µL RNA, and 10 µL master mix was incubated at 50 °C for 60 min followed by 5min incubation at 95 °C in a 20 µL reaction. SYBR green-based quantitative real-time polymerase chain reaction (RT-PCR) was performed using mastermix (BioFACT™ 2X Real-Time PCR master mix) on a LightCycler® 96 System (Roche, Basel, Switzerland). Primers sequences were as followed: RUNX2 F-GGAGTGGACGAGGCAAGAGTT, R- GGTTCCCGAGGTCCATCTACT; OPN F-CACATGGCCTCCAAGGAGTAA, R-TGAGGGTCTCTCTCTTCCTCTTG, OCN F-TCACACTCCTCGCCCTATTG, R- GCTCCCAGCCATTGATACAG. Beta-actin was used

as an internal control. The fold change was calculated using the 2-ΔΔCt method to compare the expression of target genes.

Statistical analysis

All experiments were performed three times triplicate. All analysis was performed using one way ANNOVA followed by dunnett's post hoc test using GraphPad Prism (version 5.03).

Results:

Morphological examination of cells extracted from the human dental pulp revealed that these cell lines have a spindle-shaped appearance and are also adherent to the bottom of the cell culture flask.

Cell viability results

To investigate the effect of urokinase and amiloride on cell viability and proliferation status different concentrations of uPA (8, 4, 2, 1, 0.5, 0.25 nM) and amiloride (1.5 to 400 µM) was applied to the cultured MS cells for 7 days. Our results showed that amiloride at concentration more than 0.125 mM has toxic effect while uPA showed significant increase in cell proliferation at 1, 2, 4, 8 nM (Fig 1). uPA at 1 and 3 nM and amiloride at 1.5 and 0.75 µM were selected for evaluating their osteogenic effects in the next experiments.

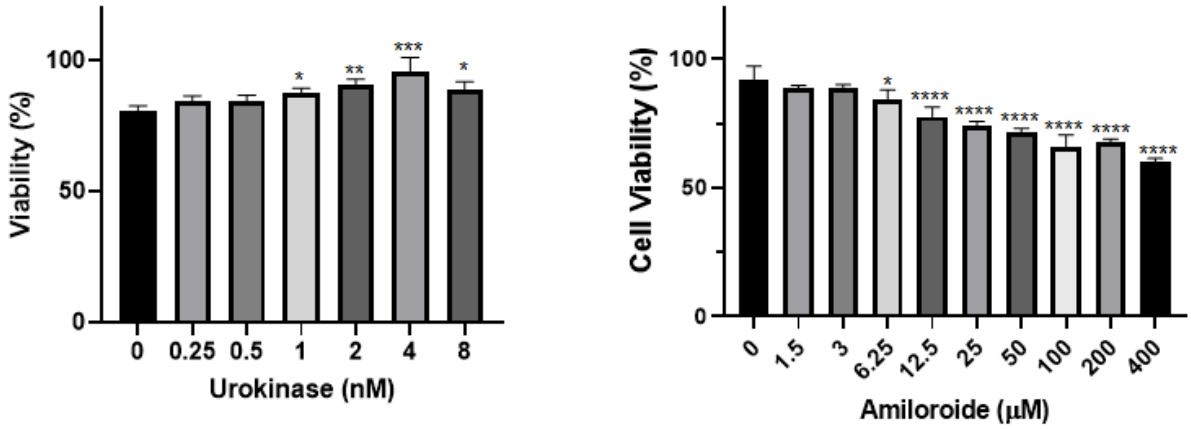

Fig 1. The effect of different concentrations of urokinase and amiloride on pulp MSc viability using MTT assay. *Significantly different from control ($p< 0.05$), **Significantly different from control ($p< 0.01$), ****Significantly different from control ($p< 0.0001$).

Alkaline phosphatase (ALP) activity

To find out the maximum Alp activity of pulp MScs in osteogenic medium we evaluated the MSCs Alp at different days after osteogenic stimulation. The results showed that the lowest alkaline phosphatase activity is on the third day of differentiation and the highest activity is on days 9 to 14, after which the enzyme activity decreased (Fig 2). We further evaluated the effect of urokinase and its inhibitor, these cells were exposed to osteogenic environment for seven days at concentrations of (1.5 and 3 nM) urokinase and (0.75 and 1.5 µM) amiloride. The results showed that the activity of alkaline phosphatase enzyme increased in the presence of urokinase concentrations while its activity decreased in the presence of amiloride concentrations (Fig 3).

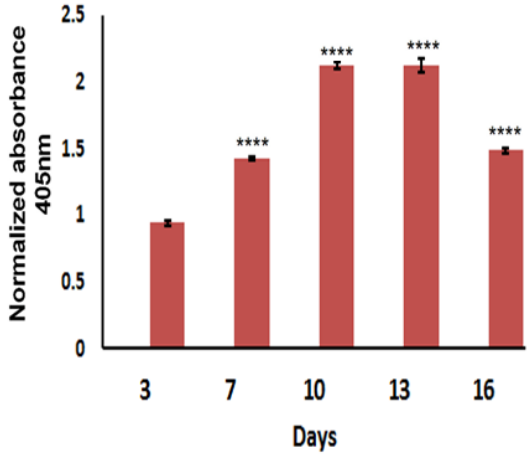

Fig 2. Alkaline phosphatase activity of MScs after 16 days culture in osteogenic medium. ****Significantly different from control ($p< 0.0001$).

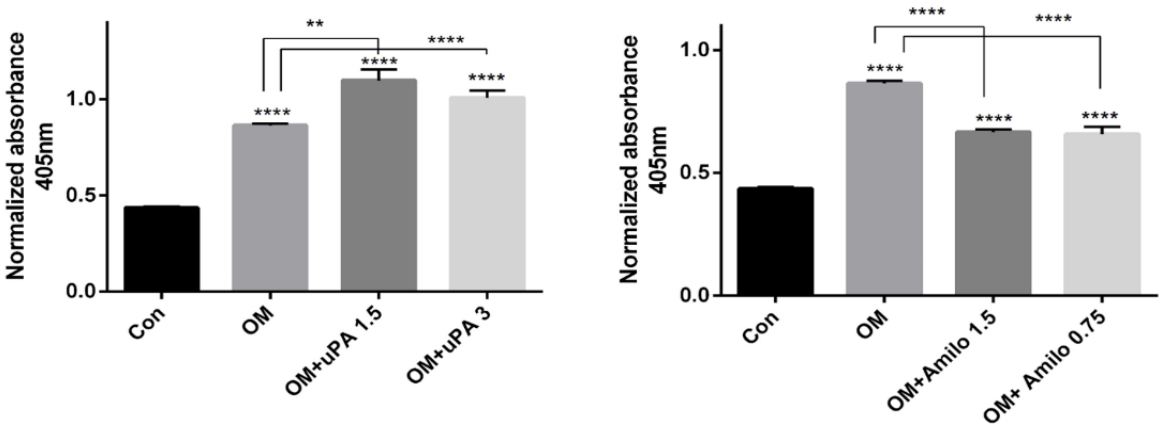

Fig 3. Alkaline phosphatase activity of MScs after 7 days exposure to uPA (1.5 and 3 nM) or amiloride (1.5 and 0.75 µM). **Significantly different from control ($p< 0.01$), ****Significantly different from control ($p< 0.0001$).

Alizarin red mineralization test

Alizarin red test was used to evaluate the mineralization and calcium deposition indicating the osteoblast differentiation of mesenchymal stem cells. The data showed that cells treated with osteogenic medium in the presence of urokinase did not have a positive effect on osteogenic differentiation while amiloride decreased mineralization (Fig 4).

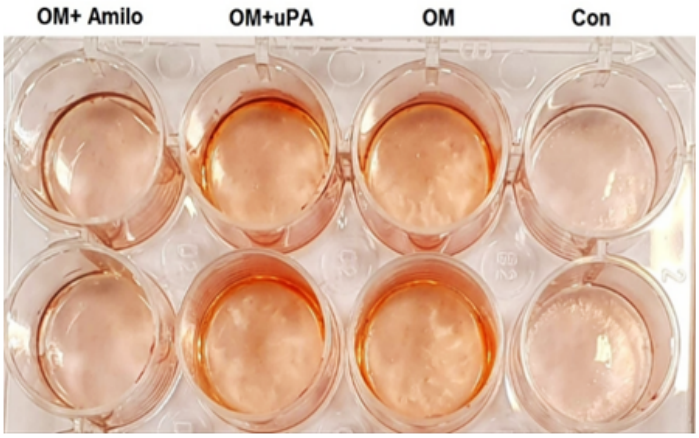

Fig4. Alizarin red staining after MScs differentiation in the presence of uPA and amiloride.

Expressions of osteogenic genes

Based on the obtained results the expression of osteopontin, RUNX2 and osteocalcin were increased in the osteogenic medium. It also shows that a concentration of 1.5 µl of urokinase did not have a significant effect on osteopontin and osteocalcin gene expression. Also RUNX2 expression decreased in the presence of uPA. It was shown that amiloride significantly reduced the expressions of osteopontin, RUNX2 and osteocalcin genes ($p< 0.01$) (Fig 5).

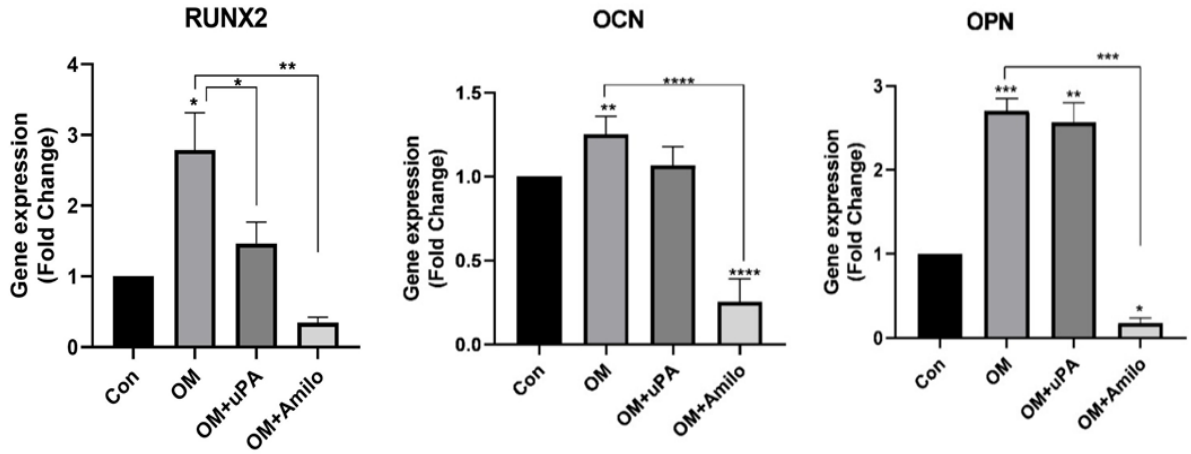

Fig 5. Evaluation of the effect of urokinase and amiloride on osteogenic related gene expression including RUNX Family Transcription Factor 2 (RUNX2), osteopontin (OPN), and osteocalcin (OCN) of mesenchymal stem cells. **Significantly different from control ($p< 0.01$), ****Significantly different from control ($p< 0.0001$).

Discussion

Urokinase is a key serine proteinase involved in the conversion of inactive plasminogen to active plasmin, as well as a range of physiologic and pathologic events (5). It is part of urokinase system consist of the plasminogen activator urokinase (uPA, urokinase), its receptor (uPAR), and inhibitors including PAI-1 and PAI-2 that involved in regulating cell migration and cell proliferation and invasion. In human, urokinase is secreted by a variety of cells including monocytes, macrophages, tumor cells, fibroblasts, smooth muscle cells, and endothelial cells.

Studies on uPAR and its uPA ligand have shown the involvement of both proteins in bone hemostasis (6) and the pathogenesis of bone associated disease including osteoporosis (7) and osteoblastic bone metastasis (8). The role of uPA has been also shown in the regulation of osteoblast and osteoclast function (6). Recent studies suggested that urokinase receptors are involved in osteogenic differentiation in mesenchymal stem cells. Their studies on the C5aR receptor suggest that uPAR regulate the C5aR expression therefore affecting differentiation of human mesenchymal stem cells into osteoblasts and that C5aR inhibition impairs bone differentiation (4). Due to the current evidence on the role of uPA in osteogenesis we aimed to evaluate the extra cellular application of uPA on osteogenic differentiation of MScs. So far no study has been done on the effect of uPA on osteogenic differentiation potential and due to the fact that the urokinase receptor is activated independently of its ligand (urokinase) through interaction with other receptors, so the study of the role of urokinase in the process of differentiation is essential.

In this study we used amiloride as a uPA inhibitor (9). Amiloride is a chemical medication with the brand name of Midamor that is used to decrease edema in patients with congestive heart failure (10). Based on researches amiloride could inhibit the function of uPA protein and down regulate the expression of PLAUR mRNA (11, 12).Therefore in this study we used amiloride to assess the effect of intracellular inhibition of uPA on cells differentiation. Based on MTT assay data the nontoxic doses of 1.5 and 0.75 μM amiloride and 1 and 3 nM of uPA were selected to assess their osteogenic effects. Our study showed that uPA at high doses stimulate cell proliferation which was consistent with previous studies in cancerous and stem cells (13, 14).

To evaluate the effect of uPA and it inhibitor on MScs differentiation, alkaline phosphatase activity and alizarin staining test were applied. Also, the expression of osteogenic related genes including RUNX2, osteopontin and osteocalcin was assessed. The process of osteoblast differentiation is controlled by specific osteoblast transcription factors such as RUNX2 (15) in coordination with other transcriptional mediators (16, 17).

The activity of alkaline phosphatase enzyme increased at day three of differentiation and reached to its maximum level at day ten followed by dramatic decrease in the next days. Therefore, in this study the activity of alkaline phosphatase enzyme was measured on the day seven. Our data showed that alkaline phosphatase activity increased in the presence of uPA, however there were no significant changes in the mineralization level according to the alizarin red data. Also there were no significant changes in the expression of osteogenic related genes. Based on this results it was concluded that adding extracellular uPA could not stimulate the process of mineralization. On the other hand, adding uPA inhibitor suppressed the mineralization process and reduces alkaline phosphatase activity that further indicate the important role of intracellular uPA in osteogenic differentiation.

In conclusion we found that urokinase is an essential factor in osteogenic differentiation of mesenchymal stem cells and its inhibition leads to disruption of this process, however treatment of cells with urokinase has no effect on osteogenic potential.

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Declarations of interest:

None

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