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Activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) is needed for the TGFβ-induced chondrogenic and osteogenic differentiation of mesenchymal stem cells

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ABSTRACT

The role of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway on the osteogenesis of progenitor and stem cells has received a lot of attention due to conflicting results in the literature. ERK1/2 has been reported to be both activating and inhibitory to the osteogenesis of different cell types under varying culture conditions. This study focused specifically on the role of ERK1/2 on the chondrogenesis and osteogenesis of mesenchymal stem cells (MSC) induced by cytokine exposure. Bone marrow-derived MSC were cultured in three-dimensional fibrin gel scaffolds and stimulated down the chondrogenic and osteogenic programs by addition of TGF-β3 to and osteogenic buffer media. Cells were cultured under control conditions (no cytokine supplementation), treated with TGF-β3 or treated with PD98059 + TGF-β3 for 7 days. RT-PCR results show that addition of TGF-β3 significantly upregulates the phosphorylation of ERK1/2 and induces the cells down the chondrogenic and osteogenic pathways (as demonstrated by the significant upregulation of aggrecan, sox9, collagen types 1 & 2 gene expressions). Inhibition of ERK1/2 phosphorylation with PD98059 led to the abolishment of the upregulation of chondrogenic and osteogenic-specific gene expressions. These results demonstrate that ERK1/2 is needed for the chondrogenic and osteogenic differentiation of MSC as induced by TGF-β3 supplementation.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that can be harvested from multiple types of tissues in the human body [1]. MSCs were first described as marrow stromal cells in the 1970s [2]. The emergence of MSCs as an important tool in regenerative medicine is due to their capability to repopulate and differentiate into several tissue lineages, including both cartilage and bone. MSCs are considered the natural source for tissue self-repair [3] and prove to be popular in stem cell based research because of their abundant availability and their circumvention of ethical issues that are inherent with other lineages such as embryonic stem cells. In fact, numerous clinical trials have already shown the potential and benefit of using MSCs for various disorders [4,5]. It has been shown that MSCs can effectively be differentiated into several tissue lineages when placed under specific conditions [6]. MSCs are most commonly used in bone as well as cartilage regeneration [7,8] and recent experiments show promising results that, 1 day, cellular therapies for bone and cartilage related disease(s) can become common practice in the medical field [9–11].

It is known that during the differentiation process of MSCs, one or several intracellular chemical cascades are modified (activated or inhibited) influencing the ultimate commitment of the cell. However, it remains unclear how each individual pathway affects the differentiation program of the cells and how manipulation of these pathways could lead to more efficient differentiation protocols. Understanding the underlying mechanisms that guide and control these intracellular cascades and their ultimate effect on cellular commitment could help harness the MSCs differentiation potential towards more effective tissue engineering and cellular-based therapy applications. Some of the most commonly studied pathways in mesenchymal stem cell chondro- and osteogenesis are the mitogen activated protein kinase (MAPK) pathways. The MAPK refers to a family of signal transduction pathways that have been conserved within eukaryotic cells throughout evolution. The three major MAPK pathways consist of the extracellular signal-regulated kinases (ERK), p38 MAP kinase, and the c-Jun N-terminal kinase (JNK) [12]. MAPKs respond to extracellular stimuli such as hormones, growth factors, and oxidative and mechanical stress by serial phosphorylation of proteins within the cascade. As a result, MAPKs have both activating and inhibiting roles in important

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physiological events such as proliferation, differentiation, and apoptosis [13]. A recent study from the Rensselaer Polytechnic Institute (RPI) [14] noted that inhibition of the ERK1/2 pathway led to an inhibition of chondrogenesis and to an amplification of the osteogenic program in human MSCs when stimulated by a three-dimensional type I collagen matrix culture. This study also suggested that the three-dimensional microenvironment configuration led to the upregulation of chondrogenic and osteogenic gene expression rather than the biochemical stimulation of the cells by a defined media (as no differentiating moieties were employed) or by the collagen I substrate (the predominant type of collagen in bone). ERK1/2 was the first signaling transduction pathway of the MAPKs that was heavily characterized and is considered one of the principal pathways that controls cell proliferation, differentiation, and overall survival of cells [15]. In terms of differentiation, ERK1/2 has been shown to be important both for both osteogenesis and chondrogenesis [16,17]. However, whether ERK1/2 is stimulatory or inhibitory to these processes remains unclear due to the amount of conflicting results in the field.

For example, in terms of osteogenesis, it has been suggested that ERK1/2 suppresses osteogenic differentiation in osteoblastic progenitor cell populations [18] and is at least partially non-critical for the hydrostatic pressure-induced osteogenesis of bone marrow MSCs [19]. However, it has also been suggested that inhibition of ERK1/2 abolishes the osteogenic response of adipose derived MSCs [20] which would imply that ERK1/2 is necessary for osteogenesis. Moreover, other studies with bone marrow-MSCs show that the inhibition of ERK1/2 amplifies osteogenesis [14]. Further, there is much ambiguity in the control that ERK1/2 has during chondrogenic differentiation of stem cells. For instance, Oh et al. (2000) showed that ERK1/2 phosphorylation decreased during chondrogenic differentiation of the chick mesenchyme [21]. In a similar cell system, Yoon et al. [22] showed that inhibition of chondrogenesis increased phosphorylation of ERK1/2; thus showing an anti-chondrogenic role of ERK1/2. However, there have been numerous reports of ERK1/2 being vital for chondrogenic differentiation of stem cells. Some studies revealed that the activation of ERK1/2 is necessary for the induction of the chondrogenic gene SOX-9 [23–25]. Hence, more research is required to fully understand the role of ERK1/2 in the processes of osteogenic and chondrogenic differentiation.

While it is clear that micro-environmental cues are by themselves capable of stimulating cells towards a differentiation pathway, it is unclear to what degree this induction activates the same chemical cascades and reaction kinetics observed with the biochemical induction via cytokine exposure. Successful differentiation of MSCs towards a chondrogenic and osteogenic lineage has been widely achieved in the past by means of soluble factor induction with cytokines from the TGF-β superfamily [26]. Similarly, researchers have demonstrated the need for the presence of extracellular inorganic phosphate–calcium complexes in order for successful osteogenic differentiation of progenitor cells to occur [27]. This same study also found that the exposure of the cells to the inorganic phosphate–calcium precipitates (CaPp) induced the phosphorylation of the ERK1/2 pathway in as little as 30 min of culture. The subsequent inhibition of ERK1/2 phosphorylation by means of the drug U0126 led to an elimination of the observed osteogenic marker upregulation [27].

Given the contradictory nature of the reports published on the role of ERK1/2 in the osteogenic and chondrogenic differentiation of cells as induced by either environmental/matrix interactions or by cytokine/soluble factor induction, it is necessary to further explore the role of this MAPK pathway in these processes. This project focuses on the activation of the ERK1/2 in cells stimulated by TGF-β3 and investigates the role of ERK1/2 in MSC chondrogenic and osteogenic differentiation via cytokine exposure.

2. Materials and methods

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise specified. Inhibitor PD98059, L-Ascorbic Acid, β-glycerophosphate, dexamethasone, and human recombinant TGF-β3 were all purchased from Sigma–Aldrich (St. Louis, MO).

2.1. Preparation of three-dimensional cell culture in fibrin gel

Three dimensional cell cultures were seeded in a 12-well plate format. The fibrin gels were prepared with equal parts of a fibrinogen solution (40 mg/ml) and thrombin solution (10 U/ml) and cast into each well. Cells were suspended within the thrombin solution at a concentration yielding 250,000 cells per sample. The final volume of each sample was 0.5 ml (0.25 ml fibrinogen + 0.25 thrombin cell suspension). After seeding of the cells into each well the plates were incubated for 45 min at 37 °C and 5% CO_2 to allow the gels to solidify after which all samples were treated with standard media consisting of HG-DMEM, 5% fetal bovine serum (FBS), 1% Streptomycin, and 0.1% Amphotericin B.

2.2. Cell culture treatment

Prior to treatment, all samples were cultured for 3 days with the HG-CCM to allow the cells to attach and expand. On the second day the media was replaced with serum-deprived culture media for 24 h. Following the serum deprivation period, the treatment was begun. Samples were separated into three groups: control, treated, and inhibited. Each group consisted of eight samples from which four samples were used for RNA analysis, and four were used for protein analysis. The control group was cultured with HG-CCM for the duration of the testing period. Treated groups were cultured with osteogenic buffer containing transforming growth factor (TGF)-β3 (10 ng/ml), dexamethasone (100 nM), β-glycerophosphate (10 mM), and L-ascorbic acid (vitamin C) (50 μg/ml). The inhibited groups was cultured with the same osteogenic buffer as the treated group but were further treated with the commercial ERK1/2 inhibitor PD98059 at 50 μM final concentration. Prior to treatment with the osteogenic buffer, the inhibited group was pre-incubated with PD98059 for 1 h in order to completely inhibit any initial phosphorylation of ERK1/2. All groups were treated for one week with media changes every other day. On day 7, the experiment was ended by either the addition of TRIzol reagent (Invitrogen) for RNA extraction or RIPA cell lysis buffer for protein analysis.

2.2.1. Gene expression analyses

2.2.1.1. RNA extraction, cDNA synthesis and polymerase chain reaction (PCR). Total RNA was extracted and purified using TRIzol® reagent according to the manufacturers recommended protocol. RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Complimentary DNA (cDNA) was synthesized by means of a cDNA Synthesis Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Briefly, 1 μg of total RNA was loaded per sample into a 20 μL reaction containing MuLV Reverse Transcriptase, dNTP mixture and a random hexamer solution and allowed to react for 2 h. The reaction was subsequently stopped by heating the samples to 85 °C for 5 min prior to using the synthesized cDNA in the PCR reactions. Obtained cDNA was subsequently diluted to 10 ng/μL in RNase- and DNase-free water (Sigma).

PCR was carried out using a Platinum Taq DNA polymerase PCR kit (Invitrogen) along with 20 ng total cDNA as per the manufacturer's recommended protocol. The sequences for the chondro-