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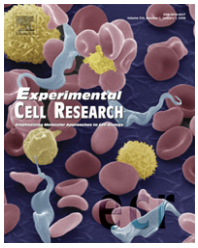

Research Article

Human mesenchymal stem cell proliferation is regulated by PGE2 through differential activation of cAMP-dependent protein kinase isoforms

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ABSTRACT

The conditions used for *in vitro* differentiation of hMSCs contain substances that affect the activity and expression of cyclooxygenase enzymes (COX1/COX2) and thereby the synthesis of prostanoids. hMSC constitutively produce PGE2 when cultivated *in vitro*. In this study we have investigated effects of PGE2 on proliferation of hMSC. We here demonstrate that one of the main control molecules in the Wnt pathway, GSK-3β, is phosphorylated at the negative regulatory site ser-9 after treating the cells with PGE2. This phosphorylation is mediated by elevation of cAMP and subsequent activation of PKA. Furthermore, PGE2 treatment leads to enhanced nuclear translocation of β-catenin, thus influencing cell proliferation. The presence of two PKA isoforms, types I and II, prompted us to investigate their individual contribution in PGE2-mediated regulation of proliferation. Specific activation of PKA type II with synthetic cAMP analogues, resulted in enhancement of proliferation. On the other side, we found that treatment of hMSC with high concentrations of PGE2 inhibited cell proliferation by arresting the cells in G_0/G_1 phase, an effect we found to be mediated by PKA I. Hence, the two different PKA isoforms seem to have opposing functions in the regulation of proliferation and differentiation in these cells.

Introduction

Adult stem cells can be isolated from a wide variety of tissues, and it is expected that their differentiation potential may reflect the local environment. Stem cells lack tissue-specific characteristics but, under the influence of appropriate signals, they can differentiate into specialized cells phenotypically different from their precursors. These cells may be reservoirs of reparative cells that are ready to mobilize and differentiate in response to inflammatory signals or disease conditions [1]. Human mesenchymal stem cells (hMSC) are tripotent cells capable of differentiating to chondrocytic, osteocytic and adipocytic lineages when cultivated under appropriate conditions. It was recently found that hMSCs constitutively produce prostaglandin E2 (PGE2) [2]. Furthermore, the conditions used for *in vitro* differentiation of hMSCs include mediators that affect the cyclooxygenase 1/2 (COX1/2) enzymes. Differentiation into adipocytes is induced by adding a cocktail of

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supplements that includes both dexamethasone and indomethacin to the culture medium while differentiation into osteocytes is induced by adding a cocktail including dexamethasone. The two latter compounds inhibit both transcription and activation of COX1/2. Prostaglandins are a group of biologically active compounds that play important roles in human physiology both in health and disease. The most abundant prostaglandin in the body is PGE2. Depending upon the context PGE2 may exert inflammatory [3] but also anti-inflammatory [4] effects. PGE2 is an important factor in inflammation caused both by tissue damage and infectious diseases. Four different PGE2 receptors exist, EP1, EP2, EP3 and EP4. They are all G-protein coupled receptors (GPCRs). The cellular response to PGE2 depends on the type of heterotrimeric G-proteins associated with the activated receptors. EP1 receptor is a well known activator of the $G\alpha_q$, eliciting enhanced intracellular Ca^{2+} levels by influencing phosphatidylinositol turnover [5]. The EP2 and EP4 receptors are generally known to activate $G\alpha_s$ which stimulates cAMP production by adenylate cyclase [6]. Finally, the EP3 receptor inhibits adenylate cyclase via the G-protein $G\alpha_i$. Three out of the four receptors are coupled to G-proteins that will affect the level of cAMP in the cell by increasing or decreasing the activity of adenylate cyclase.

Activation of PKA is achieved by the binding of cAMP to the regulatory (R) subunits which then release the active catalytic (C) subunit from the holoenzyme. There are two main isoforms of PKA, types I and II. PKA type I is soluble and preferentially located to the cytosol [7] while PKA type II is targeted to subcellular structures via A-kinase anchoring proteins (AKAPs) [8].

The Wnt signaling pathway is conserved among species and plays important roles in regulating cell proliferation, differentiation and migration [9]. The canonical β-catenin pathway is shown to be important in the regulation of proliferation in mesenchymal stem cells [10]. When Wnt is not bound to its receptor, casein kinase Iα (CKIα) and glycogen synthase kinase 3β (GSK3β) phosphorylate β-catenin in the axin complex [11]. Phosphorylated β-catenin is ubiquinated, resulting in degradation of β-catenin by proteasomes [12]. This results in a low β-catenin level in cytoplasma. When Wnt interacts with its cell surface-receptor consisting of Frizzled and lipoprotein receptor 5/6 (LPR 5/6), β-catenin will no longer be phosphorylated and can thereby escape degradation by the axin complex [13]. The accumulated β-catenin translocates to the nucleus, where it binds to the transcription factor T cell factor (Tcf)/lymphoid enhancer-binding factor (Lef) and stimulates expression of various genes [14,15]. GSK-3β kinase activity is regulated by phosphorylation on an N-terminal serine (Ser-9). This serine can be phosphorylated by several kinases, including PKA and Akt1, [16,17,17,18]. Phosphorylation will lead to a significant decrease in kinase activity [18–20]. In this study we report that PGE2 affects the Wnt pathway by phosphorylating GSK3β in hMSC-TERT20 cells. This phosphorylation is mediated by PKA type II whose selective activation can increase proliferation. We also demonstrate that PKA types I and II have opposite effects on proliferation, and the balanced activation of the two PKA isoforms are important in regulating proliferation versus differentiation in human mesenchymal stem cells.

Materials and methods

Reagents

The following antibodies were used; α-pGSK3β(Ser-9) and α-cyclin D1 from Cell Signaling, α-actin and α-β-catenin (Sigma-Aldrich). Prostaglandin E2, wortmannin, indomethacin and forskolin were all from Sigma-Aldrich. H89 was from Alexis Chemicals. The cAMP analogues sp-5,6-DCl-cBIMPS, 6-MBC-cAMP, 8-AHA-cAMP and 8-PiP-cAMP were all from BIOLOG Life Science Institute.

PGE2 quantification

PGE2 was quantified by an enzymeimmunoassay (EIA) system (cAMP Biotrack Enzymeimmunoasssay System, Amersham Biosciences, UK). Supernatants were collected from overnight cultures of $1{\cdot}10^4$ cells in 96 well plates. 50 μl culture supernatant was used per sample. The standard EIA procedure was used for measurement of PGE2 in culture supernatants. All samples were run in duplicates.

cAMP quantification

cAMP was quantified by enzymeimmunoassay (cAMP Biotrack Enzymeimmunoasssay System, Amersham Biosciences, UK). $1{\cdot}10^4$ cells were stimulated as indicated and lysed in 200 μl lysis buffer. 100 μl lysate were used per sample. Intracellular cAMP was measured using a non-acetylation EIA procedure. All samples were run in duplicates.

Cells and transfections

hMSC-TERT20 [21] were grown in RPMI-1640 supplemented with 10% fetal calf serum (FCS). Cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

For the isolation of primary human mesenchymal stem cells, bone marrow was obtained from the iliac crest of healthy volunteers after informed consent. The aspirate was diluted 1:3 in RPMI 1640 medium. After standard density-gradient centrifugation (Lymphoprep; Axis-Shield, Oslo, Norway) at 750 g for 20 min, the mononuclear cell layer was isolated, washed twice, and suspended in RPMI at $1{\cdot}10^7$ cells per ml. The cells were washed and allowed to adhere overnight at 37 °C with 5% humidified CO_2 in 175 cm^2 tissue culture flasks. On day 1, nonadherent cells were discarded and adherent cells were washed with PBS and then cultured in RPMI with 20% FCS.

At approximately 75% confluence, the cells were brought in suspension using trypsin-EDTA and replated at approximately 8500 cells per cm^2.

Cells were serum starved for 16–20 h in RPMI-1640 supplemented with 1% FCS where indicated.

The DOTAP liposome transfection reagent (Roche Ltd, CH) was used to transfect the cells with siRNA. Before transfection the cells were seeded in 24 well plates ($5{\cdot}10^4$ cells/well). DOTAP was mixed with HBS-TB (20 mM HEPES, pH 7.4, 150 mM NaCl) (1:3) and siRNA and incubated at room temperature for 1 h before it was added to the cells. The cells were grown for