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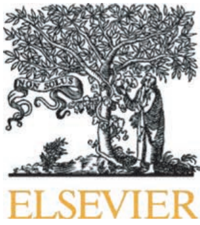

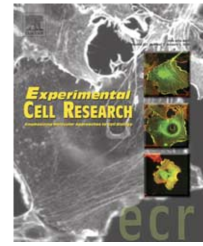

Research Article

15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 regulates leukemia inhibitory factor signaling through JAK-STAT pathway in mouse embryonic stem cells

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ABSTRACT

Embryonic stem (ES) cells are genetically normal, pluripotent cells, capable of self-renewal and differentiation into all cell lineages. While leukemia inhibitory factor (LIF) maintains pluripotency in mouse ES cells, retinoic acid and other nuclear hormones induce neuro-glial differentiation in mouse and human ES cells in culture. Peroxisome-proliferator-activated receptors (PPARs) are ligand-dependent nuclear receptor transcription factors that regulate cell growth and differentiation in many cell types. However, the role of PPARs in the regulation of ES cell growth and differentiation is not known. In this study, we show that LIF induces proliferation and self-renewal of mouse D3-ES cells in culture. However, treatment with 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15d-PGJ2), a natural ligand for PPARγ, or all-*trans* retinoic acid (ATRA) results in a dose-dependent decrease in proliferation and self-renewal in D3-ES cells. Immunoprecipitation and Western blot analyses showed that LIF induces tyrosine phosphorylation of JAK1, TYK2 and STAT3 in 30 min and treatment with 15d-PGJ2 or ATRA results in a dose-dependent decrease in LIF-induced phosphorylation of JAK1 and STAT3 in D3-ES cells. However, treatment of D3-ES cells with Ciglitazone or 15d-PGJ2 for 48 h in culture resulted in a dose-dependent increase in PPARγ protein expression. These results suggest that PPARγ agonists regulate LIF signaling through JAK-STAT pathway leading to growth and self-renewal of ES cells.

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Introduction

Embryonic stem (ES) cells derived from the inner cell mass of blastocyst stage embryos are genetically normal, pluripotent cells capable of indefinite growth and differentiation into all three embryonic germ layers even after prolonged culture [1–3]. ES cells have been derived from different animals including rabbits, pigs, mice and human [2,4–7]. D3-ES is a murine cell line isolated from day 4 129/Sv blastocysts that form embryoid bodies and differentiate spontaneously into visceral yolk sac, blood islands and myocardiocytes in vitro [8]. While various ES cell lines show different characteristics in their establishment and maintenance [9], mouse ES cells require leukemia inhibitory factor (LIF) for self-renewal. LIF was initially identified as a cytokine capable of inducing differentiation of M1 myeloid

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leukemia cells [10] and later shown to have differentiation inhibitory activity on ES cells [11–13]. LIF is a member of IL-6 family cytokine that induces growth and inhibits differentiation of ES cells for a prolonged period of time [12–15]. The effect of LIF is mediated through a cell surface receptor complex composed of a low affinity LIF receptor (LIFR) and gp130, a common receptor subunit of the IL-6 family of receptors [16,17]. Binding on its receptor, LIF induces dimerization of LIFR/gp130 complex, leading to the activation of JAK-STAT, Ras-MEK-ERK and other signaling pathways [18–20]. While the activation of LIF/gp130/STAT3 signaling pathway efficiently supports self-renewal of mouse ES cells, it failed to induce self-renewal in human and other primate ES cells, despite the activation of STAT signaling pathway [19]. However, the role of specific signaling pathways required to maintain pluripotency or self-renewal and their regulation leading to neuro-glial differentiation of ES cells are not well defined.

Peroxisome-proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-dependent transcription factors that mediate response to steroids, retinoids and thyroid hormones and regulate cell growth and differentiation. PPARα, δ and γ are the three known members of the PPAR family proteins, encoded by distinct genes [21]. PPARα is expressed abundantly in tissues with high capacity for lipid metabolism such as liver, kidney, heart and adrenal gland, whereas PPARδ is expressed ubiquitously in all tissues [22]. PPARγ is expressed in adipose tissue, heart, kidney, pancreas, spleen, intestine, colon epithelial cells and skeletal muscle, and the targeted disruption of PPARγ is embryonically lethal and dies by day E10 due to defects in the development of placental, cardiac and adipose tissue [23–25]. The 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15d-PGJ2) is a natural ligand for PPARγ [26]. Thiazolidinediones are synthetic compounds functioning as high affinity agonists for PPARγ that has been used in the treatment of type II diabetes, obesity and cancer [27]. Upon activation with specific ligands, PPARγ heterodimerizes with 9-*cis* retinoic acid receptor (RXR) and the PPARγ/RXR complex binds on direct repeats of AGGTCA hexanucleotide sequence in the promoter region of target genes [28–30]. Earlier studies have shown that all-*trans* retinoic acid (ATRA) signals through RAR/RXR complex and induces neuro-glial differentiation of ES cells [31]. However, the role of PPARγ in the regulation of growth and differentiation of ES cells is not known. In this study, we show that PPARγ agonists regulate growth and self-renewal in association with the inhibition of LIF signaling through JAK-STAT pathway in ES cells.

Materials and methods

Culture and maintenance of D3-ES cell

The D3-ES cells were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS, 1 mM Sodium pyruvate, 100 U/ml penicillin G, 100 μg/ml Streptomycin, 2 mM glutamine, 1 mM MEM nonessential amino acids, 50 μM 2-mercaptoethanol, 100 mM MTG and 10 ng/ml of LIF in 5% CO_2 incubator at 37°C. The cells were subcultured once in 5–7 days with a medium change of once in 3 days.

Reagents

The murine recombinant LIF was purchased from Chemicon International (Temecula, CA). 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 was purchased from Calbiochem (La Jolla, CA). Anti-JAK2 Ab and anti-phosphotyrosine mAb 4G10 were purchased from Upstate Biotechnology, Inc. (Lake placid, NY). Antibodies specific to JAK1, JAK3, TYK2, STAT1, STAT3, STAT4, STAT5 and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and anti-PPARγ1/2 antibody was purchased from Biomol International LP. (Plymouth Meeting, PA). The HRP-conjugated secondary Abs and other chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO).

Proliferation assay

The proliferation of D3-ES cells in culture was determined by ^{3}H thymidine incorporation assay. Briefly, D3-ES cells (5×10^4/200 μl/well) were cultured in 96-well tissue culture plates in DMEM containing 10% fetal bovine serum (FBS) in the presence of 0, 2.5, 5 and 10 ng/ml LIF or 10 ng/ml LIF in the presence of 0, 1.58, 3.16, 7.9 and 15.8 μM 15d-PGJ2 or 0, 16.64, 33.29, 83.21 and 166.42 nM ATRA. ^{3}H thymidine (0.5 μCi/ml) was added at 24 h, and the cells were harvested after 48 h using a Tomtech harvester 96. The amount of ^{3}H thymidine uptake was counted on a Wallac betaplate liquid scintillation counter as a measure of proliferation [32,33].

Preparation of whole cell extract for PPARγ analysis

D3-ES cells (1×10^6/3 ml/well) were cultured in DMEM containing 10% fetal bovine serum (FBS) and 0 or 10 ng/ml LIF in the absence or presence of Ciglitazone, 15d-PGJ2 and ATRA in 5% CO_2 incubator at 37°C. After 48 h, the cells were harvested, washed in ice-cold PBS and the total protein was extracted by boiling in 250 μl of denaturing Laemmli sample buffer for 5 min. The supernatants were collected by centrifugation at 10,000 rpm in a Beckman refrigerated micro-centrifuge for 10 min, and the total protein was estimated by Bradford assay (Bio-Rad) according to the manufacturer's instruction. The protein samples were resolved on 10% SDS-PAGE, and the expression of PPARγ analyzed by Western blot using anti-PPARγ antibody as described below.

Immunoprecipitation of JAK and STAT proteins

The immunoprecipitation and Western blot analyses of JAK and STAT proteins were performed as described earlier [32,33]. Briefly, D3-ES cells (5×10^6/lane) were cultured in medium alone or with 10 ng/ml LIF in the absence or presence of nuclear receptor agonists at 37°C for 30 min. The cells were then washed in ice-cold PBS and solubilized by gentle rocking in 250 μl of lysis buffer (Tris–HCl 50 mM, pH 7.4; NP-40, 1%; Sodium deoxycholate, 0.25%; TritonX-100, 0.2%; NaCl, 50 mM; EGTA, 1 mM; PMSF, 1 mM; Aprotinin and Leupeptin, 1 μg/ml; Na_3VO_4, 1 mM; NaF, 1 mM; Sigma, MO) at 4°C for 30 min. The

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