INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass, a component of the blastocyst. They can be maintained in permanent culture without losing their pluripotent phenotype; however, ESCs can also be prompted to differentiate into particular lineages by supplementation of specific agents or molecules. Several studies have shown that an alkaline environment is needed in order to force ESCs to secrete a mineralized matrix. This environment has been achieved with diverse osteogenic media formulations enriched with β-glycerophosphate (BGP). Addition of 1α,25-dihydroxyvitamin D3 (1,25D) has been shown to trigger the expression of bone marker genes like Runx2, collagen type I, osteocalcin (OCN) and bone sialoprotein. Effects of 1,25D in osteoblasts are mediated by two vitamin D receptors, the nuclear VDR and a membrane associated 1,25D binding protein called protein disulfide isomerase A3 (PDIA3), also known as ERp60, ERp57, Grp58, and 1,25-MARRS. Whether one or both of these receptors are present in ESCs is not known, nor is it known if they are modulated by the external alkaline environment of the osteogenic media formulations. To test this, we took advantage of a novel ESC culture system in which ESCs are grown under rotary culture conditions, forming embryoid bodies (EBs). The objective of this study was to elucidate the presence of nVDR and PDIA3 in ESCs and EBs and to examine their expression during EB formation in osteogenic media.

METHODS

Mouse ESCs (D3) were cultured on 0.1% gelatin-coated 100 mm polystyrene cell culture dishes with Dulbecco’s modified Eagle medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin and 0.1 mM β-mercaptoethanol. To maintain the cells in their undifferentiated state, 10 mM leukemia inhibitory factor (LIF) was added to the culture media at each feeding. Cells were passaged every 2-3 days before reaching 70% confluence. To initiate EB culture, ESCs were dislodged from the gelatin-coated dishes and dissociated with 0.05% Trypsin/0.03 mM EDTA. 200,000 cells/mL were inoculated into 10 mL EB cultures in 100 mm bacteriological grade polystyrene Petri dishes using differentiation media (media without LIF). Cultures were placed on rotary orbital shakers at 40 rpm at 37°C in 5% CO₂, for the entire duration of suspension culture. EBs were cultured with or without addition of 10 nM 1,25D alone, 10 mM BGP alone, or 1,25D+BGP in suspension for 14 days and fed every other day by collecting the EBs via gravity sedimentation in 15 mL conical tubes. Old media was aspirated and the cultures were replenished with 10 mL of fresh differentiation media before being placed back in the Petri dishes and onto the rotary orbital shakers. nVDR and PDIA3 were assessed by real-time PCR and Western blot. PDIA3 and nVDR spatial distribution were analyzed by immunocytochemistry and immunohistochemistry. Total DNA, alkaline phosphatase specific activity (ALP), OCN levels, and PKC activity were measured.

RESULTS

ESCs and EBs expressed mRNA and protein for both vitamin D receptors and this was confirmed by immunocytochemistry. After 14 days of treatment with 1,25D or BGP, DNA content decreased in the treatment groups when compared with control cultures suggesting a decrease in cell proliferation and an increase in differentiation. PDIA3 expression increased at 7 days of BGP treatment but decreased at 14 days; meanwhile, nVDR was slightly higher at 7 days and increased significantly at 14 days of BGP treatment (Fig 1). Alkaline phosphatase specific activity increased in all groups treated with BGP; 1,25D treatment by itself had no effect on ALP, but 1,25D+BGP caused the greatest increase in ALP, and the effect was synergistic. OCN levels increased in EBs treated with BGP; the greatest stimulation was seen in cultures treated with 1,25D+BGP (Fig 2). PKC activity increased in the groups treated with BGP ≥ 1,25D, but 1,25D alone had no effect.

DISCUSSION

ESCs and EBs expressed significant levels of nVDR and PDIA3, suggesting that vitamin D receptors are essential for ESC function and possibly for embryonic germ lineage and tissue formation as well as embryo development. The alkaline environment created by the BGP treatment increased nVDR expression and decreased expression of PDIA3. PDIA3 mediates rapid effects of 1,25D via a phospholipase A2-dependent PKC signaling pathway, suggesting that early responses to the vitamin D metabolite are regulated in this manner. In contrast, expression of proteins associated with osteoblast differentiation is regulated via nVDR mechanisms, commensurate with VDREs present in the promoter regions of these genes. 1,25D treatment by itself did not modulate nVDR or PDIA3 expression, but its effects were synergistic with BGP, suggesting that it potentiates but does not initiate changes in their levels within the cells or the differentiation process induced by BGP, allowing the cells to produce bone extracellular matrix proteins like OCN and increasing ALP activity. This is the first evidence showing the importance of vitamin D receptors and the mechanism of action of BGP in osteogenic media.

REFERENCES


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