REDUCING EXPRESSION OF Gs\textalpha  INDUCES EARLY MARKERS OF BONE FORMATION IN HUMAN MESENCHYMAL STEM CELLS

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Introduction  Ectopic formation of intramembranous bone is a characteristic feature of three developmental disorders, Albright’s hereditary osteodystrophy (AHO), progressive osseous heteroplasia (POH), and osteoma cutis (OC) in which patients have heterozygous inactivating mutations in the GNAS1 gene (2, 4). These mutations lead to reduced expression or function of the alpha chain of the heterotrimeric signaling protein G\textalpha , the G-protein that couples heptathelial receptors to stimulation of adenyl cyclase. Previous studies have demonstrated that activation of adenyl cyclase or the cAMP-dependent pathways leads to suppression of osteoblastic differentiation and function via the enhanced proteolytic degradation of the osteoblast-specific transcription factor Cbfa1 (3). Thus, it is conceivable that the development of ectopic bone in patients with reduced Gs\textalpha expression occurs as a result of increased Cbfa1 activity in undifferentiated cells. We therefore hypothesized that decreasing cAMP signaling by decreasing Gs\textalpha levels in mesenchymal stem cells would lead to increased expression of markers of osteoblastic differentiation.

Methods  Pluripotential adult human mesenchymal stem cells (MSC’s) were obtained from Osiris and grown to approximately 85% confluence in non-osteogenic medium growth medium (GM) with 10%FBS. The cAMP signaling was decreased by antisense oligonucleotides or protein kinase A (PKA) inhibition. For antisense transfection, control and antisense Morpholino oligonucleotides in EPEI were added to the cells in serum free, antibiotic free medium and the cells were incubated for 3 hours at 37 degrees. The antisense oligodeoxynucleotide was complimentary to the AUG translational start site and the 22 bases 3’ to that site. In the PKA inhibition experiments, KT5720 or Rp-8-Br-cAMPS, Na (Calbiochem, Carlsbad, California) were used to inhibit the PKA pathway. Immunoblotting was performed on membrane and nuclear isolates with Gs\textalpha and Cbfa1 antibodies, respectively. Electrophoretic mobility shift assays (EMSA) were performed with nuclear protein isolates after incubation with \textsuperscript{32}P-radio labelled double-stranded oligonucleotide probes. Total RNA was isolated using the Trizol reagent and microarrays were performed using the GF211 membranes (ResGen, Huntsville, Alabama) as recommended by the manufacturer. The filters were analyzed in pairs on the Pathways 3.0 software (ResGen). Results from these experiments were corroborated by northern blot. Semiquantitative RT-PCR was also used to assess levels of Cbfa1.

Essential Results  Immunoblot to Gs\textalpha revealed a 9-fold decrease in signal at day 1 and 3-fold decrease at day 3.5 in the MSC’s treated with the Gs\textalpha antisense oligonucleotide compared to the cells treated with a negative control (inverted sequence oligonucleotide) (Figure 1). Levels of the osteoblastic transcription factor Cbfa1 increased concomitant with the decrease in Gs\textalpha (Figure 1). To examine the mechanism by which decreased Gs\textalpha expression led to increased Cbfa1, MSC’s were treated for 3.5 days with PKA inhibitors (KT5720 0.4 or 4 \muM or Rp-8-Br-cAMPS, Na. 15 or 150 \muM). Levels of Cbfa1 were also increased after PKA inhibition (Figure 2). The increase in Cbfa1 in groups with lowered PKA corresponded with an appropriate decrease in phosphoCREB, indicating the efficacy of PKA inhibition (Figure 2). EMSA using a radio labelled OSE2 sequence (the Cbfa1 DNA binding site) showed that MSC’s that had been treated with either Gs\textalpha antisense oligo or PKA inhibitors yielded a more intense gel retardation complex than in untreated MSC’s. To determine other genes that were altered by oligo or PKA inhibitors yielded a more intense gel retardation complex site) showed that MSC’s that had been treated with either Gs\textalpha antisense oligo or PKA inhibitors yielded a more intense gel retardation complex than in untreated MSC’s. To determine other genes that were altered by Gs\textalpha antisees the formation of ectopic bone in patients with inactivating Gs\textalpha mutations. The decrease in Gs\textalpha could lead to several downstream effects such as a decrease in adenyl cyclase activity or an effect on calcium channels. A decrease in adenyl cyclase activity would in turn decrease PKA. We therefore examined the effect of the decrease in PKA activity using two different inhibitors and both increased Cbfa1, suggesting that the mechanism for an increased Cbfa1 after a decrease in Gs\textalpha is thru the cAMP pathway. Further work is needed to elucidate how a decrease or inhibition of PKA effects an increase in Cbfa1.

Discussion  Ectopic production of membranous bone occurs in at least three human disorders that are associated with mutations in the GNAS1 gene that reduce the expression or function of Gs\textalpha protein(4). Using antisense oligonucleotides to Gs\textalpha mRNA, we reduced levels of both Gs\textalpha mRNA and protein without apparent serious cellular toxicity, allowing us to further examine the role of Gs\textalpha in regulating bone formation. Reduction of Gs\textalpha protein was associated with enhanced expression of the obligate osteogenic transcription factor Cbfa1 as determined by immunoblot analysis and EMSA after greater than 3 days of continuous treatment. Moreover, using microarray analysis, we showed that changes in Cbfa1 were accompanied by an increase in mRNA encoding for collagen type I alpha 2. This finding is consistent with the known OSE2 sequence in the type I alpha 2 promoter. The increase in Cbfa1 with decreases in the stimulatory G-protein pathway in MSC’s potentially explains the formation of ectopic bone in patients with inactivating Gs\textalpha mutations. The decrease in Gs\textalpha could lead to several downstream effects such as a decrease in adenyl cyclase activity or an effect on calcium channels. A decrease in adenyl cyclase activity would in turn decrease PKA. We therefore examined the effect of the decrease in PKA activity using two different inhibitors and both increased Cbfa1, suggesting that the mechanism for an increased Cbfa1 after a decrease in Gs\textalpha is thru the cAMP pathway.

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