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Original Article

Hylonome

NFATc1 directly induces the human β_3 integrin gene in osteoclast differentiation

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Background

RANKL acts through its receptor RANK to initiate a signaling cascade that is crucial for osteoclast differentiation and activation. The transcription factor nuclear factor of activated T cells (NFATc1) is upregulated by RANKL and has been showed to play a crucial role in osteoclast differentiation and function[1-3]. Overexpression of NFATc1[2] and ectopic expression of constitutively active NFATc1[3] are able to bypass the requirement for RANKL for osteoclast differentiation. To date, however, the key transcriptional targets of NFATc1 in osteoclasts are unknown.

Recent findings demonstrate the ability of NFATc1 to induce the expression of various osteoclast genes, including the β_3 integrin[3]. The integrin $\alpha_v\beta_3$ (the vitronectin receptor)[4] is expressed on bone-resorbing osteoclasts[5] and their precursors, and evidence suggests that it is involved in the attachment of osteoclasts to bone[6]. β_3 is the regulatory component of $\alpha_v\beta_3$, and the requirement for β_3 in normal osteoclast function is clearly demonstrated by genetic ablation of the β_3 gene in mice[7]. Osteoclasts from β_3 knockout mice fail to spread *in vitro*[7], supporting the results from blocking experiments using anti-β_3 antibodies[6]. *In vivo* osteoclasts in β_3 knockout mice fail to form actin rings and do not form ruffled membranes, impeding bone resorption[7] and resulting in an osteosclerotic phenotype. Thus far, little is known regarding the transcriptional mechanisms mediating β_3 integrin expression during osteoclast differentiation. The present studies were undertaken to characterize the regulatory region of the human β_3 integrin gene and to define the potential role of RANKL-induced NFATc1 in regulating the β_3 integrin promoter.

Results and Discussion

Alignment of 15kb of the mouse and human promoter sequences by Pustell matrix (using MacVector) identified a region encompassing 1.2 kb (Figure 1A) of the promoter, upstream of the TSS (transcription start site), that was of high identity. Alignment of this region of the human β_3 integrin gene (-1242 to +29) to the mouse orthologous sequence, using the VISTA database, revealed regions of greater than 80% sequence homology (Figure 1B). Multiple conserved transcription factor binding elements are present within the region –1242 to + 29 bp relative to the TSS (as determined using MATCH in the TRANSFAC transcription factor database). Of note, 8 putative NFAT binding sites were identified within this region (Figure 1C). Alignment of the –1242 to + 29 bp human β_3 fragment to the mouse β_3 promoter (Figure 1C) showed that of the NFAT binding sites, 3 three that are 5′ in the promoter, and one site immediately upstream of the 5′ UTR, are spatially conserved between mouse and human (conservation indicated by asterix). Notably, a single NFAT:AP-1 consensus site was identified in the human β_3 promoter (Genomatix Software GmbH).

To identify sequences within the human β_3 promoter associated with RANKL-mediated gene expression, the region –1242 to +29 was cloned as a luciferase reporter construct (pB_3-1.3). In addition, 245 bp of the promoter was deleted, truncating the –1242 fragment to –997 (pB_3-1). This removed 3 conserved NFAT binding sites including the NFAT:AP-1 site. Consistent with a direct effect of RANKL-induced transcription factors on the β_3 promoter, RANKL treatment dose-dependently induced the full-length pB_3-1.3 reporter construct in RAW264.7 cells (data not shown). Co-transfection with human NFATc1 also induced the pB3-1.3 construct dose-dependently (data not shown). The pB3-1 deletion construct was significantly less responsive to RANKL treatment.

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Figure 1. Identity between the mouse β_3 promoter region to the human β_3 promoter region.

In addition, the pB3-1 deletion construct was virtually unresponsive to co-transfected NFATc1. These experiments identified the conserved region, -1242 to -997 upstream of the TSS in the human β_3 gene as the promoter region responsible for RANKL and NFATc1 induction.

While these studies identified the human β_3 promoter region responsive to RANKL and NFAT induction, they do not demonstrate direct NFAT-promoter interaction and allow the possibility for indirect/secondary transcriptional effects. Direct NFAT binding to 2 of the conserved NFAT sites within the –1242 to –997 region of the β_3 promoter was demonstrated by EMSA and competition assay. The identity of the shifted complexes was confirmed by supershift experiments with anti-NFATc1 antibodies (Santa Cruz). Mutation of both of the NFAT sites within the 20nt oligonucleotide EMSA probe, corresponding to a region in the –1242 to –997 fragment, prevented competition. These results suggest that these two sites play an important role in NFATc1 regulation of the β_3 integrin gene.

To investigate the role of the NFAT sites identified with EMSAs, in the context of the full-length human β_3 reporter, we mutated the two conserved NFAT sites in the fragment, pB3-1.3, (pB3-1.3 M1&M2). Mutation of the NFAT binding sites resulted in a decrease in RANKL induction and a striking reduction in NFATc1 transactivation (data not shown). Although these studies show that NFATc1 signaling is the predominant transcription factor in RANKL-mediated induction of the β_3 integrin promoter, there are likely to be additional transcriptional elements that mediate β_3 integrin expression in osteoclast differentiation.

To assess the effect of blockade of NFAT/calcineurin signaling on osteoclast morphology and expression of the endogenous β_3 integrin gene, we generated cell-permeable TAT-dominant-negative (dn)NFATc1 fusion proteins. TAT-fusion proteins rapidly cross the cell membranes of all cell types and have been shown to effectively transduce osteoclasts and osteoblasts[8]. The pTAT-dominant negative (dn) NFATc1 constructs were generated by deleting the DNA binding domain of NFATc1 from the construct, as described by Northop et al.[9]. The TAT-dnNFATc1 protein acts by binding to calcineurin so that after dephosphorylation and translocation to the nucleus it is unable to bind to the DNA domain in the β_3 promoter. Proteins were denatured and purified, as described by Dolgilivich et al.[8].

Transduction with TAT-dnNFATc1 (50nM-500nM) dose-dependently decreased formation of RANKL-induced bone marrow (BM) derived osteoclasts, as assessed by TRAP staining and cell morphology. The inhibition of cell spreading in cells treated with TAT-dnNFATc1 implies an integrin effect. In addition, TAT-dnNFATc1 dose-dependently repressed expression of the endogenous β_3 integrin gene, as assessed by RT quantitative (Q) PCR (data not shown). Transfection experiments verified these findings as transduction with TAT-dnNFATc1 inhibited RANKL induction of the β_3 integrin promoter (data not shown). Results were confirmed using a cell-permeable NFAT/calcineurin inhibitor, 11R-VIVIT.

Discussion and conclusion

RANKL selectively induces the transcription factor NFATc1 expression via the TRAF6 and c-Fos pathways, triggering a sustained NFATc1-dependent transcriptional program during osteoclast differentiation[2]. Functional NFAT sites have been identified in the Cath-K, TRAP, CTR and OSCAR promoters[10-12] but the role of NFATc1 in regulation of the β_3 integrin gene had not been investigated. We identified a 1.2kb region of the β_3 integrin promoter that is sufficient to direct RANKL-induced signaling and NFATc1 transactivation in RAW264.7 cells. RANKL and NFATc1-induction was reduced by mutation of 2 upstream NFAT sites, which are conserved from mouse to human. Blockade of the NFAT/calcineurin interaction using TAT-dnNFATc1 and the 11R-VIVIT demonstrated an inhibitory effect on the activation of the β_3 integrin promoter, osteoclast cell morphology and gene expression, consistent with a direct effect on β_3 integrin activity. Together, these results confirm the role of the NFATc1 signaling pathway in osteoclast differentiation

and establish the β_3 gene as a direct target of NFAT in RANKL-dependent osteoclast formation. Future studies will include identification of transcriptional factors other than NFATc1 that may be involved in regulation of the β_3 integrin.

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