Role(s) of Twist in RANKL/RANK signaling- mediated osteoclastogenesis

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INTRODUCTION

RANK (receptor activator of nuclear factor kappa B) signaling through activation of NFκB pathway is critical in osteoclast formation (osteoclastogenesis). Twist is an evolutionarily conserved basic helix-loop-helix (BHLH) transcriptional factor, and acts as a negative regulator of muscle and osteoblast differentiation by regulation of NFκB pathway. The purpose of this study was to determine the role(s) of Twist in RANK signaling- mediated osteoclastogenesis.

MATERIALS AND METHODS

Mouse RAW 264.7 macrophage cell line Raw cells were maintained in DMEM containing 10% FBS at 37°C with 5% CO_2.

Mouse bone marrow dependent macrophage cells (BMDM) were collected from BALB/c mice and cultured in the presence of both M-CSF (10 ng/ml) and RANKL (30 ng/ml) for 8 to 10 days (changed every three days).

Transient Twist gene transfection Both Raw cell and BMDM cells were transiently transfected with pcDNA3.1 vectors (Invitrogen) with the insert of Twist (Dermo-1 cDNA) kindly provided by Dr.Li Li.

Osteoclast formation was determined by TRAP staining. Osteoclast activity was evaluated by Pit formation assay. Twist expression (mRNA and protein) was measured by both western blot and real time RT-PCR. Immuno fluorescence staining (IF) was used to determine the protein expression of RANK, TRAF6 and NFκB.

Data analysis Data were expressed as Mean ± SD. Data was analyzed using one-way ANOVA by SPSS software. A p value of less than 0.05 was considered as significant.

RESULTS

Twist is constitutively expressed and increased in response to RANKL stimulation in macrophages Western blot analysis(Fig.1) indicate that Twist protein is constitutively expressed in both RAW and BMDM cells. A brief treatment of RANKL significantly increases Twist protein production. However, the stimulatory effect of RANKL is transient, because Twist protein level returned to the basal level after 48 hours (Data not shown). RANKL-induced transient up-regulation of Twist gene production was further confirmed by Real time RT-PCR analysis (Fig. 2).

Over expression of Twist gene un-regulates protein production of RANK, TRAF6 and NFκB We successfully transfected Twist gene into both RAW and BMDM cells (Data not shown). As shown in Fig.3, IF staining demonstrated that over expression of Twist increased the gene production of RANK, TRAF6 and NFκB, which are more significant when treated with RANKL.

Twist plays an orchestral role in RANKL- induced osteoclastogenesis and osteoclast activity Our finding that over expression of Twist increased the gene expression of RANK, TRAF6 and NFκB led us to ask whether Twist is involved in the process of osteoclast formation and function. To address this question, we cultured BMDM cells in the continuous presence of both RANKL and M-CSF with or without twist transfection for up to 10 days. As seen in Figure 4, transfection of twist plasmid alone could not induce $TRAP^+$ cells (well 3), but significantly increased the number of $TRAP^+$ cells when the cells were treated with RANKL. Pit formation assay further confirmed that those $TRAP^+$ cells are functional.

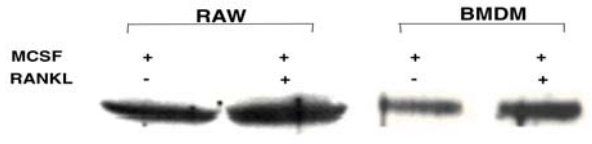

Fig. 1. Expression of Twist in macrophages. Both Raw cells and BMDM cells were treated with or without RANKL (100 ng/ml) for 24 hours. Cell lysates (50 μg) were subjected to Western blot analysis with anti-Twist Ab.

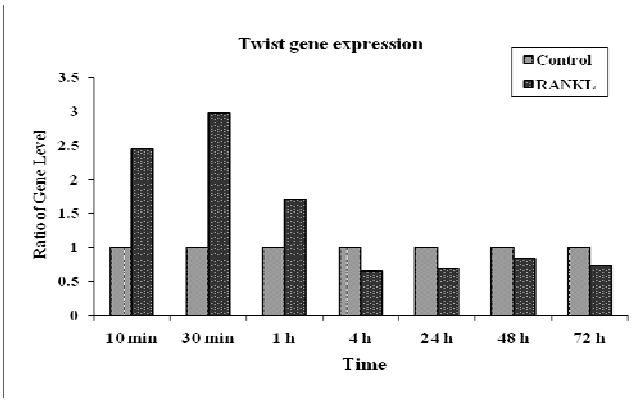

Fig. 2. Effect of RANKL on Twists gene expression (time course study)

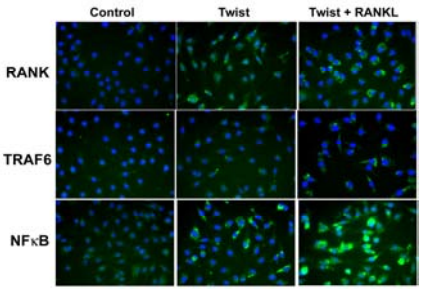

Fig. 3. Effects of Twist gene on gene production of RANK, TRAF6, and NFkB IF image analysis showed that Twist increased the gene production of RANK, TRAF6 and NFkB, which are more significant in the presence of RANKL.

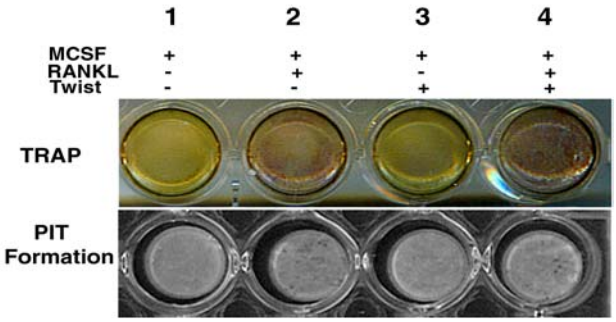

Fig. 4 Effect of Twist on osteoclast formation and activity

DISCUSSION

Our data indicated that Twist is involved in RANKL/RANK signaling-mediated osteoclastogenesis, as manifested by (1) Twist is constitutively expressed in macrophages, and increased in response to RANKL stimulation; (2) Transient Twist transfection results in increased production of RANK, TRAF6 and NFκB, and (3) Twist is involved in the process of osteoclast formation and activity. Further understanding the role(s) of Twist in these processes will provide new insights into the controlling process involved in RANKL/RANK signaling, and may lead to develop novel drugs to disarm unwanted osteoclast activity in patients with pathological bone loss.

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