NELL-1-dependent mineralisation of Saos-2 human osteosarcoma cells is mediated via c-Jun N-terminal kinase pathway activation

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Received: 16 April 2012 / Accepted: 22 May 2012 / Published online: 14 July 2012
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Abstract

Purpose NELL-1 is a novel osteoinductive growth factor that has shown promising results for the regeneration of bone. Moreover, NELL-1 has been used successfully in bone regeneration in the axial, appendicular and calvarial skeleton of both small and large animal models. Despite increasing evidence of NELL-1 efficacy and future usefulness as an alternative to traditional bone graft substitutes, much has yet to be understood regarding the mechanisms of action of this novel protein. The activation of the mitogen-activated protein kinase (MAPK) pathway has been well studied in the setting of growth factor-mediated changes in osteogenic differentiation.

Methods In this study, we provide evidence of the involvement of MAPK signalling pathways in NELL-1-induced terminal osteogenic differentiation of Saos-2 human osteosarcoma cells. Activation of extracellular signal-regulated kinase (ERK1/2), P38 and c-Jun N-terminal kinase (JNK) pathways were screened with MAPK signalling protein array after recombinant human (rh)NELL-1 treatment. Next, the mineralisation and intracellular phosphate levels after

rhNELL-1 stimulation were assessed in the presence or absence of specific MAPK inhibitors.

Results Results showed that rhNELL-1 predominantly increased JNK pathway activation. Moreover, the specific JNK inhibitor SP600125 blocked rhNELL-1-induced mineralisation and intracellular phosphate accumulation, whereas ERK1/2 and P38 inhibitors showed no effect.

Conclusions Thus, activation of the JNK pathway is necessary to mediate terminal osteogenic differentiation of Saos-2 osteosarcoma cells by rhNELL-1. Future studies will extend these in vitro mechanisms to the in vivo effects of NELL-1 in dealing with orthopaedic defects caused by skeletal malignancies or other aetiologies.

Introduction

NELL-1 was first appreciated to have osteogenic properties as it is upregulated in inappropriately fusing (or craniosynostotic) cranial sutures [1]. Subsequent studies found that mice overexpressing NELL-1 possessed cranial bone overgrowth similar to human craniosynostosis [2, 3]. Since this time, NELL-1 signalling has been successfully used in numerous skeletal tissue engineering applications, across mammalian species and in the calvarial, axial and appendicular skeleton [4–9]. Recently, our research group has extended NELL-1 efficacy studies to a large animal model of spinal fusion in sheep [9], demonstrating its clinical relevance. Despite this accumulating translational research, the basic mechanisms whereby NELL-1 signalling increases bone formation remain incompletely understood.

NELL-1 is a secreted protein of 810 amino acids with a molecular weight of about 90 kDa before *N*-glycosylation and oligomerisation. Recombinant human (rh)NELL-1 has

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a molecular weight of about 140 kDa under reducing conditions and has been reported to be secreted as either trimers or pentamers [10]. NELL-1 contains several structural motifs, including a secretory signal peptide, a thrombospondin-1 (TSP-1)-like module (TSPN, overlapping with a laminin G domain), four chordin-like cysteine-rich (CR) domains and six epidermal growth factor (EGF)-like domains. NELL-1 has recently been reported to bind the APR3, an intracellular receptor [11]. In addition, our laboratory recently reported that the effects of NELL-1 on osteoblastic differentiation and proliferation are associated with binding to APR3 [11]. Recently, preliminary data suggested that NELL-1 also binds to integrin β 1 on the cell surface (data in submission).

The mitogen-activated protein kinase (MAPK) signalling pathway is composed of three main subfamilies: the c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK1/2) and P38. MAPK signalling governs numerous cell processes, including proliferation, differentiation and apoptosis. For example, transforming growth factor (TGF)- β stimulates the ERK1/2 and JNK cascades to negatively regulate alkaline phosphatase (ALP) activity and mineralisation in osteoblasts [12]. Bone morphogenetic protein (BMP)-2, however, induces mineralisation specifically through the JNK, but not the p38 or ERK1/2, pathways [13]. Other signalling cascades such as fibroblast growth factor (FGF) [14, 15] and TGF- α signalling [16] are also known to influence osteogenic differentiation via MAPK signalling modulation.

In a previous study, NELL-1 signalling was shown to activate the JNK cascade of the MAPK pathway during mineralisation of C2C12 myoblast cells [17]. Also, in primary mouse calvarial cells, we have demonstrated that the activation of JNK1 and ERK are responsible for Runx2 phosphorylation and enhanced mineralisation [18]. Interestingly, NELL-1 has been recognised as a potential tumour suppressor in several previous reports [19, 20]. We sought here to examine the effects of NELL-1 signalling on an immortalised cell line derived from primary human osteosarcoma, Saos-2 cells. Specifically, we sought to define the extent to which MAPK signalling plays a role in NELL-1-induced differentiation in Saos-2. In this report, we confirmed NELL-1 signalling is capable of inducing Saos-2 cell terminal osteoblastic differentiation, via a JNK-dependent mechanism.

Materials and methods

Cell culture

Saos-2 cells were treated with both basal and osteogenic media. Basal media consisted of DMEM, 10 % foetal bovine serum, 100 IU/ml penicillin and 100 IU/ml streptomycin.

Osteogenic media consisted of DMEM, 10 % foetal bovine serum (FBS), 50 μ g/ml ascorbic acid, 10 mM β -glycerol phosphate, 10^{-8} μ M dexamethasone and penicillin/streptomycin.

MAPK protein array

The MAPK Array Kit (R&D Systems, Minneapolis, MN, USA) detects phosphorylated forms of all three major subfamilies of the MAPK. Saos-2 cells were cultured on 24 plates and were stimulated with 100 ng/ml rhNELL-1 in differentiation media for 15 and 45 min. The dose of rhNELL-1 was based on previous dose curves. Cells were suspended at 1×10^7 cells/ml in radioimmunoprecipitation assay (RIPA) cell lysis buffer. After protein quantification, a 5:1 ratio of array buffer to lysis buffer was used. Software was then used to analyse intensity (Quantity One through Bio-Rad).

Western blot analysis

Saos-2 cells were cultured on 24 plates and were stimulated with 100 ng/ml rhNELL-1. Cell lysate was harvested in RIPA buffer. Then 40 μ g total protein was separated on a 12 % sodium dodecyl sulphate (SDS) gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto a nitrocellulose membrane. The membranes were blocked in a blocking buffer for one hour and then incubated with primary antibodies against MAPK proteins for one hour. After washing with phosphate-buffered saline (PBS) containing anti-rabbit ERK, P38 or JNK, secondary antibodies (Pierce Biotechnology, Rockford, IL, USA) were applied to the membranes. The membranes were then exposed to Super-Signal ECL detection reagents (Pierce Biotechnology) and visualised. Protein loading and transfer efficiency were determined using the MemCode Reversible Protein Stain Kit (Pierce Biotechnology).

Intracellular phosphate detection

Saos-2 cells (2×10^6) were seeded at subconfluence level in 96-well culture plates. After 12 hours, the cells were treated with 100 ng/ml rhNELL-1 in osteogenic media. Additional components included 10 μ M U0126 (ERK1/2 pathway inhibitor), 25 μ M SP600125 (InSolution JNK inhibitor II solution, Calbiochem) and 10 μ M SB203580 (P38 inhibitor). At one and three days, cells were washed with PBS and lysed in 100 μ l lysis buffer with 0.2 % NP-40. Intracellular phosphate levels were quantified when exposed to 0.2 % malachite green; 20 μ l cell lysate was incubated with 28 mM ammonium molybdate. After adding 10 μ l 0.2 %/0.76 mM malachite green, absorbance was measured at 610 nm. Experimental conditions were repeated four times.

in triplicate and normalised with the concentration of total cellular proteins.

Quantitative measurement of alizarin red staining

Saos-2 cells (2×10^6 cells/well) were seeded at subconfluence into 24-well culture plates. After 12 days the cells were treated with 100 ng/ml rhNELL-1 in the presence of osteogenic media with additional components including 10 μ M U0126, 25 μ M SP600125 and 10 μ M SB203580. Concentrations of NELL-1 for in vitro studies, including 100, 800 and 2,000 ng/ml, have been previously used in our research laboratory [21, 22]. These have been used in both cell lines and primary cells. We have found consistently that 100 ng/ml is a reliable dosage to have pro-osteogenic effects in most cell types examined, including Saos-2 cells [11, 23]. Media were replenished after three days for two hours and then replaced with osteogenic media. The experiment was run for 12 days. Cells were fixed with 10 % formalin before incubation with 1 % alizarin red. Free dye was cleared with water and cells were dried. Alizarin red stain was leached from cells in 10 % acetic acid, heated to 85 °C for ten minutes and then iced. Supernatant was then quantified at 450 nm. Experimental conditions were performed in quadruplicate and repeated three times. Means and standard deviations were calculated from numerical data. Statistical analysis was performed using an appropriate analysis of variance (ANOVA) test. Statistical analysis between groups was assessed by a post hoc Student's *t* test; $*p \leq 0.05$ was considered to be significant.

Results

NELL-1 activates the JNK pathway in Saos-2 cells

We first sought to determine the overall relative importance of ERK1/2, P38 and JNK pathways in Saos-2 osteosarcoma cells (MAPK Array Kit, R&D Systems) (Fig. 1a). Cells were exposed to rhNELL-1 for 15 and 45 min to examine immediate MAPK signalling activation. JNK pathway activation was apparent after 15 min, including an increase in JNK phosphorylation. In contrast, only a slight increase in ERK1/2 and a slight decrease in P38 pathway activation were noted. After 45 min, all MAPK pathways showed reduced activation in comparison to control. Western blot was used to confirm these findings at both 15 and 45 min post-stimulation (Fig. 1b).

NELL-1 increases intracellular phosphate via JNK pathway activation

Phosphate levels were further examined in the presence of NELL-1 (Fig. 2). Phosphate levels have been shown to have

Fig. 1 Effects of rhNELL-1 on MAPK signalling activation in Saos-2 cells. **a** MAPK signalling activation as assessed by the MAPK Array Kit. Saos-2 cells were treated with or without rhNELL-1 (100 ng/ml) for 15 and 45 min before assessment. **b** Results were subsequently confirmed by Western blot, measuring specific phosphorylated MAPK proteins

a direct role in matrix mineralisation. For example, BMP-2 has been shown to use the JNK pathway to induce its phosphate transport in MC3T3-E1 cells and p38 and ERK in C2C12 cells. Saos-2 cells were tested using rhNELL-1 at a concentration of 100 ng/ml. At day one, rhNELL-1 showed a significant increase in intracellular phosphate levels. In contrast, JNK inhibitor addition reduced rhNELL-1-mediated increase of phosphate levels. At day three, phosphate levels normalised across all treatment groups (Fig. 2).

Fig. 2 Effects of rhNELL-1 and MAPK inhibitors on intracellular phosphate levels in Saos-2 cells. Intracellular phosphate levels were determined after 1 and 3 days stimulation with rhNELL-1 (100 ng/ml) and/or MAPK inhibitors. Specific inhibitors included: 10 μ M U0126 (ERK1/2 pathway

inhibitor), 25 μ M SP600125 (JNK inhibitor) and 10 μ M SB203580 (P38 inhibitor). Intracellular phosphate levels were quantified when exposed to 0.2 % malachite green, a colouring method for determining subnanomolar amounts of phosphate in cells as an easy and reliable way. * $p < 0.05$

Fig. 3 Effects of rhNELL-1 and MAPK inhibitors on microscopic mineralisation in Saos-2 cells. Alizarin red staining was performed to detect matrix mineralisation at 7 (a) and 12 (b) days post-differentiation with

rhNELL-1 (100 ng/ml) and/or MAPK inhibitors. Specific inhibitors included: 10 μ M U0126 (ERK1/2 pathway inhibitor), 25 μ M SP600125 (JNK inhibitor) and 10 μ M SB203580 (P38 inhibitor). *Diff.* differentiation

NELL-1 induces Saos-2 mineralisation via JNK pathway activation

We next investigated the extent to which rhNELL-1 induced mineralisation, or terminal osteoblastic differentiation, in Saos-2 cells by alizarin red staining (Figs. 3 and 4). Mineralisation was assessed after seven and 12 days of differentiation (Fig. 3a and b, respectively). Microscopical images are shown in Fig. 3, while Fig. 4a depicts scanned whole-well images performed in quadruplicate. Results showed that rhNELL-1 drastically increased matrix mineralisation in comparison to standard differentiation medium (Figs. 3 and 4). When added in combination with rhNELL-1, the JNK inhibitor fully reversed the pro-osteogenic effect of rhNELL-1. In stark contrast, no reduction in mineralisation was observed when ERK or P38 inhibitors were added to rhNELL-1 under differentiation conditions. Results were next quantified by leaching of alizarin red staining and measurement of absorbance (Fig. 4b). An approximate

threefold increase in mineralisation was observed with rhNELL-1 addition to osteogenic medium. As already demonstrated by alizarin red staining (Fig. 4a), a complete inhibition of rhNELL-1-induced differentiation was achieved by addition of the JNK inhibitor.

Discussion

Classically, MAPK signalling pathways are divided into ERK1/2, P38 and JNK signalling. In this study we found that NELL-1 induces transient activation of JNK to mediate osteoblastic differentiation of Saos-2 cell towards the terminal mineralisation stage. This finding is significant because of the malignant nature of the target cells. NELL signalling was first appreciated in neural tissues and at one time was thought to be upregulated in brain tumours including neuroblastoma and other embryonic neuroepithelial tumours [24]. However, since that time it has been appreciated that

Fig. 4 Effects of rhNELL-1 and MAPK inhibitors on gross mineralisation in Saos-2 cells. **a** Mineralisation by alizarin red staining in Saos-2 cells at 12-day experiments treated with or without rhNELL-1 and/or MAPK inhibitors. Specific inhibitors included: 10 μ M U0126 (ERK1/2 pathway inhibitor), 25 μ M SP600125 (JNK inhibitor) and 10 μ M SB203580 (P38 inhibitor). **b** Quantitative measurement of alizarin red staining at 450 nm absorbance. *Diff.* differentiation. * $p < 0.05$

NELL-1 has clear tumour suppressor properties [19, 20]. For example, in a genome-wide search of molecules with epigenetic silencing in colon cancer, NELL-1 was found to have frequent methylation along with seven other genes [20]. Likewise, NELL-1 has been found to be epigenetically silenced in oesophageal adenocarcinoma [19]. In the light of these potential tumour suppressor properties, NELL-1 may have as yet undescribed tumour suppressor activities in osteosarcomas and other skeletal malignancies.

BMP-2 has also been reported to mediate some of its pro-osteogenic effects via MAPK signalling, and both P38 and ERK1/2 inhibitors have been found to antagonise BMP-2's pro-osteogenic effects in C2C12 cells [25]. Clear and substantive differences exist between the effects of rhNELL-1 and rhBMP-2. For example, BMP signalling has pleiotropic effects on mesenchymal cell proliferation and differentiation, including positive regulation of osteogenic, chondrogenic and adipogenic differentiation among others [26, 27]. With respect to tumourigenesis, BMP-2 has been identified to positively regulate tumourigenesis in multiple studies and organ systems. For example, genetic variation in BMP signalling has been shown to increase one's risk of colon cancer [28], or decrease one's risk of survival after a diagnosis [29]. In comparison, rhNELL-1 appears to be more 'osteochondral' specific. Thus, NELL-1 may represent a promising alternative as skeletal defect therapeutics in conditions where BMP-2 is contraindicated, prohibited or not sufficient.

Several limitations exist toward the broader extrapolation of the results presented herein. First, Saos-2 cells have been described as having phenotypic instability in long-term culture [30]. Specifically at later passages, Saos-2 cells exhibit increased rates of proliferation. However, NELL-1 signalling has been observed to increase the differentiation of numerous cell types in vitro, including another human osteosarcoma cell line U2OS (data in submission) [3, 4, 17, 18]. Thus, we consider it highly unlikely that the effects of rhNELL-1 via JNK signalling are specific only to Saos-2 cells although its transient activation pattern on Saos-2 might be relatively specific. Second, MAPK inhibitors have generally been reported to be cytotoxic in a cell-type and concentration-dependent manner. We previously reported that MAPK inhibitors could induce certain apoptosis in mouse calvarial cells [18]. However, screening for cytotoxicity via trypan blue staining using the concentrations described in this study did not reveal any significant difference between treatment and control Saos-2 cells (data not shown). Nevertheless, the Saos-2 cells were exposed to MAPK inhibitors for only two hours at media replenishment during long-term mineralisation experiment in this study to reduce the risk of cytotoxicity. Therefore, we infer that the effects of the JNK inhibitor are specific to antagonising rhNELL-1's osteogenic induction effects, rather than more

broadly inhibiting cellular processes in the Saos-2 cell line. Finally, further studies are needed to confirm that JNK activation via NELL-1 is not an observation strictly confined to the in vitro setting but rather a phenomenon observed in vivo as well.

In summary, rhNELL-1 induces terminal osteoblastic differentiation of the human osteosarcoma cell line Saos-2. RhNELL-1-mediated osteogenic differentiation is both accompanied by and requires intact JNK signalling pathway. Further research to discover the precise cell surface binding receptors for NELL-1 will further elucidate the mode of action of the newly appreciated osteoinductive and potentially anti-tumourigenic molecule.

Acknowledgments This work was supported by the NIH/NIDCR (grants R21 DE0177711 and RO1 DE01607), UC Discovery Grant 07-10677, T32 training fellowship to A.W.J. (5T32DE007296-14), Eli & Edythe Broad Center of Regenerative Medicine and Stem Cell Research at UCLA Innovation Award, and the Thomas R. Bales Endowed Chair.

Conflict of interest The authors declare that they have no conflict of interest.

Disclosure Drs. X.Z, K.T, and C.S. are inventors of NELL-1 related patents. Drs. X.Z, K.T, and C.S. are founders of Bone Biologics Inc. which sublicenses NELL-1 patents from the UC Regents.

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