Delivery of Lyophilized Nell-1 in a Rat Spinal Fusion Model

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Nell-1 (Nel-like molecule-1; Nel: protein strongly expressed in neural tissue containing epidermal growth factor-like domain) is a promising osteoblast-specific growth factor for osteoinductive therapies that may circumvent adverse effects, such as nonspecific function and ectopic bone formation, associated with more established osteogenic growth factors such as bone morphogenetic proteins. Beta-tricalcium phosphate (β -TCP), an osteoconductive, biodegradable ceramic biomaterial, has been used successfully to deliver osteoinducers for bone regeneration. The aim of this study was to develop a carrier system for efficiently delivering biologically active Nell-1 protein. After a 40% initial burst release, β -TCP particles retained the majority of adsorbed Nell-1 protein *in vitro*. To test this system *in vivo*, L4/L5 spinal fusion was performed in three groups of rats ($n = 8$ each): (1) 5 μ g Nell-1 in β -TCP/demineralized bone matrix putty (DBX); (2) 2.5 μ g Nell-1 in β -TCP/DBX; (3) β -TCP/DBX only. Fusion was assessed by radiography, palpation, microcomputed tomography, and histological analysis. After 4 weeks, 75% of Nell-1-treated animals exhibited fusion, with a significant increase in new bone volume, whereas only 25% of Nell-free control animals exhibited fusion. Our findings suggest that β -TCP/DBX can increase both the biochemical stability and biological efficiency of Nell-1 protein.

Introduction

EACH YEAR, ~200,000 SPINAL FUSION surgical procedures are performed in the United States,¹ ranking it among the most commonly performed bone grafting procedures. Autogenous bone, the gold standard for bone grafts, is limited in its application due to host bone availability and associated complications such as significant donor-site morbidity, paresthesia, and pain.^{2,3} Failure rates can occur in up to 35% of single-level fusions and are even greater in fusions of multiple levels.⁴ A nonunion may result in inadequate resolution of a patient's clinical symptoms, which can translate into increased probability of additional surgical procedures, with associated morbidity and medical costs.⁵ These problems have prompted surgeons to search for alternatives for stimulating bone formation.

To reduce the need for autogenous bone, various osteoinductive growth-factor-based therapies have been developed in an attempt to improve fusion rates and eliminate possible morbidity risks from bone grafting procedures. Bone morphogenetic proteins (BMPs) have demonstrated extraordinary osteogenic potential *in vivo*,⁶ and BMP-2 has

already been approved for spinal fusion and long bone fractures in humans.^{7,8} Unfortunately, these applications currently require high doses of BMP, and undesirable effects such as heterotopic bone formation, bone resorption, and bone remodeling at the graft site have been reported.^{9,10} BMP-2 is reported to induce osteoblastic gene expression by targeting the transcription factor Runx-related transcription factor 2 (Runx2/Cbfa1), which is essential for osteoblast and hypertrophic chondrocyte differentiation.^{11–13} Runx2 has been actively investigated for potential translational applications because it signals downstream of BMPs and is osteoblast specific.¹³ However, Runx2 is a transcription factor and thus its use is restricted to gene therapy. Further, BMPs can also signal upstream of Runx2, so their effects are not limited to cells of the osteoblast lineage.¹⁴ Deleterious effects such as formation of excessive bone or nonbone cysts have limited the use of BMPs and underscores the need for osteoblast-specific stimulants.^{15,16} Therefore, a more practical approach is to use an osteogenic protein that acts downstream of Runx2.

The Nell family of molecules was first identified as proteins highly expressed in neural tissues and possessing

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epidermal growth factor-like domains.^{17–20} The first identified member of this family, Nell-1, is a novel growth factor postulated to target-specific cells destined for the osteochondral lineage.²¹ The relationship of Nell-1 to osteogenesis was first defined in craniosynostosis patients, where it was specifically upregulated within prematurely fusing sutures.^{18,19,21,22} Bone overgrowth at these sites suggested that Nell-1 was involved in osteoblast differentiation. In transgenic mice overexpressing Nell-1, sutural overgrowth resembled that of human craniosynostosis,²¹ confirming a distinct role of Nell-1 in bone formation. Conversely, mice with *N*-ethyl-*N*-nitrosourea-induced point mutations at the Nell-1 locus exhibited cranial and vertebral skeletal defects.²³ Importantly, we showed that Runx2 directly binds osteoblast-specific binding elements in the Nell-1 promoter.²⁴ This suggests that Nell-1 likely operates downstream of Runx2 on cells already expressing Runx2. Indeed, Nell-1 upregulation accelerates osteogenic differentiation and bone formation in committed osteoblasts.^{19–22,25} Because Nell-1 is a secreted molecule and can act more specifically on further differentiated osteogenic cells, Nell-1 may be a more viable alternative to BMPs. Indeed, our *in vivo* studies demonstrated that 200 ng of either Nell-1 or BMP-2 protein (equivalent to 0.5 pmol Nell-1 and 7.7 pmol BMP-2) induced comparable amounts of rat calvarial bone regeneration.²⁶

Previous studies indicated that Nell-1 has the ability to successfully fuse the posterior lateral rat lumbar spine at an increased rate via adenoviral gene delivery mixed with demineralized bone matrix (DBM) as a carrier.²⁷ Although direct application of adenoviral Nell-1 significantly enhanced spinal fusion rates, its use in humans is limited. In clinical practice, low immunogenicity, effective handling, storage, and efficient release of Nell-1 protein must be achieved to maximize its biological effectiveness.

Beta-tricalcium phosphate (β -TCP) is a biocompatible ceramic material that is bioresorbable and can be replaced by bone tissue *in vivo*.^{28,29} It has been widely used as a platform for osteogenic growth factor delivery and bone regeneration due to its attractive osteoconductive properties.^{30,31} The aims of this study were (1) to assess whether Nell-1 protein can be adsorbed to and released from β -TCP particles *in vitro* and (2) to determine *in vivo* whether bone formation and spinal fusion rate can be improved by direct implantation of Nell-1-loaded β -TCP microparticles incorporated into a DBM putty (DBX) carrier.

Materials and Methods

Preparation of microparticles

β -TCP granules 1.4–2.8 mm in diameter (Synthes Spine) were ground and sieved into particles 200–300 μ m in diameter. Microparticle surface morphology was observed by scanning electron microscopy (SEM; JEOL JSM-6700). Before SEM analysis, the samples were mounted on aluminum stubs and carbon coated.

In vitro protein release

Ten micrograms of recombinant human Nell-1 protein (Aragen Bioscience), produced and purified from Chinese hamster ovary cells, was lyophilized onto 50 mg of β -TCP particles. The protein-adsorbed particles were immersed in

1 mL of 10 mM phosphate-buffered saline (PBS, pH 7.4) at 37°C under gentle shaking. The particles were centrifuged for medium collection and the supernatant was replaced with 1 mL of fresh solution after 0, 1, 3, 5, 7, 10, and 14 days. The released Nell-1 protein in the supernatant was measured using the 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde protein assay (Invitrogen). Measurements were performed in triplicate and the amount of protein released was summed with the amount from previous time points and expressed as cumulative release.

Preparation of implants

PBS (control) or Nell-1 (2.5 or 5 μ g) was lyophilized onto 50 mg of β -TCP particles and stored for up to 1 week at room temperature before implantation. PBS or Nell-1-loaded β -TCP particles were resuspended in PBS and mixed with 0.3 mL of sheep DBX, a DBM-based product prepared by the Musculoskeletal Transplant Foundation (MTF; Edison) immediately before implantation. DBX is composed of DBM particles between 212 and 850 μ m in diameter mixed with sodium hyaluronate to form a moldable putty. A single batch of DBX was used as the carrier for all implants, which provides consistency and minimizes variability compared to using human DBX, which would require multiple batches.

Surgical procedure and study groups

Approval from the UCLA Chancellor's Animal Research Committee was obtained before commencing animal experiments. Athymic rats were used to avoid immunological reaction to DBX. Twenty-four animals, ranging from 12 to 14 weeks of age, were randomly divided into three experimental implant groups: (1) β -TCP/DBX with 5 μ g of Nell-1; (2) β -TCP/DBX with 2.5 μ g of Nell-1; and (3) β -TCP/DBX with PBS only. Bone formation in these groups was compared to our previously established positive control of absorbable collagen sponges impregnated with 10 μ g recombinant human BMP-2.^{32–35} After anesthesia induction by isoflurane inhalation, the surgical site was shaved and prepped. A posterior midline incision was made over the skin of the lumbar spine. Two separate paramedian incisions were made in the lumbar fascia 3 mm from the midline. The transverse processes of the L4 and L5 vertebrae were exposed by separating the back muscles and decorticated with an electric-driven burr (Stryker) until a blush of cancellous bone was observed. Two implants, each 0.3 mL in volume, were placed over the decorticated transverse processes on each side. Fascia and skin incisions were closed with 4-0 Vicryl absorbable sutures. After surgery, animals were housed in separate cages and allowed to eat and drink *ad libitum*. About 0.05 mg/kg buprenorphine was administered twice daily for 2 days postoperatively for analgesia, and 48 mg/mL trimethoprim/sulfamethoxazole antibiotic was administered for 10 days postoperatively. All animals were sacrificed at 4 weeks.

Radiographic assessment

Posteroanterior radiographs were performed at 2 and 4 weeks after implantation using a Faxitron LX60 cabinet radiography system at a resolution of 10 lp/mm. Observers, blinded to the treatment variables, scored the spines as fused

FIG. 1. Scanning electron micrographs of the surface of beta-tricalcium phosphate (β -TCP) granules (A, 50 $\times$; B, 1500 $\times$). The particles are 200–300 μ m in diameter and porous, providing a large surface area favorable for protein adsorption.

or not fused. Fusion was determined by the presence of a continuous bridge of bony trabeculation between the L4 and L5 transverse processes on either side.

Manual palpation

Spines were dissected and harvested after euthanasia. Three blinded observers scored spines either fused or not fused by applying flexion and extension forces to the intervertebral space. Only specimens with no mobility against the applied forces at the L4/L5 region were judged as a successful fusion. Any motion detected at either side between the transverse processes was considered a failure of fusion. At least two observers needed to have scored a spine fused in order for it to be deemed fused.

Three-dimensional microcomputed tomography evaluation

After manual palpation, spines were fixed in 10% buffered formalin (Fisher Scientific) for a minimum of 48 h. After fixation, three-dimensional microcomputed tomography (μ CT) scanning in the axial plane was performed using a μ CT 40 scanner (Scanco Medical) at 20 μ m resolution. μ CT 40 software was used to measure the bone volume between the level of the distal metaphysis of L4 and the proximal metaphysis of L5 in each group. Spines were considered fused if continuous bone bridges between the transverse processes on serial 0.4-mm-thick coronal sections were observed.

Histological analysis

After μ CT scanning, spines were decalcified using Cal-Ex solution (Fisher) and embedded in paraffin, and coronal sections encompassing the implants were collected. Sections were stained with hematoxylin and eosin and Masson trichrome stains. For immunohistochemistry, primary antibody to osteocalcin (Santa Cruz Biotechnology) was used at 1:100, and biotin-conjugated anti-IgG secondary antibody was used at 1:200, as previously described.²⁶ For observation, the ABC streptavidin-peroxidase kit (Vector Laboratories) and AEC chromogenic substrate (Dako) were used to produce a red-brown positive signal. Nuclei were counterstained blue/purple with hematoxylin.

Statistics

The two-tailed, equal-variance Student's *t*-test was used to compare bone volume measurements pairwise between the

control group and each Nell-1 group. $p \leq 0.05$ was considered statistically significant.

Results

In vitro protein release

SEM observation of the prepared β -TCP particles (Fig. 1) showed that the particles were 200–300 μ m and exhibited micropores, providing a large surface area for protein adsorption. To study the potential of β -TCP particles as an osteogenic protein carrier, the release profile of Nell-1 from the particles was investigated by incubating lyophilized rhNell-1/ β -TCP particles in PBS. A burst release of 40% of the Nell-1 from the β -TCP particles was observed in the first 24 h. The cumulative amount of Nell-1 released reached a maximum of $\sim 45\%$ over 2 weeks (Fig. 2).

Radiography and manual palpation

Fusion rates were compared to our previously published results using BMP-2 as a positive control (Table 1).^{32–35} Radiographic analysis (Fig. 3) revealed that the new bone mass between the L4 and L5 transverse processes in the

FIG. 2. *In vitro* release of Nell-1 from porous β -TCP particles in phosphate-buffered saline (PBS). Gradual release of Nell-1 from the particles was observed. The majority of adsorbed Nell-1 remained associated with the particles ($n = 3$, mean $\pm$ standard deviation).

TABLE 1. SPINAL FUSION EVALUATION OF BETA-TRICALCIUM PHOSPHATE/DEMINERALIZED BONE MATRIX PUTTY AND NELL-1 FORMULATIONS BY RADIOGRAPHY, MANUAL PALPATION, AND THREE-DIMENSIONAL MICROCOMPUTED TOMOGRAPHY

Experimental group	Radiography	Manual palpation	3D μ CT
PBS only in β -TCP/DBX	4/8 (50%)	2/8 (25%)	2/8 (25%)
2.5 μ g Nell-1 in β -TCP/DBX	7/8 (88%)	6/8 (75%)	6/8 (75%)
5.0 μ g Nell-1 in β -TCP/DBX	8/8 (100%)	7/8 (88%)	7/8 (88%)
10 μ g rhBMP-2 in ACS	10/10 (100%)	10/10 (100%)	10/10 (100%)

Number of spines deemed fused/total spines operated and percentage deemed fused by evaluation method shown. Fusion data for ACS impregnated with recombinant human BMP-2 are adapted from Miyazaki *et al.*³⁴ and used as a positive control.

ACS, absorbable collagen sponge; rhBMP-2, recombinant human bone morphogenetic protein 2; DBX, demineralized bone matrix putty; PBS, phosphate-buffered saline; β -TCP, beta-tricalcium phosphate; 3D μ CT, three-dimensional microcomputed tomography.

Nell-1 (5.0 μ g) group was the most radiopaque, and a continuous bony bridge formed between the transverse processes. In the Nell-1 (2.5 μ g) group, the spines also exhibited bony fusion, though bone mass radiopacity was reduced. Radiolucent clefts, indicating pseudoarthroses, were present between the L4 and L5 transverse processes in all animals of the Nell-free group (Fig. 3B).

We employed multiple techniques to assess spinal fusion because we have found that DBX may be radiopaque due to incomplete demineralization (Fig. 3A), and residual DBX can remain unresorbed at the implantation site for up to 8 weeks, increasing radiopacity and precluding accurate assessment by radiography alone.^{36–38} As a result, we predicted that radiographic fusion may be an overestimation of actual fusion. To support our radiographic analysis, spines were dissected after sacrifice and palpated by three independent observers (Table 1). Both Nell-1-treated groups achieved increased fusion rates by manual palpation compared to the Nell-free group, corroborating our radiographic findings.

Three-dimensional μ CT

μ CT results were consistent with radiographic and manual palpation findings for all spinal fusion samples (Table 1). Three-dimensional μ CT evaluation of complete reconstructions and coronal sectional cuts (Fig. 4) revealed that new bone formation in all groups was limited to the L4 and L5

axial levels without encroachment onto other axial levels. Nell-free spines exhibited minimal new bone formation with a lack of bone bridging between the transverse processes. In contrast, an increased amount of new bone filled the inter-transverse process region in the Nell-treated spines, and masses were evenly distributed on the dorsal surfaces of the processes. μ CT-based quantitation of bone volume between the L4 and L5 transverse processes revealed that both doses of Nell-1 treatment significantly increased bone volume compared to no treatment (Fig. 5).

Histological analysis

To characterize bone formation and fusion at the cellular level, we prepared coronal sections of spines for hematoxylin and eosin histological analysis (Fig. 6). DBX typically appears as bone particles with cell-free lacunae. In contrast, osteocytes are present in the lacunae of newly formed bone matrix, and osteoblasts surround the border of new bone matrix.^{39,40} In the Nell-free group, there is a lack of bone bridging between the transverse processes, indicating a failure of fusion. At higher magnification, DBX particles exhibited distinct boundaries and were surrounded by muscle and fibrous tissue, with only small amounts of new bone between the transverse processes, indicating a low level of bone remodeling. In contrast, in the Nell-1-treated groups, transverse process bone was continuous with DBX,

FIG. 3. Representative radiographic images of lumbar spines immediately (A) and at 4 weeks (B–D) after surgery. Radiolucent clefts (arrows) were present in the bone formed between the transverse processes in the Nell-free control group (B), indicating a pseudoarthrosis. Continuous bony bridging between the L4/L5 transverse processes was evident in the Nell-treated groups (C, D).

FIG. 4. Microcomputed tomography images at 4 weeks after surgery. (A–C) Complete three-dimensional reconstructions, and (D–F) coronal sectional cuts. The Nell-free group (A, D) exhibited a smaller bone mass without bone bridging. In the Nell-1-treated groups (B, C, E, F), spines were fused, with new bone limited to the dorsal surface of the L4/L5 transverse processes. Color images available online at www.liebertonline.com/ten.

FIG. 5. Microcomputed tomography quantification of bone volume between the L4 and L5 transverse processes for each experimental group at 4 weeks after surgery. Bone volume in each Nell-1-treated group was significantly greater than that in the Nell-free control group. No significant difference in bone volume was observed between the two Nell-1-treated groups. Each marker represents an individual data point ($n=16$, left and right implant for each animal). Larger markers and bars indicate average $\pm$ standard deviation. $*p \leq 0.001$.

forming a bony bridge with no clear boundaries visible between DBX and the native bone. Higher magnification revealed that the DBX particles resembled remodeling bone, with bone marrow cavities forming around the particles, irregular boundaries resulting from osteoclastic activity, and hypertrophic chondrocytes suggestive of a process resembling endochondral ossification. Masson's Trichrome staining confirmed that Nell-1-treated spines contained more new bone matrix and bone remodeling than Nell-free spines (Fig. 7). In addition, immunohistochemical staining for osteocalcin, a known marker of mature osteoblasts and bone matrix deposition,⁴¹ revealed increased expression in the new matrix of Nell-1-treated groups compared to the Nell-free group (Fig. 8).

Discussion

Nell-1 expression is downstream of, and directly regulated by, the master osteogenic transcription factor Runx2.²⁴ Functionally, Nell-1 acts as a critical downstream mediator of Runx2 in regulating osteoblast and bone formation.⁴² Our previous work further suggests that Nell-1 acts more specifically than BMPs on cells further differentiated along the osteogenic lineage.⁴³ We have shown that Nell-1 overexpression selectively increases osteoblast differentiation (increased osteopontin and osteocalcin expression and increased alkaline phosphatase activity) and can increase premature suture fusion.²⁶ We explored the translational

FIG. 6. Hematoxylin and eosin staining of coronal sections of the L4-L5 intertransverse region at 4 weeks after surgery. Demineralized bone matrix putty (DBX) particles (X) appear as bone matrix with empty lacunae. In the Nell-free control group (A–C), adjacent transverse processes (TP) are not contiguous with bone matrix. Higher magnification reveals DBX particles with sharp boundaries bridged by fibrous tissue, indicating little bone remodeling. In the Nell-1-treated groups (D–F, G–I), TP are bridged by bone. Eroded boundaries of DBX particles and formation of bone-marrow-cavity-like spaces (white arrows) suggest active bone remodeling. Hypertrophic chondrocytes (black arrows) represent bone formation by endochondral ossification. Magnification: left column, 8.4 $\times$; middle, 40 $\times$; right, 200 $\times$. Boxes indicate region magnified in successive images. Scale bars: left, 1 mm; middle, 250 μ m; right, 125 μ m. Color images available online at www.liebertonline.com/ten.

research aspect by showing that adenoviral delivery of Nell-1 successfully provided a continuous source of the protein for spinal fusion²⁷; however, clinical application is limited because of virus safety and immunogenicity concerns. In addition, although very small quantities of Nell-1 are required for osteoinduction, high doses of protein are necessary for therapeutic applications, as free proteins in solution are susceptible to a variety of conditions such as peptide bond hydrolysis, aggregation, conformational changes, and diffusion away from the therapeutic site. Indeed, Nell-1 protein has a half-life of only 30 h as predicted by software.⁴⁴ Therefore, a more practical delivery of Nell-1 protein in terms of handling and storage is required for effective clinical application.

DBX is a commercially available mixture of DBM and sodium hyaluronate (as a DBX carrier) used for bone grafting.³⁷ In previous studies, Nell-1 protein was mixed with DBX at the time of implantation, and 50% of spines treated with 10 μ g free Nell-1 exhibited fusion after 4 weeks (unpublished data). However, using free unbound Nell-1 protein requires low temperature storage, resulting in poor shelf life.^{45,46} In addition, the *in vivo* solubility of unmodified hyaluronan carrier is <24 h,⁴⁷ suggesting that Nell-1 mixed with DBX may diffuse prematurely after hyaluronan dissolution and degradation, effectively resulting in a burst release. Modification of hyaluronan can change physical characteristics,⁴⁸ but the need for chemical reactions under physiological conditions is undesirable.

Collagen is a major component of bone tissue and is also used for bone grafts,⁴⁹ but we have found that Nell-1 protein

undergoes a rapid burst release *in vivo* when compared to other growth factors such as BMP-2 (unpublished data). This is presumably due to electrostatic repulsion between Nell-1 and collagen, both of which are negatively charged at physiological pH.^{17,50}

Delivery of proteins by encapsulating or adsorbing them onto release systems is widely used to prevent polypeptide chain unfolding, which can lead to chemical inactivation or aggregation.⁴⁴ Calcium phosphates are widely used as delivery carriers for DNA and polypeptides due to their high affinity for macromolecules.⁵¹ To achieve localized release of Nell-1, we chose β -TCP, which is often clinically used as a bone substitute material due to its biocompatibility and osteoconductivity.^{52,53} β -TCP has been widely used as a carrier for local delivery of BMP-2.⁵⁴ In addition, the relatively slower *in vivo* resorption rate of β -TCP ($\sim$ 4–6 weeks) compared to hyaluronan or collagen results in protein delivery for an extended period with an enhanced biological effect.⁵⁵ Indeed, we found no histological evidence of β -TCP after 4 weeks, suggesting that the carrier has been largely biodegraded by this time.

In this study, we have loaded Nell-1 protein onto β -TCP particles. Varying particle size did not significantly alter initial release kinetics (data not shown), and a particle size of 200–300 μ m was chosen for subsequent experiments because it provided the highest yield after grinding and sieving of the initial β -TCP granules. A maximum of 40% of initially loaded rhNell-1 protein was released from the granules after day 1 *in vitro*, followed by gradual release over the next 14 days.

FIG. 7. Masson's Trichrome staining in coronal sections of the L4-L5 intertransverse region at 4 weeks after surgery. Mineralized tissue appears red, while connective tissue appears blue. The Nell-free group (A) contained mostly unremodeled DBX particles (mineralized bone with empty lacunae) bridged by fibrous connective tissue and little new bone matrix. In the Nell-1 (2.5 µg) group (B), DBX particles exhibited eroded borders, suggesting bone remodeling, and connective tissue containing hypertrophic chondrocytes (white arrow) is consistent with dense cartilaginous matrix deposition before mineralization during endochondral ossification. In the Nell-1 (5 µg) group (C), the columnar appearance of proliferating chondrocytes and abundant cartilaginous matrix resembles endochondral ossification in long bones. Mineralizing trabecular bone containing nascent bone marrow cavities (black arrows) is visible, suggesting bone maturation. Magnification: 200 $\times$. Scale bar: 125 µm.

The remaining protein stayed associated with the β -TCP granules, suggesting that β -TCP may provide a good reservoir for the protein in a biological setting, where local pH changes result in degradation of β -TCP particles.^{53,55} Our lyophilized Nell-1/ β -TCP particle formulations are highly relevant from a manufacturing and clinical product perspective because it can significantly increase the shelf life of Nell-1 and permit storage of the particles at room temperature. We have found that Nell-1 stored in the lyophilized state for over 3 months retained biological activity (unpublished data), but further studies are required to systemati-

cally and quantitatively compare the biological activity of lyophilized Nell-1/ β -TCP stored for periods much longer than 3 months.

To determine whether Nell-1 delivered from β -TCP particles can induce bone formation, we mixed Nell-1-loaded β -TCP granules with DBX, which resulted in an easily molded and handled dough-like composite. Combined with Nell-1 protein, we achieved significantly higher rates of bone formation in an *in vivo* spinal fusion model. Performance of the implant was assessed by posterolateral intertransverse process spinal fusion in rats, a well established *in vivo* assay of

FIG. 8. Osteocalcin immunohistochemistry in coronal sections of the L4-L5 intertransverse region at 4 weeks after surgery. Positive signal associated with new bone matrix appears as reddish-brown staining (arrows) at the boundaries of, and in areas between DBX particles (X, boundaries marked with dashed lines). (A, D) In the Nell-free control group, minimal osteocalcin expression was present in the fibrous tissue between DBX particles. (B, C, E, F) In the Nell-1-treated groups, osteocalcin expression was present at the edges of remodeling DBX particles and in the mineralizing new bone matrix bridging them. Magnification: (A–C), 200 $\times$; (D–F), 400 $\times$. Boxes indicate region magnified in successive images. Scale bars: 125 µm.

bone growth. Radiographic, manual palpation, μ CT, and histology results correlated well with each other and together support the conclusion that Nell-1 induced accelerated spinal fusion with active bone formation and remodeling. Over 75% of the animals implanted with carrier containing either 2.5 or 5.0 mg Nell-1 had solid spinal fusion at 4 weeks postsurgery. In contrast, 75% of the spines of animals implanted with the Nell-free carrier failed to fuse (Table 1). We implanted Nell-1/ β -TCP particles without washing weakly bound Nell-1 because our previous studies have shown that Nell-1 burst-released during the first 24 h had beneficial biological effects. Five, 10, and 50 μ g of free Nell-1 in the same DBX carrier, putatively released as a single burst, was able to induce spinal fusion with success rates of 28%, 54%, and 87%, respectively (unpublished data). Thus, the burst of unbound Nell-1 in our previous experiment had no detrimental effects and even had beneficial effects. In the present study, we were able to achieve the same rate of radiographic fusion (87% with 50 μ g free Nell-1) using 10-fold less Nell-1 protein (5 μ g) by associating the Nell-1 protein with β -TCP particles. To further increase delivery efficiency of Nell-1, additional studies are needed to develop delivery carriers providing a more sustained release and reducing initial burst. This can be obtained by surface modification of the β -TCP particles with acid treatments or apatite coatings, as reported in our previous study.⁵⁶

In conclusion, we demonstrated that the novel growth factor Nell-1 significantly improved spinal fusion rate in rats when lyophilized onto β -TCP granules and mixed with DBX. This implant material simultaneously provides a platform for Nell-1 protein delivery and acts as an osteoconductive and osteoinductive bone graft material. Future studies will evaluate the bone-forming ability of this formulation in other animal models more amenable to biomechanical testing such as rabbits, sheep, and nonhuman primates.⁵⁷ We envision this material as a protein delivery platform that can be used clinically for spinal fusions and other bone-building applications as well. Our ultimate goal is to establish Nell-1 as an osteospecific growth factor that significantly improves spinal fusion in humans without compromising safety and patient quality of life.

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Disclosure Statement

Bone Biologics, Inc., licensed Nell-1-related patents from UCLA. C.S., K.T., B.M.W., and X.Z. are founders of Bone Biologics, Inc., and inventors of the related patents.

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