Distal tibial fracture repair in a neurofibromatosis type 1-deficient mouse treated with recombinant bone morphogenetic protein and a bisphosphonate

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Congenital pseudarthrosis of the tibia is an uncommon manifestation of neurofibromatosis type 1 (NF1), but one that remains difficult to treat due to anabolic deficiency and catabolic excess. Bone grafting and more recently recombinant human bone morphogenetic proteins (rhBMPs) have been identified as pro-anabolic stimuli with the potential to improve the outcome after surgery. As an additional pharmaceutical intervention, we describe the combined use of rhBMP-2 and the bisphosphonate zoledronic acid in a mouse model of NF1-deficient fracture repair.

Fractures were generated in the distal tibiae of neurofibromatosis type 1-deficient (*Nf1*$^{+/-}$) mice and control mice. Fractures were open and featured periosteal stripping. All mice received 10 µg rhBMP-2 delivered in a carboxymethylcellulose carrier around the fracture as an anabolic stimulus. Bisphosphonate-treated mice also received five doses of 0.02 mg/kg zoledronic acid given by intraperitoneal injection.

When only rhBMP but no zoledronic acid was used to promote repair, 75% of fractures in *Nf1*$^{+/-}$ mice remained ununited at three weeks compared with 7% of controls ($p < 0.001$). Systemic post-operative administration of zoledronic acid halved the rate of ununited fractures to 37.5% ($p < 0.07$).

These data support the concept that preventing bone loss in combination with anabolic stimulation may improve the outcome following surgical treatment for children with congenital pseudarthoris of the tibia and NF1.

Congenital pseudarthrosis of the tibia (CPT) is difficult to treat[1] and many children who have the condition also have neurofibromatosis type 1 (NF1),[2] with associated osteoblast and osteoclast dysfunction.[3,4] These cellular deficiencies may cause the focal bone defects and systemic osteopenia associated with NF1, although other cell types and interactions with homozygous null cells may also contribute to the pathophysiology of CPT.[5]

Critical for the successful surgical management of children with CPT is adequate fixation, which may involve external (e.g. Ilizarov frame[6-8]) or internal fixation (e.g. intramedullary nailing[9,10]) alone or in combination.[11] In terms of the timing of surgery, bracing is increasingly used, particularly before three years of age, in order to delay surgery.[12] A combination of bracing and a cortical bypass allograft has recently been reported with a good outcome in ten patients at a mean follow-up of 6.5 years.[13] Cancellous bone graft and more recently cortical bone graft[13,14] have been used to promote healing. Bone morphogenetic proteins (BMPs) may also be used to promote new bone formation in large diaphyseal defects,[15,16] and can be used as replacement or in conjunction with bone grafting. In the context of the treatment of children with NF1 and CPT, isolated cases and small clinical case series treated with recombinant human BMPs (rhBMP-7(OP-1)[17-19] and rhBMP-2[20]) have yielded encouraging outcomes in some cases. Nevertheless, a study assessing ectopic bone formation induced by BMP in the NF1-deficient mouse indicated that the setting of NF1 is less amenable to BMP-based interventions.[21] This study also indicated that better outcomes with rhBMP-2 could be obtained by suppressing catabolism using a bisphosphonate (zoledronic acid), systemically. This work is also consistent with treatment of nonunion in a rat model with rhBMP-7 and the bisphosphonate pamidronate.[22]

We have previously described a mouse model of NF1-deficient fracture healing and shown significant differences in open distal tibial fracture repair in *Nf1*$^{+/-}$ mice.[23] In this

Fig. 1

Intra-operative radiograph showing a pinned tibial fracture.

model a closed fracture was generated by three-point bending that was subsequently opened and subjected to local tissue trauma and periosteal stripping. For the current study we modified this model to apply 10 µg rhBMP-2 locally to the fracture site at the time of surgery as an anabolic stimulus. However, rhBMP-2 is also known to stimulate osteoclasts,[24] and this may be further exacerbated in an NF1 setting. Consequently, additional experimental groups also received 0.1 mg/kg zoledronic acid systemically. The primary outcome measure was radiological union, with tissue histology as a secondary outcome measure. This study aimed to support the concept of dual anabolic/anti-catabolic therapy for NF1/CPT.

Materials and Methods

NF1 knockout mice that are uniformly deficient for one NF1 allele were sourced from L. Parada (UT Southwestern, Dallas, Texas).[25] The mice were maintained on a C57BL/6J background and housed with food and water supplied *ad libitum*. Genotyping was performed using a polymerase chain reaction-based method. All experiments were approved by the Sydney West Area Health Service Animal Ethics Committee. Mice were assigned to treatment groups prior to surgery.

The methodology was based upon our previous study that showed a difference in distal tibial fracture healing between wild type ($Nf1^{+/+}$) and NF1-deficient mice ($Nf1^{+/-}$).[23] Surgical procedures were performed in a sterile fashion by a single operator. Anaesthesia was induced with ketamine (35 mg/kg) and xylazine (4.5 mg/kg) via intraperitoneal injection and maintained using inhaled isofluorane. A transverse fracture was manually created in the distal tibia using three-point bending tweezers adapted from surgical staple removers. It was stabilised using a 0.3 mm stainless steel pin with a second pin inserted at an angle to minimise loss of fixation at the ankle (Fig. 1). The fracture was then opened with a scalpel and the periosteum stripped 2 mm each side using a rasp. Opening the operative site prior to fracture increased the variability and incidence of comminution with three-point bending. A 10 µg dose of rhBMP-2 (Medtronic Australasia, North Ryde, Australia) was delivered in a hydrated carboxymethylcellulose carrier that was packed around the fracture site. The wound was closed using 5-0 nylon sutures (Ethicon Inc., Somerville, New Jersey). Pain was managed using buprenorphine (0.05 mg/kg to 0.1 mg/kg subcutaneously post-operatively, then every 12 hours as required). Dehydration was managed by saline injection as required. Mice receiving bisphosphonate were given five doses of 0.02 mg/kg zoledronic acid twice weekly by intraperitoneal injection as previously described.[21]

Fractures were assessed radiologically using a digital x-ray machine (Faxitron X-ray Corp., Wheeling, Illinois). Mice with comminuted or angular fractures were culled and excluded. Mice were monitored by weekly radiographs and any loss of fixation or evidence of infection resulted in the mouse being culled and excluded. Mice were killed three weeks post-operatively for analysis.

A total of 76 mice were operated on with 20 exclusions (26%); 12 were culled at the time of operation or died during the procedure and eight were excluded post-operatively. Of the 56 mice included in the study, 28 (50%) were of the $Nf1^{+/+}$ genotype and 28 (50%) of the $Nf1^{+/-}$ genotype.

Radiological and histological outcomes. Fracture grading was determined from the radiographs at three weeks with tibiae scored as completely, partly, or not bridged,[23] similar to the Johnston criteria used for clinical assessment.[9]

Fractured tibiae and surrounding soft-tissue were removed and fixed overnight in 10% formalin and stored in 70% alcohol at 4°C. Bones were scanned by micro-computed tomography (microCT) using a SkyScan 1174 compact microCT scanner (SkyScan, Kontich, Belgium) at 17 µm pixel resolution with 0.5 mm aluminium filter, 50 kV radiological tube voltage and 800 µA tube electric current. Maximum intensity projection models of three-dimensional (3D) representative fracture callus were generated using the CT Analyser Program (version 1.10.05; SkyScan).

Following microCT analysis, six or seven representative samples per group were fixed in 4% paraformaldehyde and stored in 70% ethanol prior to processing for decalcified (paraffin) histology. Sections were stained with picrosirius red and Alcian blue for bone and cartilage. Analysis for bone volume/total callus volume, percentage of non-bony callus and percentage of cartilage content were calculated using BIOQUANT Nova Prime histomorphometry analysis software (Bioquant, Nashville, Texas). In order to calculate the bone volume and total volume for each callus, the total area and bone area within the calluses were measured. The areas of cartilage tissue and fibrous tissue were similarly measured and compared to the total callus volume (cartilage tissue/total volume, fibrous tissue/total volume).

Table I. Radiological union in a mouse model of tibial fracture treated with recombinant human bone morphogenetic protein (rhBMP-2) with and without treatment with zoledronic acid (ZA)

Group	Genotype*	Treatment	Bridged (n, %)	Partially bridged (n, %)	Not bridged (n, %)
1 (n = 15)	$Nf1^{+/+}$	rhBMP-2 only	10 (*66.7*)	4 (*26.7*)	1 (*6.7*)
2 (n = 12)	$Nf1^{+/-}$	rhBMP-2 only	1 (*8.3*)	2 (*16.7*)	9 (*75.0*)
3 (n = 13)	$Nf1^{+/+}$	rhBMP-2 + ZA	5 (*38.2*)	5 (*38.5*)	3 (*23.1*)
4 (n = 16)	$Nf1^{+/-}$	rhBMP-2 + ZA	7 (*43.8*)	3 (*18.8*)	6 (*37.5*)

* *Nf1*(+/+), control group; *Nf1*(+/-), NF1-deficient

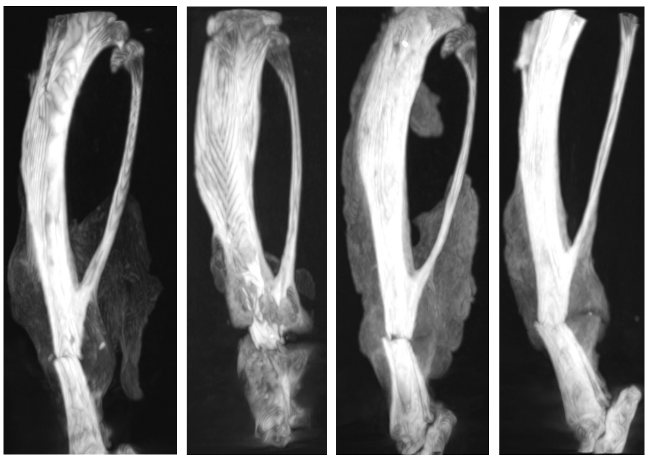

Fig. 2a Fig. 2b Fig. 2c Fig. 2d

Maximum intensity projection microCT images showing the tibiae of wild type (a and c) and NF1-deficient mice (b and d), treated with bone morphogenetic protein (BMP-2) alone (a and b) or with BMP-2 and zoledronic acid (c and d). As can be seen in these representative images, bone healing was superior in wild type mice compared with NF1-deficient mice, and treatment with zoledronic acid led to a more dense fracture callus.

Statistical analysis. Kruskal-Wallis and Mann-Whitney U tests were performed using SPSS version 17 (SPSS Inc., Chicago, Illinois). Union rates between those fractures completely/partly bridged *versus* not bridged were statistically tested by Fisher's exact test as described previously, with a value ≤ 0.05 being considered statistically significant.[23]

Results

Radiological union. Only one of 15 (7%) fractures in control mice ($Nf1^{+/+}$) remained not bridged after three weeks. In contrast, the $Nf1^{+/-}$ mice showed an inferior response to rhBMP-2, with nine of 12 (75%) not bridged at three weeks (Table I). The difference in bone healing between $Nf1^{+/+}$ and $Nf1^{+/-}$ mice with rhBMP-2 only was extremely significant ($p < 0.001$).

When administered with anti-catabolic treatment, $Nf1^{+/-}$ mice had an improved outcome with the proportion of not bridged fractures being halved (six of 16, 37.5%; p = 0.06). Although the density of the callus was greater in both control mice and $Nf1^{+/-}$ mice when zoledronic acid treatment was administered, control mice showed a small but not significant decrease in rate of union (p = 0.24).

The differences in callus size with zoledronic acid treatment were reflected on 3D images reconstructed using micro-CT software (Fig. 2). Without zoledronic acid treatment the bone surface of $Nf1^{+/-}$ mouse fractures also appeared highly mottled (Fig. 2b). Treatment with zoledronic acid produced a larger callus with a smooth surface (Fig. 2d).

Fracture histology. Two representative specimens from each group were selected for descriptive histology (Fig. 3). Samples were decalcified, embedded in paraffin, sectioned, and stained for bone and cartilage. In the control $Nf1^{+/+}$ mice treated with rhBMP-2 alone, bone was seen bridging the fracture site but also extending into the proximal tibia. Little cartilage was present, indicating a completion of endochondral ossification (Fig. 3a). In contrast, $Nf1^{+/-}$ fractures treated with rhBMP-2 alone had much cartilage and fibrous tissue in the fracture gap; little bone remained (Fig. 3b). The persistence of cartilage was a feature of all $NF1^{+/-}$ fractures, with cartilage or cartilage-remnants present in all fractures treated with zoledronic acid including those that had and had not bridged (Fig. 3d). The treatment with zoledronic acid led to a larger, denser callus in both $Nf1^{+/+}$ and $Nf1^{+/-}$ specimens (Figs 3b and 3d). This indicates that even in fractures without an NF1-deficiency, much of the rhBMP-2 induced bone can be resorbed within three weeks.

Fracture calluses were analysed by quantitative histomorphometry for bone, cartilage, and fibrous tissue at the fracture site (Table II). Treatment with zoledronic acid led to a mean increase of 33% in bone volume/total volume for $Nf1^{+/+}$ mice ($p < 0.01$) and a mean increase of 16% in $Nf1^{+/-}$ mice (p = 0.09). This was associated with mean increases in trabecular number of 54% ($p < 0.01$) and 30% ($p < 0.01$) for $Nf1^{+/+}$ and $Nf1^{+/-}$ mice, respectively. Cartilage quantities were highly variable and while an overall mean increase of 85% was seen in cartilage/total volume between $Nf1^{+/+}$ and $Nf1^{+/-}$ mice with rhBMP-2 alone, this was not statistically significant (p = 0.21). A major difference was seen in the amount of fibrous tissue within the callus, with an overall mean increase of 171% ($p < 0.02$) seen in fibrous tissue/total volume between $Nf1^{+/+}$ and $Nf1^{+/-}$ mice with rhBMP-2 alone. For some $Nf1^{+/-}$ samples this tissue included tissue that more resembled persistent inflammation than fibrosis, which was not observed in the $Nf1^{+/+}$ group. Treatment with zoledronic acid led to a reduction in $Nf1^{+/-}$ fibrosis. $Nf1^{+/+}$ and $Nf1^{+/-}$ groups treated with zoledronic acid were not significantly different for fibrous tissues/total volume (p = 0.41).

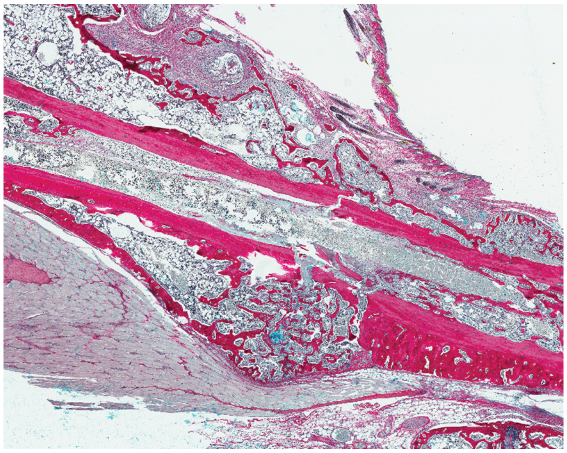

Fig. 3a

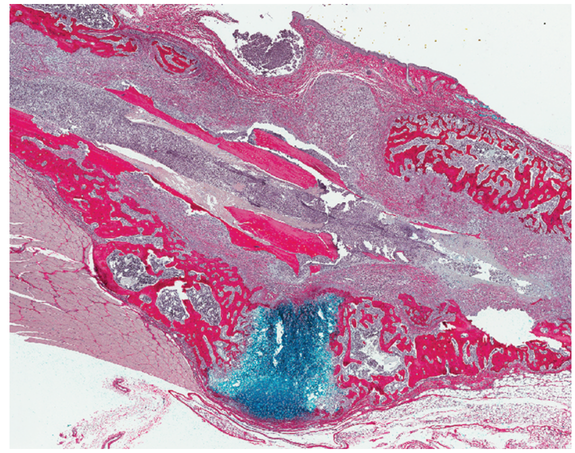

Fig. 3b

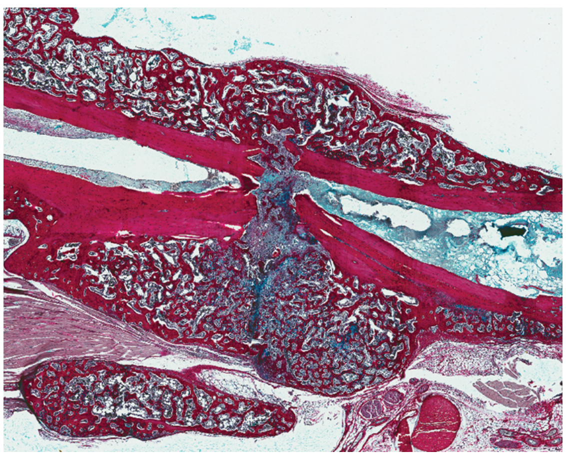

Fig. 3c

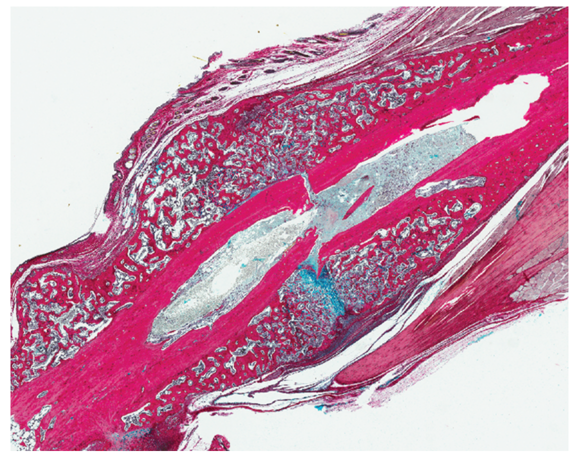

Fig. 3d

Histological assessment of the fracture was performed for wild type (a and c) and NF1-deficient mice (b and d), treated with bone morphogenetic protein (BMP-2) alone (a and b) or with BMP-2 and zoledronic acid (c and d) (picrosirius Red/Alcian Blue, × 200 magnification; Aperio Scanscope Software). Increased callus can be seen in samples treated with zoledronic acid. Cartilage was retained in NF1-deficient mice at three weeks, more so in samples not treated with zoledronic acid.

Discussion

The underlying molecular pathology of CPT in patients with NF1 is poorly understood. The characteristic anterolateral bowing can be associated with a tapering of the tibial diaphysis or with cystic or dysplastic lesions, which may or may not be due to the localised loss of the second NF1 allele.[26,27] Nevertheless, tibial dysplasia and the deformity are progressive, and fracture is usually inevitable. No consensus has been established on the optimal method for treating CPT; conservative treatment with plaster and/or bracing does not usually achieve union, but has been advocated to avoid operations in very young children.[12] However, a recently published case series reported that intramedullary nailing with transfixation of the ankle and cortical bone grafting was successful in 12 of 13 children under three years of age.[14,28] In addition, there is no universally accepted method of fixation with both Ilizarov ring fixators and several designs of intramedullary nail being described.[6-11] Most agree that all tissue should be resected from the pseudarthrosis at operation and a strong anabolic stimulus applied to promote healing.

It seems logical to use BMPs to boost the potential for bone healing in these patients. Nevertheless, a combination of rhBMP-7, bone grafting, and intramedullary nailing and external fixation has been reported surprisingly as unsuccessful with resorption of the rhBMP-7 bone graft composite and only one of five cases healing within one year.[19] Better results were more recently reported using a standardised rhBMP-2 regimen with healing in five of seven cases.[20] However, a recent case report has highlighted a potential risk of increased transformation of tissue into a neurofibroscarcoma with rhBMP treatment, and this will need to be monitored as the use of BMPs increases.[29]

Another concern is that BMPs are reported to stimulate osteoclastic resorption[24,30] and NF1-deficient osteoclast progenitors are particularly sensitised to pro-osteoclastic

Table II. Histomorphometry parameters (mean, range) in the fracture callus at three weeks

Group	Genotype and treatment*	BV/TV†	Cgv/TV‡	Ftv/TV§
1	*Nf1*$^{+/+}$ rhBMP-2	0.29 (0.02 to 0.35)	0.05 (0.00 to 0.16)	0.09 (0.00 to 0.22)
2	*Nf1*$^{+/-}$ rhBMP-2	0.33 (0.27 to 0.39)	0.09 (0.02 to 0.14)	0.23 (0.13 to 0.46)¶
3	*Nf1*$^{+/+}$ rhBMP-2 + ZA	0.38 (0.32 to 0.46)	0.05 (0.00 to 0.15)	0.08 (0.00 to 0.17)
4	*Nf1*$^{+/-}$ rhBMP-2 + ZA	0.38 (0.29 to 0.43)	0.07 (0.01 to 0.17)	0.12 (0.02 to 0.27)

* *Nf1*(+/+), control group; *Nf1*(+/-), NF1-deficient; rhMBP, recombinant human bone morphogenetic protein; ZA, zoledronic acid

† BV/TV, bone volume/total callus volume

‡ Cgv/TV, cartilage volume/total callus volume

§ Ftv/TV, fibrous tissue volume/total callus volume

¶ includes cellular inflammatory tissue

stimuli.[4] NF1-deficient osteoblasts are also reported to secrete an excess of paracrine factors such as osteopontin that can increase osteoclast recruitment.[31] In a previous study we reported that rhBMP-2 induced osteoclasts were upregulated in an NF1-deficient mouse model.[21] Taken together, we hypothesised that intervention with an anti-catabolic agent may improve bone healing in a mouse model of NF1-deficient fracture repair.

We have again shown poor distal tibial fracture healing in the *Nf1*$^{+/-}$ mouse, where only 8.3% of fractures treated with rhBMP-2 alone completely bridged within three weeks. While mice show more rapid and vigorous healing than humans, with most control mice bridging a tibial fracture in three weeks, the capacity to generate large numbers of consistent fractures at the same anatomical site make it advantageous to obtain a satisfactory model for such a rare and heterogeneous condition as CPT. In addition to fibrosis, we observed a persistence of cartilage in the fracture gap of *Nf1*$^{+/-}$ mice with rhBMP-2 treatment. Although this has been previously observed in untreated fractures,[23] it appears that this phenomenon is exacerbated by the addition of rhBMP-2. It is unclear whether this is due to the increased formation or impaired resorption of cartilage and a further detailed analysis would be required to investigate this finding.

A dramatic improvement in NF1-deficient bone repair was seen with systemic treatment with zoledronic acid, where the number of fractures which were not bridged at three weeks was halved and the callus was larger and more dense. Bisphosphonate treatment has been safely and successfully used in children with osteogenesis imperfecta for many years.[32] There are some reports of bisphosphonate inhibiting the repair of fractures, but only in patients on long-term treatment that may result in reduced bone turnover affecting both anabolic and catabolic responses.[33,34] In our series, treatment with zoledronic acid started three days after surgery, following the initiation of bone repair. For clinical use, a similar delay in starting treatment might be advantageous.

Thus, this study comprehensively illustrates that in a mouse model of NF1-deficient bone repair, improved outcomes can be obtained using a combined pro-anabolic and anti-catabolic approach. This work complements our recent case series where seven children with CPT were successfully treated with rhBMP and bisphosphonates (pamidronate and zoledronic acid).[35]

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