The Regulation of Bone Formation by the Met-5-enkephalin-Opioid Growth Factor Receptor Signaling Axis

Nikhil Thakur, MD, Sean D. DeBoyace, BS, Bryan S. Margulies, PhD.
SUNY Upstate Medical University, Syracuse, NY, USA.

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Introduction: Our understanding of the signaling milieu that regulates the spatial and temporal balance between bone absorption by osteoblasts versus bone re-absorption by osteoclasts remains incomplete. Previous work demonstrated that the opioid receptor antagonist naloxone increased osteogenesis.(1, 2) However, very little is known about opioid receptors in bone. We endeavored to determine the role that opioid receptors and the enkephaline ligands play in regulating osteogenesis. Only the canonical δ-opioid receptor and the non-canonical opioid growth factor receptor (OGFr) were identified. Both receptors bind the opioid growth factor met-5 enkephalin (Met5). Based on our earlier work, we hypothesized that the addition of an OGFr antagonist would promote osteogenesis and accelerate bone healing in a unicortical defect mouse model. Here we show that opioid-receptor antagonists promote osteogenesis and accelerate bone healing in a unicortical defect model.

Methods: Bone marrow was collected from consenting adult patients (n=5) undergoing elective total joint arthroplasty as a part of an IRB approved study. The adherent fraction of bone marrow cells (mesenchymal stem cells; MSC) was derived from bone marrow aspirates. MSC were assayed for patterns of gene expression and examined for their capacity to differentiate into osteoblasts or adipocytes. The non-adherent fraction of bone marrow cells (monocytes) was cultured with 100-ng/ mL M-CSF; with 1x106 monocytes subsequently cultured with 25-ng/ mL of M-CSF and 25-ng/ mL RANK-ligand to promote osteoclast formation. Human MSC, osteoblasts, adipocytes and osteoclasts were assayed for gene expression: μ-opioid receptor, κ-opioid receptor, δ-opioid receptor, the OGFr, pro-enkephaline (PENK), proopiomelanocortin (POMC), carboxypeptidase A1 (CPA1), prohormone convertase-1 and -2 (PSK1 and PSK2). Patterns of gene expression were confirmed using Western blots, immuno-cytochemistry and immuno-histochemistry. Mouse brain lysates were used as positive expression controls (mB). MSC were then induced to become osteoblasts and treated with 1-pM, 1-nM, 1-μM or 1-mM of naloxone or naltrexone. In parallel, cultures were also treated with 5- or 50-μM doses of Met5 alone and with naloxone. In order to test the therapeutic potential of OGFr inhibition, a unicortical defect was surgically administered to the proximal tibias of 3-week old mice (n=5 per treatment group of 1-mM of naloxone or Met 5). Tibia were scanned with a μCT (μCT 40, Scanco), sectioned and stained for the Met5-ligand or the OGFr and with tomato lectin for osteoclasts. The SUNY IACUC approved all animal studies. Prism statistical software (Graphpad) was used to analyze data with one-way ANOVA using the Holm-Sidak post-hoc correction for multiple comparisons with p<0.05.

Results: The μ-opioid receptor, the κ-opioid receptor, the Met5 precursor POMC and the CPA1 enzyme gene expression were never observed. In contrast, the Met5 precursor PENK was expressed in osteoblasts, adipocytes and osteoclasts. (Figure 1A) This expression was confirmed in three human MSC cultures and corresponding osteoblast (OB) cultures protein lysates using a Western blot (mouse brain lysates, mB, served as positive expression controls). (Figure 1A) Likewise, increased PSK1 and PSK2 gene
expression were demonstrated in osteoblasts and adipocytes. The δ-opioid receptor gene expression was significantly decreased 3-fold in osteoblasts cultures versus MSC (p<0.0138). OGFr gene expression was observed to increase in osteoblasts (*, p<0.031), adipocytes (Not Significant, p<0.244) and osteoclasts (*, p<0.034) (Figure 1B) Met5 staining (arrow) was rare in MSC cultures. (Figure 1C) OGFr staining was abundant in MSC cultures. (Figure 1D) Met5 staining was observed adjacent to the growth plate, which included a sub-set of cells that stained with tomato lectin and we identified as osteoclasts. (Figure 2A) Intense OGFr staining was observed adjacent to the growth plate that included tomato lectin-positive staining cells. (Figure 2B) In addition, we also identified OGFr staining within the reserve and hypertrophic zones of the growth plate. (Figure 2B) Met5 staining was rarely observed within the bone marrow alongside abundant tomato lectin staining in myeloid cells. (Figure 2C) In addition, Met5 staining was observed in the osteoblasts of the endosteal surface. OGFr staining was abundant throughout the bone marrow and in the osteoblasts of the endosteal surface. (Figure 2D) The addition of 1-mM and 1-μM naloxone substantially increased mineral accumulation in MSC cultures induced to become adipocytes. (Figure 3A) Met5 had no effect on mineral accumulation; however, Met5 was able to reduce the effects of naloxone. Unicortical defects were surgically administered to mouse tibias that were then treated with naloxone (1-mM) or Met5 (50-μM). Naloxone increased bone healing while Met5 had no effects. (Figure 3B) Naloxone treatment increased bone mass (Bv/Tv) 1.53-fold (*, p<0.001). (Figure 3C) The elevated bone mass that we measured was driven by a 1.2-fold increase in trabecular number (TbN) (*, p<0.047). (Figure 3D)

**Discussion:** Growth factors administered during orthopedic procedures carry the risk of stimulating tumor growth or of triggering malignant transformation. While Met5-ligand and the OGFr have been observed in bone, very little is known about the functional significance of the opioid-receptor signaling axis in bone.(3, 4) MSC and osteoblasts were observed to express both the Met5-ligand precursor PENK and the OGFr. The OGFr staining that we observed in the chondrocytes of the growth plate is a novel finding. The expression of the Met5 precursor PENK, in parallel with the lack of the other potential Met5 precursor POMC, suggests that PENK is the only source of Met5 in bone. The OGFr staining in hypertrophic chondrocytes and in osteoblasts of the endosteal surface suggests that the Met5-OGFr signaling axis regulates differentiation in MSC. The ability of naloxone to promote mineral accumulation in MSC cultures suggests that the Met5-ligand functions to maintain a non-differentiated state in MSC through the OGFr. The administration of naloxone to unicortical defects promoted bone healing. These finding suggests that naloxone may be a promising candidate for stimulating bone formation in the context of orthopedic interventions that require accelerated bone healing. Nevertheless, further work will be necessary to fully elucidate the mechanism through which OGFr promotes osteogenesis and bone healing.

**Significance:** Opioid antagonists offer a promising alternative to growth factor adjuvant therapies that are currently employed to stimulate bone formation during orthopedic surgical interventions.
Figure 1

A. Gene Expression (Gene X Nj / GAPDH Nj)

B. Gene Expression (Gene X Nj / GAPDH Nj)

C. DAPI Met5

D. DAPI OGFr
Figure 3

A. 
- Induced Naloxone
- 1 mM
- 1 μM
- 1 nM
- 50 μM Met5

B. 
- Control
- Naloxone
- Met5

C. 
- Bone Volume/Total Volume (BvTv)

D. 
- Trabecular Number (TbN)

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