Beta-Aminopropionitrile Treatment Effects on MC3T3-E1 Osteoblast Gene Expression and Type I Collagen Production

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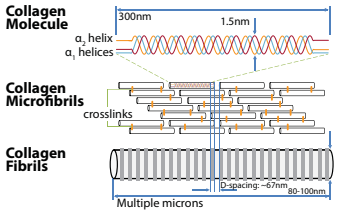
INTRODUCTION

Type I Collagen

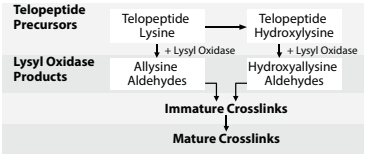
- Cells secrete three alpha helices which form a helical procollagen molecule
- Terminal telopeptide ends of molecules are cleaved by proteinases
- Inline self-assembly of molecules forms microfibrils
- Microfibrils arrange in a quarter-staggered array into fibrils with repeating gap and overap regions and are stabilized by crosslinks
- Periodicity of the gap and overlap region is referred to as the D-spacing and exists as a distribution of values



- D-spacing provides information on the state and internal structure of tropocollagen as well as post-translational modifications
- Changes in the D-spacing distribution are reflective of disease, tissue type, and drug treatment

Collagen crosslinking

- Crosslinks stabilize collagen molecules within the fibrillar structure and the staggered array
- Enzymatic crosslink formation initiated in telopeptides by lysyl oxidase (LOX) enzyme reaction



Osteolathyrism

- Disease characterized by crosslink deficiency resulting in mechanical defects to bone and connective tissues
- Caused by high dietary consumption of osteolathyrogenic compounds such as beta-aminopropionitrile (BAPN)
- BAPN irreversibly binds to the active site of the LOX enzyme, preventing it from acting on telopeptide precursors

Study motivation

- Induce collagen synthesis by osteoblasts and study collagen in its native state
- Research the effects of BAPN on nanoscale collagen morphology and expression of genes relating to collagen synthesis

HYPOTHESIS

BAPN will alter the morphology of type I collagen produced *in vitro* by osteoblasts and drive upregulation of lysyl oxidase to compensate for the reduction in crosslink formation

MATERIALS AND METHODS

Cell culture

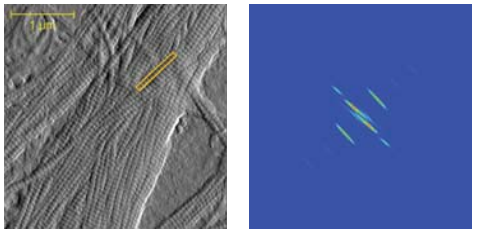
- Murine preosteoblasts (MC3T3-E1) cultured in proliferation medium:
  - α-MEM medium
  - 10% fetal bovine serum
  - 0.05% penicillin/streptomycin
  - 1% L-glutamine
- Cells were differentiated in proliferation medium supplemented with 50 µg/mL ascorbic acid
- Experimental cultures were supplemented with 0.25 mM BAPN-fumarate
- 500,000 cells were seeded into 60 mm dishes (density: 177 cells/mm^2)

Collagen synthesis

- MC3T3-E1 cells were cultured in a T75 flask and allowed to proliferate until reaching 80% confluence

Atomic force microscopy

- Cells seeded into 8 dishes, 4 dishes per group (control or BAPN, n=4 each), and differentiated for 2 weeks
- Media was removed and cells were treated with 10 mM EDTA to promote detachment from the extracellular matrix
- Matrix was rinsed with water and air-dried
- 5 locations per dish were imaged in air by atomic force microscopy
- 3.5 µm x 3.5 µm images from each location
- 2D Fast Fourier Transform (2D-FFT) performed on 10 collagen fibrils per location for D-spacing analysis
- Minimum of 50 fibrils per dish and 200 fibrils per group



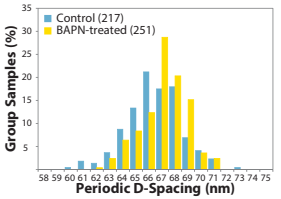
Left: AFM error image of collagen with one fibril outlined for 2D-FFT.
Right: 2D-FFT corresponding to the outlined fibril.

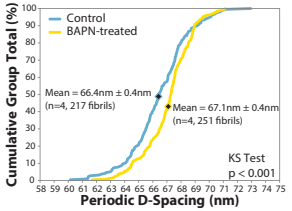
Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

- Cells seeded into 10 dishes, 5 dishes per group (control or BAPN, n=5 each), and differentiated for 1 week
- SYBR Green primers and master mix used in determining mRNA expression
- qRT-PCR performed using an ABI 7500 Fast machine
- Sample/gene combinations run in triplicate with beta-actin as reference gene
- Expression fold change found using Comparative CT method

RESULTS AND DISCUSSION

Atomic force microscopy





- Mean D-spacing
  - Control: 66.4 nm ± 0.4 nm
  - BAPN: 67.1 nm ± 0.4 nm

Non-parametric U-test revealed p=0.060

- D-spacing distribution range
  - Control: 60.2 nm - 72.9 nm
  - BAPN: 61.7 nm - 71.1 nm

Kolmogorov-Smirnov test revealed p<0.001

Quantitative reverse transcription polymerase chain reaction

Target Gene		Fold Change	p-value
Type I Collagen α_1	COL1A1	1.10	0.2208
Type I Collagen α_2	COL1A2	-1.02	0.8133
Lysyl Oxidase	LOX	1.30	0.3610
	COL1A1:COL1A2	-1.13	0.3410

- No significant effect of BAPN treatment on mRNA expression of any of the target genes in BAPN samples relative to controls

Discussion

- Fewer crosslinks are initialized and formed due to BAPN binding to LOX active site
- BAPN caused an increase in the D-spacing distribution of collagen produced *in vitro*
  - Crosslinks may compress fibrils driving lower D-spacing in normal collagen
  - Fewer crosslinks in the BAPN-treated group could account for the increase in D-spacing
- BAPN treatment of mice *in vivo* causes a decrease in D-spacing distribution in bone (Hammond and Wallace, 2015)
- Post-hoc size analysis of marginally significant mean D-spacing (n=4, p=0.060) indicated a sample size n=6 would be required to detect a statistical significance between groups at 80% power
- Lack of LOX gene upregulation shows no compensatory response to LOX inhibition
- BAPN causes no significant change in expression of genes encoding either α protein helix

CONCLUSION

BAPN alters the nanoscale morphology of type I collagen produced *in vitro* by osteoblasts by causing an upward shift in the D-spacing distribution, but induces no significant change in gene expression relating to collagen synthesis or LOX regulation