

Quantitative Expression of Bone Morphogenetic Protein and Bone Matrix Protein in Human Carotid Plaque

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Abstract

Objectives
Bone morphogenetic proteins (BMPs) regulate differentiation of progenitor cells towards bone and lipid containing adipocytes. Demonstration of ectopic bone formation and lipid accumulation in atherosclerotic plaques has implicated BMPs and other bone matrix proteins in the pathogenesis of atherosclerosis. We investigated the presence of those mineralization-regulating proteins in atherosclerotic plaques and plasma.

Methods
Archival carotid endarterectomy samples were analyzed by quantitative RT-PCR for expression of BMP-2, BMP-4, BMP-6 and matrix proteins osteoprotegerin and osteopontin. Samples of the plaque were also submitted for routine H&E staining and plaque characterization. Chi-square analysis was performed to assess the presence or absence of each transcript relative to a plaque characteristic. ELISA for BMP-4 was performed on atherosclerotic patients and volunteers without a history of atherosclerosis.

Summary of Findings
High concentration of BMP-4 protein ($p < 0.002$) was found in patients undergoing cardiac catheterization for suspected coronary disease (497.9 ± 107.8 ng/ml) compared to volunteer controls (35.5 ± 28.78 ng/ml). Age correlated strongly $r = 0.6$ plasma BMP-4 levels. Of 8 carotid plaques tested, three specimens had detectable BMP-6, two specimens had detectable osteoprotegerin, one specimen had detectable BMP-4 mRNA and one specimen had detectable osteopontin. There was no relation between plaque characteristics including detectable calcification and the presence of any of the transcripts. All three BMP-6 positive specimens (100%) were obtained from male patients.

Clinical Application of Findings

1. Mineralization regulating proteins are present in atherosclerotic plaque and represent a target for slowing the disease.
2. A plasma assay of bone morphogenetic protein may be a useful biomarker for assessing risk of atherosclerosis.



Introduction

Atherosclerotic arterial disease is the number one cause of death in the United States. Many of the recognized risk factors for atherosclerosis have a common pathway, termed oxidative stress, that results in endothelial dysfunction. This leads to a histopathologic process associated with fatty streaks, lipid accumulation, calcification and inflammation. Increasing research interest has focused on arterial calcification in the setting of atherosclerosis. Understanding the molecular and genetic determinants of plaque calcification can provide the basis for the development of novel therapeutic approaches to favorably detect and alter plaque phenotype and structure, thereby altering the risk of clinical events.

Noninvasive calcium scores in coronary arteries, carotid arteries and aortas are independent predictors of cardiovascular morbidity. Vascular cells are induced to become osteogenic by inflammatory and atherogenic stimuli. Proteins controlling bone mineralization are related to a family of growth factors named bone morphogenetic proteins (BMP's) that are involved in the regulation of vascular calcification. Several BMP members and non collagenous bone matrix proteins are capable of converting mesenchymal stromal precursor cells into osteoblasts or adipocytes. Expression of BMP's and bone matrix proteins have been reported in atherosclerotic plaque. BMP-2, BMP-4 and BMP-6 and osteopontin are notable osteogenic and adipogenic factors.

The specific goals for this study are 1) to measure BMP-4 in plasma from patients undergoing coronary or peripheral angiograms; 2) to investigate the presence of BMPs and bone matrix proteins in archival carotid plaques.

Materials and Methods

Clinical Specimens

A total of 56 archival carotid artery sections collected from patients that had endarterectomy surgery at Ochsner Clinic in 2000/2001 were analyzed. Age range of the patients was 43-91 years, average 71 years. Fifty eight percent (58%) male and 42% female. Those specimens were utilized for a previous study on the presence of Chlamydia pneumoniae and other infections and were stored frozen.

RNA Extraction

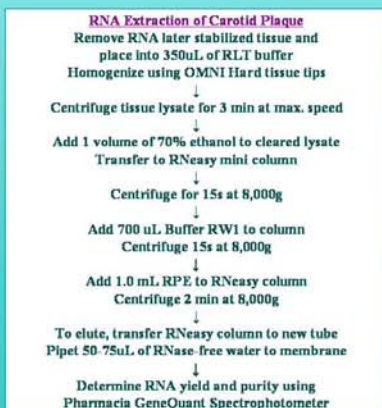
Approximately 2ug of purified RNA were reverse-transcribed for one hour at 42°C using random hexamers, RNase inhibitor and MuLV RT according to standard procedures

PCR analysis.
Synthesized cDNA was utilized for gene expression of BMPs-1,-2, -3,-4,-6, -7, -8,-9,-10,-11,-12 and matrix proteins osteopontin, osteonectin and osteoprotegerin. Primers for PDH containing intron were used as in-house keeping gene. All PCRs were hot-started; positive (cDNA from human cell line osteogenic sarcoma:SaOS-2) and negative controls were included in each run.

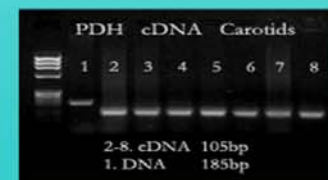
Quantitative PCR analysis
Real time quantitative PCR was used for quantitation of positive amplified BMPs. The amount of target cDNA in a sample was interpolated from a standard curve run simultaneously with the unknown samples. Three µl of cDNA was added to 12 µl Dynamo™ SYBR® qPCR mix containing primers at a concentration of 0.35µM and run for 40 cycles on a DNA Engine Opticon™ real-time fluorescence detection instrument. Standard curves generated from the SaOS-2 standard were examined for linearity using the coefficient of determination R². Optimal R² value is the closest to 1.

ELISA
DuoSet™ ELISA Development System for human BMP-4 kit from R&D Systems was used for measurement of BMP4 in plasma according to manufacturers instructions.

Statistical analysis.
Comparison of mean BMP-4 plasma levels was assessed using t-test ($p < 0.01$). Chi-square analysis was performed to assess the presence or absence of each transcript relative to a plaque characteristic.

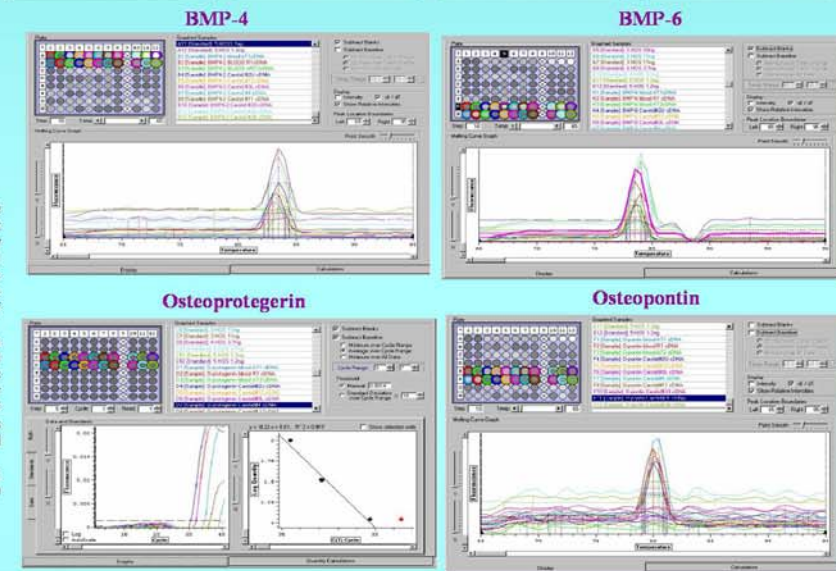


| Primer | Forward and Reverse 5'-3' | Target |
|----------------------|---|-------------------------|
| BMP 4 | F-GCA GCA TCC CTG AGA ACG AG R-CCG CAG GGC TCA CAT CAA AAG | 229 bp |
| BMP6 | F-ACA CGG AAG CAC AGT TGG AGG R-GGG TAG GAA GAG CTT CAC GG | 215bp |
| Osteo- protegerin | F-AAA AAT GGC GAC CAA GAC AC R-GCC TCA AGT GCC TGA GAA AC | 205bp |
| Osteo- pontin | F-TGA AAC GAG TCA GCT GGA TG R-TGA AAT TCA TGG CTG TGG AA | 162bp |
| PDH | F-CTT CCA CAG CCC TCG ACT AA R-GGT ATG GAT GAG GAC CTG GA | 185bp DNA 105bp cDNA |



| ELISA BMP-4 | | | | | |
|--------------------------|--------------------|-------------|------------------------------|-------------------|---------------|
| Atherosclerotic Patients | | | Non-Atherosclerotic Patients | | |
| Age/Sex | Clinical | BMP (pg/mL) | Age/Sex | Clinical | BMP-4 (pg/mL) |
| 80 yr male | >50% CAD | 881 | 23 yr female | Dialysis patient | 0 (ND) |
| 65 yr male | DM, PAD, RAS | 845 | 67 yr male | <50% CAD | 3 |
| 75 yr male | >50% CAD, LVH | 659 | 35 yr male | healthy volunteer | 120 |
| 66 yr female | >50% CAD | 645 | 41 yr male | healthy volunteer | 22 |
| 69 yr male | >50% CAD, PAD, HTN | 601 | 35 yr female | healthy volunteer | 0 (ND) |
| 75 yr male | >50% CAD, HTN | 472 | 30 yr female | healthy volunteer | 0 (ND) |
| 66 yr male | >50% CAD, PAD, HTN | 375 | | | |

CAD=Coronary Artery Disease, PAD=Peripheral Artery Disease, RAS=Renal Artery Stenosis, LVH=Left Ventricular Hypertrophy, HTN=Hypertension



RESULTS

- Heavily calcified carotid plaques yielded degraded RNA
- BMP-4 is elevated in atherosclerotic patients
- BMP-4, BMP-6, Bone related proteins are present from carotid plaque
- Qualitative presence of BMP-3, BMP-5, BMP-7, BMP-8 transcripts were detected in most plaques

| Presence of BMPs According to Carotid Atherosclerotic Plaque Phenotype | | | | | | | | | | |
|--|-----|-----|-------|-------|------|-------|-------|--------|--------|--------|
| # Pt | AGE | SEX | Ulcer | Calc. | Fib. | Chol. | BMP 4 | BMP 6 | OPN | OP |
| 2 | 71 | M | 0 | 0 | 2 | 1 | <10fg | 2.4 ng | <1.0pg | <1.0ng |
| 3 | 74 | M | 1 | 2 | 3 | 1 | <10fg | <1.0pg | <1.0pg | <1.0ng |
| 4 | 73 | F | 2 | 3 | 2 | 2 | <10fg | <1.0pg | 1ng | <1.0ng |
| 11 | 83 | F | n/d | 0 | 2 | 2 | <10fg | <1.0pg | <1.0pg | <1.0ng |
| 12 | 72 | M | 0 | 0 | 1 | 0 | 14fg | 5.0 ng | 3.7 ng | 236 ng |
| 30 | 74 | M | n/d | 0 | 0 | 0 | <10fg | 1.0 ng | <1.0pg | <1.0ng |
| 33 | 68 | F | 0 | 0 | 2 | 1 | <10fg | <1.0pg | <1.0pg | <1.0ng |
| 36 | 89 | F | n/d | 0 | 0 | 0 | <10fg | <1.0pg | <1.0pg | <1.0ng |

Fib=Fibrosis, Chol=Cholesterol, OPN=Osteoprotegerin, OP=Osteopontin Ng=ngligible

CONCLUSIONS

1. Mineralization regulating proteins are present in atherosclerotic plaque and represent a target for slowing the disease.
2. A plasma assay of bone morphogenetic protein may be a useful biomarker for assessing risk of atherosclerosis