BMP-7 TREATMENT DIFFERENTIATES INFILTRATED MONOCYTES INTO M2 MACROPHAGES MEDIATED THROUGH PI3K AND MAP KINASE PATHWAYS IN APOE KO MICE

Dinender Singla, Heidi Shoulders and Kaley Garner

ABSTRACT

Aim: Atherosclerosis involves the infiltration of inflammatory cells monocytes; however, the fate of infiltrated monocytes to become deleterious M1 or beneficial M2 macrophages at acute stage of the atherosclerosis (ATH) is not fully understood. Moreover, whether bone morphogenetic protein (BMP)-7 has any influence on infiltrated monocytes in acute atherosclerosis needs investigation.

Methods: ApoE KO mice (10±2 weeks old) using the partial left carotid artery ligation (PLCA) model which induces low blood flow stress. Blood velocity function was examined with echocardiography, and carotid arteries were collected for histological, biochemical and further analysis in acute stages of atherosclerosis at D5.

Results: A decreased trend in the plaque formation in PLCA+BMP-7 group compared with PLCA was observed. A significant increase ($p<0.05$) in infiltrated monocytes, and M1 macrophages whereas a decrease in M2 macrophages and arginase 1 was observed in PLCA group compared to control. Furthermore, BMP-7 treatment decreased inflammatory monocytes, M1 macrophages but an increase in anti-inflammatory M2 macrophages, and arginase 1. Moreover, our data shows significant ($p<0.05$) increase in cell signaling proteins (pP38 and pJNK pathway) and a decrease in pro-survival proteins (PI3K and pERK) in PLCA group and these protein expression was reversed with BMP-7 treatment. A significant ($p<0.05$) decrease in blood velocity in the PLCA group was improved with BMP-7 treatment.

Conclusion: Our data in acute stages of atherosclerosis suggest a significant increase in infiltrated monocytes which BMP-7 differentiates into M2 macrophages, and this process is mediated through the PI3K and MAP Kinase pathways.

MATERIALS AND METHODS

Experimental Design - In Vivo Study

- Apolipoprotein E (ApoE) $-/-$ mice (11±1 weeks of age, Male and Female)
- Study Groups:

SHAM	PLCA	PLCA+BMP7
Surgical procedure, but left carotid artery is not ligated	Surgical procedure with Partial Left Carotid Artery (PLCA) ligation	Surgical procedure with artery ligation + 3 Serial Injections of BMP-7 (200 µg/kg of body weight, i.v.)

- Sacrificed at Day 5 (Acute) and Day 28 (Mid-stage)
- Analysis – Western Blot, Systolic Blood Flow Analysis, IHC, Western Blot, and ELISA

RESULTS

Plaque Area

Figure 1: BMP-7 Significantly Reduces Plaque Accumulation by D28 in ApoE KO Mice. Hematoxylin and Eosin staining of arterial sections show regions of plaque development in the left carotid artery. **A** shows representative arteries from SHAM, PLCA, and PLCA + BMP7 groups, there was no significant difference between groups at D5. In contrast, by D28 PLCA animals had significantly more plaque accumulation compared to SHAM and PLCA + BMP7 animals. Bar graph (**B**) shows quantitative analysis done using Image J software. Blue circle indicates region of plaque formation. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 6$ animals for both D5 and D28.

Blood Flow Velocity

Figure 2: BMP-7 Improves Blood Flow Velocity Through the Carotid Artery at D5 and D28. At D5 and D28, PLCA animal's systolic velocity was significantly lower compared to SHAM animals. PLCA + BMP7 animals showed significant improvement in systolic velocity compared to PLCA animals at D5 and D28. PLCA + BMP7 animals did show a significant decrease in systolic velocity compared to SHAM animals at D28. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 8-9$ (D5) and $n = 9$ (D28).

Monocytes

Figure 3: Monocyte Recruitment to the Area of Injury. Photomicrographs of immunohistochemistry performed using antibody against the characteristic monocyte marker CD14 at D5 (left) and D28 (right). CD14⁺ cells shown in red (a, e, i), DAPI stained nuclei in blue (b, f, j), merged images (c, g, k), and selected enlarged areas of merged images (d, h, l). Bar graph depicts quantitative analysis indicating that animals treated with BMP-7 showed significantly less initial recruitment of monocytes to the area of inflammation (D5) but that significance was lost by D28 compared to PLCA animals. At both D5 and D28 PLCA animals had significantly more monocytes present compared to SHAM animals. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 6-7$ for both D5 and D28. Scale bar = 100µm.

M1 Macrophages

Figure 4: Reduced Monocyte Differentiation into the Inflammatory M1 Macrophage. Monocytes differentiated into the inflammatory M1 phenotype were identified using antibody against iNOS. iNOS⁺ cells are shown in green (a, e, i), DAPI stained nuclei in blue (b, f, j), merged images (c, g, k), and selected enlarged areas (d, h, l) for both D5 (left) and D28 (right). Quantitative analysis indicates that animals treated with BMP-7 showed a significant reduction of M1 macrophages present at both D5 and D28 compared to PLCA animals (bar graph). PLCA animals had significantly more iNOS⁺ cells vs. SHAM at D5 and D28. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 6$ (D5) and $n = 6-7$ (D28). Scale bar = 100µm.

RESULTS

M2 Macrophages

Figure 5: BMP-7 Directs Monocyte Differentiation Toward the Anti-Inflammatory Phenotype. CD206 and Arginase-1 are characteristic markers of M2 macrophages. At D5, there was not a significant increase in CD206⁺ macrophages of PLCA + BMP7 animals compared to PLCA animals, although the PLCA group was significantly lower than SHAM animals. By D28, there were significantly more M2 macrophages in PLCA + BMP7 animals compared to PLCA animals. PLCA animals also had significantly less M2 macrophages compared to SHAM animals at D28. **A** shows representative images show CD206 positive cells in red (a, e, i), DAPI stained nuclei in blue (b, f, j), merged images (c, g, k) and selected enlarged areas of merged images (d, h, l) (D5 and D28). Bar graph **B** shows results of quantitative analysis using Image J software. **C** shows representative photos of Arginase-1⁺ cells in green (a, e, i), DAPI stained nuclei in blue (b, f, j), merged images (c, g, k), and enlarged selected areas of merged images (d, h, l) (D5 and D28). Quantitative analysis in bar graph (**D**) shows at D5 there is no significant difference between groups. By D28 there is a significant increase of Arginase-1⁺ macrophages in the PLCA + BMP7 group compared to the PLCA. PLCA group was significantly less than the SHAM animals as well (D28). *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 6-7$ animals (D5 and D28). Scale bar = 100µm.

Pro-Inflammatory Cytokines

Figure 6: BMP-7 Reduces Inflammatory M1 Cytokines at D5 and D28. ELISA of blood serum collected immediately post-sacrifice of cytokines IL-6 (**A**), TNF-α (**B**), and MCP-1 (**C**). Results show that animals treated with BMP-7 had significantly less inflammatory cytokines present at both D5 and D28 compared to PLCA animals. PLCA animals also had significantly more IL-6 (D28 only) and MCP-1 (D5 and D28) compared to SHAM animals. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 5-7$ (D5 and D28).

Anti-Inflammatory Cytokines

Figure 7: BMP-7 Increases Levels of Anti-Inflammatory Cytokines. ELISA of IL-1RA (**A**) and IL-10 (**B**). At D5, PLCA animals had significantly less IL-1RA compared to SHAM. PLCA + BMP7 animals had significantly more IL-1RA vs. PLCA. By D28, treatment animals had significantly less IL-1RA compared to SHAM and there was no significant difference between PLCA + BMP7 and PLCA animals. ELISA also showed that at D5, the PLCA group had significantly less circulating IL-10 compared to SHAM while D28 showed no significant difference. IL-10 was significantly higher in PLCA + BMP7 animals vs. PLCA at D5 and D28. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 5$ (D5), $n = 4-5$ (D28).

p-p38, p-JNK, p-ERK

Figure 8: BMP-7 Decreases Activation of p38 Pathway. Western blot analysis of carotid artery tissue demonstrates a significant reduction in the activated form of p38 protein, p-p38, in BMP-7 treated animals compared to PLCA at both D5 and D28. PLCA + BMP-7 was also significantly lower compared to SHAM animals at D5. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 4$ (D5) and $n = 4-5$ (D28).

Figure 9: BMP-7 Decreases Activation of JNK. Animals who received BMP-7 showed significantly less activation of inflammatory kinase JNK vs. PLCA animals at both D5 and D28. PLCA animals showed significantly more JNK activation compared to SHAM (D5 and D28). *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 3-4$ (D5) and $n = 4-5$ (D28).

p-ERK

Figure 10: BMP-7 Increases Activation of ERK at D5 and D28. Results of Western blot analysis of carotid artery tissue show a significant increase in ERK activation at both D5 and D28 compared to PLCA animals. PLCA animals also had significantly less p-ERK vs. SHAM (D5 and D28). *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, $n = 4$ (D5) and $n = 4-5$ (D28).

SUMMARY AND CONCLUSION

H and E Stain	At D5, plaque area was not significantly different between groups; however, at D28, mice receiving arterial ligation demonstrated a significant increase in plaque formation vs. sham. Following BMP-7 treatment plaque area was significantly reduced vs. PLCA.
Echocardiography	At D5 and D28, BMP-7 demonstrated a significant improvement in systolic blood flow through the left carotid artery vs. PLCA.
Immunohistochemistry (IHC)	PLCA significantly increased monocyte recruitment compared with sham animals. Treatment with BMP-7 significantly decreased these levels at D5; however, by D28 these levels were not significantly different than PLCA although a decreasing trend was observed. At both D5 and D28, BMP-7 significantly decreased pro-inflammatory M1 macrophages per artery. BMP-7 improved levels of anti-inflammatory macrophages at D5 vs. PLCA although levels were not significant. By D28, BMP-7 significantly increased M2 macrophages vs. PLCA.
ELISA	BMP-7 administration significantly decreased inflammatory cytokines IL-6, TNF-α, and MCP-1. IL-1RA was significantly higher in BMP-7 treated mice at D5 vs. PLCA; however, by D28 this significant increase was lost. Characteristic M2 cytokine IL-10 was significantly higher in BMP-7 treated animals at both timepoints.
Western blot	Significantly less activation of inflammatory kinases p38 and JNK at D5 and D28, while p-ERK significantly increased at both D5 and D28.