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Bone morphogenetic proteins: Novel mediators of atherothrombosis

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Table of Contents

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SUMMARY

Atherosclerosis is a major cause of morbidity and mortality in western world. Bone morphogenetic proteins are secreted regulatory proteins that regulate various processes throughout human body. Recent studies indicate their presence in atherosclerotic plaques and emerging role in plaque development. Since monocytes are the key effector cells in atherosclerosis, the aim of this thesis was to investigate the actions of BMP-2 and -7 on monocyte thrombogenicity and motility.

In our first paper we investigated the effects of BMP-2 on TF expression in human mononuclear cells (MNCs). We showed that BMP-2 induced phosphorylation of Smad 1/5/8, thus activating the canonical BMP signaling pathway. Though BMP-2 had no effect on the baseline TF expression, it was able to significantly reduce LPS-induced TF expression. When MNCs were pretreated with BMP-2 prior to LPS stimulation, a marked decrease in phosphorylation of ERK1/2, JNK and p38 was observed. BMP-2 also blocked the activation of AP-1 transcription factor, as was shown by use of AP-1 or NFkB sensitive luciferase constructs. This study shows that BMP-2 reduces LPS-induced TF expression in human MNCs by reducing activation of ERK1/2, JNK and p38 as well as blockade of AP-1 transcription factor.

In our second and third papers we investigated the signaling pathways behind the ability of BMP-7 to induce TF in human MNCs. We showed that BMP-7 upregulates both TF protein levels, surface presentation and procoagulant activity as well as mRNA levels. BMP-7 was able to induce phosphorylation of ERK1/2, JNK and p38, signaling kinases essential in regulation of TF gene expression. Using luciferase constructs driven by either wildtype or mutated *F3* gene promoters we showed that intact NFkB binding site on the *F3* promoter is necessary for BMP-7-induced TF expression. Experiments with NFkB inhibitor, JSH-23, supported this finding.

In our forth paper we present a novel function for BMP-7 – regulation of monocyte motility. We showed that human monocytes pretreated with BMP-7 crawl for longer distances, attach to endothelium more readily and migrate faster through endothelial monolayers and show higher levels of active β2 integrins on the cell surface. The observed effects were dependent on the activation of Akt/FAK signaling pathway and were not dependent on *de novo* integrin expression. Finally, the effect of BMP-7 on monocyte motility can be blocked by either natural BMP antagonist Noggin or synthetic BMP type 1 receptor inhibitor, Dorsomorphin.

LIST OF PAPERS

I. BMP-2 inhibits TF expression in human monocytes by shutting down MAPK signaling and AP-1 transcriptional activity. Egorina EM, **Sovershaev TA**, Hansen JB, Sovershaev MA. *Thromb Res. 2012 Apr;129(4):e106-11. doi: 10.1016/ j.thromres.2011.10.024.*

II. Increased expression of TF in BMP-7-treated human mononuclear cells depends on activation of select MAPK signaling pathways. Sovershaev MA, Egorina EM, **Sovershaev TA**, Svensson B, Hansen JB. *Thromb Res. 2011 Dec;128(6):e154-9. doi: 10.1016/j.thromres.2011.07.027.*

III. BMP-7 induces TF expression in human monocytes by increasing *F3* transcriptional activity. **Sovershaev TA**, Egorina EM, Unruh D, Bogdanov VY, Hansen JB, Sovershaev MA. *Thromb Res. 2015 Feb;135(2):398-403. doi: 10.1016/j.thromres.2014.11.031.*

IV. A novel role of bone morphogenetic protein-7 in the regulation of adhesion and migration of human monocytic cells. **Sovershaev TA**, Unruh D, Sveinbjørnsson D, Fallon JT, Hansen JB, Bogdanov VY, Sovershaev MA; *Thromb Res. 2016 accepted manuscript, doi: 10.1016/j.thromres.2016.09.018.*

ABBREVIATIONS

ActRIA – activin receptor type IA
ActRIB – activin receptor type IB
ActRIIA – activin receptor type IIA
ActRIIB – activin receptor type IIB
Alk – activin receptor-like kinase
AP-1 – activator protein 1
ApoB100 – apolipoprotein B100
ApoE – apolipoprotein E
asTF – alternatively spliced tissue factor
BAMBI - BMP and activin membrane-bound inhibitor
BMP – bone morphogenetic protein
BMPR2 – bone morphogenetic protein receptor type 2
BMPRIa – BMP receptor type Ia
BMPRIb – BMP receptor type Ib
CD – cluster of differentiation
cDNA – complementary DNA
Co-Smad – common mediator Smad
COX2 – cyclooxygenase 2
CSF – colony stimulating factor
CX3CR1 – C-X3-C motif chemokine receptor 1
DNA – Deoxyribonucleic acid
ELISA – enzyme-linked immunosorbent assay
eNOS – endothelial nitric oxide synthase
ERK – extracellular signal-regulated kinase
FAK – focal adhesion kinase
flTF – full-length tissue factor
FRET – fluorescence resonance energy transfer
FV – coagulation factor V
FVa – coagulation factor V activated
FVII – coagulation factor VII
FVIIa – coagulation factor VII, activated
FIX – coagulation factor IX
FX – coagulation factor X
FXa – coagulation factor X activated
GDF – growth and differentiation factor
ICAM – intercellular adhesion molecule
ID – inhibitor of DNA-binding
IFNγ – interferon gamma
IκBα - NFκB inhibitor alpha
IL – interleukin
JAM – junctional adhesion molecule
JNK – c-Jun N-terminal kinase
LDL – low density lipoprotein
LDLR – low density lipoprotein receptor
LFA-1 – lymphocyte function-associated antigen 1
LPS – lipopolysaccharide
MAC-1 - macrophage-1 antigen
MAPK – mitogen activated protein kinase
MCP-1 – monocyte chemoattractant protein 1
MH – Mad homology domain
MKK – mitogen-activated protein kinase kinase
MNCs – mononuclear cells
mTOR – mammalian target of rapamycin
NADPH – nicotinamide adenine dinucleotide phosphate-oxidase
NFκB – nuclear factor kappa B (nuclear factor kappa-light-chain-enhancer of activated B cells)
NOX1 – NADPH Oxidase 1
OP-1 – osteogenic protein 1, BMP-7
oxLDL- oxidized LDL
PI3K – phosphoinositide 3-kinase
PSGL-1 – P-selectin glycoprotein ligand 1
RANTES – regulated on activation, normal T cell expressed and secreted
ROS – reactive oxygen species
TβRI – TGFβ receptor type I
TF – tissue factor
TGFβ – transforming growth factor β
TNF – tumor necrosis factor
TRAF6 – TNF receptor associated factor 6
VCAM-1 - vascular cell adhesion molecule 1
VLA-4 – very late antigen 4
VSMC – vascular smooth muscle cells

1. INTRODUCTION

1.1. Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) family of cytokines.

The name BMP comes from papers by Urist et al. published as early as 1965 and 1971 (1, 2). They isolated the protein component of demineralized rat bone matrix and showed its ability to induce bone formation in rat muscle. Human bone morphogenetic protein was first isolated as a 17 kDa fraction from human bone in 1983 (3). Since then, over 30 proteins have been identified.

BMPs are highly conserved between species, with human BMP-4 gene sequence sharing a significant portion with *Drosophila* analogue *dpp*(4), and human BMP-5, -6 and -7 harbor 73% homology of their sequence with *Drosophila* gene *glass bottom boat (gbb,* also known as *60A)* (5).

The functions of BMPs in human development are extensive, and include bone and cartilage tissue formation, axonal growth, development of the kidney and the eye (6-16), as well as the nervous system (17). The importance of BMPs in development is illustrated by knockout studies in mice (14): BMP-2 knockout mice are nonviable due to extensive developmental malformations (12, 18). Knockout of BMP-7 in mice leads to skeletal abnormalities, kidney agenesis (7), and eye defects (6, 10).

Mutation in genes coding BMPs or elements of their signaling pathways were shown to be associated with various diseases, the most prominent being non-thromboembolic pulmonary artery hypertension (PAH). This condition is associated with mutations in the genes coding BMP receptors Alk1, Alk6, BMPR2, and signaling protein Smad8 (19-21). These mutations account for the majority of familial cases and 10-40% of idiopathic cases of PAH. Another example is hereditary hemorrhagic telangiectasia (HHT, or Rendu–Osler–Weber syndrome), known to be associated with mutations in genes coding Alk1, Smad4 and BMP-9 (22).

BMPs may also have an important regulatory function and role in pathobiology of the cardiovascular system, as measurable levels of BMPs are present in vasculature, especially within atherosclerotic plaques (23, 24). Furthermore, levels of different BMP antigens correlate with plaque morphology and extent of plaque calcification (25, 26). It has been shown that BMP-2 is responsible for calcification of arteries via activation of Wnt/b-Catenin signaling (27). On the other hand, BMP-7

has an opposite effect on vascular calcification in mice (28). The effect of BMPs in regulation of plaque calcification has been substantiated by the studies where the blockade of BMP signaling with a specific inhibitor LDN-193189 reduced both plaque calcification and the extent of atherosclerosis in LDLR-/- mice (29). In humans, levels of BMP-2 and -4 correlate with atherosclerotic burden in diabetic patients (30, 31). Moreover, BMP-4 was shown to up-regulate eNOS uncoupling and expression of COX2 and NOX1 genes in endothelial cells, thus contributing to endothelial dysfunction, inflammation and, possibly, oxidation of LDL within the vessel wall (32). In addition, BMP-2 is upregulated in endothelial cells by pro-inflammatory stimuli such as TNF and in turn increases ROS production by activating NADPH oxidase (33).

1.1.1. Classification

The Transforming Growth Factor (TGF) β family of proteins is divided into several groups on the basis of homology/receptor interactions: 1) TGFβs, 2) BMPs, and 3) activins. The BMP subgroup alone, being the most numerous, contains 33 members.

BMP-1 is a metalloproteinase and not technically a member of the TGFβ family. However, the genuine BMPs share structural similarities, namely cysteine knot structures, that consist of six cysteines, of which four are arranged in the motif C2-X-G-X-C3 and C6-X-C7-X-Stop, thus establishing an eight-membered ring. A third disulfide bond formed between the first and the fifth cysteine residue penetrates this ring, thereby tying the knot.

Human BMP-2 and -4 share 86% of amino acid sequence, BMP-5, -6, and -7 share 71-80% homology (34). Based on the sequence similarity and receptor affinity, BMPs are subdivided into the following subgroups:

- BMP-2/4 group,
- OP-1 group (BMP-5, -6, -7, -8),
- BMP-9 (BMPs -9 and -10),
- Growth and differentiation factors (GDF)-5 group (GDF-5, -6, -7).

BMPs, as all TGFβ ligands except GDF15, are synthesized as large pre-proproteins comprising a signal peptide, a large prodomain (200 aa) and a mature region of 100–150 aa in length. For activation, propeptides are cleaved at conserved Arg-XX-Arg dibasic sequence by specific convertases (35). The data on the role of

these massive prodomains in BMPs are sparse, with only some showing distinct roles as in BMP-9, where the prodomain covers receptor epitopes on the mature peptide (36), effectively blocking the formation of the ligand-receptor complex.

While BMPs act mainly as homodimers, heterodimers of BMP-2/6 and BMP-2/7 have been identified *in vitro* (37). These findings were confirmed in an *in vivo* system (38).

1.1.2. BMP signaling pathway

1.1.2.1 *BMP receptors*

BMPs, as other members of the TGFβ family, exert their effects through cell surface dimer receptors, consisting of type 1 and type 2 receptors.

Type 1 receptors include: BMPRIa, BMRIb, ALK1, ALK2, ALK3, ActRIb.

Type 2 receptors are: BMPRII, ActRIIA, ActRIIB. BMPRII is specific for BMPs, while ActRIIA and ActRIIB can bind BMPs, activins, and myostatin.

Type 3 receptors are: endoglin, betaglycan, and repulsive guidance molecules (39, 40).

Under normal conditions, BMPs have very low affinity to standalone type 2 receptors, while having higher affinity to type 1 receptors (41). This is significantly increased when type 2 receptors form complexes with type 1 receptors (8). Moreover, the structure of the receptor heterodimer appears to regulate different functions, depending on the type 1 receptor subunits. Oligomerization of the receptors may explain the distinct functions of BMPs in different organs and/or at different time-points in human development (42).

The main difference between type 1 and type 2 receptors is the presence of the membrane-proximal serine-glycine rich sequence (GS-region) located towards the N-terminus to the intrinsic serine/threonine kinase domain in type 1 receptors, although ligand-binding sites differ significantly between receptors.

BMPR2 is considered to be constitutively active, as it is capable of autophosphorylation *in vitro* (43).

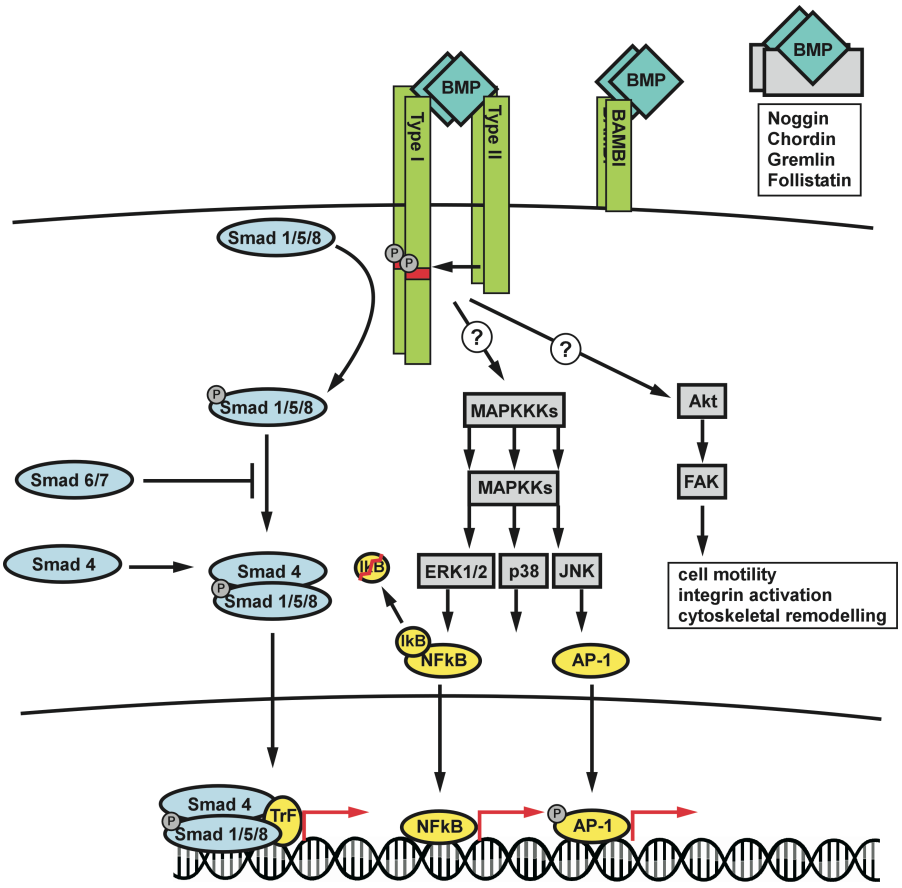

Figure 1. BMP signaling pathway. Binding of BMPs to BMP receptor heterodimer initiates phosphorylation of type 1 receptor, which in turn leads to activation of Smad signaling as well as activation of several alternative pathways, including MAPK and Akt-FAK, which ultimately results in activation of transcription factors. BMP signaling can be inhibited by extracellular inhibitors (Noggin, Chordin, Gremlin, Follistatin), decoy receptor BAMBI and intracellularly by inhibitory Smads 6 and 7. TrF – transcription factors

The exact mechanisms for regulation of cellular responses to BMPs are studied not as extensively as those of TGFβs (Figure 1). Most of the BMP receptors can bind several ligands with varying affinity. The promiscuity of the BMP receptor – ligand interactions is not well characterized today, however, studies in BMP-2 – BMP type 2 receptor interaction show that, unlike TGFβs, there are only few hydrogen bonds formed between receptors and the ligand, while most of the interaction is attributed to hydrophobic interactions (44). This may explain the low affinity interaction with type 2 receptor.

Upon BMP dimer binding to the receptor, the GS domain in type 1 receptor is phosphorylated by constitutively active type 2 receptor, thus changing its conformation to the active form (45). Mutations in these regions can lead to constitutively activated type 1 receptors. The receptor-ligand interaction is different from TGFβ, where the type 2 receptor is capable of binding ligands on its own and

subsequently recruits the type 1 receptor to propagate signaling events (46). When in the active conformation, the type 1 BMP receptor extends the L45 loop that can bind receptor Smads (R-Smads) and phosphorylate them by interaction with MH2 domain of Smads (47), shown previously on an example of Smad1 phosphorylation by Alk2 receptor (48).

1.1.2.2 *Smad family of proteins*

Smad (orthologues of Mad in *Drosophila* and Sma in *Caenorhabditis elegans*) proteins are common signaling molecules for TGFβ and BMPs. Smads 1, 5 and 8 possess a high degree of structural similarity and are generally activated by BMP signaling (48), while they can also be transiently activated by other members of TGFβ protein family (49).

The main feature common to all regulatory Smads (R-Smads) is the presence of two Mad homology domains (MH1 and MH2). MH2 domains are preserved among all SMADs, while MH1 domains are specific for R-Smads and the common mediator Smad (co-Smad) Smad 4 (50). MH2 domains are responsible for interaction with receptors and transcription factors (51), while MH1 is capable of directly binding to DNA. The phosphorylation of SSxS (ser-ser-x-ser) motif, common to R-Smads, by the type 1 receptor disrupts the interaction between the MH domains present in the inactive state and enables the interaction with co-Smad.

To facilitate Smad-receptor interactions, R-Smads can be connected to the cell membrane by a variety of anchoring proteins (52-54). Upon binding of a BMP dimer to a heterodimeric receptor, the constitutively active type 2 receptor serine-threonine kinase site phosphorylates the intracellular part of type 1 receptor, which, in turn, induces phosphorylation of R-Smads (1/5/8), that forms a heteromeric complex with co-Smad4, which is then translocated to the nucleus (55). Within the nucleus, Smads spend most of the time in complex with transcription factors such as Runx2, NFκB, AP-1.

Phosphorylation of R-Smads by receptors increases the affinity for nuclear factors thus adding to the transport of the factors to the nucleus. Dephosphorylation by phosphatases reverses this effect, transporting R-Smads closer to the cell membrane and receptors (56, 57). R-Smads have a sequence that is capable of binding DNA on its own, albeit weakly. There are several genes that incorporate so-called BMP responsive elements and are regulated by BMPs: Id1, Id2, Id3, and Hex, to

name a few (58, 59). However, BMP responsive elements are evolutionary conserved and may be found in a vast variety of genes.

1.1.2.3 *Non-canonical BMP signaling*

There is abundant evidence that BMPs and TGFβs in general exert their biological effects through means other than just canonical Smad signaling. To name the classical example, the p38 MAPK signaling pathway was shown to be strongly activated by the Alk2 BMP receptor (60).

TGFβs were shown to be able to activate ERK kinase in epithelial cells (61), breast cancer cells (62), and fibroblasts (63). ERK activation in response to TGFβ signaling varies greatly, with activation times ranging from minutes to days, suggesting that ERK can be activated either directly by receptor interaction or through gene regulation. ERK is able to phosphorylate a BMP-specific R-Smad1 (64), suppressing its activity. In turn, the interplay between ERK and Smads seems to regulate the activity of AP-1 transcription by interacting with c-Jun/c-Fos subunits (65).

TGFβ can rapidly activate JNK through MKK4 and p38 MAPK through MKK3/6 in various cell lines (66-68), and JNK signaling appears to be Smad-independent (66). Mutation in the Smad- binding epitope in TGFβ type I receptor (TβRI) effectively blocks Smad signaling, while preserving the ability of the receptor to induce JNK and p38 kinases(68, 69). Interestingly, activation of JNK and p38 depends on the interaction of the receptor with TRAF6 protein (70, 71). Members of the TGFβ family, including BMPs, are also known to activate the PI3K/Akt/mTOR pathway (72-75) independently of SMAD activation.

1.1.2.4 *Regulation of BMP signaling*

BMP signaling is tightly regulated by a number of mechanisms, including decoy receptors, extracellular antagonists, as well as intracellular inhibitors.

BAMBI (BMP and Activin Membrane-Bound Inhibitor homolog) is a decoy receptor for BMPs, similar in structure to type 1 receptor, but lacking the intracellular domain for signal propagation (76). It is upregulated by BMPs as part of the negative feedback loop for autoregulation, and is co-expressed with BMP-4 during embryonic development (13, 16).

BMP signaling can be inhibited extracellularly by BMP antagonists (Noggin, Gremlin, Follistatin, Dan family proteins, Chordin). These antagonists bind BMP dimers with high affinity, efficiently blocking the formation of the BMP-receptor complex (11, 77-80).

Recently, another member of the TGFβ family, activin A, was shown to have high affinity for the ActRIIA and ActRIIB type 2 BMP receptors, competing with BMP-6 and -9 for the receptor, thus inhibiting signaling through those receptors (81). Activin A has low affinity towards BMPR2, thus not affecting BMP-2 and -4 signaling, providing a mechanism of regulating TGFβ effects through differential expression patterns and TGFβ members antagonism.

Inhibitory Smads (I-Smads 6 and 7) bind to BMP type I receptors, thus preventing the activation of R-Smads. They also compete with Smad1 for Smad4 binding intracellularly, and, finally are able to disrupt the binding of R-Smad-co-Smad complexes to DNA (82-84).

1.2 Atherosclerosis

Atherosclerosis is the number one cause of non-communicable disease morbidity and mortality in the world (85). It is characterized by localized deposition of lipids and inflammatory cells mainly in the tunica intima in the walls of arteries, generally in areas with disturbed blood flow.

1.2.2 Classification

Currently, the predominant classification used in practice is the American Heart Association classification (86, 87). It classifies atherosclerosis as a staged process - lesions are marked according to severity by a roman numeral from I to VI (Figure 2). Stages I-II are initial lesions, defined by minimal lipid deposition in the intima and isolated groups of macrophage foam cells (type I lesion, or initial lesion).

Type II lesions include fatty streaks; they are subdivided into type IIa, or progression prone, and type IIb, or progression-resistant lesions, depending on the composition and location in the vasculature.

Type III is an intermediate transitional lesion or preatheroma. These lesions contain more free cholesterol, fatty acids, sphingomyelin, lysolecithin, and triglycerides than type II lesions.

Type IV lesions (atheroma) are distinguished by accumulation of the characteristic lipid core. This is the first advanced lesion, with intimal deterioration, though without immediate danger for rupture.

Type V is characterized by prominent fibrous tissue and calcification. Type Va lesion is called fibroatheroma, with predominant development of fibrous tissue, type Vb is characterized by extensive calcification, and type Vc by minimal or no lipid core.

Type VI (a-c) lesions – unstable, vulnerable lesions, characterized by the presence of hematoma, hemorrhage, and / or thrombosis. Type VIabc indicates the presence of all three features.

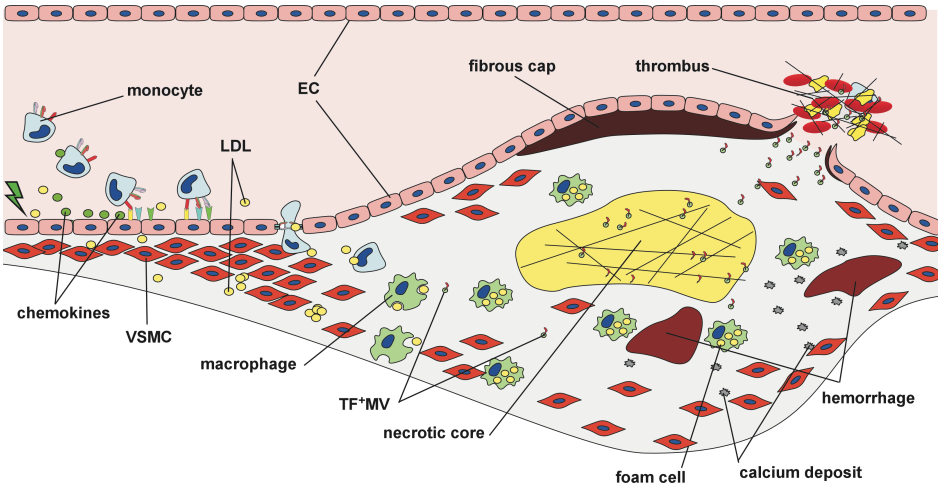

Figure 2. Progression of atherosclerotic lesion. The initial stages of atherosclerosis are characterized by deposition of lipids in the vascular wall and recruitment of leukocytes. In later stages, formation of necrotic core and foam cells leads to thickening of the vessel wall and consequent rupture of the fibrous cap that ultimately leads to thrombosis. $TF^{+}MV$ – TF positive microvesicles.

Virmani et al. (88) proposed in 2000 a modification of AHA classification based on morphological appearance of the plaque and specifically defining Type I and Type II lesions as "non-atherosclerotic" to emphasize their relation to atheromas (advanced lesions) as non-continuous.

Naghavi et al. (89) proposed the term "vulnerable plaque" for those plaques prone to become culprit lesions, and they put forward several criteria for defining a vulnerable plaque.

1.2.3 Lipids

At the site of the future atherosclerotic plaque, activated endothelium becomes permeable for lipids, which, in turn, leads to the formation of reactive oxygen species (ROS) and further enhances endothelial dysfunction (90-92).

Retention of lipids in the vascular wall is initiated either in places with disrupted endothelium (93), where the elastin layer is absent and matrix proteoglycans become exposed to blood flow, or by passive diffusion. ApoB100-LDL binding to exposed proteoglycans starts the accumulation of lipids in the vessel wall, where they are oxidized in the presence of free superoxide anions that are produced by a number of oxidases (NADPH and xanthine oxidases) highly present in atherosclerotic lesions (94).

Oxidized LDL (oxLDL) are able to induce integrin activation in endothelial cells and serve as chemoattractants for monocytes/macrophages (95). Phagocytes, in turn, engulf oxLDL and become foam cells, which produce chemokines to recruit more phagocytes, thus promoting the formation of the lesion.

1.2.4 Vascular smooth muscle cells (VSMCs)

VSMCs, along with endothelial cells and leukocytes, comprise the main cellular components of atherosclerotic plaque. They produce collagen and other extracellular matrix proteins, creating and maintaining the fibrous cap. Induction of VSMC apoptosis leads to the enlargement of the necrotic core and thinning of the fibrous cap in plaques, leading to the development of unstable plaques (96). Disruption of VSMC apoptosis and proliferation by deletion of Akt1 also leads to the formation of vulnerable plaques and severe atherosclerosis in mice (97). It is possible that VSMC-macrophage transition occurs in the plaque, with smooth muscle cells engulfing lipids and transforming into foam cells (98). Studies show that, upon lipid loading, VSMCs acquire characteristic CD68 macrophage markers in mice and humans, and that a significant part of foam cells in atherosclerotic lesions are of VSMC lineage (99-101). It is possible that as much as 50% of foam cells in human atherosclerotic lesions are of VSMC lineage (102-104).

1.2.5 Monocytes and macrophages

Monocytes are phagocytic leukocytes comprising 2-10% of the total blood leukocyte population. In early publications they were defined as short-lived

precursors of macrophages (105), yet their actions are varied and include antigen presentation and phagocytosis of microorganisms, apoptotic cells, etc.

Monocytes originate in the bone marrow from common myeloid progenitor cells (CMP) through a series of precursor cells (106), although there is evidence of extramedullary monocytopoiesis (107, 108) during atherosclerosis and myocardial infarction.

One of their most important functions is to provide a pool of precursors for tissue macrophages and dendritic cells.

In humans, there are three main populations of monocytes, divided by the expression of surface markers. In a recent study it was shown that those subsets have distinct protein expression profiles (109). Different subpopulations of monocytes can have unique roles in coronary artery disease (110).

Three monocyte subsets in humans are $CD14^{++}CD16^{-}$(classical), $CD14^{++}CD16^{+}$(intermediate) and $CD14^{+}CD16^{++}$ (non-classical) (111), that apperar to differ significantly in their biological roles. For example, long-range crawling behavior is characteristic for classical and non-classical monocytes, but not for the intermediate subset. $CD14^{+}CD16^{++}$ and $CD14^{++}CD16^{+}$ monocytes showed a preference for adhering to microvascular over macrovascular endothelium, in contrast to classical subset (112). $CD14^{+}CD16^{++}$ monocytes are dependent on fractalkine (CX3CR1) to adhere and interact with endothelium (113). Knockout of CX3CR1 in mice showed a reduction in atherosclerotic burden by minimizing monocyte extravasation. Furthermore, CX3CR1 upregulates MMP-9, IL-6, CCL2, attributing to further vascular injury and recruitment of inflammatory cells to the lesion (114).

It is believed that monocytes are attracted to the atherosclerotic lesions by a combination of factors, including endothelial dysfunction, chemoattractants, and oxidized lipoproteins in the vessel wall, where they become macrophages, one of the main types of inflammatory cells in the plaques. While traditionally considered to be the final step in the differentiation of blood monocytes (105), there is evidence of possible macrophage proliferation in plaques (115-118). Studies in mice show that tissue resident macrophages can either multiply by cell division or be recruited directly form bone marrow precursors (116, 118).

Macrophages play a central role in the development of atherosclerotic plaques and regulate the plaque milieu in various ways – by engulfing and oxidizing lipids, contributing to the formation of foam cells, regulating cell apoptosis and the

formation of necrotic core, as well as secreting an array of pro- and anti-inflammatory cytokines (TNF, interleukins (IL)) and chemokines (MCP-1, RANTES), and acting as antigen presenting cells. Cytokines released by macrophages facilitate further recruitment of monocytes and lymphocytes into the plaque (119). They are also thought to be the major source of tissue factor (TF) in atherosclerotic plaques (120).

Lipid uptake by macrophages via scavenger receptors, namely Scavenger Receptors Class A-I/II and CD36, is a major pathogenic mechanism in the development of atherosclerosis (121). Knockout of CD36 protects against atherosclerosis in ApoE knockout mice (122), suggesting a pivotal role for this scavenger receptor in plaque formation.

Macrophages can be subdivided into several groups, according to their surface marker/cytokine production. The first classification included M1 (proinflammatory, classical) and M2 phenotypes (anti-inflammatory, non-classical) (123-126), based on the response to various stimuli (IFN-γ, IL-4, LPS). This division was later challenged, due to the fact that macrophage responses do not entirely fit within this dichotomy (127-129). Evidence exists for macrophages changing their "polarization" by continuously adjusting to their environment (129, 130). In 2014, a group of experts proposed a unified classification of macrophages(131) framed around the two extremes observed in vitro by polarizing macrophages using either IFN-γ or IL-4, and further named according for the predominant stimulus used (e.g. M(IL-4), M(IFN-γ), M(LPS), etc.). Worthy of mentioning is the concept of a macrophage "spectrum of activation", which may be the best to reflect *in vivo* conditions (132). This concept marks three distinct populations (classically activated, wound-healing, and regulatory macrophages) with any amount of interplay in-between.

1.2.6 Monocyte extravasation

Adhesion of leukocytes to the vascular wall is enhanced by several conventional cardiovascular risk factors such as smoking, high blood pressure, and hyperglycemia through the regulation of adhesion molecules on monocyte and endothelial cell surface. It was shown that endothelial dysfunction caused by smoking and inflammation dramatically upregulates the expression of adhesion molecules on the endothelium and additionally changes their affinity status (133-135).

The extravasation process starts with the interaction between E- and P-selectins on the activated endothelium and P-selectin glycoprotein ligand 1 (PSGL-1) on the

monocytes (136, 137). PSGL-1 is the predominant ligand for all three types of selectins (138).

In animal models, knockout of P-selectin and ICAM-1 expression on the endothelium was shown to be protective against atherosclerosis (139). The expression of endothelial adhesion molecules VCAM-1 and ICAM-1 is enhanced in atherosclerotic lesions (140).

Selectin-PSGL-1 interaction does not only regulate rolling on the endothelium, but also serves as a signaling input for cytokine production (*e.g.* TNF) and integrin activation (141-143). While rolling over inflamed endothelium, monocytes come in contact with pro-inflammatory cytokines (ILs, TNF, MCP-1), which, in turn, prepare the adhesion machinery for the next step – firm adhesion (144).

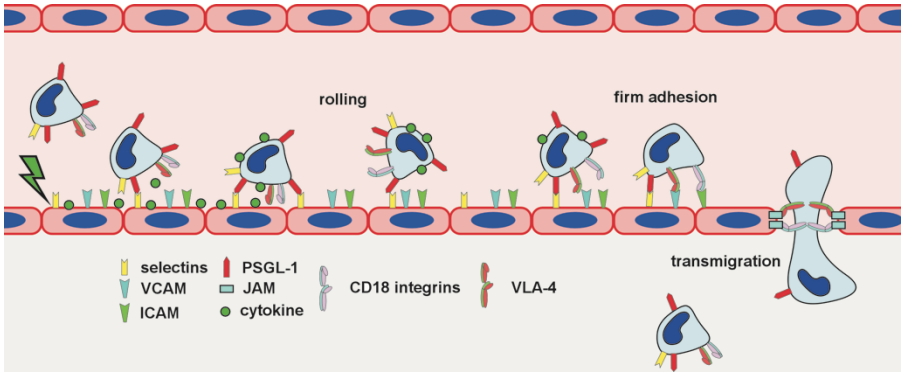

Figure 3. Monocyte extravasation. Monocyte extravasation begins with PSGL-1- selectin interaction. Monocyte come in contact with chemokines. This activates migratory machinery in the cells, leading to firm adhesion and transendothelial migration.

The main classes of monocyte integrins involved in extravasation are CD18 (β2) integrins LFA-1 (CD11a CD18; αLβ2) and MAC-1 (CD11b CD18; αMβ2), and VLA4 (CD49d CD29, α4β1) (145, 146). Absence of either LFA-1 or MAC-1 results in a decrease in numbers of adhered leukocytes, with LFA-1 reducing the adhesion by 80%, suggesting a pivotal role of this integrin in creating a firm adhesion(147, 148), MAC-1 regulates monocyte rolling on the endothelium. VLA4 binding to vascular cell adhesion molecule 1 (VCAM-1) mediates rolling as well as firm adhesion (149). VLA-4 also contributes to mediate cell arrest on the endothelium (150). The role of VLA-4 is supported by *ex vivo* studies in ApoE$^{-/-}$ mice, where functional blockade of the integrin resulted in 75% decrease in cell adhesion to endothelium (151). General integrin activation mechanisms involve conformational change from a bent conformation to an extended one (152). High-affinity (extended) conformation significantly increases the adhesive properties of integrins, especially LFA-1 (153).

The final step of leukocyte extravasation is transendothelial migration, when monocytes traverse the endothelial lining of the vessel to get into the lesion. Junctional adhesion molecules (JAM) are one of the crucial players, with LFA-1 binding JAM-A and MAC-1 interacting with JAM-C (154, 155) on the endothelium. JAM-C interacts with β2 integrins on the endothelial surface and opens intercellular contacts for leukocytes. Of note, JAM-C is upregulated by oxLDL(156) and is responsible for preventing backwards migration of monocytes through the endothelium (157).

Other cell types that are involved in atherosclerosis are dendritic cells, lymphocytes and neutrophils (158). Dendritic cells are often confused with plaque macrophages as some of them come from the same lineage and possess similar surface markers. Dendritic cells are found in the normal intima and accumulate in the plaques (159-161). They possess CD11c and provide stimuli for immune response and activation of the T-lymphocyte system (162, 163). They are also capable of engulfing lipids and generating foam cells (164).

1.3 Tissue Factor

Tissue factor (TF) is a 47 kDa transmembrane glycoprotein, comprised of 263 amino acids. The cDNA sequence was discovered in 1987 simultaneously by three independent groups (165-167), shortly followed by the discovery of the full gene sequence in 1989 (168). TF is encoded by the *F3* gene located on chromosome 1 in the p22-p21 region. The molecular weight of TF is heavily influenced by posttranslational modifications (169).

The TF molecule consists of three domains (170-172): the extracellular domain that binds FVII (Fig. 3, pink), the transmembrane domain (Fig. 3, green) and the cytoplasmic carboxyterminal domain (Fig. 3, blue).

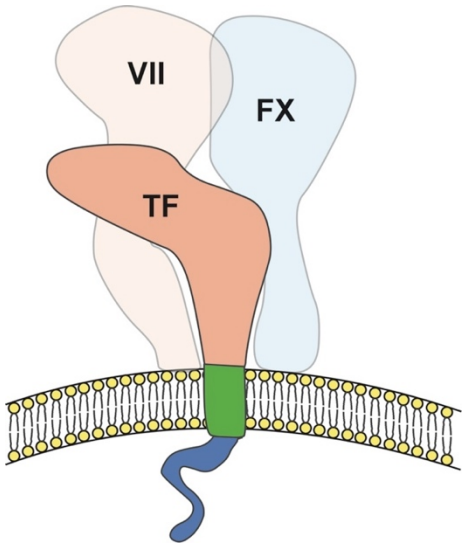

Figure 4. Tissue factor molecule. Schematic representation of TF interaction with FVII and FX. The TF extracellular domain is shown in pink, the transmembrane domain in green, and the intracellular domain in blue.

TF is present in two isoforms, full-length TF (flTF), and alternatively spliced TF (asTF). The mRNA sequence of the latter lacks the exon 5 sequence, and exon 4 is directly linked to exon 6(173). Thus, the mature protein sequence of asTF lacks the transmembrane domain and is not capable of procoagulant activity.

TF undergoes a variety of posttranslational modifications, including glycosylation (169) and palmitoylation (174) which were shown to be important for the regulation of its procoagulant activity (175), although some controversy still exists (176, 177).

Under physiologic conditions, TF does not come into contact with blood, existing as a ”hemostatic envelope” in the vasculature (178) aimed at stopping the bleeding in the event of vessel rupture. This leads to an abundance of TF on the surface of vascular smooth muscle cells, fibroblasts and pericytes (178). TF is also abundant in atherosclerotic plaques. The amount of TF in atherosclerotic plaques increases with the progression of the lesion (179, 180).

TF expression patterns show increased TF prevalence in brain (astrocytes), heart (cardiac myocytes), kidney, placenta (178, 181, 182) as well as stromal cells in human endometrium (183), reflecting the evolutional adaptation for the increased risk of bleeding in these organs. The primary source of TF in blood is thought to be monocytes, yet there are some publications showing TF expression in neutrophils (184), eosinophils (185) and lymphocytes (186). Monocytes may potentially serve as the primary source of TF in whole blood, later transferring it to granulocytes and platelets (187, 188). An important point to bear in mind in *in vitro* studies is that cultured cells show significant changes in TF activity throughout the culturing process, and may not represent the *in vivo* situation (189).

TF association with the cell membrane is necessary for its function as forms lacking the transmembrane domain are not capable of executing its procoagulant function due to the lack of anchoring to the cellular membrane (171). The TF procoagulant activity is induced by Ca^{2+} influx in the cell and influenced by the phosphatidylserine distribution in the membrane (190). Phospholipids are essential for full TF procoagulant activity (191).

The primary function of flTF is to be a cellular receptor for FVII and FVIIa, which it binds with high affinity (192). This interaction triggers the coagulation cascade by activating FIX (193) and FX (194). Activated FXa in association with its cofactor Va, the prothrombinase complex, cleaves prothrombin to thrombin, which in

turn cleaves fibrinogen to fibrin(195). TF-induced FVII activation is blocked by TFPI, that blocks the formation of TF:FVII complex and also inhibits FXa (196).

The promoter of the TF gene has binding sites for Sp1, AP-1 and NFκB transcription factors (197). Deletion studies show that binding sites for Sp1 are responsible for basal expression of TF, while induced expression is dependent on AP-1 and NFκB. The F3 gene promoter also contains a binding site for a transcriptional repressor. Activation of AP-1 and NFκB transcription factors is essential for TF expression (198-200). To our knowledge, there are no previous reports showing BMP-responsive elements or Smad-binding sequences in the human TF promoter.

1.3.2 Non-coagulant functions of tissue factor

Apart from activation of FIX and FX in complex with FVIIa, TF exerts a number of non-procoagulant functions. Over 20 years ago, TF was found to be heavily expressed in tumors, with a significantly higher prevalence in malignancy (201) and in correlation with thrombotic complications. Later, TF expression was found to be associated with metastasis in patients with non-small-cell lung carcinoma (202). It also strongly correlates with the histological grade of pancreatic cancer (203). An elegant study in mice using specific TF mutants provided evidence that TF contributes to cancer metastasis by a pathway independent of coagulation (204). TF inhibits cellular apoptosis and promotes cell survival, thus contributing to tumor growth (205). Blockade of non-coagulant TF signaling by antibodies suppresses tumor growth by reducing TF association with β1 integrins and inhibiting PAR2 mediated signaling (206). PAR2-independent TF signaling has a potential role in angiogenesis in the tumor environment by integrin ligation (207).

TF-FVIIa interaction is able to induce activation of several signaling kinases, including ERK1/2 by activation of PAR2 (208) in human colon cancer cell line, while FVIIa and FX binding to TF increases phosphorylation of ERK1/2, p38 and JNK in human keratinocytes (209). TF activates PAR2 by the TF-FVIIa complex and indirectly via activation of FX (210). Activation of PAR2 by TF-FVIIa enhances migration of human coronary smooth muscle cells (211) and fibroblasts (212). TF also colocalizes with cytoskeletal proteins in lamellipodia of migrating SMCs (213) and endothelial cells (214), suggesting involvement in cell migration.

TF-FVIIa interaction results in upregulation of IL-8 and IL-6 in human keratinocytes (215) and endothelial cells (216) in cell cultures.

2 AIMS OF THE STUDY

Overall aim

- To investigate the actions of BMP-2 and -7 on monocyte thrombogenicity and motility

Specific aims

1. To investigate signaling events behind BMP-2-mediated inhibition of tissue factor activity
2. To study the involvement of ERK1/2, p38, and JNK in BMP-7 induced TF expression in human MNCs
3. To study the effect of BMP-7 on TF protein, mRNA expression, and surface presentation in human MNCs
4. To study the transcriptional regulation of the *F3* gene by BMP-7 in human MNCs
5. To investigate the effect of BMP-7 on motility of human MNCs
6. To investigate signaling events behind BMP-7-attenuated motility of human MNCs

3 SUMMARY OF THE RESULTS

3.1 Summary paper I

BMP-2 inhibits TF expression in human monocytes by shutting down MAPK signaling and AP-1 transcriptional activity.

Egorina EM, **Sovershaev TA**, Hansen JB, Sovershaev MA.

Thromb Res. 2012 Apr;129(4):e106-11. doi: 10.1016/j.thromres.2011.10.024.

Bone morphogenetic protein-2 (BMP-2) is a known inducer of vascular calcification. BMP-2 is abundant in stable, calcified atherosclerotic lesions. We have previously shown that BMP-2 is capable of downregulating induced TF expression in human monocytes. In this paper, we investigated signaling mechanisms behind the observed events.

LPS (strain 026:B6) stimulation led to a 5-fold increase in TF surface presentation, which was accompanied by an increase in TF-positive cells from 1.3±0.3% to 67.1±13.0%. Pretreatment of whole blood with BMP-2 prior to LPS stimulation prevented the increase in TF surface presentation and significantly reduced the number of TF positive cells.

By means of confocal microscopy, we showed that treatment of human mononuclear cells (MNCs) isolated by density gradient centrifugation with 300 ng/ml BMP-2 for 2 hours leads to an 11-fold increase in phosphorylation of Smads 1/5/8 ($p<0.05$), revealing activation of canonical BMP signaling. Of interest, when MNCs were treated with a combination of LPS and BMP-2 for two hours, the phosphorylation of Smad proteins was significantly reduced. BMP-2 did not change basal TF expression, but was capable of significantly reducing LPS-induced TF expression.

We then looked at the activation status of ERK1/2, JNK, and p38 signaling kinases. As judged by immunoblotting, LPS is capable of inducing phosphorylation of all three kinases. Pretreatment with 300 ng/ml BMP-2 prior to addition of LPS resulted in significant reduction of ERK1/2, JNK, and p38 phosphorylation. Next, in order to look at the effects of BMP-2 on AP-1 and NFκB transcription factors, we transfected MNCs with synthetic constructs where the luciferase gene was driven by either AP-1 or NFκB sensitive promoter, and the activation was assessed by measuring luciferase activity by means of luminescence-based assay. LPS was able to

induce both AP-1 and NFκB sensitive plasmids. Treatment with BMP-2 abolished the effect of LPS on activation of AP-1 sensitive plasmid. Interestingly, while BMP-2 did reduce activation of NFκB sensitive plasmids it was unable to completely negate the effects of LPS. This is in line with our data on the weak decrease of IκB degradation, suggesting that BMP-2 has little effect on NFκB activation.

In this study we sought to shed some light on the signaling mechanisms behind the effects of BMP-2 on LPS-induced TF upregulation. We discovered that BMP-2 is capable of downregulating phosphorylation of MAPKs ERK1/2, JNK, and p38. In our luciferase experiments we showed that BMP-2 negates the effect of LPS on activation of AP-1 transcription factor and, while significantly reducing the activation of NFκB, is not capable of completely reversing its activation. The presence of BMP-2 in atherosclerotic plaques, therefore, contributes to plaque stability not only by inducing calcification of the plaque, but may also hamper thrombogenicity by downregulating monocyte TF production.

3.2 Summary paper II

Increased expression of TF in BMP-7-treated human mononuclear cells depends on activation of select MAPK signaling pathways

Sovershaev MA, Egorina EM, **Sovershaev TA**, Svensson B, Hansen JB.

Thromb Res. 2011 Dec;128(6):e154-9. doi: 10.1016/j.thromres.2011.07.027.

BMP-7 is present in lipid-rich, rupture-prone atherosclerotic plaques. It is a known inhibitor of vascular calcification. BMP-7 can contribute to plaque thrombogenicity by activation of TF. TF is present in atherosclerotic plaques and is a major contributor to plaque thrombogenicity. Monocytes are a major cellular component of atherosclerotic plaque and the source of TF in blood. MAPKs can be activated by BMPs and contribute to the induction of TF by LPS. BMPs are capable of activating MAPK signaling alongside with canonical Smad pathway. In this study, we explored whether the activation of the aforementioned pathways is connected with TF upregulation by BMP-7.

Treatment of whole blood with 300 ng/ml of BMP-7 for 2 hours resulted in a 7-fold increase over vehicle ($p<0.001$) in TF protein levels as measured by western blotting. The observed rise in TF protein levels was accompanied by an increase in TF

functional activity (16- fold vs vehicle control, $p<0.001$). LPS was used as a positive control.

When lysates of MNCs treated with vehicle or BMP-7 where subjected to western blotting, we observed that BMP-7 increases phosphorylation of Smads 1/5/8, thus confirming the activation of canonical signaling. This data was also proven by confocal microscopy of MNCs stained for intracellular p-Smads.

Next, we looked into activation status of ERK1/2, JNK, and p38 signaling kinases. BMP-7 was capable of significantly increasing phosphorylation of those kinases (western blotting) to the level comparable with LPS stimulation.

To prove that observed kinase activation is relevant for TF upregulation, we utilized In-Cell Western assay to study the effect of kinase inhibition. The use of ERK1/2 inhibitor PD98059 led to a significant reduction in BMP-7-induced TF expression, while p38 inhibitor SB203580 reduced it almost to baseline levels.

Taken together, out data suggests that TF regulation by BMP-7 is dependent on the activation of ERK1/2 and p38 kinase pathways, with p38 playing the major role in the observed effects, while ERK1/2 is only partially responsible for TF induction by BMP-7.

3.3 Summary paper III

BMP-7 induces TF expression in human monocytes by increasing *F3* transcriptional activity.

Sovershaev TA, Egorina EM, Unruh D, Bogdanov VY, Hansen JB, Sovershaev MA.

Thromb Res. 2015 Feb;135(2):398-403. doi: 10.1016/j.thromres.2014.11.031.

In over previous work, we showed that BMP-7 is capable of upregulating TF expression in human mononuclear cells. This effect is dependent on the activation of MAPKs p38 and ERK1/2. In this study, we aimed to delineate the involvement of two transcription factors responsible for TF gene regulation, AP-1 and NFκB.

First, we measured the response of monocytes to BMP-7 stimulation by means of Western blotting and TF procoagulant activity assay. Stimulation with 300 ng/ml BMP-7 resulted in a time-dependent TF upregulation that was accompanied by an

increase in TF procoagulant activity. The increase in TF protein levels was confirmed by ELISA.

On the basis of those experiments, we utilized the 2-hour time point in subsequent experiments. LPS was used as a positive control in all experiments.

To investigate the underlying mechanisms responsible for the upregulation of TF expression by BMP-7, we studied the activation status of two transcription factors, AP-1 and NFκB, known to be essential for the regulation of *F3* expression. BMP-7 markedly increased the levels of phosphorylated c-Jun in MNCs. However, BMP-7 did not change the transcriptional activity of a synthetic AP-1-sensitive reporter construct.

BMP-7 also led to a loss of IκB-α protein in the lysates of MNCs, which was similar to that measured in the samples stimulated with LPS. This was accompanied by increased transcriptional activity of an NFκB-sensitive luciferase reporter construct.

Next, we used a reporter construct in which luciferase expression was driven by either wild type *F3* promoter, or *F3* promoter mutated in the NFκB or AP-1 binding sites. Cells transfected with the NFkB mutant reporter construct did not respond to BMP-7, while both the wild type and the AP-1 mutant constructs responded with a 6- and 1.5- fold increase, respectively.

Having established that BMP-7 increases transcriptional activity of NFκB, we subjected monocytes or THP-1 cells to BMP-7 stimulation in the presence of JSH-23, a small molecule inhibitor of NFκB. Two hours of BMP-7 stimulation led to a significant increase in TF mRNA levels in human monocytes and THP-1 cells, while pretreatment with JSH-23 abolished the BMP-7-induced increase in TF mRNA. We also investigated the effect of BMP-7 and NFκB inhibition on the surface presentation of TF and got similar results. While BMP-7 was capable of enhancing TF surface presentation more than 2-fold, inhibition of NFκB with JSH-23 abolished the increase in BMP-7-induced fraction of CD14 + TF+ cells.

Taken together, in this study we show that the *F3* gene upregulation and TF protein production by BMP-7 is mediated via the NFκB transcription factor, with involvement of AP-1.

3.4 Summary paper IV

A novel role of bone morphogenetic protein-7 in the regulation of adhesion and migration of human monocytic cells

Sovershaev TA, Unruh D, Sveinbjørnsson B, Fallon JT, Hansen JB, Bogdanov VY, Sovershaev MA; *accepted, Thromb Res 2016. doi: 10.1016/j.thromres.2016.09.018*

BMP-7 is present in atherosclerotic plaques. It is abundant in lipid-rich vulnerable plaques, and can regulate plaque thrombogenicity via upregulation of monocyte / macrophage TF. Monocyte extravasation is a crucial step in the formation of atherosclerotic plaques. BMP signaling pathways are involved in the regulation of integrins in endothelial cells. BMPs are chemotactic for human monocytic cells, and, recently, BMP-2 and -4 were found to be able to recruit monocytes into atherosclerotic lesions (217). In this paper we investigated the effects of BMP-7 on integrin function in monocytes and its effect on the regulation of monocyte migration.

First, using immunostaining of human carotid atherosclerotic plaques, we identified the spatial relation between BMP-7 and macrophages in human atherosclerotic plaques. Of note, BMP-7 is highly present in the intimal region of the diseased vessel, possibly contributing to activation of endothelial cells as well as patrolling monocytes. We then explored the functional effects.

First, we utilized a live cell imaging system, where we recorded videos of THP-1 cells or human monocytes crawling with or without BMP stimulation. Indeed, when treated with BMP-7, monocytes crawled faster and for longer distances (1.76±0.21-fold increase in crawling distance vs vehicle control, $p<0.001$).

Next, we observed that, under orbital shear conditions, monocytes attach more readily to microvascular endothelium when treated with BMP-7 (2.57±0.97-fold vs control, $p <0,001$).

As a final step, we examined transmigration of monocytes and THP-1 cells through microvascular cell monolayers towards MCP-1 gradient (10 ng/ml) in a modified Boyden chamber assay. BMP-7 increased the amount of monocytes transmigrated 2.96±0.65 fold ($p<0.001$) over vehicle control. Thus, BMP-7 is capable of inducing all three major steps of monocyte extravasation.

We then sought to investigate the effect of BMP-7 on integrin activation by means of highly selective monoclonal antibody to high-affinity conformation of β2

integrins, which have a pivotal role in all stages of monocyte recruitment into the plaque. By means of confocal microscopy (2-fold increase in MFI over vehicle, $p<0.001$) and flow cytometry (more than 3-fold increase) we showed that BMP-7 is capable of activating integrins as early as after 30 minutes of stimulation.

Next, to explore signaling pathways behind the observed events, we investigated the activation status of Akt/FAK signaling kinases, known to be central in cell migration.

BMP-7 was capable of increasing the phosphorylation of AKT kinase as early as 15 minutes after stimulation, which was then followed by a dramatic increase in phosphorylation of focal adhesion kinase at 30 minutes post-stimulation.

To prove that observed effects are BMP-specific, we utilized Noggin and Dorsomorphin to inhibit either BMP-7 binding to the receptor or the phosphorylation of the BMP receptor. Indeed, the use of both inhibitors resulted in a significant reduction of BMP-7 effects on monocyte crawling and adhesion.

Lastly, to see whether Akt and FAK kinases are involved in the observed functional effects, we utilized specific kinase inhibitors for Akt 1/2 and FAK. Application of either of the two resulted in significant reduction in cell crawling distance and adhesion to endothelium under orbital shear.

To summarize, in this paper we present a novel function for BMP-7 – regulation of monocyte motility. For the first time, we showed that BMP-7 rapidly activates β2 integrins on the monocyte cell surface. Increased presence of BMP-7 in intimal lining over atherosclerotic plaques clearly shows that BMPs are present at the very onset of monocyte extravasation.

Next, we show that BMP-7 makes monocytes more motile and significantly enhances their adhesive and migratory properties, enhancing all step of monocyte extravasation. This suggests a novel role for BMP-7 in regulation of plaque formation and monocyte extravasation.

4 DISCUSSION OF MAJOR FINDINGS

Since the discovery in the 1960s, bone morphogenetic proteins were extensively studied and now adhere to the name only historically. A wide variety of BMP functions are now recognized both during embryonic development as well as in several diseases. BMPs were first described in atherosclerotic lesions over twenty years ago (23). BMP expression patterns correlate well with the morphology of the plaques, with calcified plaques having more BMP-2 than lipid rich plaques, and an inverse relation with BMP-7 (25, 26). Thrombogenicity of atherosclerotic plaques is heavily dependent on monocyte / macrophage TF (180). Recently, we and others showed that levels of BMP-2 positively correlate with vascular calcification in atherosclerosis and negatively regulate TF expression in human MNCs (25, 26, 31).

Our data further clarifies the involvement of BMP signaling in the development of atherosclerosis.

In **our first paper,** we provide evidence implying that some of the MAPK signaling pathways are involved in the inhibition of LPS-induced TF upregulation in monocytes. Monocytes have functional BMP receptors on their surface (218) and BMP-2 is capable of activating canonical BMP signaling, which is evident from increased phosphorylation of Smads 1, 5, and 8. By means of flow cytometry and confocal microscopy we show that BMP-2 does not affect resting TF levels in human MNCs. Although LPS is a well-known regulator of TF activity in mononuclear cells (219), we confirmed that LPS increases TF expression by flow cytometry and confocal microscopy. We also show that LPS is capable of inducing several signaling pathways (ERK1/2, p38, JNK) that are relevant for TF gene expression. Moreover, LPS activated AP-1- and NFkB-sensitive constructs, showing the activation of transcription factors is vital for TF expression (168). BMP-2 pretreatment resulted in significant reduction of LPS-induced TF expression. The plausible mechanisms include BMP-2 acting through dampening of the activation of the aforementioned signaling kinases (ERK, p38, JNK).

While we clearly observe the negative effect of BMP-2 on the activation of kinases in human MNCs, recent data indicates that BMP-2 can induce phosphorylation of the MAPKs in the gastric cancer cell lines (220) and also activate NFκB transcription factor. This may be attributed to either cell-type specific

differences, specifically the probability of signaling through different surface receptors that can activate various downstream pathways (41).

One may question the relevance of LPS as a TF regulator in atherosclerosis. There are several lines of evidence that infectious agents are present in atherosclerotic lesions (221-223) and may play a role in the pathogenesis of the disease. However, we suppose that one may study the inhibitory effects of BMP-2 on human monocytes, activated via LPS, since LPS stimulation of monocytes starts with CD14 and TLR4 (224) and activates a variety of signaling pathways; the most relevant for TF regulation are MAPK kinases (225) and activation of AP-1 and NFkB transcription factors (226). Further studies may shed more light on BMP-2-mediated regulation of TF in monocytes activated by oxLDL, TNF, and interleukins.

BMP-2 is present in atherosclerotic plaques and is able to recruit monocytes into the lesions (217). BMP-2 therefore has a double role in atherogenesis by increasing monocyte influx into the plaque, and, in the later stages of atherosclerosis, by adding to the stabilization of the process by lowering the thrombogenicity via downregulation of tissue factor and induction of calcification. It would be interesting to look deeper into the association between various subsets of monocytes and macrophages in the plaque and BMP-2 chemotactic activity, since BMP-2 may be a differential stimulus for also recruiting *anti-inflammatory* cells into the plaque.

In **our second and third papers,** we sought to identify the signaling pathways responsible for the BMP-7-mediated TF (*F3*) gene regulation. We found that BMP-7 induces TF surface presentation, upregulates F3 mRNA production, and increases TF protein levels in a time-dependent manner, as well as TF procoagulant activity.

We found that stimulation with BMP-7 induces phosphorylation of Smads 1/5/8, thus indicating the activation of canonical signaling. BMP-7 also significantly increased the phosphorylation of ERK1/2, JNK, and p38. Pharmacological blockade of ERK1/2 and p38 signaling resulted in a significant reduction of TF expression, stressing the importance of the aforementioned pathways in TF regulation by BMP-7.

Next, we studied the activation of transcription factors involved in the regulation of *F3* expression. We found that BMP-7 increases phosphorylation of c-Jun, a measure of AP-1 activation. This was accompanied by increased degradation of the NFκB inhibitory subunit IκBα, thus enabling the NFκB complex to be transferred into the nucleus. When we utilized a luciferase construct driven by the TF promoter,

we showed that both AP-1 and NFκB binding sites are required for full-scale activation of *F3* expression by BMP-7. The NFκB binding site is essential for TF upregulation by BMP-7. To further substantiate our findings, we utilized a small-molecule compound, JSH-23, that prevents NFκB translocation to the nucleus (227). This compound completely negated the effect of BMP-7 on both TF surface presentation as well as *F3* mRNA production.

These data show that the signaling pathway involved in the regulation of TF expression by BMP-7 includes non-canonical BMP signaling through MAPKs and activation of AP-1 and NFκB transcription factors.

The effects of BMP-7 on the activation of NFκB in atherosclerosis may extend beyond the regulation of TF expression. There is a significant amount of data on the activation of NFκB in atherosclerosis, and specifically, coronary artery disease (228). The activation of this transcription factor leads to induction of vascular inflammation (229) and its inhibition protects from atherosclerosis (230).

Activation of NFκB is central in proinflammatory response (231) and leads to the expression of a variety of genes, including TNF, IL-6, IL-8 and matrix metalloproteinases. This may have implications far beyond thrombogenicity of the plaque. Recently, the BMP-specific antagonist Noggin was shown to have an anti-inflammatory effect on endothelial cells (232). The use of small molecule inhibitors of BMP signaling can affect various pathogenetic mechanisms of atherosclerosis (29), including plaque formation, vascular calcification, and plasma LDL levels in murine models, possibly by reducing BMP-2-induced ApoB100 expression.

In our **fourth paper** we present a novel function for BMP-7 – regulation of monocyte motility, adhesion, and transendothelial migration by acting solely on monocytes and not through endothelium / chemoattractant activity. In our present work we demonstrate that BMP-7 is expressed in the intimal region of the vessels as well as inside the plaques. We show that BMP-7 induces a high-affinity conformation in β2 integrins as early as 30 minutes after stimulation, allowing for enhanced firm adhesion and transendothelial migration. Activation of β2 integrins is known to potentiate adhesion, crawling, and transendothelial migration of leukocytes (233, 234). Exposure to BMP-7 triggers intracellular signaling events leading to the activation of the Akt-FAK pathway crucial to integrin-mediated cell migration (235).

The use of specific inhibitors of BMP signaling, Noggin and Dorsomorphin, proves that the observed effects are BMP-specific.

When we used FAK inhibitor, the effects of BMP-7 on monocyte crawling were abolished, and blockade of Akt eliminated BMP-7-induced crawling and adhesion to endothelium. Blockade of Akt reduced phosphorylation of FAK at Tyr397. Interestingly, while abolishing the effects of BMP-7 on cell motility, small molecule inhibitor of FAK (FAK inhibitor 14) did not affect phosphorylation of the kinase at Tyr397. We note that Akt directly regulates FAK through physical association of the two kinases and phosphorylation of FAK on Tyr397 (236), and our findings may suggest that both Akt and FAK are essential for the observed effects. Our results agree with the previous reports on the involvement of PI3K/Akt pathways in BMPR2-mediated chemotaxis in cultured mouse WEHI 274.1 and human THP-1 cells (237). Previous data indicates that BMPs act as chemotactic factors for monocytic cells (217, 238) and induce a proinflammatory phenotype in the endothelial cells (33). It is noteworthy that, in the present study, monocytes were pretreated with BMP-7 and then allowed to interact with untreated endothelial cells. This substantiates the notion that BMP-7 activates intracellular machinery within monocytes, regardless of endothelial involvement. In addition, monocytes preconditioned with BMP-7 migrated faster towards the MCP-1 gradient in the absence of BMP-7 in the medium.

In this work, we outline the importance of bone morphogenetic proteins in the pathogenesis of atherosclerosis. Our results suggest that BMP-7 takes part in regulation of major events in the development of atherosclerotic plaques. First, they enhance the extravasation of monocytes into the vascular wall – a crucial event in the formation of early atherosclerotic lesions (95).

Activation of β2 integrins in monocytes is in line with the data on involvement of BMP signaling in integrin activation on endothelial cells (239). We observe a certain degree of colocalization between β2 integrins and BMPR2 in our work (unpublished data) and together these findings suggest a possible new mode of interaction between BMP and integrin signaling. Our data is limited to the observations obtained from immunostaining of the two proteins *in vitro* using fluorescent microscopy. Further delineation of this interaction is needed using techniques suitable to verification of the genuine protein-protein interaction e.g. FRET and co-immunoprecipitation.

In our studies we also observed a degree of activation of VLA4 (unpublished data) by BMP-7 in monocytes. These data further substantiate our conclusions on the role of BMP-7 in monocyte migration.

Interestingly, a study in ApoE$^{-/-}$ mice showed an inverse relation between BMPR2 in the endothelium and atherosclerosis (240). This may also be attributed to the negative feedback between BMPs and BMP receptor expression in the endothelium.

BMP-7 actions are quite diverse in atherosclerosis – the protein is a known inhibitor of calcification, helps monocytes to enter the plaque, and in later stages of the diseases contributes to thrombogenicity via upregulation of tissue factor expression and its procoagulant activity. However, some reports show that BMP-7 promotes alternative activation of macrophages(241), shifting polarization towards expression of anti-inflammatory markers, and may reduce atherosclerotic burden in mice (242). Moreover, BMP-7 reduced vascular calcification in a murine model of chronic kidney failure and atherosclerosis (28). However, we have not looked into the effects of BMP-7 on macrophages in the context of atherosclerosis. Moreover, the data presented in the studies mentioned were obtained from murine models and THP-1 cells, which may not necessarily reflect the behavior of human macrophages (243-245).

One of the naturally occurring BMP antagonists, Noggin, has recently been shown to reduce inflammation and leukocyte chemotaxis (217, 232), and it is thus plausible to postulate the existence of anti-atherogenic properties of Noggin, as well as other BMP antagonists. In the **fourth paper** we show that Noggin is capable of suppressing of BMP-7-induced monocyte motility. This again suggests a novel role of BMP signaling in atherogenesis.

4.1 Methodological considerations

There are several limitations to the observations in our study. First, data on the regulation of tissue factor expression in mononuclear cells was produced in density gradient-isolated human mononuclear cells that contain a significant portion of lymphocytes. The best option would have been to use a population of monocytes negatively selected from buffy coats. However, monocytes are the major source of blood-borne TF, with only one report indicating the expression of TF by lymphocytes (186). Moreover, our data on BMP-7-induced TF expression in monocytes is

substantiated by findings in THP-1 cells, the most widely used human monocytic cell line.

Another consideration is the use of synthetic AP-1- or NFκB-sensitive promoters. The expression of TF is tightly regulated by an interplay between several transcription factors and thus the use of synthetic promoters may not reflect the *in vivo* gene expression, yet will provide data on the activation of specific transcription factors. A more correct way is to use a wild-type gene promoter incorporated into the luciferase construct, thus providing a more "real" system, which we utilized in our **third paper**.

We did not evaluate the effect of BMP-2 on TF procoagulant activity. While we observe that BMP-2 does not change TF surface presentation or total protein levels in resting monocytes, the TF procoagulant activity may not directly correlate with protein levels due to possible encrypted TF on the cell membrane.

In our fourth paper, we utilized attachment to plastic to isolate human monocytes from blood. A recent study show that, after 2 days in culture monocytes still bear characteristic CD14 and CD16 surface markers (246). Another study showed no difference in surface marker expression between MNCs isolated by adherence and CD14 positive selection, yet demonstrated loss of $CD14^{dim}$ population when isolating with CD14-positive beads (247), and one showed no difference between either bead-isolated or attachment isolated monocytes in their ability to form DCs (248). While negative selection would most probably be the best option, adhesion may serve as an easy and reliable alternative.

Thus, while monocytes may be activated by plastic (249), an overnight incubation will not significantly alter the phenotype of human monocytes. Moreover, relevant controls were utilized, and similar results were obtained in the human monocytic cell line THP-1.

5 CONCLUSIONS

1. BMP-2 inhibits LPS-induced TF expression in human MNCs by inhibition of phosphorylation of ERK1/2, p 38, and JNK signaling kinases and inhibition of LPS-induced activation of AP-1 and NFκB transcription factors.
2. BMP-7-induced phosphorylation of ERK1/2, p38, and JNK in human MNCs plays an important role in TF induction by BMP-7.
3. BMP-7 increases TF protein and mRNA expression, procoagulant activity, and TF surface presentation in human MNCs.
4. BMP-7-mediated *F3* upregulation is mediated by activation of NFκB and AP-1 transcription factors. NFκB is essential for the *F3* gene upregulation by BMP-7, and AP-1 is necessary for full-scale activation of the *F3* gene promoter by BMP-7.
5. BMP-7 enhances crawling, adhesion, and transendothelial migration of human monocytes and THP-1 cell line.
6. BMP-7 rapidly induces the active conformation in β2 integrins. BMP-7-induced crawling and adhesion are dependent on activation of Akt and FAK.
7. Noggin and Dorsomorphin completely reverse the effects of BMP-7 on monocyte crawling and adhesion.

6 FUTURE DIRECTIONS

This work can be expanded in several ways. First of all, a study on the role of other members of the BMP family in atherosclerosis may be beneficial. There is now sufficient evidence to support their role in atherosclerotic processes.

The observed effects of BMPs on monocytes are performed *in vitro*. It would be compelling to confirm our findings *in vivo* in a mouse model and observe how administration of BMPs / BMP inhibitors can change the morphology of plaques, monocyte / macrophage burden, and plaque thrombogenicity. The resulting data may provide a certain basis for developing new treatment strategies. Moreover, data on the role of BMP signaling in atherosclerotic burden can further be substantiated by the use of conditional knockouts of BMP receptors/signaling molecules.

While in our fourth paper we focused on the CD18 integrins, a study on the effects of BMPs on the activation of various adhesion molecules in monocytes, including VLA-4, PSGL-1 and selectins is warranted to provide additional data on the effects of BMPs on leukocyte migratory machinery.

It would be also of interest to elaborate on the BMP receptor – integrin interaction. In our work we observed a rapid increase in colocalization of BMPR2 and CD18 integrins on the surface of THP-1 cells activated with BMPs. The interaction was previously shown in endothelial cells under shear stress (239), yet no studies so far have examined the role of such an interaction in activation of leukocyte motility and its role in monocyte extravasation. A variety of techniques can be utilized, including yeast 2 hybrid (250), fluorescence resonance energy transfer (251), and immunoprecipitation.

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