Osteopontin deficiency dampens the pro-atherogenic effect of uraemia

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1. Introduction

End-stage renal disease leads to accumulation of waste products in plasma, i.e. uraemia, and is treated with dialysis or kidney transplantation. The 5-year mortality rate in dialysis patients is ~60%, mainly reflecting cardiovascular disease (CVD).¹ To a large extent, this probably reflects uraemic vasculopathy, which includes accelerated atherosclerosis and arterial calcifications. The classical risk factors, i.e. hypertension, diabetes, and hypercholesterolaemia, cannot explain the extreme risk of cardiovascular death in uraemic patients.² This prompts the need to understand the unique pathogenic mechanisms leading to acceleration of CVD in uraemia.

Moderate uraemia accelerates atherosclerosis in the 5/6 nephrectomized (NX) apolipoprotein E knockout (E KO) mouse model³⁴—providing a valuable mouse model of uraemic atherosclerosis. Indeed, uraemia causes a distinct gene expression profile in atherosclerotic aortas of E KO mice. In our previous study, osteopontin (OPN) was the gene most up-regulated by uraemia in the aorta.⁶ OPN has multiple effects that could accelerate atherosclerosis in uraemia and is highly expressed in lesion macrophages, vascular smooth muscle cells (SMCs), and endothelial cells.⁷,⁸ OPN increases recruitment, migration, and adhesion of macrophages and modulates expression of pro-inflammatory cytokines [e.g. monocyte chemo-attractant protein 1 (MCP-1) and interleukin (IL)-6], i.e. processes well-known to be crucial in atherogenesis.⁹,¹⁰ In SMC’s, OPN expression is increased as a response to arterial injury in vivo,¹⁰ and to stimulation with oxidized low-density lipoprotein in vitro.¹¹ OPN expression is increased during SMC de-differentiation; a key process in atherogenesis.¹²,¹³ SMC de-differentiation is characterized by a phenotypic change from a contractile to a synthetic phenotype. This is associated with specific changes in gene and protein expression patterns, i.e. the transcription factor myocardin (Myocd) and several of its target genes, including α-smooth muscle actin (α-SMA), are down-regulated in...
synthetic when compared with contractile SMCs.\textsuperscript{14,15} OPN modifies SMC migration and proliferation\textsuperscript{10,16–18} and also affects other processes with putative effects during atherogenesis, such as modulation of the immune response towards a pro-atherogenic Th1 response, and migration of endothelial cells.\textsuperscript{19–21} Accordingly, previous studies in mice indicate a pro-atherogenic effect of OPN in classical and angiotensin (Ang)-II-accelerated atherosclerosis.\textsuperscript{22–27} Interestingly, plasma OPN is increased in uremic patients\textsuperscript{28,29} and serum isolated from uremic patients up-regulates OPN protein levels in bovine vascular SMCs.\textsuperscript{30} Nevertheless, it remains to be determined whether there is a causal relationship between increased plasma OPN and development of vascular disease in uremic patients.

In the present study, we have used the NX mouse model to explore the effect of OPN deficiency on atherosclerosis in a uremic setting.

### 2. Methods

#### 2.1 Mice

ApoE\textsuperscript{2–/–} mice (CS7BL/6J/bom-Apoe\textsuperscript{ext/1Inc}, Taconic M&B laboratory Animals and Services for Research, Ry, Denmark) were crossed with OPN\textsuperscript{2–/–} mice (CS7BL/6J background).\textsuperscript{31} The resultant heterozygous mice were bred to generate apoE\textsuperscript{2–/–} (E KO) and apoE\textsuperscript{2–/–}, OPN\textsuperscript{2–/–} (E/OPN KO) mice. Mice were kept on a 12-h light/dark cycle in a temperature-controlled room at 21–23°C with free access to water and standard mouse chow (Altromin 1314, Altromin, Lage, Germany). Mice were genotyped for apoE and OPN as described in Supplementary material online. Female mice were used.

Two separate mouse studies were performed. In the first study, we analysed the effect of whole-body OPN KO on atherosclerosis in control (CTRL) and uremic E KO (n = 15 CTRL, n = 20 NX) and E/OPN KO (n = 18 CTRL, n = 12 NX) mice. After detecting uraemia-specific effects of whole-body OPN deficiency, we investigated whether this was mediated via bone marrow-specific OPN expression. Thus, we transplanted E KO or E/OPN KO bone marrow to E KO mice prior to the induction of uraemia. Littermate CTRL mice were studied in parallel (see Supplementary material online, Figure S1 for an overview of mouse studies).

Moderate uraemia was induced by 5/6 nephrectomy in two operations as previously described\textsuperscript{3} with the modifications described in Supplementary material online. Anaesthesia was achieved with a mixture of fentanyl (0.079 mg/mL), fluanisone (2.5 mg/mL), and midazolam (1.25 mg/mL) (hypnorm/dormicum) at a dose of 0.1 mL/10 g body weight, subcutaneous. During surgery, each mouse was monitored closely visually to assure adequate anaesthesia. After surgery, analgesia (buprenorphine 0.1 mg/kg body weight) was given subcutaneously for 2–3 days. Thirty weeks after the second surgery, mice were anaesthetized with hypnorm/dormicum as detailed above, and perfused with ice-cold saline. The heart and aortic arch were retrieved and prepared as described previously.\textsuperscript{22} Furthermore, we isolated blood samples. The overall mortality in the whole-body OPN study was 24% (21 mice).

For the bone marrow transplantation (BMT) study, 80 E KO mice (age: 9–10 weeks) were irradiated (9.5 Gy) in a Gammacell 40 Exactor (MDS Nordion). The following day, bone marrow was isolated from femurs and tibias of littermate E KO (n = 4) or E/OPN KO (n = 4) donor mice and administered to irradiated recipients as detailed in Supplementary material online. Uraemia was induced 9 and 12 weeks after BMT as detailed in Supplementary material online. Seventeen weeks after the second surgery, the BMT study was terminated in the same manner as the whole-body OPN KO study. The transplanted mice appeared to be more susceptible to surgery-related mortality and general survival after the induction of uraemia. Thus, a total of 23 mice (29%) were lost during the study period.

All animal experiments were performed according to the principles stated in the Danish law on animal experiments and were approved by the Animal Experiment’s Inspectorate, Ministry of Justice, Denmark. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the European Parliament (EU directive 86/609/EEC (from 1.1.2013 directive 2010/63/EU)). The ethical policy of the University of Copenhagen complies with that of the NIH (AS846-01).

#### 2.2 In vitro studies

Bone marrow cells were isolated from E KO (n = 4) and E/OPN KO (n = 4) mice as detailed in Supplementary material online. For detailed protocols regarding in vitro studies, see Supplementary material online.

#### 2.3 Real-time PCR

Real-time PCR on a LightCycler (Roche) or a TaqMan (Applied Biosystems) was used for gene expression analyses as specified in Supplementary material online.

#### 2.4 Plasma biochemistry

Blood was collected in heparinized microtubes (Capiject; Terumo Medical, Elkton, MD, USA) and centrifuged at 4000 rpm for 10 min at 4°C. All plasma markers were measured as detailed in Supplementary material online.

#### 2.5 Evaluation of atherosclerosis and plaque composition

Evaluation of atherosclerosis in the aortic arch and the aortic root was performed as described in Supplementary material online. Plaque composition was determined histologically as detailed in Supplementary material online.

#### 2.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA) as specified in Supplementary material online. P < 0.05 was considered significant.

### 3. Results

#### 3.1 OPN promotes uraemia-induced atherosclerosis

Uraemia was induced by NX in female E KO and E/OPN KO mice at the age of 12–16 weeks. NX increased plasma urea (~2.5 times), creatinine (~1.5–1.8 times), and cholesterol (~1.6 times) to a similar extent in E KO and E/OPN KO mice (Table 1). Hence, NX resulted in moderate uraemia in mice, as described previously.\textsuperscript{33}

After 30 weeks, the average surface plaque area in the aortic arch was higher in E KO NX compared with E KO CTRL mice (Figure 1A). In contrast, uraemia did not accelerate atherosclerosis in E/OPN KO mice (Figure 1A). Similar results were seen in analyses of cross sections of the aortic root: the relative lipid content, as determined after Oil-Red O staining, was increased in NX vs. CTRL E KO mice, but not in NX vs. CTRL E/OPN KO mice (Figure 1B). Total plaque area in the aortic root was not affected by uraemia and/or genotype (Supplementary material online, Figure S2). Likewise, there was no significant effect of NX or whole-body OPN deficiency on the plaque content of α-SMA positive cells as judged by immunohistochemistry, or fibrosis as judged by trichrome staining (Supplementary material online, Figure S3).

In the E KO mice, NX increased OPN mRNA expression levels in the thoracic portion of the aorta ~2.3-fold (P < 0.04, Figure 2A).
OPN mRNA was undetectable in the E/OPN KO mice (Figure 2A). Plasma OPN concentration was increased ≏1.5-fold in uraemic E KO mice (Figure 2B), and we found a positive correlation between plasma OPN and aortic atherosclerosis within the group of uraemic E KO mice (Figure 2C), which was not seen within the group of non-uraemic CTRL E KO mice (Figure 2D). Combined, these data strongly suggest that OPN facilitates uraemia-induced acceleration of atherosclerosis.

3.2 OPN deficiency reduces the inflammatory response of macrophages

To assess whether the effect of OPN on atherosclerosis in the uraemic mice could result from effects on the differentiation of macrophages into foam cells or a dampening of the inflammatory response of macrophages in the pro-inflammatory uraemic environment, bone marrow cells from E KO and E/OPN KO mice were differentiated into macrophages in vitro.

Foam cell formation, as judged from the cellular cholesterol accumulation upon incubation for 24 h with acetylated low-density lipoprotein (acLDL), was similar in macrophages from E KO and E/OPN KO mice (Supplementary material online, Figure S4). Upon differentiation of the macrophages into foam cells, OPN deficiency was accompanied by lower expression of MCP-1 and IL-6 mRNA (Supplementary material online, Figure S5A). Moreover, OPN deficiency led to reduced IL-6 and MCP-1 mRNA in both macrophages and foam cells, when the cells were stimulated with lipopolysaccharide (LPS). LPS was applied to mimic the pro-inflammatory milieu in uraemia (Supplementary material online, Figure S5B).

The in vivo expression of IL-6 and MCP-1 mRNA in the thoracic aorta of E/OPN KO CTRL mice was 33 ± 8% (P = 0.04) and 55 ± 15% (P = 0.10), respectively, of that in E KO CTRL mice.
A similar trend towards lower expression of IL-6 and MCP-1 in OPN deficiency was seen in the NX mice, albeit the differences were not statistically significant (Supplementary material online, Figure S5C). Notably, in accordance with the pro-inflammatory effect of uraemia, NX increased the expression of IL-6 and MCP-1 by 309±59% (P = 0.004) and 267±54% (P = 0.017), respectively, without any difference in the response to uraemia between E KO and E/OPN KO mice (data not shown).

3.3 Macrophage deficiency of OPN does not abolish the pro-atherogenic effect of uraemia

To analyse whether the observed effects of OPN on the inflammatory response in macrophages might influence uraemic atherosclerosis in vivo, we transplanted bone marrow from donor E KO or E/OPN KO mice to lethally irradiated recipient E KO mice (n = 40 recipients of each genotype). Uraemia was induced by 5/6 NX 9 weeks later (n = 24 mice/genotype). The remaining transplanted mice served as CTRL mice (n = 16 mice/genotype). NX increased plasma urea (∼3.3–3.8 times), creatinine (∼2.5 times), and cholesterol (∼1.5 times) to a similar extent in mice transplanted with E KO or E/OPN KO bone marrow (Table 1B). Uraemia increased aortic atherosclerosis both in mice transplanted with E KO bone marrow and in mice transplanted with E/OPN KO bone marrow (Figure 3A). In the aortic root, neither uraemia nor the transplant genotype affected total plaque area, macrophage content, or the relative accumulation of lipid (Supplementary material online, Figure S6A and B).

OPN mRNA expression in the thoracic aorta was reduced by ∼80% in recipients of E/OPN KO bone marrow when compared with recipients of E KO bone marrow (Figure 3B). On immunohistochemistry, the OPN protein was seen widespread in the lesions. A substantial portion of OPN was extracellular—particularly in necrotic areas (Figure 4). Regardless of the transplant genotype, endothelial cells did not appear to contain OPN protein. Although we cannot exclude that some SMCs were OPN-positive, most SMCs did not contain OPN protein reactivity (Figure 4B). In mice transplanted with E KO bone marrow, lesion macrophages expressed OPN (Figure 4A, top). As expected, luminal macrophages did not contain OPN protein in mice transplanted with E/OPN KO bone marrow (Figure 4A, bottom).

Plasma OPN levels were not affected by BMT in neither E KO nor E/OPN KO mice (Supplementary material online, Figure S7; baseline vs. post-BMT). Uraemia increased plasma levels of OPN in mice
transplanted with E KO bone marrow as well as E/OPN KO bone marrow (Supplementary material online, Figure S7). Thus, even though OPN protein is found in some lesion macrophages, expression of OPN in bone marrow cells, including macrophages, is not important for uraemia-mediated acceleration of atherosclerosis, and macrophages do not contribute significantly to plasma content of OPN.

3.4 SMC phenotype is altered by uraemia

To explore mechanisms underlying the pro-atherogenic effect of OPN in uraemia, we performed microarray analyses on thoracic aortas isolated from CTRL and NX mice from the whole-body OPN KO study. Although most analyses of individual genes did not reveal statistically significant differences, these analyses combined provided some indication that uraemia led to a de-differentiation of SMCs in the arterial wall and that OPN deficiency dampened this effect (data not shown). To explore this idea, we used real-time PCR to analyse the expression of the transcription factor Myocd, a key regulator of SMC differentiation, and Myocd target genes in the thoracic aorta from both the whole-body OPN KO and the OPN-BMT KO study.

In the whole-body OPN KO study, uraemia led to a down-regulation of Myocd and its target genes α-SMA (Figure 5A) and smooth muscle myosin heavy chain (αMHC) (Supplementary material online, Figure S8A) in the thoracic aorta of E KO mice. Also, the Myocd target gene calpain 1 (Cnn1) tended to be down-regulated, although not statistically significant (Supplementary material online, Figure S8A). Interestingly, none of these genes were affected by uraemia in whole-body OPN-deficient mice (Figure 5A and Supplementary material online, Figure S8A), suggesting that whole-body OPN deficiency dampens the effect of uraemia on genes controlling...
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SMC phenotype. Quantification of α-SMA protein in aortic root plaques from the whole-body OPN KO study (Supplementary material online, Figure S3A) showed a non-significant tendency towards less α-SMA content in plaques from E KO NX compared with E KO CTRL mice, whereas similar α-SMA levels were seen in E/OPN KO NX vs. E/OPN KO CTRL mice (Supplementary material online, Figure S3A).

In the OPN-BMT study, uraemia also led to lower levels of Myocd, α-SMA, and smMHC in the thoracic aorta of mice transplanted with E KO bone marrow (Figure 5B). Likewise, uraemia tended to reduce expression of Myocd, α-sma, smMHC, and Cnn1 in mice transplanted with E/OPN KO bone marrow (Figure 5B and Supplementary material online, Figure S3B), although these differences did not reach statistical significance. Thus, deficiency of OPN in bone marrow-derived cells did not appear to dampen the uraemia-mediated effect on SMC phenotype to the same extent as whole-body OPN deficiency.

4. Discussion

This study demonstrates a link between increased OPN and accelerated atherosclerosis in a uraemic milieu. The data strongly support the idea that uraemia increases plasma OPN levels, and that OPN contributes to increased development of atherosclerosis in a uraemic setting. Thus, OPN represents both a potential new biomarker and a target for specific intervention in uraemic vascular disease.

Several studies have suggested pro-atherogenic effects of OPN. The effect, however, seems dependent on experimental circumstances and we did not see any effect of OPN deficiency on atherosclerosis in non-uraemic CTRL mice. This result is compatible with a previous observation by Bruemmer et al. that whole-body OPN deficiency has no effect on atherosclerosis in E KO mice unless the mice are infused with Ang-II. Interestingly, uraemia is characterized by activation of the renin–angiotensin system and increased Ang-II production. Moreover, pharmacological blockade of angiotensin converting enzyme or the Ang-II-receptor prevents oxidative stress, vascular inflammation, and development of atherosclerosis in uraemic E KO mice, suggesting that increased Ang-II signalling is a key accelerator in uraemic atherosclerosis. The results of the present study and the previous study in Ang-II-infused mice combined, thus, support the hypothesis that OPN is particularly atherogenic during activation of the renin–angiotensin system, such as in uraemia.

How does OPN increase atherosclerosis in uraemic E KO mice? Our in vitro studies indicated that the expression of inflammatory genes is decreased in OPN-deficient foam cells especially after stimulation with LPS. These results are compatible with the notion that macrophage OPN expression is pro-inflammatory. Nevertheless, the lack of effect of OPN deficiency in the OPN-BMT study suggests that OPN expression in macrophages (and other haematopoietic cells) has no major effect on the development of uraemic atherosclerosis, despite the fact that expression of OPN in haematopoietic cells accounted for ~80% of the total aortic mRNA expression (as determined in the BMT study, Figure 3B). Even though this result could reflect that macrophage OPN expression has no effect on the pro-atherogenic effect of uraemia, it should be noted that the surface plaque areas were quite different in the whole-body and the BMT KO study. Hence, the data do not exclude an effect of macrophage OPN in more developed lesions as observed in the whole-body OPN study than those observed in the BMT KO study.

Alternatively, the pro-atherogenic effect of OPN in uraemia is mainly caused by OPN expression in non-haematopoietic vascular cells (e.g. endothelial and vascular SMCs) in the vascular lesions or by the increased plasma OPN concentrations. The immunohistochemical analyses indicated that endothelial cells covering atherosclerotic lesions are practically devoid of OPN protein and only few SMCs contain OPN protein to an extent detectable by immunohistochemistry. A substantial part of the OPN present in the atherosclerotic lesions was derived from the increased plasma OPN concentrations and increased SMC expression of inflammatory proteins due to OPN. Conversely, the immunohistochemical analyses indicated that OPN expression in macrophage OPN expression is pro-inflammatory. The effect, however, seems dependent on experimental circumstances and we did not see any effect of OPN deficiency on atherosclerosis in non-uraemic CTRL mice. This result is compatible with a previous observation by Bruemmer et al. that whole-body OPN deficiency has no effect on atherosclerosis in E KO mice unless the mice are infused with Ang-II. Interestingly, uraemia is characterized by activation of the renin–angiotensin system and increased Ang-II production. Moreover, pharmacological blockade of angiotensin converting enzyme or the Ang-II-receptor prevents oxidative stress, vascular inflammation, development of atherosclerosis, despite the fact that expression of OPN in haematopoietic cells accounted for ~80% of the total aortic mRNA expression (as determined in the BMT study, Figure 3B). Even though this result could reflect that macrophage OPN expression has no effect on the pro-atherogenic effect of uraemia, it should be noted that the surface plaque areas were quite different in the whole-body and the BMT KO study. Hence, the data do not exclude an effect of macrophage OPN in more developed lesions as observed in the whole-body OPN study than those observed in the BMT KO study.

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The decreased expression of SMC-specific genes in aortas of uraemic mice suggests that uraemia indeed is accompanied by de-differentiation of aortic SMC’s from a contractile to a synthetic phenotype. Such de-differentiation of SMCs is believed to occur on various forms of vascular injury, e.g. atherosclerosis, hypertension, and formation of aortic aneurysms. Thus, the arterial damage(s) induced by uraemia may be preceded and/or accompanied by a de-differentiation of SMCs, which is stimulated by OPN. In combination with other molecular mechanisms, i.e. activation of the endothelium and increased vascular inflammation, this might lead to acceleration of atherosclerosis in a uraemic setting. The abrogation of the effect of uraemia on markers of SMC de-differentiation by whole-body OPN deficiency suggests that OPN promotes de-differentiation of SMC’s in a uraemic setting. Indeed, previous in vitro studies have linked OPN to de-differentiation of SMCs. Hence, the switch from contractile to synthetic SMC phenotype is associated with increased OPN expression. OPN can also directly affect functional characteristics of SMC’s such as adhesion and spreading, and migratory ability, all of which are potentially important during atherogenesis. The effect of OPN on SMC phenotype in NX vs. CTRL mice may be due to changes in the local expression of OPN in the arterial wall and/or to changes mediated via the increased plasma OPN levels.

The results of the present study should be interpreted with several limitations in mind. Most importantly, lack of statistical power may cause type II statistical errors, mouse studies cannot necessarily be extrapolated to humans, and morphological analyses of atherosclerotic lesions are often subject to considerable variation, which may have affected the present results. Finally, we observe an unexplainable decrease in plasma OPN in control E KO animals over time.

Assessment of cardiovascular risk in uraemic patients has been hampered by the lack of predictive power of traditional cardiovascular risk factors such as plasma cholesterol. Plasma OPN is increased in uraemic patients, and increased plasma OPN has been suggested as a risk factor for CVD. With the before mentioned limitations in mind, the present data suggest that OPN likely plays a causal role in uraemic atherosclerosis and hence could be a new biomarker to assess cardiovascular risk specifically in uraemic patients. Further development of this idea obviously requires prospective clinical studies in humans.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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