BMP-7 Is an Efficacious Treatment of Vascular Calcification in a Murine Model of Atherosclerosis and Chronic Renal Failure

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Abstract. Chronic renal failure is complicated by high cardiovascular mortality. One key contributor to this mortality is vascular calcification, for which no therapy currently exists. Bone morphogenetic protein 7 is an essential renal morphogen that maintains renal tubular differentiation in the adult and is downregulated in renal failure. Several studies have demonstrated its efficacy in treating various renal diseases in rodents, and it was hypothesized that it would also be an effective treatment of vascular calcification in this setting. Uremia was imposed on LDL receptor null mice (a model of atherosclerosis), which were then treated with bone morphogenetic protein 7 for 15 wk. Uremic animals had increased vascular calcification by histology and chemical analysis. Calcification in treated animals was similar to or less than non-uremic control animals. Cells exhibiting an osteoblast-like phenotype in the vessel wall may be important in the etiology of vascular calcification. Expression of osteocalcin was assessed as a marker of osteoblastic function, and it is shown that it is increased in untreated uremic animals but downregulated to levels similar to non-uremic control animals with treatment. The data are compatible with bone morphogenetic protein 7 deficiency as a pathophysiologic factor in chronic renal failure, and they demonstrate its efficacy as a potential treatment of vascular calcification.

Vascular calcification (VC) is common and severe in chronic renal failure (CRF) (1), and it is predictive of both increased cardiovascular mortality and morbidity (2,3). Calcification throughout the medial vascular layer is particularly characteristic of CRF (4,5), and changes in the biophysical properties of the vasculature stemming from this (6,7) lead to reduced aortic compliance, increased cardiac afterload, and left ventricular hypertrophy (LVH), which is in turn highly predictive of mortality. The demonstration of several proteins characteristic of normal endochondral bone formation, including the critical osteoblast transcription factor CBFA1 and bone morphogenetic protein 2 (BMP-2), as well as the presence of matrix vesicles in association with VC suggests the presence of an osteoblast-like cell in the vessel wall (8,9), and it is inferred that the expression of BMP-2 is an initiating event. This concept is supported by the isolation of spontaneously calcifying clones of cells expressing a similar range of proteins from healthy aortic medial tissue in several species including humans with CRF (10,11). These osteoblast-like cells in the vasculature share many characteristics of vascular smooth muscle cells (VSMC), including morphology and the expression of α-smooth muscle actin (α-SMA), and may in fact originate from them (9), because both VSMC and osteoblasts derive from the same mesenchymal precursors, and VSMC retain sufficient pluripotentiality to transdifferentiate into osteoblasts (12–14).

Bone morphogenetic protein 7 (BMP-7), a member or the transforming growth factor–β (TGF-β) superfamily, is widely expressed during embryonic growth (15) and is an essential morphogen in renal, skeletal, and eye development (16–18). The BMP-7 null mouse dies of perinatal renal failure due to hypoplastic dysplastic kidneys. In the skeleton, BMP-7 deficiency produces a patterning defect causing extra digits and rib to be formed and leads to a deficiency of precursor cell commitment to the osteoblastic differentiation and mineralization program. In the adult, BMP-7 is expressed in the distal collecting tubule and podocytes of the kidney (19), where it maintains differentiated phenotype of tubular cells through autocrine and paracrine signaling (20). Its ongoing role in osteoblast function suggests a hormonal role, and circulating levels are detectable. BMP-7 expression is downregulated early in CRF (21–23), and the concept of CRF as a state of BMP-7 deficiency is supported by studies showing that therapy with BMP-7 is efficacious in several rodent models of renal disease (21,23–27). BMP-7 may also play a role in maintaining VSMC differentiation and preventing transdifferentiation of VSMC into an osteoblast phenotype. In one study, BMP-7 upregulated expression of α-SMA in peritubular capillaries (21), a marker of smooth muscle phenotype. Similarly, BMP-7 has been shown to preserve smooth muscle phenotype and

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reverse changes consistent with transdifferentiation into an osteoblastic phenotype in vascular smooth muscle cells *in vitro* (28). Thus, despite its role in promoting normal osteoblast function and despite the possible role of BMP, such as BMP-2 in initiating VC, we hypothesized that BMP-7 would be able to prevent transdifferentiation of smooth muscle cells into osteoblast-like cells in the vessel wall and thereby prevent vascular calcification in the context of uremia and atherosclerosis. We also hypothesized that osteocalcin (OC), regarded as a marker of osteoblast function, would be upregulated in the context of VC and downregulated by BMP-7 treatment.

We tested these hypotheses in LDL receptor null (LDLR−/−) mice, a genotype homologous to familial hypercholesterolemia in humans and a model of atherosclerosis characterized by VC (29). We imposed uremia on 10-wk-old mice by a surgical technique and started a high-cholesterol, high-fat diet to induce atherosclerosis. Control animals received standard chow and sham surgery. Animals then received BMP-7 therapy or vehicle for 15 wk. We show by histologic and chemical data that CRF in the context of hypercholesterolemia is associated with significantly increased vascular calcification and that BMP-7 therapy successfully prevents this. OC expression in vascular tissues is also upregulated in animals with CRF and hypercholesterolemia, and this change is also reversed by BMP-7 therapy. Thus, the mechanism of BMP-7 action in preventing vascular calcification is in part related to its effect of maintaining normal tissue restricted expression of factors involved in mineralization.

Materials and Methods

Animals and Diets

LDLR−/− mice in a C57Bl/6J background (gift from Dr C. Semenkovich, Division of Atherosclerosis Nutrition and Lipid Research, Washington University School of Medicine, St Louis) were maintained according to local and national animal care guidelines. Animals were weaned at 3 wk to a standard chow diet. At 10 wk, animals either continued chow or started a high-cholesterol (0.15%) diet containing 42% calories as fat (Product No.TD88137; Harlan Teklad, Madison, WI). Animals had access to water *ad libitum*.

Surgical Procedures

We used a two-step procedure to create uremia (30). Briefly, we applied electrocautery to the right kidney through a 2-cm flank incision at 10 wk and performed left total nephrectomy 2 wk later. Control animals received sham operations. Intraperitoneal anesthesia (13 mg/kg xylazine and 87 mg/kg ketamine) was used for all procedures. Femoral vein blood samples were taken 1 wk after the second surgery. We sacrificed animals under anesthesia and dissected the heart and aorta *en bloc* to the iliac arteries. For histologic analysis, heart and aorta to the arch were separated from more distal aorta; for chemical analysis, the aorta was separated from the heart at the level of the aortic valves and then further processed in its entirety.

Treatment Groups

We allocated animals to six groups: chow-fed animals received either uremic (Chow CRF) or sham (Chow Sham) surgery; high cholesterol-fed animals received uremic (Fat CRF) or sham (Fat Sham) surgery, and each of these groups received either intraperitoneal 10 μg/kg BMP-7 (Curis Inc, Cambridge MA) in 100 μl of vehicle once weekly or 100 μl of vehicle alone. Treatment began on the day after postsurgical phlebotomy.

Blood Tests

Serum was separated on the day of blood draw, stored at −80°C and assayed for blood urea nitrogen (BUN), cholesterol, and glucose by standard laboratory methods.

Tissue Preparation

For histology, we fixed resected specimens in formalin and then divided as follows, The heart and proximal aorta up to and including the arch were separated from the descending aorta and bisected sagittally through the aortic outflow tract. Five-micrometer-thick slices were stained with Alizarin Red and von Kossa. For the chemical calcification assay, we washed resected specimens in PBS, removed extraneous tissue by blunt dissection, desiccated tissues at 55°C for 18 h, and then crushed them in a pestle and mortar. Samples were stored at −80°C until assayed. For RNA extraction, we flash-froze resected tissues in dry ice/ethanol slurry and stored them at −80°C until processed.

Calcification Score

In the absence of an accepted histologic method of VC quantification, we developed a scoring system for the presence or absence of calcification. For each animal, we scored each of three slides stained with Alizarin Red for the presence or absence of calcification in two areas, the intimal and medial compartments (maximal score of three in each compartment) of the proximal aortic sections. We derived a total vascular score (maximal six) by combining the individual compartment scores for each animal. To maximize objectivity in this scoring system, no attempt was made to grade the degree of calcification; the system is thus more sensitive in differentiating between no calcification, which appears to be the effect of BMP-7 and some calcification than it is at differentiating between various degrees of calcification where present. One investigator developed the scoring system, and one investigator scored all sections in a blinded fashion. The correlation between these two scorers was >0.9. Where differences were present, an arithmetic mean was taken.

Immunohistochemistry

Sections were deparaffinized in xylene, rehydrated in graded ethanols and incubated in 3% hydrogen peroxide to block endogenous peroxidase activity and in a solution of casein in PBS (Background SNIPER; Biocare Medical, Walnut Creek, CA). For OC, antigen retrieval with 5-min incubation in citrate buffer (Decloaker; Biocare Medical, Walnut Creek, CA) at 1000°C was performed. Primary antibody (polyclonal goat anti-mouse OC; Biogenesis Inc, Brentwood, NH) was incubated overnight; slides were developed with a biotin-streptavidin-horseradish peroxidase system (all reagents: Biocare Medical, Walnut Creek, CA) then counterstained with hematoxylin. For anti–α-SMA staining, we incubated primary monoclonal antibody (Sigma, St Louis, MO) with anti-mouse Ig secondary antibody (Universal Link; Biocare Medical, Walnut Creek, CA) overnight at 40°C (31) to form immune complexes, which were applied to sections prepared as before overnight at 40°C and then processed as above.

Chemical Calcification Quantitation

Calcium was eluted from desiccated, crushed tissue in 10% Formic acid (10:1 vol/wt) for 24 h at 4°C. Assay of eluate calcium content used a Cresolphthalein complexone method (Calcium Kit; Sigma, St Louis, MO), according to manufacturers instructions and values corrected for dry tissue weight (32).

RT-PCR and Real Time RT-PCR

mRNA extraction used the RNAqueous-4PCR kit (Ambion), according to manufacturer's instructions. RT-PCR used the Onestep RT-PCR Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Murine OC primers: sense: 5'-CAAGTCCCACACACAGCAGCTT-3'; antisense:5'-AAGCCGAGCTGCCAGAGCTGCCAGAGTT-3'. Conditions: 50°C for 30 min, 95°C for 15 min, 35 to 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and then 72°C for 10 min.

Statistical Analyses

Comparisons across all groups together were performed with ANOVA; where significant trends were detected, significance was allocated to a specific group or groups by comparison to control (group 1) using Dunnett post hoc test of ANOVA. Comparisons between individual pairs of groups used *t* test. Data are presented as mean ± SD. Analyses were performed using Instat statistical software (GraphPad Software, San Diego, CA).

Results

Animals and Groups

The study design was planned to sequentially enter animals into the various groups as follows: five animals in a chow-fed sham-operated group (group 1) as controls, and ten animals in groups (groups 2 to 6) receiving uremic surgery (group 2, group 4, and group 6), high fat (groups 3 to 6) or treatment with BMP-7 (groups 5 to 6) (Table 1). When some animals in groups 2, 3, and 4 (animals 2, 3, and 1, respectively) did not survive surgery, it was decided to enter 12 animals in the final two groups (groups 5 and 6). Two animals did not survive surgery in group 5, but all animals survived in group 6. This led to uneven numbers in each group, attributable to the surgical program, but not to either the treatment regime or diet (Table 1). Approximately equal numbers of male and female animals were entered into each group. Initial weights were comparable across all groups (Table 1). Untreated uremic animals (groups 2 and 4) tended to have a smaller weight gain than untreated non-uremic animals (groups 1 and 3), but these differences were NS. Weight gain in the BMP-7–treated fat-fed sham-operated animals (group 5) was significantly greater than in other groups. Weight gain in BMP-7–treated fat-fed uremic animals (group 6) did not differ from untreated uremic animals (group 4) or chow-fed sham-operated animals (group 1). We did not observe any toxicity attributable to BMP-7 therapy.

Biochemical Data

All animals undergoing uremic surgery developed significant renal impairment, and there were no significant differences in the degree of renal failure in CRF groups (groups 2, 4, and 6) (Table 1). Renal function deteriorated during the treatment period, as evidenced by a rise in BUN between surgery and sacrifice (median, 5 mg/dl; range, −16 to +75 mg/dl); however, the increment was small and similar in magnitude to that in sham-operated animals (1 mg/dl; range, −5 to +14 mg/dl). Animals fed the high-cholesterol diet (groups 3 to 6) were significantly hypercholesterolemic compared with chow-fed animals (Table 1) and were observed to have dramatic atherosclerotic plaque formation in the proximal aorta compared to chow-fed animals. All animals had mild glucose intolerance, but this did not vary significantly across the groups.

Histology

All of the LDLR−/− animals fed the high-fat diet developed an increase in the number and size of atheroma of the proximal aorta and aortic arch. No difference was observed between male and female animals, and no effect of BMP-7

Table 1. Biochemical parameters by group[a]

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Diet	Chow	Chow	Fat	Fat	Fat	Fat
Surgery	Sham	CRF	Sham	CRF	Sham	CRF
Rx	—	—	Vehicle	Vehicle	BMP-7	BMP-7
n (males)	5 (2)	8 (5)	7 (4)	9 (4)	10 (5)	12 (8)
Pre-surgical weight	19.8 ± 3.4	22.1 ± 2.3	19.3 ± 2.6	24.4 ± 4.2	22.0 ± 1.1	20.5 ± 3.3
Sacrifice weight	24.8 ± 4.0	22.3 ± 2.1	25.0 ± 3.9	26.2 ± 5.4	34.8 ± 2.8[b]	25.8 ± 6.0
Post-surgical BUN	24.2 ± 5.2	69.8 ± 16.2[c]	21.5 ± 8.3	86.0 ± 12.1[c]	23.8 ± 5.0	63.5 ± 16.4[c]
Sacrifice BUN	23.0 ± 5.0	76.5 ± 11.0[d]	25.5 ± 6.6	88.0 ± 10.5[d]	29.0 ± 6.0	71.9 ± 21.3[d]
Total cholesterol	261 ± 28	168 ± 24	1071 ± 370[e]	940 ± 386[e]	871 ± 192	1097 ± 348[e]
Glucose	172 ± 31	183 ± 21	192 ± 23	190 ± 46	211 ± 66	197 ± 59

[a] All values are mean ± SD. BUN, cholesterol, and glucose are in mg/dl.
[b] Group 5 sacrifice weight was higher than group 1.
[c] The CRF groups 2, 4, and 6 had significantly higher post-surgical BUN than group 1 ($P < 0.01$).
[d] Groups 2, 4, and 6 had significantly higher sacrifice BUN than group 1 ($P < 0.01$).
[e] Group 3 to 6 had significantly higher cholesterol than group 1 ($P < 0.01$).

therapy on atheromas was observed. Punctate calcification was seen in the aortic intima and media in all untreated groups (Figure 1, A through D). The high cholesterol diet produced severe atherosclerosis and calcification of aortic intima and media (Figure 1, C and D). Addition of CRF (Figure 1, E and F) increased the number of punctate calcium deposits detected by the histochemical stain as shown in Figure 1E. Calcification was strikingly reduced in BMP-7-treated, fat CRF animals (group 6) (Figure 1, G and H); the extent of atherosclerosis (number and size of atheromas) was unchanged by BMP-7 treatment. Scoring of the histologic slides for the presence or absence of calcification confirmed these findings (Table 2). Total calcification scores and scores for the intimal vascular compartment were significantly higher in untreated fat CRF animals (group 4) than for chow sham animals (group 1). These scores for fat CRF animals treated with BMP-7 (group 6) demonstrated significantly less calcification than either fat CRF vehicle-treated animals (group 4) or chow sham animals (group 1). Scores for the medial compartment revealed that BMP-7–treated fat CRF animals had significantly less calcification here than either untreated fat CRF or chow sham animals (groups 4 and 1). There was a nonsignificant benefit of BMP-7 on vascular calcification in fat-fed sham animals (group 5 *versus* group 3). Our scoring system did not measure differences in size or number of calcifications among groups where calcification was present. In particular, the effect of fat to increase size of medial calcifications (Figure 1, C and D) and CRF to increase number of medial calcifications (Figure 1E) were not measured in the scoring system, but it was the most objective system for the effect of BMP-7, which was to eliminate calcification (Figure 1, G and H; Table 2). No attempt to grade the severity of the calcification was performed. Thus, specimens with a few small punctate lesions scored similarly to large areas of confluent calcification.

To assess calcification quantitatively, six additional animals were added to group 1 and groups 3 to 6 (but not chow-fed CRF animals, group 2), and the calcium content of the proximal aorta was assayed by an established chemical method.

Chemical Quantitation of Vascular Calcification

Calcification was detectable in chow sham animals (group 1) (Figure 2), and there was a nonsignificant twofold increase in fat sham animals (group 3). BMP-7 therapy (group 5) insignificantly decreased calcification in the fat sham animals. The proximal aortas of the fat CRF animals (group 4) had a significant increase in calcification, approximately sixfold over chow sham animals. BMP-7 therapy reduced calcification (group 6), and calcium levels in this group were not different statistically from chow sham animals (Group 1).

Immunohistochemistry

OC is regarded as a marker of the osteoblastic phenotype. Immunohistochemistry demonstrated low-grade baseline staining of vascular tissues in LDLR−/− chow-fed sham-operated animals (Figure 3B) compared with wild-type animals (Figure 3A). OC immunostaining was diffusely increased in fat sham (not shown) and Fat CRF animals (Figure 3C). In addition, OC staining was also noted on other tissues fortuitously present on the same slides, including atheroma plaques (Figure 3C), lung, and esophagus (not shown). In BMP-7–treated fat CRF animals, OC staining was greatly reduced in vascular (Figure 3D) and other tissues (not shown). This is in contrast to expression of α-SMA, a marker of smooth muscle cell phenotype, which remained confined to smooth muscle layers in pulmonary, esophageal (not shown). The intensity of SMA immunostaining in vascular tissues was consistent across all experimental groups (Figure 3, E through H).

RT-PCR

To confirm the finding of widespread extraosseous OC expression, we extracted RNA from the organs of 14-wk-old LDLR−/− mice fed chow or the high-fat diet for 4 wk, and performed RT-PCR.

OC mRNA was detectable in a number of organs in LDLR−/− animals and was found to be even more widely expressed in fat-fed animals (Figure 4). This confirmed the immunohistochemistry results that a heretofore-unknown component of the LDLR−/− phenotype is loss of tissue-specific expression of OC, which was restored by BMP-7 therapy.

Discussion

We show here that BMP-7 is an efficacious treatment for VC in the context of CRF and atherosclerosis, an effect that appears to be general to both medial and intimal calcification on histologic grounds. There is no current therapy for VC, which is undoubtedly an important contributor to cardiovascular morbidity and mortality in CRF; this may therefore represent a significant advance in the management of the uremic syndrome. This finding also reinforces the concept of uremia as a syndrome of BMP-7 deficiency (21–23). While this concept remains speculative, we suggest that BMP-7 may be viewed as a third renal hormone, along with erythropoietin and 1,25-dihydroxyvitamin D. The majority of adult expression of BMP-7 is renal and this protein has not been shown to be expressed locally within the vessel wall. The dose of BMP-7 used in these studies was low, and the dosing interval was relatively long. We believe that effects mediated via influences on cellular differentiation programs such as in this study are likely to require lower and less frequent dosing than conventional pharmacotherapy, because mediation of these effects may not be dependent on continuous interaction between ligand and receptor.

There were some apparent discrepancies between the two methods used here to quantify calcification. First, it appeared on histologic grounds that BMP-7 in fat-fed CRF group (group 6) was able to reduce vascular calcification to levels lower under non-uremic baseline conditions (group 1 and group 3). This is likely to reflect limitations of the scoring system, which is based simply on the presence or absence of VC, but made no attempt to grade differing degrees of VC where present, to maximize objectivity. A consequence of this is that the difference in score among samples with small amounts of calcification, such as group 1 and samples with extensive VC, such as 3 or 4, was minimized, but the difference among samples with

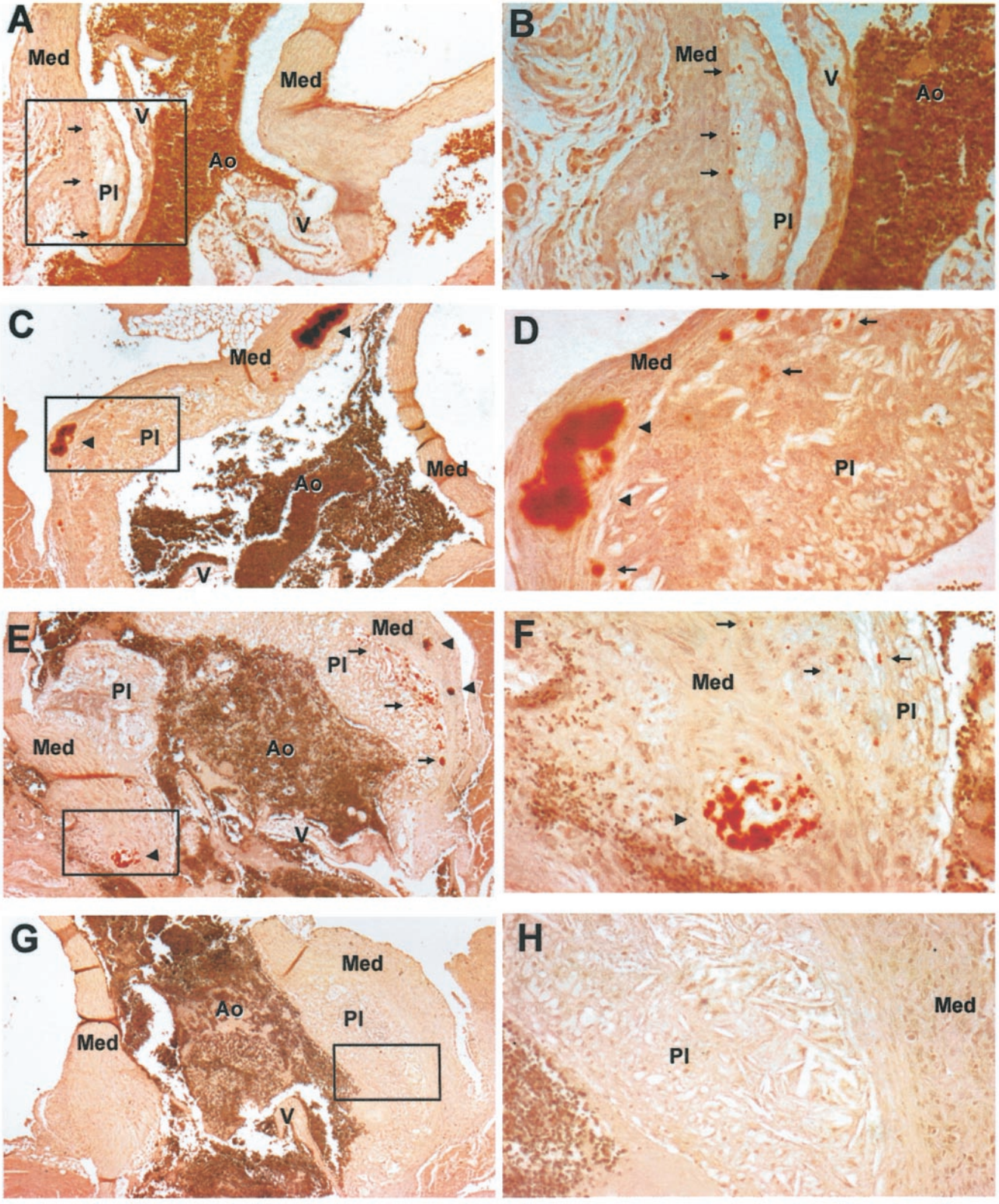

Figure 1. Effect of chronic renal failure (CRF) and bone morphogenetic protein–7 (BMP-7) treatment on proximal aortic calcification. Aortic outflow tract sections defined by two vascular walls and intervening aortic valves stained for calcification (Alizarin Red-S). Boxes in left-hand panels show area enlarged in corresponding right-hand panel. (A and B) Punctate intimal calcification in an area of plaque (arrows) in chow sham animal (group 1). (C and D) Intimal (arrows) and medial (arrowheads) calcification in fat sham animal (group 3). (E and F) Intimal (arrows) and medial (arrowheads) calcification in fat CRF vehicle animal (group 4). (G and H) Absence of intimal or medial calcification in Fat CRF BMP-treated animal (group 6). Magnifications: ×5 in A, C, E, and G; ×10 in B; ×20 in D, F, and H. Ao, aortic lumen; Pl, intimal plaque; Med, layer; Va, Valve leaflet.

Table 2. Effect of BMP-7 on proximal aortic calcification scores by group[a]

	Group1	Group2	Group3	Group4	Group5	Group6
Diet	Chow	Chow	Fat	Fat	Fat	Fat
Surgery	Sham	CRF	Sham	CRF	Sham	CRF
Rx	-	-	Vehicle	Vehicle	BMP-7	BMP-7
n	5	8	7	9	10	12
Total Score	3.6 ± 1.6	3.8 ± 1.0	4.4 ± 2.1	5.7 ± 0.4[b]	2.9 ± 2.9	0.5 ± 0.5[c]
Intimal Score	1.0 ± 1.3	1.2 ± 0.9	2.0 ± 1.3	2.8 ± 0.4[d]	1.5 ± 1.5	0.1 ± 0.2[e]
Medial Score	2.6 ± 0.4	2.6 ± 0.4	2.4 ± 1.0	2.9 ± 0.2[f]	1.4 ± 1.4	0.4 ± 0.5[g]

[a] The maximal total calcification score is six, the sum of the maximal intimal and medial scores. Three sections were analyzed from each animal for the presence (one) or absence (zero) of calcification yielding a maximal score of three for each compartment - media and intima. All values are mean ± SD, in arbitrary units (see text).
[b] Total score for group 4 is significantly higher than for group 1 ($P < 0.01$).
[c] Total score for group 6 is significantly lower than for group 1 ($P < 0.01$).
[d] Intimal score for group 4 is significantly higher than for group 1 ($P < 0.01$).
[e] In addition, the intimal score for group 6 is significantly lower than group 4 ($P < 0.0002$).
[f] Medial score for group 6 differs from group 1 ($P < 0.01$).
[g] Group 4 and 6 differ from each other ($P < 0.0003$).

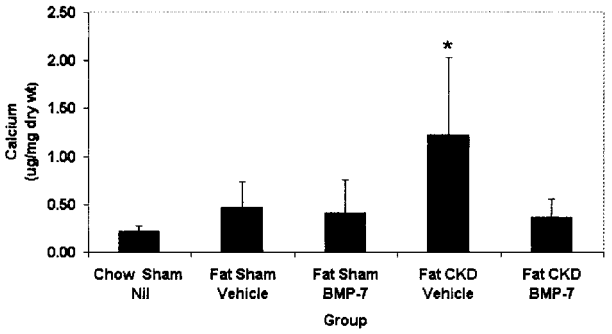

Figure 2. Chemical assessment of effect of BMP-7 on vascular calcification by treatment group. Total aortic calcium content measured in a 10% formic acid eluate of crushed. Data are mean ± SD. Trend is significant by ANOVA, $P = 0.008$. *Fat-fed uremic animals treated with vehicle have significantly higher levels than chow-fed sham controls. ($P < 0.01$, by Dunnetts post hoc test.) Fat-fed uremic animals treated with BMP-7 are indistinguishable statistically from control (chow sham animal).

small amounts of VC and nearly no VC (groups 1 and 6) was exaggerated. This scoring system is also prone to sampling errors that are difficult to quantify. These limitations led us to use the chemical quantitation assay of further samples; while this assay provides no information on the anatomical distribution of VC, it nevertheless confirms the striking effect of BMP-7 on overall VC in uremia. A further inconsistency was the apparent lack of effect of BMP-7 on VC in the absence of uremia (group 3 *versus* group 5). It is possible that the mechanisms underlying the VC in the uremic and non-uremic situations may differ, with only uremic-related disease amenable to BMP-7 therapy, but it is also possible that pharmacokinetics of BMP-7 therapy may be importantly different. First, animals in group 5 appeared to put on more weight than other groups, for reasons that are not clear (Table 1). Second, the therapy was given via the intraperitoneally route, and it has been shown that peritoneal permeability is increased in uremia (33). Third, BMP-7 is bound in the circulation to α_2-macroglobulin, which is removed from the circulation by the receptor LRP1 in the liver; it has been shown that this protein may be upregulated in the context of the LDLR−/− genotype (34), and so BMP-7 may be present for shorter periods in the context of this model. The dose we have used is a small one, and it may be that the combination of these factors has rendered the dose sub-therapeutic in non-uremic animals. Further work is needed to clarify these issues.

Given the data supporting a cell expressing an osteoblastic phenotype in the vessel wall (8–11), it may seem paradoxical that a BMP would reduce VC, given the known role of BMP-7 in osteoblast biology. However, it is clear that the effects of individual BMP are highly dependent on the target cell type, the receptors expressed, and the state of differentiation and maturity (35). In this respect, the nomenclature of these proteins may be superficially confusing. These proteins are developmental morphogens critical in a wide range of tissue differentiation. BMP-5, -6, and -7 together are critical morphogens for the heart and vasculature. Important to our results is the data presented above that BMP-7 has a positive influence on VSMC differentiation, and our data support the hypothesis that transdifferentiation of these cells is the critical first step in the etiology of VC. However, beneficial effects of therapy could be explained by other mechanisms, such as secondary benefits of improved osteodystrophy, calcium phosphate homeostasis, or nutrition, and further studies are indicated to clarify the exact mechanisms behind the benefits seen.

Important issues raised by the results of this study include the relationship between VC and renal osteodystrophy in the animals and the effect of therapy on measurable parameters of cardiovascular function such as BP and pulse wave velocity. While we do not have data at present, we hope to address these issues in future studies.

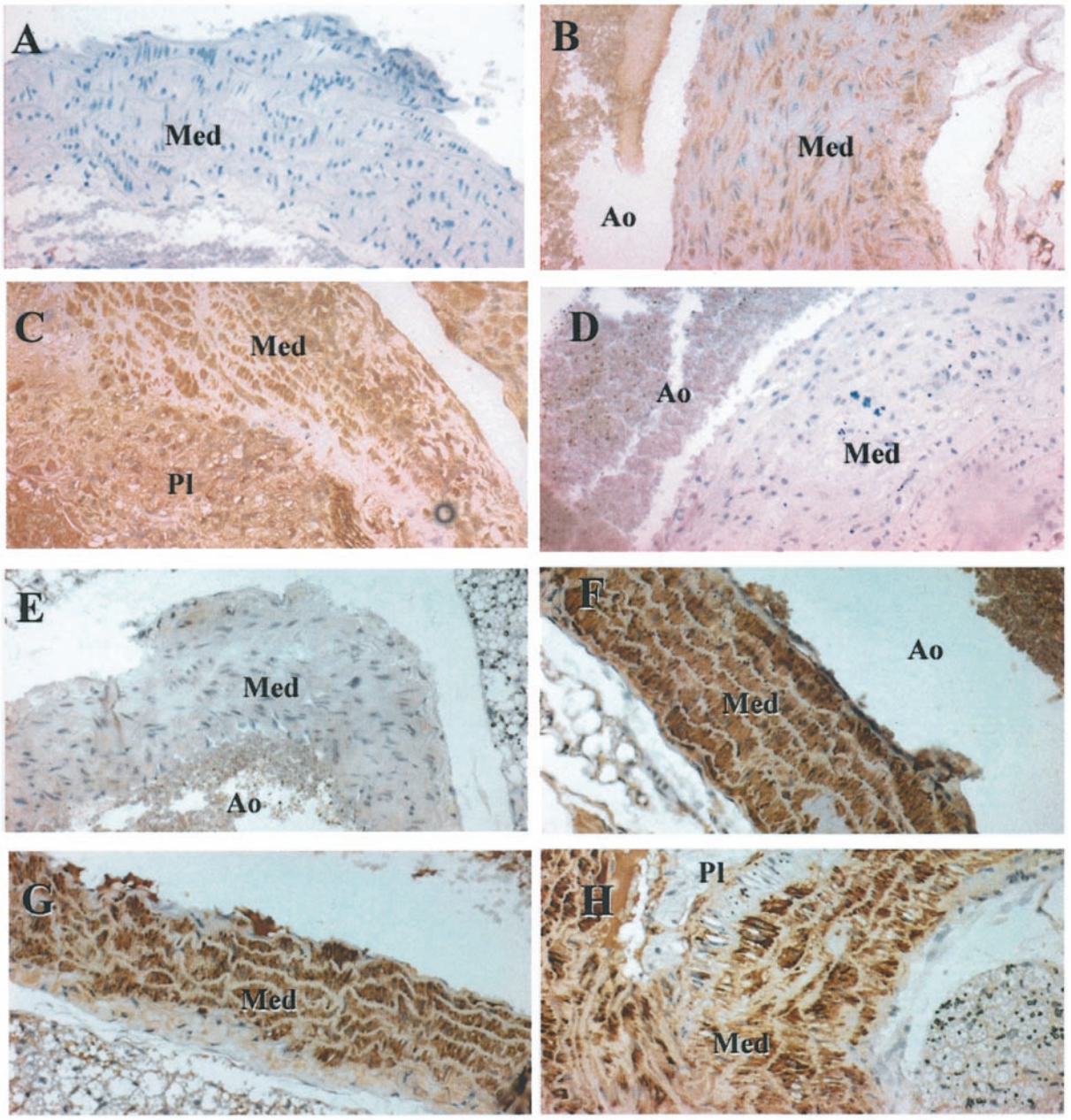

Figure 3. Effect of BMP-7 on osteocalcin (OC) expression. Immunohistochemistry of proximal aortic sections for OC (A through D) and α-smooth muscle actin (E through H). (A) Negative control (vascular media of wild-type mouse); (B) OC expression (brown staining) in medial layer of a chow sham animal; (C) more intense medial and plaque OC staining in a fat CRF animal; (D) striking reduction in medial and plaque staining for OC in a fat CRF BMP-7-treated animal; (E) negative control for α-smooth muscle actin. (F through H) Consistently intense SMA staining in the medial layer of chow sham (F), fat CRF (G), and fat CRF BMP-7–treated animals (H). Magnification, $\times$20 in all panels. Med, medial layer; Pl, intimal plaque; Ao, aortic lumen.

The study of VC *in vivo* has been hampered by the lack of adequate animal models because rodents are generally resistant to VC. However, the C57/Bl6 strain of mouse has been documented to develop spontaneous VC in up to 25% of observed animals on a high-fat, high-cholesterol diet (36), and VC was consistently seen in LDRL$-/-$ mice in this background (29). This seemed the most appropriate model to investigate VC in CRF. The surgical technique used is analogous to 5/6 nephrectomy model in rats (30) and was previously used to model renal osteodystrophy (37). In contrast to other models of CRF, it is relatively stable over time; it is debatable whether the small but statistically significant increase in BUN during the study is clinically significant, because a similar increase was seen in sham-operated animals. It is also evident that atherosclerotic plaque formation is dependent on dietary manipulation; nevertheless, the diet used is similar in content to a Western high-fat diet, containing 0.15% cholesterol wt/wt. BMP-7 therapy had no effect on extent of atherosclerosis. Despite its

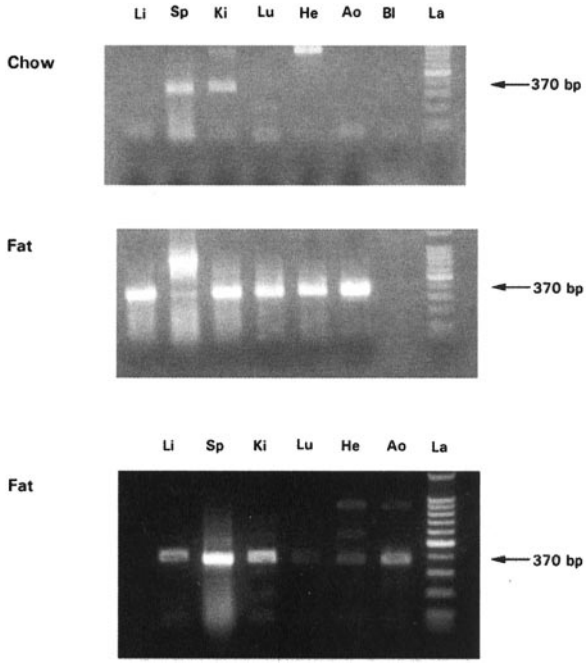

Figure 4. RT-PCR demonstrates OC mRNA at extraosseous sites. (Top panel) Samples from a chow-fed LDLR−/− mouse showing OC product at 370 bp in spleen and kidney samples. (Middle and bottom panels) Samples from two cholesterol-fed LDLR−/− mouse showing OC product at 370 bp in liver, spleen, kidney, lung, heart, and aorta. Li, liver; Sp, spleen; Ki, kidney; Lu, lung; He, heart; Ao, aorta; Bl, blank; La, 100-bp ladder.

complexities, this model is relatively simplistic compared with the human clinical setting, where additional factors contributing to VC include medications, such as Vitamin D analogues and calcium-containing phosphate binders, as well as the effects of a high dietary phosphate (9).

We chose to study OC because it is a marker of the osteoblastic phenotype, and we had anticipated that immunohistochemistry (IHC) would display local expression in relation to areas of calcification. The finding of widespread expression on IHC was unexpected. However, analysis of RNA from various tissues of LDLR−/− mice (Figure 4) supported the IHC findings. We observed that OC expression was increased in fat CRF LDLR−/− animals, in parallel with increased calcification, and that both OC expression and VC are strikingly suppressed by BMP-7 treatment. We conclude that interpreting OC expression as a specific marker of OB function is overly simplistic. We believe these findings are consistent with the concept of OC as an endogenous limiter of calcification and extend its role beyond bone to include other tissues, including but not limited to the vasculature, with upregulation occurring under certain circumstances such as the pro-calcific milieu of CRF, LDLR deficiency, and high-fat diet. Established data support this interpretation. In the mouse, OC exists as a cluster of three genes, OC1, OC2, and OC-related gene, or ORG (38). It has been shown by other methods that OC1 is strongly expressed in bone and that OC2 and ORG, which is synonymous with nephrocalcin, are expressed elsewhere albeit at lower levels (38). High structural homology between these proteins is consistent with functional homology, and they may be regarded as isoforms. All three proteins bind to calcium via gamma carboxylated residues and act to limit the size of calcium crystal formation. The OC null mouse is characterized by an enlarged though otherwise normal skeleton with increased bone formation rate (39); nephrocalcin expression in the kidney tubule serves to limit renal stone formation (40). Their high structural homology also makes it impossible to distinguish between the three gene products by IHC or RT-PCR (38). We believe that our data show an increase in expression of extraosseous isoforms of OC in the face of the LDLR−/− genotype and a pro-calcific environment caused by a combination of hypercholesterolemia, atherosclerosis, and CRF, and that BMP-7 ameliorates that pro-calcific tendency, leading to downregulation of this response. In this analysis, calcification of the vasculature in CRF represents a special circumstance, homologous to endochondral bone formation, where pro-calcific tendencies orchestrated by osteoblasts or osteoblast-like cells outweigh local anti-calcific mechanisms represented by OC and related proteins.

In summary, we show for the first time that BMP-7 is an effective treatment of VC in the context of a murine model of atherosclerosis and CRF, a finding that may have important implications for the development of future therapies for this condition in humans both with and without uremia, because this is a condition currently without treatment and with strong negative influences on mortality.

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