http://www.kidney-international.org

The mechanisms of uremic serum-induced expression of bone matrix proteins in bovine vascular smooth muscle cells

NX Chen[1], D Duan[1], KD O'Neill[1], GO Wolisi[1], JJ Koczman[1], R LaClair[1] and SM Moe[1,2]

[1]*Indiana University School of Medicine, Indianapolis, Indiana, USA and* [2]*Richard L Roudebush VAMC, Indianapolis, Indiana, USA*

We have previously found that uremic human serum upregulates RUNX2 in vascular smooth muscle cells (VSMCs), and that RUNX2 is upregulated in areas of vascular calcification *in vivo*. To confirm the role of RUNX2, we transiently transfected a dominant-negative RUNX2 (ΔRUNX2) construct in bovine vascular smooth muscle cells (BVSMCs). Blocking RUNX2 transcriptional activity significantly decreased uremic serum induced alkaline phosphatase (ALP) activity (268 ± 34 vs 188 ± 9.5 U/g protein, $P < 0.05$) and osteocalcin expression (172 ± 17 vs 125 ± 9 ODU, $P < 0.05$). To determine the mechanism by which uremic serum upregulates RUNX2, we examined cell signaling pathways. BVSMCs were incubated in the presence or absence of inhibitors and RUNX2 expression and ALP activity were determined. The results demonstrate that the cyclic AMP (cAMP)/protein kinase A (PKA), but not protein kinase C, signaling pathway is involved in uremic serum-induced RUNX2 expression and ALP activity in BVSMCs. To examine potential uremic 'toxins', we measured bone morphogenetic protein (BMP)-2 concentration and found that uremic serum contained increased BMP-2 (uremic serum = 169 ± 33 pg/ml, normal serum = 117 ± 15 pg/ml, $P < 0.05$). The incubation of BVSMCs with noggin, an inhibitor of BMP, decreased RUNX2 expression. In addition, BMP-2 secretion progressively increased during calcification and uremic serum enhanced its secretion compared to normal serum. In conclusion, this study demonstrates that RUNX2 transcriptional activity is critical in uremic serum-induced bone matrix protein expression in BVSMCs and that the cAMP/PKA pathway is involved. BMP-2 is also increased in uremic serum and can upregulate RUNX2 and calcification *in vitro* in VSMCs.

Kidney International (2006) **70,** 1046–1053. doi:10.1038/sj.ki.5001663; published online 12 July 2006

KEYWORDS: core binding factor α1 (RUNX2); bone morphogenetic protein 2 (BMP-2); vascular calcification; chronic kidney disease (CKD)

Correspondence: *NX Chen, Indiana University School of Medicine, 1001 West 10th Street, WD, OPW 526, Indianapolis, Indiana 46202, USA. E-mail: xuechen@iupui.edu*

Received 11 January 2005; revised 23 March 2006; accepted 18 April 2006; published online 12 July 2006

Cardiovascular disease and stroke are the leading causes of death in patients on dialysis, at a risk that is 10–20-fold the age- and sex-matched general population.[1] Vascular calcification, in both the intima and media, is also more prevalent and severe in patients with chronic kidney disease (CKD), and is associated with increased morbidity and mortality (reviewed by Moe and Chen[2]). For many years, vascular calcification in CKD patients was thought to occur predominantly by unregulated, purely physiochemical mechanisms, as disorders of bone and mineral metabolism are common in CKD. However, recent data demonstrate that vascular calcification in CKD patients is a regulated process.

Important work in the 1990s demonstrated the presence of bone proteins in areas of calcification in pathologic specimens from both coronary and peripheral arteries.[3–7] Furthermore, it was demonstrated that vascular smooth muscle cells (VSMCs) isolated from human or bovine arteries were capable of mineralizing *in vitro*[3,8] in a similar manner to osteoblasts.[9] We have confirmed these findings in arteries from dialysis patients, demonstrating the presence of 'bone' proteins such as osteopontin, bone sialoprotein, type I collagen, and alkaline phosphatase (ALP), and even an osteoclast-like cell, in arteries from patients with CKD.[10–12] These data confirm a cell-mediated, osteogenic process in vascular calcification in CKD patients on dialysis, similar to that in non-CKD patients, and suggest that VSMCs can phenotypically behave like osteoblasts.

Mesenchymal stem cells are capable of differentiating into osteoblasts, chondrocytes, adipocytes, and myoblast/smooth muscle cells by expression of one set of transcription factors and repression of another. Selective deletion of RUNX2 in mice leads to a failure to form mineralized bone by either endochondral or intramembranous ossification,[13–15] confirming the critical role of RUNX2 in osteoblast differentiation. One of the initial markers of osteoblast differentiation is the upregulation of RUNX2 followed by the expression of matrix proteins, and then mineralization, the former considered the best marker of the differentiated osteoblast. In addition to RUNX2, bone morphogenic proteins are also important early in mineralization and are known to upregulate RUNX2 expression.[15,16] Exogenous bone morphogenetic protein (BMP)-2 implanted into animals can induce

extraskeletal calcification.[17] However, there are several BMPs with some redundancy in activity such that targeted deletions of single BMPs does not cause phenotypic alterations in bone mineralization.

Jono *et al.*[18] first demonstrated that exogenous phosphate induced the expression of RUNX2 and calcification of human VSMCs. We have also demonstrated that the expression of RUNX2 and its downstream proteins osteopontin and type I collagen was found in both the medial and intimal layer of calcified arteries obtained from patients undergoing renal transplantation.[19] Similar findings were observed in arteries from non-CKD patients.[20] Furthermore, pooled serum from patients undergoing dialysis (uremic serum) upregulated RUNX2 and its downstream proteins osteopontin and ALP activity in bovine vascular smooth muscle cells (BVSMCs) compared to serum from non-CKD (normal) individuals,[19,21] regardless of the phosphorus concentration. Uremic human serum also leads to accelerated and increased calcification in BVSMCs *in vitro* compared to normal serum.[21] Taken together, these findings suggest that RUNX2 may be a key regulatory factor in the vascular calcification of CKD, with VSMCs transforming to osteoblast-like cells in response to uremic toxins. There are many potential uremic toxins that, individually, have been shown to induce calcification *in vitro* such as oxidized proteins, phosphorus, lipids, parathyroid hormone-related peptide, and calcitriol (reviewed by Moe and Chen[2]). However, it is most likely that a combination of factors in uremic serum is responsible for the upregulation of RUNX2. The purpose of the present study was to (1) confirm the direct role of RUNX2 in bone matrix protein expression, (2) determine the cell signaling mechanism by which uremic serum upregulates RUNX2 in BVSMCs, and (3) examine the role of bone morphogenic proteins in the upregulation of RUNX2.

RESULTS

Role of RUNX2 in uremic serum-induced 'bone' protein expression from BVSMCs

As detailed in the Materials and Methods, this RUNX2 DNA-binding domain (ΔRUNX2) has been well characterized by Ducy *et al.*[22] and has no transactivation ability on its own. We first examined ALP activity in dominant-negative RUNX2 (ΔRUNX2) transiently transfected or empty vector and non-transfected (control) BVSMCs treated with human normal or uremic serum for 72 h. As shown in Figure 1a, uremic serum significantly increased ALP activity in non-transfected BVSMCs compared to normal serum (normal-134 ± 10 U/g protein; uremic $= 268 \pm 34$ U/g protein, $P < 0.05$). Transient transfection with ΔRUNX2 significantly decreased uremic serum-induced ALP activity in BVSMCs (268 ± 34 vs 188 ± 9.5 U/g protein, $P < 0.05$), but had no effect on cells treated with normal serum (Figure 1a). Transfection with the empty vector control (pEFBOS) had no effect on ALP activity in BVSMCs treated with normal or uremic serum (Figure 1a). Furthermore, uremic serum, but not normal serum, significantly increased the expression of the osteoblast marker osteocalcin, whereas ΔRUNX2 transfection significantly reduced uremic serum-induced osteocalcin expression in BVSMCs (Figure 1b). In contrast, transfection with the empty vector had no significant effect on osteocalcin expression in BVSMCs treated with normal or uremic serum (Figure 1b). These results suggest that RUNX2 is critical for uremic serum-induced transformation of BVSMCs to osteoblast-like cells, capable of expression of 'bone' proteins.

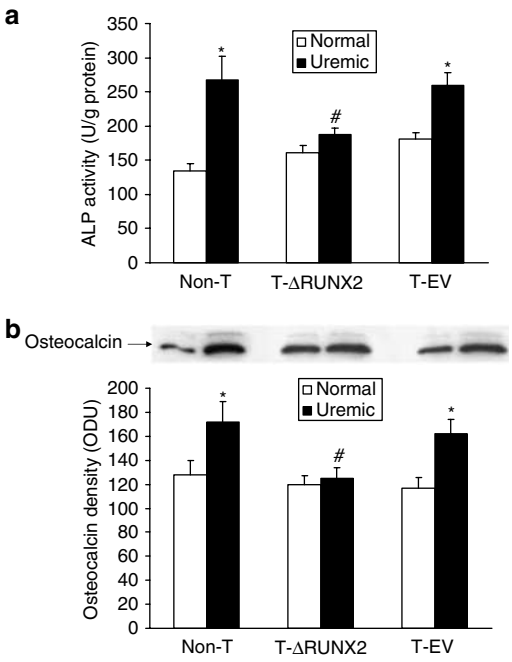

Figure 1 | ALP activity and osteocalcin expression in BVSMCs treated with normal or uremic serum after transient transfection with the dominant-negative RUNX2 (ΔRUNX2) construct. (**a**) ALP activity and (**b**) osteocalcin expression in BVSMCs treated with normal or uremic serum after transient transfection with the dominant-negative RUNX2 (ΔRUNX2) construct. BVSMCs were transfected with a dominant-negative RUNX2 or with pEF-BOS empty vector as a negative control. After transfection, cells were incubated in the presence of 10% normal serum or uremic serum for 72 h. Cellular proteins were solubilized and supernatants were assayed for ALP activity normalized to **(a)** cellular protein content or **(b)** osteocalcin expression by Western blotting. Data are shown as mean ± s.d. from three experiments. Non-T: non-transfected; T: transfected; EV: empty vector. $^{*}P < 0.05$, uremic vs normal; $^{\#}P < 0.05$, uremic/NT vs uremic/T.

Cell signaling pathways

To examine the potential signaling mechanism involved in uremic serum-induced expression of RUNX2 and ALP activity in BVSMCs, cells were treated with normal or uremic serum in the presence or absence of protein kinase A (PKA) inhibitor KT5720 (10 μmol/l), an inhibitor of cyclic AMP (cAMP) production SQ22536 (500 nmol/l), or protein kinase C (PKC) inhibitor GF10923X. Uremic serum significantly increased RUNX2 expression by 85% in BVSMCs at 48 h. Inhibition of PKA activity by KT5270 blocked uremic serum-induced RUNX2 expression ($P < 0.05$; Figure 2a), but had no effect on BVSMCs treated with normal serum