Translational Nephrology

Calcification and the usual suspect phosphate: still guilty but there are other guys behind the scenes

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In patients with renal failure, the high prevalence of vascular, valvular and soft-tissue calcifications and their consequences for cardiovascular outcomes have recently received much attention. Several studies documented that the calcification burden is associated with increased morbidity and mortality in uraemia. In vitro and in vivo research has demonstrated that tissue calcification is not just based on passive calcium and phosphate precipitation, but that active cellular processes such as osteogenic differentiation of vascular smooth muscle cells (VSMC) are involved and that a number of local and systemic calcium-regulatory factors control and prevent unwanted extra-osseous calcification. An important finding was the new understanding that calcium and phosphate are immediate inducers of osteogenic VSMC differentiation and that this particular calcification process starts intracellularly [1,2]. In addition, various extracellular calcification inhibitors such as fetuin-A, matrix Gla protein (MGP) and pyrophosphates were identified via genetic manipulation of mice [3]. Of these, so far only fetuin A deficiency has been found to be associated with increased mortality, cardiovascular calcifications and calciphylaxis in uraemic patients [4–6].

Given that various studies identified serum phosphate levels as a major predictor of mortality in dialysis patients [7,8], its role in the development of calcification remains at the center stage of research. A recent groundbreaking study by Murshed et al. has addressed this particular issue in an admirably comprehensive experimental approach [9]. They set out to clarify the interplay of (a) extracellular phosphate, (b) pyrophosphate, a small molecule made of two phosphate ions that prevents incorporation of inorganic phosphate into nascent hydroxyapatite crystals and (c) tissue non-specific alkaline phosphatase (Tnap), that can degrade pyrophosphate. In this study, the following models were used:

- Primary murine osteoblast cell cultures;
- Knockout mice for MGP (MGP−/−; a local vitamin K-dependent calcification inhibitor expressed in VSMCs), Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (Enpp1−/−; the rate-limiting enzyme of pyrophosphate synthesis), ANK (Ank−/−; a specific transmembrane transporter protein for shuttling pyrophosphates into the extracellular environment), and PHEX (Hyp; a mutant hypophosphataemic mouse strain resembling X-linked hypophosphataemic rickets);
- Constructs for transgenic overexpression of the tissue non-specific alkaline phosphatase subcloned downstream of a dermis-specific α2(I) collagen promoter-enhanced fragment (facilitating collagen I co-expression, i.e. the major extracellular matrix (ECM) protein in bone);
- Phosphate-modified diets.

These approaches were used in distinct combinations in order to explore the ‘hierarchy’ of factors such as calcium, phosphate, ECM composition and calcification inhibitors in their involvement in the regulation of osseous and extra-osseous calcification.

In the first in vitro approach, the authors determined that the availability of phosphate (but not calcium) was the key contributor to osteoblast-dependent calcification. This finding was subsequently underlined by in vivo experiments in Hyp mice showing that the presence of hypophosphataemia led to severely decreased bone mineralization. This, in turn, could be rescued by feeding a high-phosphate diet. In parallel, an intrinsic defect of Hyp osteoblasts was excluded, because these cells did not react differently to phosphate exposure than wild-type osteoblasts in vitro. Take home message number 1: Extracellular phosphate availability rather than calcium regulates bone mineralization.

The authors next probed whether phosphate might be of similar importance for vascular and extra-osseous calcification. In support of this, they demonstrated that the lethal phenotype of severe aortic calcification in MGP−/− mice could be completely prevented by mating them with Hyp mice, i.e. by creating a...
hypophosphataemic environment. Similarly, spontaneous periarticular and inducible vascular calcifications in pyrophosphate-deficient Ank$^{-/-}$ mice were prevented by mating them with hypophosphataemic Hyp mice. Vice versa, in both pyrophosphate deficient Enpp$^{-/-}$ and Ank$^{-/-}$ mice, which under baseline conditions do not develop a vascular phenotype, severe vascular calcifications were observed following exposure to a high-phosphate diet. Vascular calcification was not observed in the appropriate wild-type controls fed phosphate.

**Take home message number 2:** Extracellular phosphate is a key inducer of extraosseous calcifications, in particular when its antagonist pyrophosphate is not around. In this respect, it is important to note that dialysis patients exhibit low serum pyrophosphate levels and that these are lowered further during a haemodialysis session [10].

Up to this point there is still one unresolved question: phosphate, pyrophosphate and Tnap are very widely distributed throughout the body, yet why does calcification develop in distinct patterns and why is it physiologically limited to bones? Murshed and colleagues [9] reasoned that type I collagen may be such a spatial regulator, since the only location, where Tnap and type I collagen chains are coexpressed, are bones and teeth. To test this hypothesis, they created mice that overexpressed membrane-bound (non-circulating) Tnap in the dermis under the control of a dermis specific α2(I)collagen promoter-enhancer fragment. By thus degrading pyrophosphate locally into phosphate plus providing the ‘right’ matrix, they indeed induced dermal calcification. In contrast, if Tnap was overexpressed in the adjacent keratinocytes, which do not release type I collagen, no calcification ensued. **Take home message number 3:** The localization of calcifications depends on the combined presence of sufficient amounts of phosphate, low amounts of pyrophosphate (for example due to high local Tnap activity) and the correct matrix consisting of type I collagen (Figure 1). In this respect, it is important to note that uraemic patients are characterized by a state of ‘advanced ageing’, which includes widespread increases in fibrotic tissue, for example in the heart [11].

The paper by Murshed et al. is an outstanding example of how by systematic permutation of pathophysiologically important factors, key mechanisms can be identified [9]. Certainly, their model is not comprehensive and important players such as local pH, magnesium (a crystal formation inhibitor), fetuin A or MGP have not yet been factored into the equation. However, the study of Murshed et al. nicely illustrates how certain events must come together to allow calcification to develop. While the work, lends further support to counteracting high phosphate levels in patients with renal failure, it also stresses, that a number of key events occur outside of the plasma and that we need a more comprehensive ‘alarm system’ of biomarkers to assess the calcification risk in uraemic patients. For example, we have recently cautioned that an apparently well controlled calcium × phosphate

<table>
<thead>
<tr>
<th>Serum phosphate</th>
<th>Tissue nonspecific alkaline phosphatase</th>
<th>Type I collagen</th>
<th>Calcification inhibitors</th>
<th>Calcification</th>
<th>Typical tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Absent (high PPi)</td>
<td>Absent</td>
<td>Absent/low</td>
<td>−</td>
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<td>Normal</td>
<td>Absent (high PPi)</td>
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<td></td>
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<td>Absent</td>
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<td>Present</td>
<td>Present</td>
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<td>nl bone</td>
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<td>High</td>
<td>Absent (high PPi)</td>
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<td></td>
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<td>Present</td>
<td>Absent/low</td>
<td>+</td>
<td>nl bone</td>
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<tr>
<td></td>
<td>Present (low PPi)</td>
<td>Absent</td>
<td>Absent/low</td>
<td>++</td>
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</tbody>
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Fig. 1. Schematic illustration of the combined roles of extracellular phosphate, pyrophosphate, tissue non-specific alkaline phosphatase (Tnap) and type I collagen in the development of calcifications.
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product in the presence of high inflammatory activity may herald massive ongoing calcium and phosphate precipitation in tissue rather than a safe clinical situation [12]. Until we have developed and refined such an alarm system, unwanted calcification will continue to be an important complication of renal failure.

Conflict of interest statement. None declared.

References

11. Amann K, Ritz E. Cardiovascular abnormalities in ageing and in uraemia—only analogy or shared pathomechanisms? Nephrol Dial Transplant 1998; 13 [Suppl 7]: 6–11

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