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COMMENTARY

Untangling the thread of life spun by αKlotho

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Since its serendipitous discovery several decades ago as a putative anti-ageing factor [1], αKlotho (αKl)—named after one of the three Fates from Greek mythology who *spins the thread of life*—has been implicated in a disparate array of homeostatic processes encompassing effects on mineral metabolism, cytoprotective functions, regenerative/self-renewal pathways, in addition to being an endogenous antagonist of growth factor signalling and a tumour suppressor. Most established is its function as one obligate component of the receptor complex for fibroblast growth factor 23, an osteocyte-derived hormone that regulates mineral ion and vitamin D homeostasis in the kidney. That αKl is not only manifestly deficient in the setting of acute and chronic renal impairment but seemingly also lost so early on in course of disease has raised the prospect that it may be amenable to restorative therapies. So far, a number of studies have addressed this possibility (recently reviewed in [2]); initially with proof-of-concept interventions using forced overexpression vectors, to more translatable strategies: administering exogenous recombinant peptide or pharmaceuticals that enhance endogenous expression by targeting so-called epigenetic and non-epigenetic mechanisms of suppression.

In this issue of the *Journal of Molecular Medicine*, Bi et al. extend their investigations of 1,8-dihydroxy-3-carbonyl anthraquinone (Rhein) [3], a monomeric anthraquinone derivative isolated from the rhubarb plant extensively used in Chinese traditional medicine, as one such restorative therapy for endogenous αKl. In their present exposition, Bi et al. elucidate a novel pathway by which restoration of endogenous αKl expression with Rhein in mice with LPS-induced acute kidney injury (AKI), counter-regulated the induction of Toll-like receptor 4 (TLR4) by LPS, prompting TLR4 internalisation, degradation, and suppression of downstream NF-κB signalling, resulting in markedly attenuated inflammation and renal injury. Potentially, this schema provides insight into novel anti-inflammatory functions of αKl but also a deeper mechanistic understanding of how Rhein might work. Nonetheless, while compelling, several aspects of the report need to be appraised carefully.

First, although renal tubular epithelial cells are viewed as the major physiological site of αKl production and action, Bi et al. implicate αKl secreted from infiltrating macrophage as the critical negative regulator of innate inflammatory signaling in this context. While this is consistent with other studies reporting expression of αKl (albeit only at the transcript level) in cells of the myeloid lineage in vitro, and further adds to an expanding body of literature that portends a much wider expression of αKl than originally envisioned, there is as yet scant evidence that these cells contribute regulatory effects in vivo. Indeed, in the present study, αKl expression was only demonstrable in cultured macrophage and not in mice and consequently the role of αKl in the macrophage remains somewhat ambiguous. Surprisingly, Bi et al. readily identified αKl in several monocyte-macrophage cell lines usually considered to have negligible endogenous expression: in the case of RAW264.7 and THP-1 cells this included both full-length (130 kDa) and supposedly "secreted" αKl (~65 kDa) isoforms. The identification of secreted αKl is especially troublesome, given the known specificity issues of the available αKl antibodies for lower molecular weight species (i.e. 60–70 kDa) [4], and quite convincing reports showing that the alternatively spliced mRNA encoding the truncated secreted isoform is not translated in humans but a target for nonsense-mediated mRNA decay [5]. Indeed, although repeatedly referred to as the "secreted" isoform by Bi et al., tangible evidence for secretion in this and other studies is lacking. In reality, the only soluble αKl isoform present is likely to be the cleaved ectodomain of αKl that is shed from its plasma membrane tethers by the action of ADAM10 and ADAM17.

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Second, how does Rhein restore αKl expression in this model? Although not explicitly addressed in the current article, clues come from previous work by the same group, where in mice with Chronic Kidney Disease (CKD), Rhein was found to correct the aberrant expression of DNA methyltransferases (DMTs) that are responsible for the suppressive hypermethylation of the αKl promoter [6]. Whether this holds true in this AKI model is untested, but it seems reasonable to suspect a similar mode of action through changes in DMTs. While this attracts the now fashionable moniker of an "epigenetic" modulator, with tacit reference to alteration in a higher level of information beyond the genome, it remains unproven that changes in αKl promoter methylation reflect a persistent change in cell fate to fulfil the classical definition of epigenesis. A more accurate, but less en vogue, description is that Rhein alters αKl expression through transcriptional regulation.

Third, it was suggested that the resultant boost in αKl levels blocked LPS-priming through deglycosylation of TLR4. To account for this, Bi et al. invoke an argument based on the supposed glycosidase activity of soluble αKl. This aspect of the study is especially controversial since αKl is now known to be devoid of such intrinsic enzymatic activity. Recent breakthrough studies by Chen et al. [7] show that the crystal structure of αKl is incompatible with it having significant glycosidase activity since both KL domains of the protein, which share homology with type I glycosidases, not only lack key catalytic residues but have active sites inaccessible to substrate or a configuration with very low binding affinity. Indeed, Chen et al. found that the purified αKl ectodomain failed to hydrolyse substrates for sialidase or β-glucuronidase [7]. Experimentally, Bi et al. convincingly confirm involvement of glycosidase activity as indicated by the nullifying effect of co-treatment with a β-glucoronidase inhibitor, as well as showing the loss of α2–6-linked sialic acid residues from TLR4, strongly implicating TLR4 deglycosylation in the process. Nevertheless, the only evidence of a physical interaction between αKl and TLR4 comes from co-immunoprecipitation studies in immortalised murine macrophage and HEK293 cells engineered to overexpress αKl; a procedure known to be fraught with false positive signals. In truth, the assertion that it is the intrinsic sugar-cleaving activity of αKl acting on TLR4 seems largely based on earlier reports of such activity against other targets. Given the current state of knowledge, these findings can only be rationalised by the involvement of another, bone fide glycosidase, acting downstream of αKl. One other possibility accounting for the apparent interaction with TLR4 is that αKl—while not acting as sugar-cleaving enzyme—may retain some residual sugar binding functionality as appears to be the case for binding to monosialoganglioside-containing lipid rafts [8]; however, whether such binding is compatible with the now defined atomic structure of the protein has yet to be determined.

In aggregate, further experimentation will be needed to tease out the role of additional players in this schema and in order to pin down precisely how reactivation of αKl by Rhein might work. Nonetheless, a consistent message emerges from this, and related studies, which is that therapeutic enhancement of αKl looks to hold promise for tackling AKI-to-CKD progression and accompanying cardiovascular sequelae and we await the next steps to see whether we can truly refill the fountain of youth.

A more fundamental question arises, however, which is that by restoring αKl what are we actually doing? Superficially, it would appear beneficial to have more of a protective factor like αKl, but from a teleological perspective it is difficult to reconcile why such a protective factor would be downregulated in the first place; at a time when it was needed the most. Indeed, αKl deficiency in the context of both AKI and CKD is not due to loss of functioning nephrons but primarily through a rapid suppression of expression induced by a host of injury-evoked inflammatory cytokines, reactive oxygen species, uraemic toxins and sustained activation of the renin-angiotensin-aldosterone system; notably some of the very same injurious pathways supposedly counteracted by αKl. While few would decry the impressive cytoprotective effects of adding exogenous αKl in culture, what often appears forgotten is that the human ageing-like phenotype of the *kl/kl* hypomorphic or *klotho* knockout mice can be entirely rescued by addressing the disturbances in mineral metabolism that result (e.g. hyperphosphataemia) [9]. Indeed, there is ample evidence to suggest that phosphate or its mediators are the driver of the pathologies for which αKl is protective. Thus, it remains open to debate whether the benefit of restoring αKl in kidney disease mediates its effects through pathways that are truly independent of mineral regulation or rather by reinstating physiological signalling networks and phosphate balance.

Compliance with ethical standards

Conflict of interest The author declares that there is no conflict of interest.

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