VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Klotho Deficiency Induces Arteriolar Hyalinosis in a Trade-Off with Vascular Calcification

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Hyalinosis is a vascular lesion affecting the renal vasculature and contributing to aging-related renal function decline. We assessed whether arteriolar hyalinosis is caused by Klotho deficiency, a state known to induce both renal and vascular phenotypes associated with aging. Histochemistry was used to assess hyalinosis in Klotho^{-/-} kidneys, compared with Klotho^{+/-} and wild-type littermates. Immunohistochemistry was used to investigate vascular lesion composition and the different layers of the vascular wall. Finally, spironolactone was used to inhibit calcification in *kl/kl* mice, and vascular lesions were characterized in the kidney. Arteriolar hyalinosis was detected in Klotho^{-/-} mice, which was present up to the afferent arterioles. Hyalinosis was accompanied by loss of α -smooth muscle actin expression, whereas the endothelial lining was mostly intact. Hyalinous lesions were positive for IgM and iC3b/c/d, indicating subendothelial leakage of plasma proteins. The presence of extracellular matrix proteins suggested increased production by smooth muscle cells (SMCs). Finally, in Klotho^{-/-} mice with marked vascular calcification, treatment with spironolactone allowed for replacement of calcification by hyalinosis. Klotho deficiency potentiates both endothelial hyperpermeability and SMC dedifferentiation. In the absence of a calcification-inducing stimulus, SMCs assume a synthetic phenotype in response to subendothelial leakage of plasma proteins. In the kidney, this results in arteriolar hyalinosis, which contributes to the decline in renal function. Klotho may play a role in preventing aging-related arteriolar hyalinosis. (*Am J Pathol* 2019, 189: 2503–2515; <https://doi.org/10.1016/j.ajpath.2019.08.006>)

Arteriolar hyalinosis, or hyaline arteriosclerosis, is a vascular lesion often found in the kidney in aging and hypertension,¹ or caused by calcineurin inhibitor use after transplantation.^{2,3} It consists of accumulated hyaline material, which is thought to be the result of subendothelial leakage of plasma proteins^{4,5} and of subsequently increased extracellular matrix production by smooth muscle cells (SMCs).⁶ In the kidney, the afferent arterioles are the most commonly affected vessels, likely because of their role of major resistance arterioles, placing great local stress on the endothelium. It is thought that hyalinosis of the afferent arterioles leads to loss of autoregulation, which, in turn, leads to glomerular damage and the decline of renal function.^{7,8}

We determined whether murine Klotho deficiency, as a model for premature aging that also leads to a spontaneous decrease in renal function and develops sequelae of chronic renal failure, is affected by hyalinosis as well. Klotho is a

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renal antiaging protein that is predominantly expressed in the distal convoluted tubule.⁹ Its marked protective effects on the kidney^{10–21} and on the vasculature^{22–26} are intricately interrelated, involving both direct effects on renal and vascular cells and indirect effects via the regulation of phosphate^{27–29} and calcium^{30–32} homeostasis. In mice, *Klotho* deficiency leads to a renal phenotype of albuminuria, elevated serum creatinine and urea nitrogen levels,³³ both interstitial and glomerular fibrosis,^{33,34} and mesangial matrix expansion.³⁵ Furthermore, *Klotho*-deficient kidneys are more prone to the development of renal disease in various damage models.^{10,14,17,20,34,35} With regard to the vasculature, full *Klotho* deficiency leads to a phenotype of medial calcification⁹ and endothelial hyperpermeability,³⁶ whereas heterozygous mice display endothelial dysfunction,^{37–39} arterial stiffening,^{40–42} and hypertension.^{33,43} Given the profound protective effects of *Klotho* on endothelial function, smooth muscle cell differentiation, and the kidney, we hypothesized that a relationship might exist between

hyalinosis as an aging-associated vascular lesion and *Klotho* deficiency as a state of accelerated aging.

Materials and Methods

Experimental Animals

All animal experiments were conducted in accordance with the NIH's *Guide for the Care and Use of Laboratory Animals*.⁴⁴ *Klotho*^{−/−} mice were housed at the central animal facility of the University Medical Center Groningen (Groningen, the Netherlands). *Klotho*^{−/−} mice were housed in individually ventilated cages with abundant nesting material and wild-type (WT) or *Klotho*^{+/-} buddies to prevent hypothermia. *Klotho*^{−/−} mice were provided with wetted or soaked food and drinking water (with long drinking nipples to ensure easy accessibility) ad libitum. They were monitored daily. At the age of 7 weeks, mice were sacrificed under deep isoflurane anesthesia by cardiac puncture. Kidneys were collected and snap frozen in liquid

Figure 1 Arteriolar hyalinosis develops in *Klotho* deficiency. **A:** Periodic acid-Schiff (PAS) staining on wild-type (WT) kidney. **Boxed area:** Includes a normal arteriole. **B:** Hematoxylin-eosin (H&E) staining on WT kidney, with a normal arteriole. **C:** PAS staining on *Klotho*^{+/-} kidney. **Boxed area:** Includes a normal arteriole. **D:** H&E staining on *Klotho*^{+/-} kidney, with a normal arteriole. **E:** PAS staining on *Klotho*^{−/−} kidney with PAS-positive hyaline lesions throughout the cortex. **Boxed area:** Includes an arteriole affected by hyalinosis. **F:** H&E staining on *Klotho*^{−/−} kidney, with arterioles affected by hyalinosis, displaying a typically eosinophilic and glassy aspect. **Arrows** indicate arterioles. Scale bars: 200 μ m (**A**, **C**, and **E**, main images); 50 μ m (**A**, **C**, and **E**, boxed areas, and **B**, **D**, and **F**). Original magnification: $\times 100$ (**A**, **C**, and **E**, main images); $\times 400$ (**A**, **C**, and **E**, boxed areas, and **B**, **D**, and **F**).

Figure 2 Determination of affected segments of the renal vasculature. **A:** α -Smooth muscle actin (α -SMA)/periodic acid-Schiff (PAS) double staining on $Klotho^{-/-}$ kidney with a large segmental artery, showing severe hyalinosis, with loss of α -SMA expression in the lesion, spanning the vascular wall. **B:** α -SMA/PAS double staining on $Klotho^{-/-}$ kidney with a terminal interlobular artery and afferent arteriole, showing hyalinosis up until halfway the afferent arteriole. **C:** Renin/PAS double staining on $Klotho^{-/-}$ kidney, showing that the terminal afferent arteriole is not affected by hyalinosis. **A–C:** Closed arrowheads indicate hyalinosis; open arrowheads, unaffected arteries. **D:** Histogram of lumen diameters of all renal α -SMA-positive arteries, showing that hyalinous arteries are generally 6 to 42 μ m in diameter, with the smallest arterioles generally being unaffected. Scale bars = 200 μ m (A–C). Original magnification, $\times 400$ (A–C).

nitrogen or fixed in formalin (after harvesting of tissues) and embedded in paraffin. $Klotho^{-/-}$ ($n = 3$), $Klotho^{+/-}$ ($n = 4$), and WT ($n = 3$) mice were analyzed for the presence of arteriolar hyalinosis. *Kl/kl* mice were housed at the Institute for Physiology at the University of Tübingen (Tübingen, Germany) and were not treated ($n = 13$) or treated with spironolactone (80 mg/L; $n = 10$) in drinking water for 8 weeks. Kidney tissue was used from 6- to 8-week-old β -actin-Cre/ $Klotho^{flox/flox}$ mice, bred as previously described.⁴⁵ Different strains of mice were used to assess whether the hyalinosis phenotype is present or can be induced independent from the genetic background.

Histochemistry

Paraffin sections (4 μ m thick) were cut, deparaffinized in xylene, and rehydrated in a graded ethanol series. Periodic acid-Schiff (PAS) staining was performed by incubating sections in 1% periodic acid for 10 minutes and in Schiff's reagent for 15 minutes (or for 5 minutes as a mild counterstaining after immunohistochemistry with diaminobenzidine), followed by hematoxylin counterstaining. Von Kossa staining was performed by incubating sections in 1% silver nitrate for 1 hour under sunlight exposure, followed by incubation in 3% sodium thiosulfate for 5 minutes and counterstaining with nuclear fast red. Masson trichrome staining was performed by incubating sections for 5 minutes in celestine blue, 5 minutes in hematoxylin, 5 minutes in a 1:2 mixture of 1% acid fuchsin and 1% xylinidyl ponceau, 5 minutes in 1% phosphomolybdic acid, and 1 minute in 1% aniline blue. Verhoeff staining was performed by incubating sections for 15 minutes in Verhoeff staining solution (5:2:2

mixture of 5% alcoholic hematoxylin, 10% ferric chloride, and 2% potassium iodide/1% iodine), followed by destaining 2% ferric chloride and staining with Van Gieson solution. Oil red O staining was performed on cryosections, which were fixed for 10 minutes in 8% formalin and dipped twice in 60% 2-propanol, followed by incubation for 10 minutes in oil red O solution, destaining in 60% 2-propanol, and counterstaining in hematoxylin.

Immunohistochemistry

Paraffin sections (4 μ m thick) were cut, deparaffinized in xylene, and rehydrated in a graded ethanol series. Antigen retrieval was performed by heating sections at 500 W for 15 minutes in 10 mmol/L citric acid (pH 6) for α -smooth muscle actin (α -SMA), CD31, and collagen I, in 170 mmol/L Tris/1 mmol/L EDTA (pH 9) for renin and S100A4, and in 1 mmol/L EDTA (pH 8) for collagen III. After blocking endogenous peroxidase [0.3% H_2O_2 /phosphate-buffered saline (PBS)] for 30 minutes, sections were incubated with primary antibodies [α -SMA: dilution 1:300; mouse monoclonal 1A4; Dako, Glostrup, Denmark; CD31: dilution 1:50; SZ31; Dianova, Hamburg, Germany; collagen I: dilution 1:100; 1310-01; Southern Biotech, Birmingham, AL; collagen III: dilution 1:75; 1330-01; Southern Biotech; renin: dilution 1:2500; polyclonal antibody kindly provided by Dr. Tadashi Inagami (Vanderbilt University School of Medicine, Nashville, TN); and S100A4: dilution 1:2000; A5114; Dako] for 1 hour in 1% bovine serum albumin/PBS. If necessary, avidin and biotin blocking solutions were applied (Vector Laboratories Inc., Burlingame, CA). Sections were incubated with the

Figure 3 Evaluation of morphology of the vascular wall in Klotho deficiency-induced arteriolar hyalinosis. **A:** CD31/periodic acid-Schiff (PAS) double staining on Klotho^{-/-} kidney. **Boxed area:** A close-up of CD31/PAS staining showing that in arteries/arterioles both unaffected and affected by hyalinosis, the endothelial lining is intact. **B:** Verhoeff staining on Klotho^{-/-} kidney. **Boxed area:** A close-up of Verhoeff staining showing that the elastic lamina is intact in unaffected arteries, which cannot be ascertained in arteries affected by hyalinosis. **C:** α -Smooth muscle actin (α -SMA)/PAS double staining on Klotho^{-/-} kidney. **Boxed area:** A close-up of α -SMA/PAS staining, showing normal media in unaffected arteries, whereas the media in arteries affected by hyalinosis mostly consist of accumulated hyaline material, with smooth muscle cells having lost their α -SMA expression. **Closed arrowheads** indicate hyalinosis; **open arrowheads**, unaffected arteries. Scale bars: 200 μ m (A–C, main images); 50 μ m (A–C, boxed areas). Original magnification: $\times 100$ (A–C, main images); $\times 400$ (A–C, boxed areas).

appropriate secondary and tertiary antibodies for 30 minutes [goat anti-mouse–biotin, rabbit anti-rat–horseradish peroxidase (HRP), goat anti-rabbit–HRP, and rabbit anti-goat–HRP; Dako]; and the chromogenic reaction was performed using diaminobenzidine in 0.03% H₂O₂/PBS, followed by hematoxylin counterstaining. IgM and iC3b/c/d stainings were performed on frozen sections (4 μ m thick), which were dried and fixed in acetone for 10 minutes at room temperature, followed by endogenous peroxidase blocking in 0.075% H₂O₂/PBS for 30 minutes and incubation with primary antibodies for 1 hour (iC3b/c/d: dilution 1:20; HM1065; Hycult Biotech, Uden, the Netherlands; IgM-HRP: dilution 1:100; 1020-05; Southern Biotech). Then, the appropriate secondary and tertiary antibodies were applied for 30 minutes (goat anti-mouse–HRP, rabbit anti-goat–HRP, and goat anti-rabbit–HRP; Dako); and the chromogenic reaction was performed with diaminobenzidine or 3-amino-9-ethylcarbazole (AEC). Nuclei were counterstained with hematoxylin. All slides were scanned using a Hamamatsu Nanozoomer 2.0HT (Hamamatsu Photonics, Hamamatsu, Japan), and scans were analyzed using Aperio ImageScope software version 12.3.2.8013 (Leica Microsystems B.V., Amsterdam, the Netherlands).

Immunofluorescence

Paraffin sections (4 μ m thick) were cut, deparaffinized in xylene, and rehydrated in a graded ethanol series. Antigen retrieval was performed in 10 mmol/L citric acid (pH 6),

followed by a blocking step with 1% bovine serum albumin and 5% donkey serum in PBS for 80 minutes at room temperature. Sections were incubated with polyclonal rabbit anti-mouse phosphorylated Smad2/3 (dilution 1:150; sc-11769; Santa Cruz Biotechnology, Dallas, TX) and polyclonal goat anti-mouse CD31 (dilution 1:200; AF3628; R&D Systems, Minneapolis, MN) for 60 minutes at room temperature, followed by donkey anti-rabbit–Alexa Fluor 555 (dilution 1:500; ab150074; Abcam, Cambridge, UK) and donkey anti-goat–Alexa Fluor 647 (dilution 1:500; A21447; Invitrogen, Carlsbad, CA) for 70 minutes at room temperature, followed by DAPI counterstaining and imaging on an EVOS fluorescence imaging system (Thermo Fisher Scientific, Waltham, MA).

Statistical Analysis

Normally distributed data are presented as means $\pm$ SD or median (interquartile range). Differences between groups were tested with analysis of variance, followed by Bonferroni post-hoc correction; *t*-test; a Kruskal-Wallis test, followed by Dunn post-hoc correction; or *U*-test, after prior Kolmogorov-Smirnov testing for normality. Spearman's ρ was used for correlation analysis. *P* < 0.05 was considered statistically significant. All data analysis was performed using SPSS version 23 (IBM, Armonk, NY) and GraphPad Prism version 5 (GraphPad Software, San Diego, CA).

Figure 4 Accumulation of plasma proteins in hyalinous lesions. **A:** Immunohistochemistry for IgM on wild-type (WT) kidney, showing interstitial staining, but no IgM in the vascular wall. **B:** Immunohistochemistry for IgM on Klotho^{-/-} kidney, showing an artery with immunoreactivity for IgM in the vascular wall. **C:** Immunohistochemistry for IgM on WT mouse spleen, as a positive control. **D:** Immunohistochemistry for iC3b/c/d on WT kidney, showing no vascular expression. **E:** Immunohistochemistry for iC3b/c/d on Klotho^{-/-} kidney, depicting immunoreactivity in the vascular wall of an arteriole. **F:** Immunohistochemistry for iC3b/c/d on brain-dead mouse kidney, as a positive control. **Arrows** indicate arteries/arterioles. Scale bars = 50 μ m (A–F). Original magnification, $\times 400$ (A–F).

Results

Klotho Deficiency Induces Arteriolar Hyalinosis

In 7-week-old Klotho^{-/-} mouse kidneys, vascular lesions that were strongly PAS positive (Figure 1, A, C, and E) and were typically eosinophilic and glassy were detected on hematoxylin-eosin staining (Figure 1F), indicative of hyalinosis. These lesions were consistently observed in all Klotho^{-/-} mice and were not observed in Klotho^{+/-} or wild-type littermates (Figure 1, A–D). The Von Kossa staining was negative in these arterioles, indicating that these lesions did not represent calcifications (Supplemental Figure S1, A–C). Oil red O staining was also negative, indicating that these lesions did not contain a fatty component (Supplemental Figure S1, D and E).

Hyalinosis Is Mostly Found in Terminal Interlobular Arteries and Proximal Afferent Arterioles

The relatively large segmental arteries generally contained affected regions (Figure 2A), and luminal measurements of all α -SMA-positive arteries indicate that this pattern remained patchy, with some parts of interlobar and arcuate arteries being affected. Most affected arteries were interlobular arteries and proximal afferent arterioles (Figure 2B), with most having a lumen with a diameter of 6 to 42 μ m

(Figure 2D). However, the vast majority of arterioles <15 μ m (including terminal afferent arterioles and efferent arterioles) were generally unaffected (Figure 2D) and renin/PAS double staining did not reveal colocalization of renin expression and hyalinosis in terminal afferent arterioles (Figure 2C).

Arteriolar Morphology

PAS/CD31 double staining indicated that the endothelial lining of affected arterioles is generally still intact (Figure 3A). Verhoeff staining indicated that the elastic lamina is intact in unaffected arterioles but cannot be discerned in affected areas (Figure 3B). PAS/ α -SMA double staining indicated that arteries and arterioles affected by hyalinosis lose their smooth muscle cell expression of α -SMA (Figure 3C).

Hyaline Depositions Contain Plasma Proteins

Using immunohistochemistry, hyalinous lesions in Klotho^{-/-} kidneys were found to be positive for IgM (Figure 4, A–C) and for activated C3 proteins iC3b/c/d (Figure 4, D–F), indicating that the integrity of the endothelial barrier function is locally compromised and that leaked plasma proteins accumulate in the subendothelial

space. Vascular positivity for IgM and iC3b/c/d was not found in WT kidneys (Figure 4, A and D).

Dedifferentiation of Smooth Muscle Cells to a Synthetic Phenotype

In *Klotho*^{-/-} mouse arteries, lesional smooth muscle cells were found to have lost expression of α -SMA, a contractile apparatus protein that is also a marker for differentiated SMCs (Figures 2A and 5H). In turn, lesional SMCs gained expression of S100A4 (Figure 5J), which is considered a marker of a synthetic phenotype in SMCs. Indeed, in *Klotho*^{-/-} kidneys, collagen I, collagen III, and Masson trichrome positivity were also detected, most prominently in hyalinized parts of segmental arteries (Figure 5, B, D, and F), indicating increased deposition of extracellular matrix proteins. WT renal arteries of similar size did not have

medial/subendothelial collagen expression (Figure 5, A, C, and E), loss of α -SMA expression (Figure 5G), or SMC expression of S100A4 (Figure 5I).

Hyalinous Lesions Are Associated with Increased TGF- β 1 Signaling

The key pathway known to be involved in the development of arteriolar hyalinosis is transforming growth factor (TGF)- β 1 signaling, of which *Klotho* is a known inhibitor. We, therefore, hypothesized that loss of *Klotho* leads to deranged TGF- β 1 signaling and promotes the development of arteriolar hyalinosis. Phosphorylated Smad2/3 expression was highly increased ubiquitously in *Klotho*^{-/-} mouse kidney, with strong nuclear expression in glomerular, tubular, and vascular cells alike (Figure 6, C–F), compared with low, constitutive expression levels in WT mice (Figure 6, A–C). Particularly high phosphorylated Smad2/3 expression,

Figure 5 Dedifferentiation of smooth muscle cells into a synthetic phenotype results in the deposition of extracellular matrix in hyalinous lesions. **A:** Masson trichrome staining on wild-type (WT) mouse kidney, showing part of a normal segmental artery. **B:** Masson trichrome staining on *Klotho*^{-/-} kidney, showing part of a segmental artery affected by hyalinosis, with increased collagen deposition (blue) at the site of the lesion. **C:** Collagen I/periodic acid-Schiff (PAS) double staining on WT mouse kidney, showing part of a normal segmental artery. **D:** Collagen I/PAS double staining on *Klotho*^{-/-} mouse kidney, showing part of a segmental artery affected by hyalinosis, with locally increased collagen I expression in the affected vessel wall. **E:** Collagen III/PAS double staining on WT mouse kidney, showing part of a normal segmental artery. **F:** Collagen III/PAS double staining on *Klotho*^{-/-} mouse kidney, showing part of a segmental artery affected by hyalinosis, with locally increased collagen III expression in the affected vessel wall. **G:** α -Smooth muscle actin (α -SMA)/PAS double staining on WT mouse kidney, showing part of a normal segmental artery. **H:** α -SMA/PAS double staining on *Klotho*^{-/-} mouse kidney, showing part of a segmental artery affected by hyalinosis, with loss of α -SMA positivity in lesional smooth muscle cells. **I:** S100A4/PAS double staining on WT mouse kidney, showing part of a normal segmental artery. **J:** S100A4/PAS double staining on *Klotho*^{-/-} mouse kidney, showing part of a segmental artery affected by hyalinosis, with S100A4-positive smooth muscle cells in the affected area. Scale bars = 25 μ m (A–J). Original magnification, $\times 400$ (A–J).

Figure 6 Increased activation of transforming growth factor- β 1 signaling in $Klotho^{-/-}$ kidney is particularly pronounced in hyalinous arterioles. **A:** Phosphorylated Smad2/3 (pSmad2/3) staining on wild-type (WT) mouse kidney, showing mild constitutive positivity. **B:** CD31 staining on WT mouse kidney, including an arteriole. **C:** Merged image of **A** and **B**, including DAPI. **D:** pSmad2/3 staining on $Klotho^{-/-}$ mouse kidney, showing ubiquitously strong, nuclear positivity in renal glomerular, tubular, and vascular structures. **E:** CD31 staining on $Klotho^{-/-}$ kidney, including an artery and an arteriole. **F:** Merged image of **D** and **E**, including DAPI, showing a hyalinous arteriole with strong pSmad2/3 expression in lesional smooth muscle cells. **G:** Conjugate control for pSmad2/3 on $Klotho^{-/-}$ kidney. **H:** Conjugate control for CD31 on $Klotho^{-/-}$ kidney. **I:** Merged image of **G** and **H**, including DAPI, showing no specific pSmad2/3 or CD31 positivity. **White arrows** indicate arterioles; **purple arrows**, CD31-positive endothelium; **orange arrows**, pSmad2/3-positive dedifferentiated smooth muscle cells. Scale bars: 200 μ m (main images); 25 μ m (insets). Original magnifications, $\times 200$ (main images); $\times 500$ (insets).

however, was found in the vascular cells associated with hyalinous lesions (Figure 6F), suggesting that not only is TGF- β 1 signaling constitutively activated in $Klotho^{-/-}$ kidney, activation of TGF- β 1 signaling could play a role in the development of arteriolar hyalinosis in $Klotho$ deficiency. Conjugate control stainings for phosphorylated Smad2/3 and CD31 were negative (Figure 6, G–I).

Vascular Lesions in Different Klotho Knockout Mouse Strains

Because not only the development of hyalinosis but also the lack of vascular calcification in $Klotho^{-/-}$ mice is an unconventional finding, it was assessed whether hyalinosis is a trait also found in other strains of $Klotho$ knockout mice. Therefore, PAS and Von Kossa staining was performed on kidney sections from kl/kl mice (which have a disrupted promoter) and β -actin–Cre/ $Klotho^{lox/lox}$ (or β -actin– $KL^{-/-}$) mice (which have a deletion of exon 2 of the $Klotho$ gene in cells that express β -actin, which is ubiquitous), to compare with

$Klotho^{-/-}$ mice (which have a deletion of exon 2 in all cells). The kl/kl mouse kidneys almost exclusively displayed vascular calcification (Figure 7, A and D), whereas β -actin– $KL^{-/-}$ mice exhibited a mix of vascular calcification and some hyalinosis (Figure 7, B and E) and $Klotho^{-/-}$ mice only had prominent hyalinosis (Figure 7, C and F). Quantitative assessment is depicted in Figure 7, G–I. These findings indicate that at least β -actin– $KL^{-/-}$ mice can also develop arteriolar hyalinosis, although their phenotype is variable, possibly because of slight variations in recombination.

Spironolactone Inhibits Vascular Calcification and Allows for Its Replacement by Hyalinosis

Finally, to further examine whether the development of arteriolar hyalinosis is a consequence of $Klotho$ deficiency in general, it was studied whether inhibition of vascular calcification in kl/kl would also potentiate the development of arteriolar hyalinosis. The kl/kl mice were treated with 80 mg/L spironolactone for 8 weeks, which was previously

Figure 7 Assessment of vascular pathologies in different Klotho knockout mouse strains. **A–C:** Periodic acid-Schiff staining shows prominent vascular calcification in *kl/kl* (**A**), mild arteriolar hyalinosis in β -actin-Cre/*Klotho*^{flox/flox} (β -actin-KL^{-/-}; **B**), and marked arteriolar hyalinosis in *Klotho*^{-/-} (**C**) mouse kidney. **D–F:** Von Kossa staining shows prominent vascular calcification in *kl/kl* (**D**), no medial calcification in β -actin-KL^{-/-} (**E**), and no vascular calcification in *Klotho*^{-/-} (**F**) mouse kidney. **G:** Quantification of arteries assessed as calcified in different Klotho-deficient mouse strains, showing marked vascular calcification in *kl/kl* mice, variable vascular calcification in β -actin-KL^{-/-} mice, and no vascular calcification in *Klotho*^{-/-} mice. **H:** Quantification of arteries assessed as affected by hyalinosis in different Klotho-deficient mouse strains, showing virtually no hyalinosis in *kl/kl* mice, some hyalinosis in β -actin-KL^{-/-} mice, and prominent presence of hyalinosis in *Klotho*^{-/-} mice. **I:** Quantification of arteries assessed as normal in different Klotho-deficient strains. **Arrows** indicate arterioles affected by hyalinosis. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bars = 50 μ m (**A–F**). Original magnification, $\times 400$ (**A–F**).

found to inhibit the development of vascular calcification.^{46,47} The *kl/kl* mice spontaneously displayed severe nephrocalcinosis (86% $\pm$ 8% calcified arteries) (Figure 8, A, B, and D) and little hyalinosis (4% $\pm$ 2%) (Figure 8G). However, spironolactone treatment allowed for the replacement of calcified arteries (51% $\pm$ 27%) with hyalinosis (32% $\pm$ 23%) (Figure 8, C–G).

Discussion

This study shows that Klotho deficiency induces arteriolar hyalinosis. The emergence of an aging-related vascular lesion as part of the phenotype of Klotho deficiency compounds the hypothesis that Klotho exerts antiaging effects. Given the prevalent view that arteriolar hyalinosis, particularly in afferent arterioles, leads to the loss of renal autoregulation and contributes to the aging-related loss of renal function,^{7,8} the potential involvement of Klotho is of

particular interest to the aging population. The aging-related decrease in Klotho expression may be causally involved in this process; and considering the protective effects Klotho has been shown to have experimentally on renal function,^{10,14,17,22} increasing Klotho levels may prove to be a promising approach to the preservation of renal function during aging.

Klotho deficiency allows for plasma proteins to accumulate in the subendothelial space in hyaline lesions, attesting to the poor barrier function of the endothelium. Indeed, endothelial hyperpermeability³⁶ and endothelial dysfunction^{37–39} have been shown before to develop in the absence of Klotho. It is, therefore, clear that adequate Klotho levels are paramount to endothelial integrity. However, more interesting in this study is the observed plasticity of the smooth muscle cell phenotype in Klotho deficiency, considering the previously reported direct *in vitro* effects of Klotho on SMC differentiation.²² The *Klotho*^{-/-} mouse is well studied with respect to its development of severe vascular calcification,^{48–52} in particular of

Figure 8 Inhibition of vascular calcification by spironolactone potentiates the replacement of vascular calcification with hyalinosis. **A:** Periodic acid-Schiff (PAS) staining on untreated *kl/kl* mouse kidney, displaying severe nephrocalcinosis throughout the cortex. **B:** Von Kossa staining on untreated *kl/kl* mouse kidney from **A**, displaying severe nephrocalcinosis (black). **C:** Correlation between the percentages of arteries assessed as calcified and as hyalinous, showing a linear, negative correlation ($P < 0.001$, Spearman ρ). **D:** Quantification of the arteries assessed as calcified on both PAS and Von Kossa staining in untreated and spironolactone-treated *kl/kl* mouse kidneys. **E:** PAS staining on spironolactone-treated *kl/kl* mouse kidney, exhibiting no vascular (or tubular) calcification, but marked hyalinosis. **F:** Von Kossa staining on spironolactone-treated *kl/kl* mouse kidney from **E**, displaying no vascular (or tubular) calcification. **G:** Quantification of the arteries assessed as affected by hyalinosis on both PAS and Von Kossa staining in untreated and spironolactone-treated *kl/kl* mouse kidneys. *** $P < 0.001$. Scale bars: 300 μ m (**A** and **E**, main images, **B**, and **F**); 50 μ m (insets). Original magnifications: $\times 80$ (**A** and **E**, main images, **B**, and **F**); $\times 400$ (insets).

the media, as occurs in chronic kidney disease or in aging. However, likely due to differences in genetic background, diet, and other environmental factors, Klotho-deficient mice displayed a certain phenotypic variability. Klotho^{-/-} mice had not developed vascular calcification in the kidney at the age of 7 weeks. We hypothesize that the accumulated procalcific triggers were not potent enough to induce vascular calcification, which, in turn, allowed for the development of arteriolar hyalinosis instead. The Klotho^{-/-} mice have a mixed genetic background, but one that is mostly C57BL/6, which is generally more resistant to the development of vascular calcification. To address this phenomenon, *kl/kl* mice (on a mixed background, which was mostly 129/Sv) and β -actin-KL^{-/-} mice (also on a mixed background, predominantly C57BL/6⁴⁵) were studied; *kl/kl* mice displayed severe vascular calcification at 7 to 8 weeks of age. The observation that β -actin-KL^{-/-} mice spontaneously display some arteriolar hyalinosis and that *kl/kl* mice do so after treatment with spironolactone, which inhibited the development of calcification by blocking vascular SMC-derived aldosterone, inducing calcification via a Pit1-dependent mechanism,^{46,47,53} allows us to speculate that the development of hyalinosis is a function of the degree to which vascular calcification develops, which can be influenced by

the genetic background, as well as by dietary factors. The development of arteriolar hyalinosis in lieu of vascular calcification in spironolactone-treated mice lets us conclude that, in the absence of sufficiently strong procalcific stimulus, smooth muscle cells dedifferentiate to a more synthetic phenotype. We hypothesize that Klotho inhibits pathologic dedifferentiation of smooth muscle cells and the consequently assumed phenotype is the net result of the stimuli to which the SMCs are exposed. In the case of hyalinosis, the sub-endothelial presence of accumulated plasma proteins likely triggers the SMC response of dedifferentiation and synthesis of ECM proteins.^{54,55} This concept is schematically depicted in Figure 9.

To expand on the topic of phenotypic variability in Klotho deficiency, it has long been known that different Klotho levels induce different vascular phenotypes. Klotho^{-/-} mice display severe vascular calcification,⁹ whereas Klotho^{+/-} mice do not do so spontaneously, although they are more prone to the development of vascular calcification on induction of chronic kidney disease.²² Although Klotho^{+/-} mice develop endothelial dysfunction, vascular function in full knockouts has proved difficult to evaluate because of severe calcification.^{37–39} Klotho^{+/-} mice develop arterial stiffening after 14 weeks

Figure 9 Paradigm of the effect of Klotho on smooth muscle cell (SMC) phenotypic transitions. Klotho inhibits SMC dedifferentiation from a contractile phenotype to either a calcifying or a synthetic phenotype. Depending on the stimuli that SMCs are exposed to, dedifferentiation can go in either direction. High phosphate and calcium levels and cellular senescence promote the development of vascular calcification, whereas subendothelial leakage of plasma proteins, potentially combined with constitutively increased transforming growth factor (TGF)- β 1 signaling, will foster the development of hyalinosis. In the absence of sufficiently strong procalsific stimuli (which can be either before these stimuli properly develop, in the context of greater resistance against these stimuli, or on inhibition of calcification, like by spironolactone), SMCs respond to endothelial hyperpermeability in contributing to the development of arteriolar hyalinosis.

of age with increased aortic collagen deposition and elastin degradation,^{40–42} which probably does not develop in *Klotho*^{−/−} mice because of the dominance of the calcification phenotype. Of note, arteriolar hyalinosis and arterial stiffening have in common the aberrant SMC behavior of excessive ECM deposition. Naturally, the short lifespan of full knockout mice also limits the window for the development of other pathologies. The same probably holds true for the development of hypertension from 15 to 16 weeks of age onward in *Klotho*^{+/-} mice,^{33,40,43} whereas *Klotho*^{−/−} mice are generally hypotensive because of hypovolemia.^{46,56,57} This study indicates that a similar example of phenotypic divergence is the uncovering of arteriolar hyalinosis if the development of calcification is mildly delayed and/or inhibited. The comparison of these phenotypes between different *Klotho* knockout genotypes under different conditions may shed light on the development of vascular disease in patients. The rapid and severe development of vascular calcification in *kl/kl* mice may make them a suitable model for vascular pathologies in chronic kidney disease patients, who are also more severely *Klotho* deficient than the general aging population. Heterozygous mice may constitute a suitable model for general aging or other patients with intermediate *Klotho* levels, displaying milder pathologies, like arterial stiffening, endothelial dysfunction, and hypertension. This study suggests that even in aging patients in whom vascular calcification in the kidney plays a limited role, *Klotho* deficiency may still contribute to the development of arteriolar hyalinosis and the subsequent decline in renal function.

The development of hyalinosis has not been widely studied in animal models, but calcineurin appears to be a key modulator. Calcineurin inhibitors, like tacrolimus and

cyclosporine A, genetic deletion of calcineurin α ,⁵⁸ and endothelial deletion of FK-506-binding protein 12 (to which tacrolimus binds)⁵⁹ readily induce hyalinosis. This effect is mostly mediated by TGF- β 1 signaling, considering the evidence that anti-TGF- β 1 antibodies mitigated hyalinization⁶⁰ and deletion of FKBP12 constitutively activates transforming growth factor beta receptor I (TGF- β RI) and downstream Smad2/3 signaling.⁵⁹ Given that *Klotho* inhibits TGF- β 1 signaling by binding to transforming growth factor beta receptor II (TGF- β RII) and decreasing its affinity for TGF- β 1,¹⁵ leading to less activation of TGF- β RI, it appears that *Klotho* may act upstream of calcineurin inhibitor-induced hyalinosis. Indications of constitutively and ubiquitously active TGF- β 1 signaling in the absence of *Klotho* and in particular of strong phosphorylated Smad2/3 expression in vascular cells associated with hyalinous lesions were also found in this study, suggesting a role for TGF- β 1 signaling. Together, these lines of evidence lead to the hypothesis that *Klotho* may be able to mitigate the TGF- β 1-induced adverse effects of calcineurin inhibitor use. Of note are also the down-regulation of *Klotho* in cyclosporine A nephropathy,^{61–65} producing functional *Klotho* deficiency, and the general renoprotective and anti-inflammatory effects of *Klotho* overexpression in cyclosporine A nephropathy.^{65,66} Investigation of *Klotho* as a therapeutic target in inhibiting development of chronic graft dysfunction in renal transplantation patients may, therefore, also be warranted. Furthermore, although interventional studies are certainly necessary for more solid conclusions, the evidence that *Klotho* and TGF- β 1 signaling may act in a common pathway in the development of arteriolar hyalinosis may be a step in expanding

our understanding of this aging-related vasculopathy and its implications in the aging process.

In summary, arteriolar hyalinosis appears to be an important feature of the Klotho deficiency phenotype, which is likely generally masked by the development of vascular calcification and is uncovered on inhibition of or resistance against vascular calcification. The finding that Klotho deficiency induces arteriolar hyalinosis raises new questions and hypotheses on the effects of Klotho on endothelial integrity and smooth muscle cell dedifferentiation, on the role of Klotho in aging and in aging-related renal function decline, and on the potential of Klotho in aging and in calcineurin inhibitor nephrotoxicity.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.08.006>.

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