ROLE OF CKD AND CASPASE-1 IN NEOINTIMAL HYPERPLASIA DEVELOPMENT

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ABSTRACT

Vascular access dysfunction is a cause of morbidity and mortality in chronic kidney disease (CKD) patients that require hemodialysis. The major cause of vascular access failure is venous stenosis due to neointimal hyperplasia (NH). Vascular smooth muscle cells (VSMC) are critical for the development of NH lesions, as they have the ability to modulate their phenotype from a "contractile" to a "synthetic" phenotype in the presence of uremia, through the regulation of sensor genes for uremia danger signals and VSMC-specific differentiation genes. Recent research indicates that Caspase-1 (casp-1) activation plays an essential role in sensing metabolic danger signal-associated molecular patterns and initiating vascular inflammation. Carbamylated LDL, a uremic toxin that has been shown to be found in higher levels in patients with CKD and in CKD murine models when compared to controls, and could play a role in casp-1 activation. Therefore, the goal of this project is to examine the role of cLDL/CKD-driven casp-1 activation in VSMC and CKD-related NH.

We have established a CKD mouse model and published on CKD-associated vascular remodeling. We exposed wild type and caspase-1 knockout mice to our CKD model, analyzed and quantified the NH lesion formed. We also examined *in vitro* and *ex-vivo* changes in VSMC-specific differentiation genes when exposed to uremic serum and cLDL, in the presence or absence of caspase-1 inhibitor.

We found that CKD serum induces with casp-1 activation and phenotypic changes in VSMCs from a "contractile" to a "synthetic" phenotype, which are reversed with casp-1 inhibition. In an *ex-vivo* model using relative quantification we found that VSMC contractile markers α -Actin, Calponin, SM-22, and Smoothelin gene expression of CKD mouse carotid VSMC were higher in casp-1 knockout mice when compared to wild-type (1.40, 1.28, 1.22, 1.41 respectively). Also using an *in-vivo* model, relative quantification of α-actin decreased from 1.0 to 0.329 when VSMCs were exposed to uremic serum and but increased back to 0.588 when Caspase-1 inhibitor is added. The relative quantification of Calponin also decreased from 1.0 to 0.394 when exposed to uremic serum and increased back to 0.601 with caspase-1 inhibitor. We also found that caspase-1 deficiency significantly reversed CKD-related vascular remodeling in casp-1 knockout mice and reduced NH volume by 50% from 1,440,023in wild-type mice to 71,069 μm^2 in casp-1 knockouts (p-value 0.002).

This evidence provides evidence that casp-1 plays a critical role in NH formation. Furthermore our results provide a novel insight over the therapeutic potential of casp-1 inhibitors for CKD induced NH and other inflammation induced vascular remodeling.

I dedicate this work to my wife Mariselis for her eternal support.

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CHAPTER 1

INTRODUCTION

Chronic kidney disease (CKD) is a disease affecting >15% of the adult population (Basnakian, Shah, Ok, Altunel, & Apostolov; Levey et al., 2003). Vascular access dysfunction is a primary reason for morbidity and mortality in CKD patients that require hemodialysis (Coresh, Longenecker, Miller, Young, & Klag, 1998) with an associated annual cost that exceeds $1 billion (H. Lee et al., 2002) in U.S. An arteriovenous fistula (AVF) is the method of choice for dialysis access in patients with end-stage renal disease (Tordoir et al., 2007). The major cause of vascular access failure in AVFs is venous stenosis due to neointimal hyperplasia (NH) (T. Lee & Roy-Chaudhury, 2009). Neointimal Hyperplasia refers to inward proliferation and migration of vascular smooth muscle cells (VSMCs) primarily in the tunica intima, resulting in the thickening of arterial and venous walls and decreased vessel lumen space. There is an urgent need to develop better therapeutic strategies to treat vascular access stenosis in hemodialysis patients, since the one year patency rates of AVF are estimated to be only 63%.

The uremic environment present in hemodialysis patients exacerbates vascular dysfunction and may be responsible for the pre-existing venous neointimal hyperplasia and medial hypertrophy that is observed in CKD patients even before the creation of AVFs(A. Biuckians, E. C. Scott, G. H. Meier, J. M. Panneton, & M. H. Glickman, 2008). The pathological factors in CKD that contribute to vascular dysfunction may include chronic inflammation and increased levels of oxidative stress(Yang, Yin, & Wang, 2008).

Several studies have shown that CKD aggravates damage and accelerates development of neointimal hyperplasia in AVFs(T. Kokubo et al., 2009). A better understanding of the cellular and molecular mechanisms that lead to the development of neointimal hyperplasia in CKD is warranted in order to develop appropriate therapeutic interventions.

CHAPTER 2

REVIEW OF LITERATURE

Vascular smooth muscle cells (VSMC) are critical for the development of NH lesions, as they have the ability to modulate their phenotype in response to environmental stimuli. These cells go through profound changes from a "contractile" to a "synthetic" phenotype in the presence of uremia, which is mediated through the regulation of uremia danger signals-sensor genes and VSMC-specific differentiation genes. The fully differentiated VSMC is associated with high expression of several specific contractile proteins including smooth muscle alpha-actin, smooth muscle myosin heavy chain, SM22 and calponin. In response to vascular injury, VSMCs exhibit a phenotypic change exemplified by loss of contractile and abnormal proliferation, migration and matrix secretion (G. K. Owens, 1995). This synthetic phenotype plays a role in the development of various vascular pathologies including atherosclerosis, hypertension and post-angioplasty restenosis (Gary K. Owens, Kumar, & Wamhoff, 2004; Regan, Adam, Madsen, & Owens, 2000) .

A feature of VSMC phenotype plasticity is transcriptional repression of the specific contractile genes (Regan et al., 2000). Several studies have shown that uremia accelerates development of NH in AVFs (Taku Kokubo et al., 2009; Langer et al., 2010). Patients who undergo hemodialysis utilizing AVFs often need to be re-intervened surgically in order to correct stenotic lesions which compromise blood flow through the fistula. This is reflected in the fact that the one year patency rates of AVF are estimated to be only 63% (Andre Biuckians, Eric C. Scott, George H. Meier, Jean M. Panneton, &

Marc H. Glickman, 2008). These repeat interventions expose the patient to an increase in morbidity and mortality associated with the procedures. Therefore, novel therapies are urgently needed to inhibit the progression of NH associated with CKD. However, the mechanisms underlying how metabolic danger signals-sensor senses uremia/CKD and accelerates NH remain largely unknown. Recent research indicates that Caspase-1 (Casp1) activation requires the formation of a protein complex termed inflammasome with Nod-like receptor protein 3 (NLRP3), which plays an essential role in sensing metabolic danger signal-associated molecular patterns (DAMPs)(Lundberg & Yan, 2011) and in initiating vascular inflammation (Yang et al., 2008; Y. Yin, Lopez Pastrana, J, Li, X, Huang, X, Mallilankaraman, K, Choi, E.T., Madesh, M, Wang, H, Yang, XF, 2013). It has also been described that in the setting of a proinflamatory state like hyperlipidemia, caspase-1 through the caspase-1-sirtrulin pathway leads to endothelial cell activation, monocyte recruitment and plaque formation.

The objective of this study is to examine the novel theory that caspase-1 plays a role in the modulation of vascular smooth muscle cell phenotype and neointimal hyperplasia formation. We used a chronic kidney disease-carotid ligation murine model, and applied it to wild-type and caspase-$1^{-/-}$ animals. We demonstrated that caspase-1 plays a critical role in the modulation of neointimal hyperplasia formation and contractile marker gene expression.

CHAPTER 3

METHODS

RNA Extraction and Quantitative Real-Time PCR.

Cells were subjected to serum starvation for 48-72 h followed by treatment with serum for 24 h. Untreated serum starved cells were used as control. Total RNA from cultured cells was extracted using the RNeasy kit (Qiagen), and cDNA was synthesized with the VILO first-strand synthesis system (Invitrogen). In the real time PCR step, cDNA was amplified with inventoried gene assay products containing two gene specific primers and one FAM dye labeled Taq Man MGB probe using the 7500 Real Time PCR System (Applied Biosystems). Relative gene expression levels were calculated after normalization with internal control eukaryotic 18S gene using the $2^{-\text{deltadeltaCt}}$ method, where Ct is the threshold value.

Mouse carotids (5) from each group were collected and frozen. Total RNA was then extracted using Trizol (Invitrogen). For arrays, 1ug of RNA was reversed transcribed using the RT Kit QIAGEN. A genomic DNA elimination step is employed before reverse transcription step. cDNAs were then used for gene expression analysis using the mouse inflammasome array (QIAGEN). For vascular smooth muscle cell gene expression, cDNA was assayed using gene specific Taqman probes as described above.

Mice Model

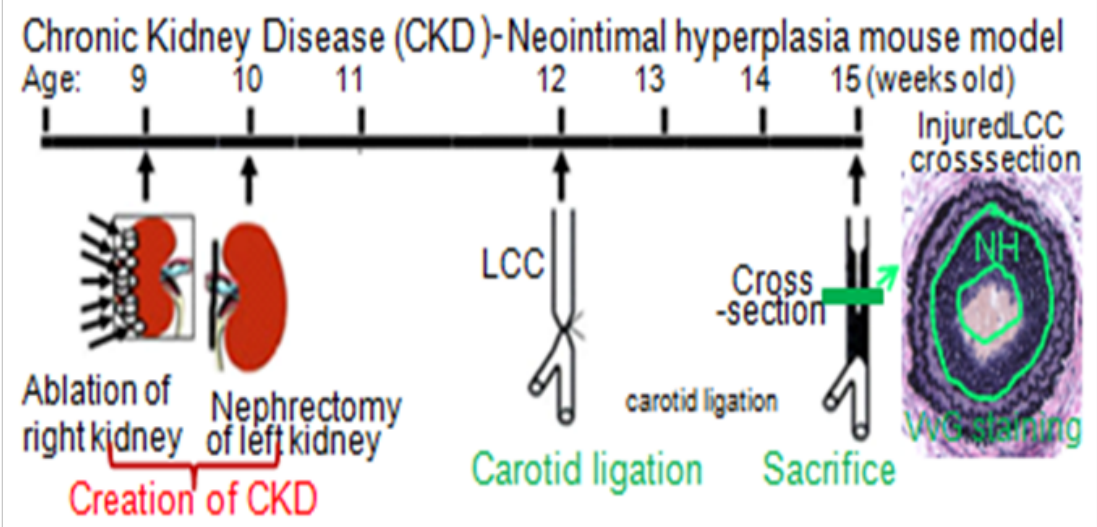

Figure 1. CKD creation and CKD-NH mouse model. Right kidney ablation is performed at 9 wks of age on C57/BL6 background mice with and without Casp1 deficiency and is followed by left nephrectomy a week later to create a CKD state. We verify we have achieved the desired BUN level the following week . On week 4 we perform a left carotid artery (LCC) ligation in order to induce neointimal hyperplasia (NH). Three weeks later the LCC is perfused and fixed with formalin at 100mm HG for 15 minutes and harvested for cross-section immunohistochemistry analysis. VvG staining oh LCC cross-section are presented with concentric NH.

All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals. Mice were housed in veterinarian-supervised AALAC-accredited facilities. The proposed experiments were approved by the Animal Care and Use Committee Institutional Review Board of Temple University School of Medicine (see attached memo). We purchased wild type mice (Caspase-1 $^{+/+}$; background, C57BL/6 mice) from The Jackson Laboratory (Bar Harbor, Maine). Caspase-1 $^{-/-}$ (background, C57BL/6) animals were obtained from Dr. Xiao-Feng Yang. Otherwise, we used C57BL/6 mice for the remaining CKD experiments. We used only 10- to 16-wk-old male mice in the study. They were housed in polycarbonate cages in a pathogen-free, temperature-controlled environment with free access to a standard chow diet and water.

We used a two-step process in the creation of a chronic kidney disease state in mice. First we ablated the left kidney of 10 to 12 wk-old male mice through a 2 cm flank incision using electro cautery. One week after this first procedure we performed a contralateral nephrectomy using a 2 cm flank incision. One week after performing the nephrectomy we performed BUN analysis from plasma samples. Sham control animals received sham operations that included decapsulation of both kidneys during the same time periods as CKD animals. After this initial steps, both CKD and sham groups underwent carotid ligation one week later.

Both CKD and sham groups underwent common carotid ligation. Under a sterile condition, we made a vertical incision in the left neck with the left common carotid artery and carefully dissected out. The carotid bifurcation was identified and a 7-0 prolene suture was used to ligate the common carotid artery at the level of the bifurcation. Three weeks after the creation of arterial injury we euthanized the animals and the common carotid was perfusion-fixed via a left ventricle puncture and perfusion of formalin at 100 mmHg for 15 minutes.

Immunohistochemistry And Histomorphometry

We harvested the left common carotid artery starting distal to the ligation all the way to the ostia at the level of the aortic arch at the time of sacrifice. Samples were processed and embedded in paraffin. We obtained serial sections of 5-μm thickness every 100 μm throughout the entire left common carotid artery including the ligation injury, and we stained them with Verhoeff elastic-van Gieson (VvG) and hematoxylin and eosin (H&E).For immunohistochemistry, adjacent sections were stained for vascular smooth muscle cells (SM alpha-actin, 1:500 dilution; Sigma-Aldrich, St. Louis, Missouri). We performed volumetric measurements for NH lesion and thrombus on digitizing images using Image J software (National Institutes of Health).

CHAPTER 4

RESULTS

Caspase-1 Inhibition In The Setting Of Uremia Increases *In Vitro* Gene Expression Of Contractile Genes

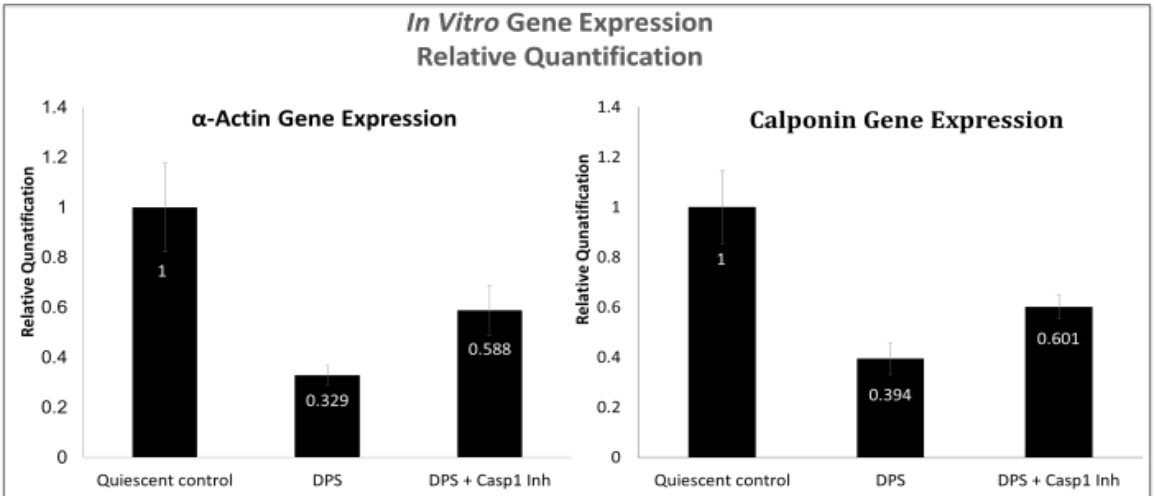

Figure 2. Exposure of HASMCs to uremic serum decreased the expression of contractile markers and this effect is reversed by Casp1 inhibitor. HASMCs were grown to subconfluence and serum starved for 24 hours at which time they were exposed to 10% uremic serum from CKD patients with and without caspase-1 Inhibitor for another 24 hours. RNA was extracted, reverse transcribed and, expression of contractile markers alpha actin, and calponin were measured by quantitative RT-PCR using Applied Biosystems 7500 and Taqman primers and probes. DPS, dialysis patient serum; Casp1 Inh, Caspase-1 inhibitor.

We demonstrated that CKD serum is associated with caspase-1 activation and phenotypic changes in VSMCs from a "contractile" to a "synthetic" phenotype and these changes are reversed with Casp1 inhibition. When human aortic smooth muscle cells are exposed to diabetic patient serum, alpha-actin gene expression decreased to a relative quantification of 0.329 when compared to quiescent cell control. When these cells that were exposed to dialysis patient serum were exposed to caspase-1 inhibitor the relative quantification increased to 0.588 when compared to quiescent control. Calponin gene expression was also decreased when cells was exposed to dialysis patient serum, to 0.394 relative quantification, and increased to 0.601 relative quantification when caspase-1 inhibitor was added.

Creation Of CKD And Sham Mice

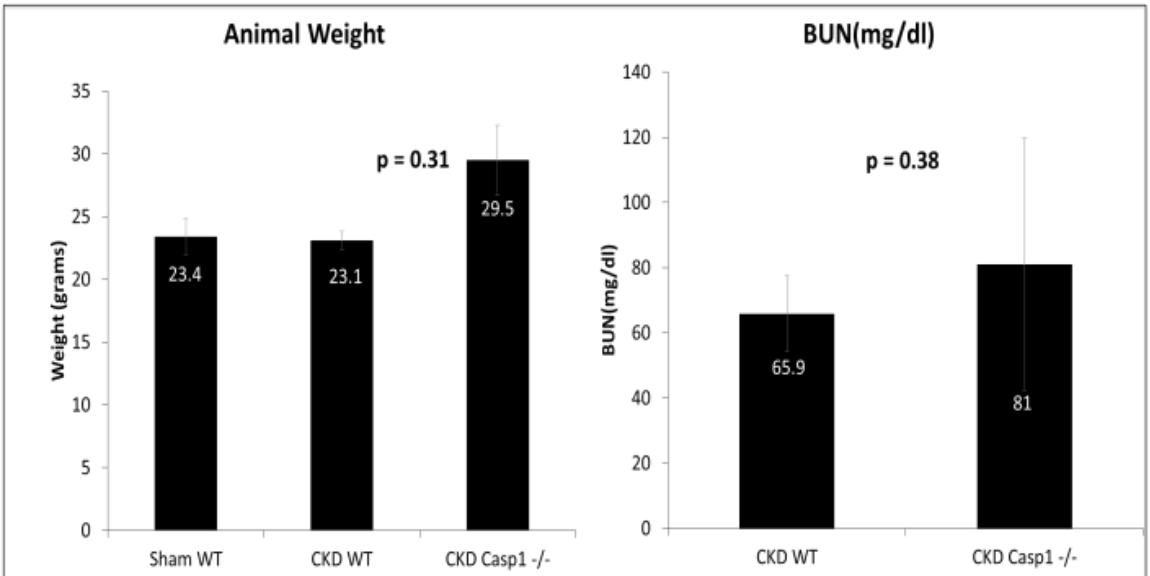

Figure 3. Animal group weight and BUN: There was no statistically significant difference in weight between groups. There was also no statistically significant in BUN levels difference between CKD groups. WT: Wild Type; CKD: Chronic kidney Disease; Casp1 $^{-/-}$:Caspase 1 knout.

To determine the effect of caspase-1 deficiency on neointimal hyperplasia formation in mice we employed our murine CKD model. There was no significant differences in the tested biological measurement between the wild type and caspase-1 knockout CKD groups (65.9 $\pm$ 11.64 mg/dL and 71.43 $\pm$ 35.5 mg/dL; p = 0.18). There was also no significant difference in weight between all groups at the time of ligation.

Caspase-1 Deficiency Significantly Decreases Neointimal Hyperplasia *In Vivo*

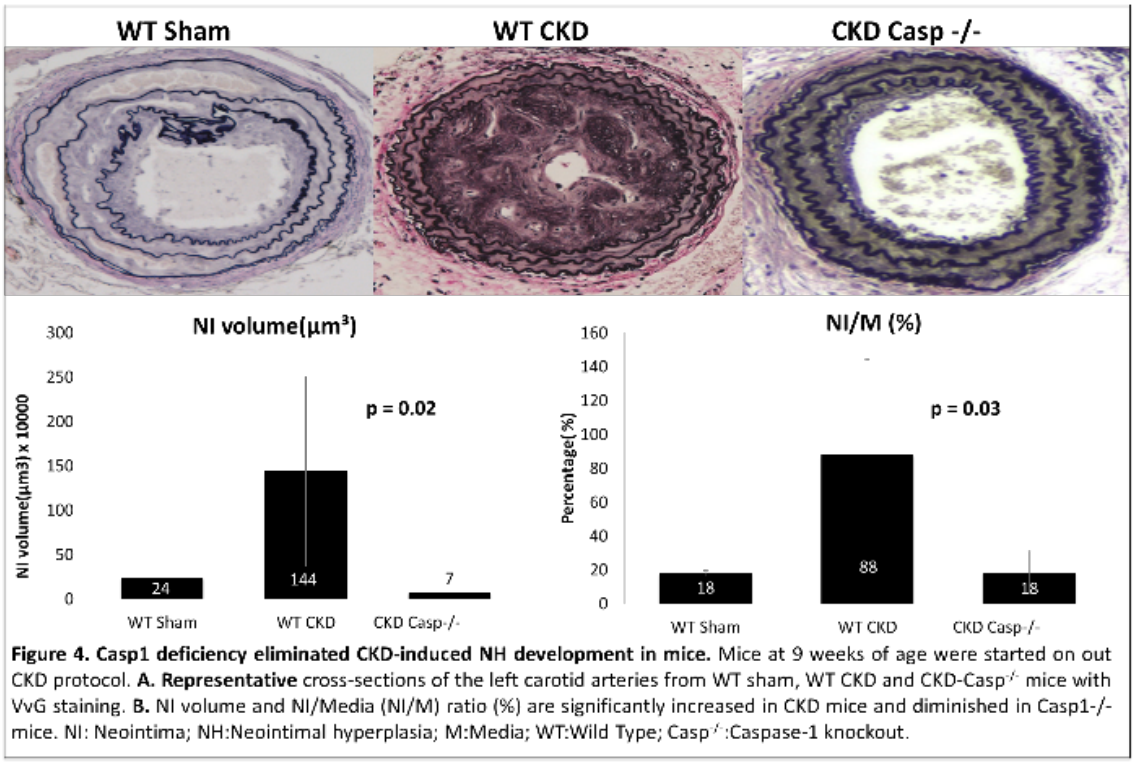

Figure 4. Casp1 deficiency eliminated CKD-induced NH development in mice. Mice at 9 weeks of age were started on out CKD protocol. **A. Representative** cross-sections of the left carotid arteries from WT sham, WT CKD and CKD-Casp$^{-/-}$ mice with VvG staining. **B.** NI volume and NI/Media (NI/M) ratio (%) are significantly increased in CKD mice and diminished in Casp1-/- mice. NI: Neointima; NH:Neointimal hyperplasia; M:Media; WT:Wild Type; Casp$^{-/-}$:Caspase-1 knockout.

Six wild type CKD, eight caspase-1 knockout/CKD and seven wild type sham mice underwent carotid ligation and survived three weeks for the histophotometric analysis. We made serial sections of 5 µm starting from the site of ligation, and proceeding every 100 µm. We observed and measured neointimal hyperplasia in each of these sections. We identified the neointimal hyperplasia lesion, along with the media, and adventitia using Verhoeff–Van Gieson staining.

The neointimal hyperplasia volume increases significantly when comparing wild type CKD mice and wild type sham animals from 711,568.66 µm³ to 9,397,450.35 µm³ ($p = 0.003$). When we compared wild type CKD mice to caspase-1 CKD mice we found that the neointimal hyperplasia volume is reduced significantly in the caspase-1 knockout

animals (9,397,450.35 μm^3 vs. 1,929,361.74 μm^3; $p = 0.008$). We found no difference in the neointimal hyperplasia volume between wild type sham animals and CKD capase-1 knockout animals. We also found a difference in the neointimal hyperplasia/media ratio. We use this measurement modality in order to account for variations caused by the process of slides. The neointimal hyperplasia/media volume ratio of the sham wild type animals is 34.7, that of the CKD wild type animals is 469.32 (sham/wild type vs. CKD/wild type; $p = 0.002$) and that of the CKD caspase-1 knockout animals is 101.2 (CKD wild type vs. CKD caspase-1 KO; $p = 0.007$). We found no significant difference when comparing wild type sham animals to CKD caspase-1 knockout animals ($p = 0.17$). The stenosis percentage was also found to significantly decrease in the caspase-1 knockout animals. Wild type sham animals were found to have a lumen stenosis percentage of 7.35%, and CKD wild type animals were found to have a lumen stenosis of 66.6% ($p < 0.001$). The lumen stenosis percentage of CKD caspase-1 knockout animals is 21.04%. We found a significant difference when we compare CKD wild type animals and CKD caspase-1 animals ($p < 0.001$) and between sham wild type and CKD caspase-1 knockout animals ($p = 0.05$). We found no difference between media volume of any of the three groups.

CKD Decreased The Expression Of Contractile Markers *In Vivo* And This Effect Is Reversed By Caspase-1 Deletion.

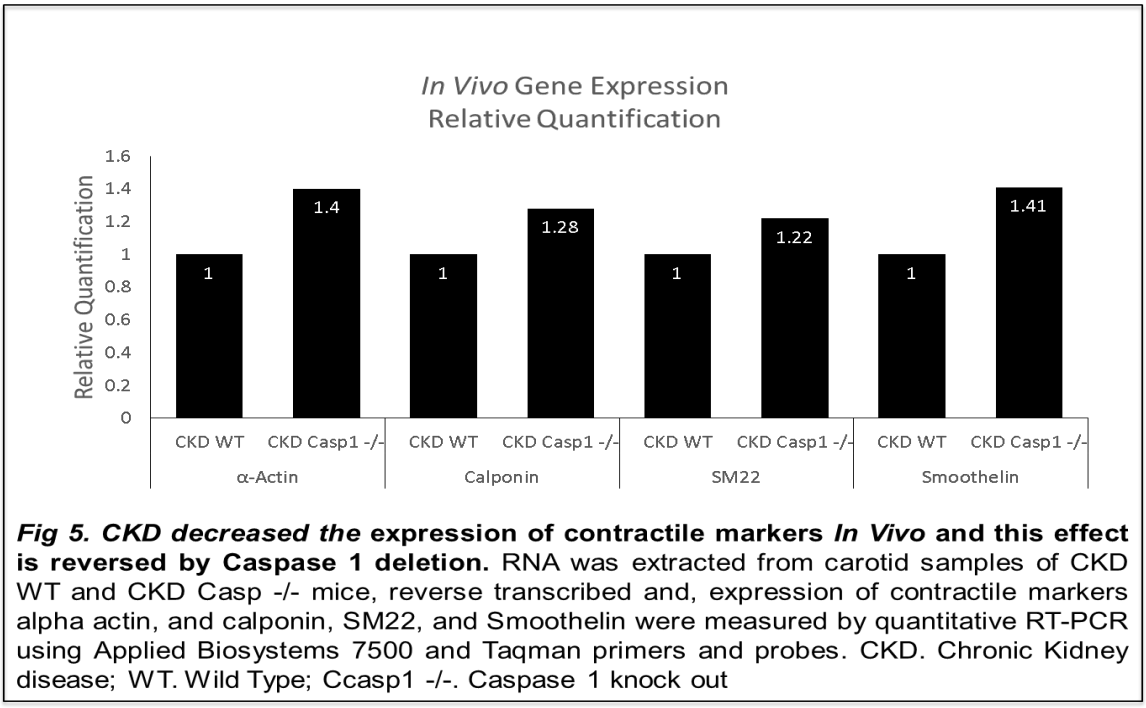

***Fig 5. CKD decreased the* expression of contractile markers *In Vivo* and this effect is reversed by Caspase 1 deletion.** RNA was extracted from carotid samples of CKD WT and CKD Casp -/- mice, reverse transcribed and, expression of contractile markers alpha actin, and calponin, SM22, and Smoothelin were measured by quantitative RT-PCR using Applied Biosystems 7500 and Taqman primers and probes. CKD. Chronic Kidney disease; WT. Wild Type; Ccasp1 -/-. Caspase 1 knock out

We found that contractile marker genes are also significantly preserved in CKD caspase-1 knockout animals when compared CKD wild type animals. We extracted RNA from carotid samples, reverse transcribed expression of contractile markers alpha actin, calponin, SM22, and smoothelin and measured them by quantitative RT-PCR. The relative quantification of alpha actin in caspase-1 knockout animals was 1.4 when compared to CKD wild type animals. The relative quantification of calponin (1.28), SM22 (1.22), smoothelin (1.41) were all elevated when compared to CKD wild type animals.

CHAPTER 5

CONCLUSION

We have demonstrated that caspase-1 pays a critical role in the modulation of neointimal hyperplasia after arterial injury in the setting of uremia. Three weeks after arterial ligation we found that caspase-1 knockout animals had a 4.8 fold decrease in the amount of neointimal hyperplasia volume measured. At this point this is the first demonstration that caspse-1 plays a critical role in the modulation of neointimal hyperplasia in CKD, the primary cause of vascular access failure in patients receiving hemodialysis. This work has led to the writing of a manuscript submitted for publication.

We demonstrated *in-vitro* and *in-vivo* that caspase-1 deficiency preserves vascular smooth muscle cell "contractile" phenotype in mice, with a higher expression of contractile gene expression, compared to cells without caspase-1 deficiency. This is significant because it has been well established that CKD promotes a phenotypical transformation from a contractile phenotype to a synthetic phenotype in VSMCs. These changes have been associated with acceleration of neointimal hyperplasia formation after the creation of arteriovenous fistulas in both humans and murine models.

Caspase-1 has been demonstrated to have a role in the formation of atherogenesis, and endothelial cell activation in the setting of hyperlipidemia. It has been demonstrated that hyperlipidemia leads to the activation of caspase-1(Gage, Hasu, Thabet, & Whitman, 2012). In this setting caspase-1 deficiency has been demonstrated to reduce inflammatory cytokine production and adhesion molecule expression by endothelial cells, leading to the

decreased recruitment of monocytes. In addition, in the setting of hyperlipidemia caspase-1 deficiency has been associated with the accumulation of Sirtulin-1, an anti-inflammatory histone deacetilaze(Y. Yin, 2014). At this time it is unknown whether the mechanisms which lead to caspase-1 activation in the setting of CKD also leads to a proinflamatory state favorable to the formation of neointimal hyperplasia and whether this change is similar to that described in the setting of hyperlipidemia. There appears to be a complicated interaction between endothelial cells, monocytes and vascular smooth muscle cells, and the role that caspase-1 plays in each of these cell lines needs to be determined in order to develop novel therapies for the treatment of vascular access dysfunction and possibly peripheral vascular disease.

The current management paradigm of vascular access stenosis due to neointimal hyperplasia formation includes endovascular and open surgical procedureswhose goal is to reestablish adecuate blood flow through the conduit. These procedures expose patients, whom often suffer from multiple comorbities, to the morbidity and mortlity associated with anesthesia and sugery. If after further research caspase-1 proves to be an important factor in the modulation of neointimal hyperplasia formation and thus vascular access disfunction, its inhibition could reduce the need for these invasive interventions and decrease the adverse outcomes associated with them.

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