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Endothelial cells and cathepsins: biochemical and biomechanical regulation

Manu O. Platt^{a,*} and W. Andrew Shockey^a

^aWallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, 950 Atlantic Drive, Suite 3015, Atlanta, GA 30332, USA

Abstract

Cathepsins are mechanosensitive proteases that are regulated not only by biochemical factors, but are also responsive to biomechanical forces in the cardiovascular system that regulate their expression and activity to participate in cardiovascular tissue remodeling. Their elastinolytic and collagenolytic activity have been implicated in atherosclerosis, abdominal aortic aneurysms, and in heart valve disease, all of which are lined by endothelial cells that are the mechanosensitive monolayer of cells that sense and respond to fluid shear stress as the blood flows across the surfaces of the arteries and valve leaflets. Inflammatory cytokine signaling is integrated with biomechanical signaling pathways by the endothelial cells to transcribe, translate, and activate either the cysteine cathepsins to remodel the tissue or to express their inhibitors to maintain healthy cardiovascular tissue structure. Other cardiovascular diseases should now be included in the study of the cysteine cathepsin activation because of the additional biochemical cues they provide that merges with the already existing hemodynamics driving cardiovascular disease. Sickle cell disease causes a chronic inflammation including elevated TNF α and increased numbers of circulating monocytes that alter the biochemical stimulation while the more viscous red blood cells due to the sickling of hemoglobin alters the hemodynamics and is associated with accelerated elastin remodeling causing pediatric strokes. HIV-mediated cardiovascular disease also occurs earlier in than the broader population and the influence of HIV-proteins and antiretrovirals on endothelial cells must be considered to understand these accelerated mechanisms in order to identify new therapeutic targets for prevention.

1. INTRODUCTION

According to a 2015 American Heart Association report, cardiovascular disease accounts for more than 17 million deaths per year and is the leading cause of death in the world [1]. It is well established that atherosclerotic plaque formation occurs preferentially at areas of low

*Corresponding Author information: Manu O. Platt, Ph.D., Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, 315 Ferst Drive, Suite 1308, Atlanta, GA 30332, manu.platt@bme.gatech.edu, Phone: +1 (404) 385-8531.

Additional Author Information: W. Andrew Shockey, Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, ashockey@gatech.edu

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and oscillatory shear stress, such as those seen at curves and bifurcations within the vasculature; while regions of high, unidirectional fluid shear stress, appear to be atheroprotected [2–8]. Shear stress can be defined as the tangential force of the blood flowing along the vascular wall, and the endothelial cells lining the blood vessel wall are directly exposed to it. These mechanical forces not only cause morphological changes in the endothelium and blood vessel wall, but also trigger biochemical and biological events. Endothelial cells form a monolayer of cells that line blood vessel walls and serve several functions, many of which are induced based on local hemodynamics and shear stress: regulation of cellular adhesion molecules on the cell surface including E-selectin, ICAM-1 and VCAM-1; monocyte recruitment, adhesion, and migration into the vascular wall; inhibition of platelet aggregation, thrombosis, and coagulation; selective transport of macromolecules from the blood into the blood vessel wall; and regulation of vascular tone by stimulating smooth muscle cell contraction or relaxation by producing endothelin-1 or nitric oxide, respectively [3, 9–14]. A number of mechanosensitive cellular mechanisms and signaling pathways in endothelial cells have been described and are comprehensively reviewed by Davies[9].

More importantly for this review, however, is the regulation of vascular structure by endothelial cells via production or inactivation of cysteine cathepsins, powerful elastases and collagenases that can remodel the arterial wall and contribute to cardiovascular disease progression. Low and oscillatory shear stress at sites of disturbed flow at bifurcations and branches activates shear-mediated cysteine proteases, the cathepsins, powerful elastases and collagenases which have the ability to remodel extracellular matrix, initiating and promoting elastic lamina fragmentation, neointimal thickening, and plaque progression, while also modifying the mechanical properties of the arterial wall [15–17]. Of note, multiple cathepsins have been implicated in the pathologies of atherosclerosis, abdominal aortic aneurysms (AAA), and heart valve disease, which all preferentially occur at these hemodynamically defined regions. Mechanisms of regulation and the physiological consequences of endothelial cell cysteine cathepsin production by both biomechanical and biochemical influences will be discussed in this review. Additional discussion of endothelial cell cathepsin regulation in HIV-mediated cardiovascular disease and in sickle cell disease vasculopathy will also be included as these are new diseases at the frontier of cysteine cathepsin activity in biochemical and biomechanically driven arterial remodeling that should garner attention.

1.1. Shear stress and endothelial cells

Shear stress is a mechanical, tangential force over the area of the endothelial monolayer as the blood drags across it, and this changes with the cardiac cycle. Differences in magnitude and frequencies of shear stress in the arteries have been linked to a number of cardiovascular health and disease mechanisms [15, 16, 18–22]. High unidirectional laminar shear stress, such as that found in the straight parts of the arteries, signals the endothelial cells to produce several atheroprotective proteins, including cystatin C, the main protein inhibitor of the cysteine cathepsins. Low or oscillatory shear stress, such as that found at bifurcations or sharp turns in the vascular tree where atherosclerotic plaques are localized, does the inverse: it induces cysteine cathepsin expression and activity as well as cell adhesion molecule

expression to promote monocyte adhesion and other local inflammation for atherosclerosis. Certain portions of the vascular tree appear to be differentially regulated by shear stress. The geometry of the proximal ascending aorta produces native regions of oscillatory shear stress. Endothelial cells on the aortic side of the aortic valve are normally subjected to oscillatory shear stress after the valves close, and a loss of this oscillatory stress results in expression of adhesion proteins and cytokines consistent with endothelial activation [23, 24]. Cathepsin expression by endothelial cells are ultimately regulated by this complex inflammation cascade and altered biomechanical hemodynamic signaling [15, 16, 25, 26].

1.2. Cathepsins expressed in endothelial cells

Cathepsins K, L, S, and V are of particular focus here; they have unique properties and homeostatic functions, but share 60% sequence homology [27–30]. They are also potent collagenases and elastases that have been highly implicated in cardiovascular diseases and have been shown to be upregulated by endothelial cells at sites of disturbed flow, increasing elastin and collagen degradation *in vitro* and in diseased human arteries [15, 16].

1.2.1. Cathepsin synthesis and regulation—Cysteine cathepsins are included in the papain family of proteases that comprises 11 members denoted by letters: cathepsins B, C, F, H, K, L, V, O, S, W, and Z (or X). In mammalian cells, cathepsins were first identified in lysosomes, but are now known to play functional roles in other cellular compartments and even in the extracellular space after secretion [31, 32]. Cathepsins are synthesized in their inactive form, and the N-terminal propeptide must be enzymatically cleaved to expose the active site for substrate catalysis. This can occur by positive feedback if other, active, proteases cleave the propeptide off of each other [28, 33]. Many are also capable of autocatalytically cleaving their own propeptide under acidic conditions to activate themselves [33–35]. Mature, active cathepsins are optimally active at acidic pH, and prefer reducing environments for the –SH group of the active site cysteine to participate in the nucleophilic attack that cleaves peptide bonds, and can be inactivated/denatured due to pH, oxidation, or other mechanisms [36]. If cathepsins escape their intracellular compartments, they are susceptible to inhibition by the cystatins, a family of protein inhibitors that regulate and inhibit intra- and extracellular cathepsin activity, and are usually produced and present in high molar excess to the cathepsins for healthy maintenance of cell and tissue proteins [37–41]. Taken together, cathepsins exist as a system of transiently active enzymes working simultaneously and quickly, but their cell/tissue specificity, substrate preferences, binding affinities, and catalytic rates differ and will be discussed next.

1.2.2. Cathepsins K and S—Cathepsin K is capable of solubilizing collagen I better than MMP-9, -1, and -13 [42]. It has the unique proteolytic capability of cleaving types I and II collagens at the telopeptides and intrahelically, which lead to its characterization as the most potent mammalian collagenase [42]. Cathepsin K also has strong elastase activity [28]. Although this protease was first characterized with osteoclasts for bone resorption, cathepsin K has since been identified as being present in atherosclerotic plaques [43, 44], and produced by endothelial cells, particularly at sites of disturbed blood flow [16, 45, 46]. *In vitro* studies with mouse aortic endothelial cells indicate that cathepsin K is secreted by endothelial cells in the procathepsin form, but can be activated to the mature form after

secretion. Mature, cathepsin K was detected in the conditioned media of endothelial cells cultured under oscillatory shear stress, but not under static, no flow conditions [16], suggesting that there was a disturbed flow mediated activation mechanism. Biochemically, Interferon (IFN)- γ and interleukins(IL)-6 and -13 have been shown to stimulate cathepsin K in vascular cells and macrophages, while TGF β and IL-10 have inhibited cathepsin K expression and activity in a number of different cell types [43, 47–51]. TNF α is an inflammatory cytokine that is upregulated at sites of disturbed flow and low, oscillatory shear stress [52] and has been shown to independently stimulate cathepsin K expression and activity in human aortic endothelial cells [45, 46].

Human umbilical vein endothelial cells (HUVECs), rat aortic endothelial cells (RAECs), and bovine aortic endothelial cells (BAECs) exposed to vascular endothelial growth factor (VEGF) increased transcription and translation of cathepsin K, and chemical hypoxia further increased these expressions[53]. This data contributes to the role of cathepsins in angiogenesis which is a process driven by endothelial cells, and also demonstrated that cathepsin K cleaved Notch1 which was involved in this signal cascade upstream of Akt phosphorylation, but downstream of VEGF receptor phosphorylation, all of which were lowered in cathepsin K-deficient mice implicating cathepsin K. All were upregulated when cathepsin K was overexpressed [53].

Cathepsin S is a potent elastase and has the special characteristic of retaining activity at neutral pH, making it unique among the papain family of cysteine proteases [28]. Cathepsin S has been identified in endothelial cells, smooth muscle cells, and macrophages [43] and shown to be regulated by shear stress in mouse and human aortic endothelial cells [15, 54]. Diseases associated with elastolytic remodeling have identified cathepsin S as a contributing culprit [55] including atherosclerosis [56–58] and abdominal aortic aneurysms [28, 59]. Biochemical regulation of cathepsin S has been accomplished by TNF α , IFN γ , VEGF, and basic fibroblast growth factor (bFGF) with observed increased mRNA and protein levels [60]. Similar to cathepsin K, cathepsin S has also been implicated in angiogenesis with ischemia induced neovascularization being partially controlled by cathepsin S regulation of PPAR- γ and VEGF/Akt signaling pathways [61]. Tumor associated endothelial cells also showed cathepsin S activity [62], and cathepsin S deficient mice had reduced neovascularization and reduced tumor size [62].

Cathepsins K and S are interesting in that a recent report indicated a new proteolytic interaction of cathepsin S hydrolyzing cathepsin K, termed cathepsin cannibalism [63]. The effect of this interactive degradation led to a reduction in total substrate degradation by the combined enzymes from what would have been expected if cathepsins K and S were inert to each other and solely focused on degrading the elastin or collagen protein present in the systems. As a therapeutic tool, this study demonstrated that excess cathepsin S could cleave cathepsin K and mitigate type I collagen degradation. However, since both cathepsins K and S cleave elastin at high catalytic rates, such excess cathepsin S would be detrimental and accelerate elastin fragmentation and degradation in the arterial wall. Both cathepsins K and S are produced and secreted by endothelial cells under low, oscillatory shear stress, but to understand the net effects in the artery wall where both collagen and elastin are the dominant

structural proteins will mandate consideration of the cannibalistic interactions, cathepsin molar ratios, and the types and amounts of substrate or cystatin inhibitors.

1.2.3. Cathepsins L and V—Cathepsins L and V have been implicated in atherosclerosis, abdominal aortic aneurysms, and heart valve disease being regulated by inflammation and biomechanical stimuli [15, 64–67]. Cathepsins L and V in humans share 80% homology [29], and because of this, parsing and identifying the activity of one over the other has been confusing. To confuse things further, mouse cathepsin L is the ortholog to human cathepsin V and there is no mouse equivalent to human cathepsin L [29, 68]; this is especially important when interpreting cathepsin L null mouse studies to make inferences about human disease. Cathepsin V (murine cathepsin L) has been deemed the most potent mammalian elastase even above cathepsins K and S [68] although it is generally believed to be intracellular with tissue-specific expression [68, 69]. Much of this powerful elastase activity had previously been ascribed to human cathepsin L, but this is now being corrected. Cathepsins L and V only function at acidic pH and lose activity quicker than other cathepsins as pH rises [70].

Cathepsin L has also been identified in endothelial cells of both mice and humans [15, 64]. Cathepsin L cleaves a variety of ECM substrates: fibronectin, laminin, and type I, IV, and XVIII collagen, even at neutral pH [71]. When it cleaves collagen XVIII, the anti-angiogenic endostatin fragment is released [72], and in bovine coronary artery endothelial cells, cathepsin L derived endostatin at high levels could cause endothelial dysfunction, NADPH oxidase activation and increased O_2^- production [73]. This may be a biological negative feedback mechanism since NADPH oxidase activity can decrease cathepsin activity [74], presumably due to oxidation of the active site cysteine.

Cathepsin L has a number of biochemical pathways that regulate its expression. Elevated glucose lowered cathepsin L mRNA production, gelatinolytic activity, and invasion of endothelial progenitor cells compared to controls [75] suggesting a role for cathepsin L in diabetes impaired angiogenesis and wound healing. Endothelial progenitor cells from patients with type 2 diabetes had significantly reduced cathepsin L activity and protein amount compared to healthy controls [75]. Besides glucose, VEGF also increased cathepsin L expression in human glioblastoma cells (U87MG) with upregulation occurring at the transcriptional level. A VEGF response element (VRE) was found in the promoter region of cathepsin L, for SP-1 and AP-4 transcription factors to bind [76]. This regulation of cathepsin L has not yet been linked in endothelial cells, but endothelial cells do express VEGF receptors and are VEGF responsive suggesting this mechanism may be involved in its biochemical regulation. Platelet derived growth factor (PDGF) also regulates cathepsin L's transport through the Golgi apparatus and secretion (through a suspected mannose-6-phosphate receptor mediated mechanism). This response was unique to cathepsin L, as increased cathepsin S synthesis occurred downstream of PDGF, but did not lead to its secretion [77]. Cathepsin L epigenetic regulation by hyper-methylation of its promoter in human peripheral blood mononuclear cells was inhibitory. When treated with 5-aza-cytidine to demethylate genomic DNA, there was an increased level of cathepsin L detected by immunolabeling of the cells. A 40 CpG site island was identified in the cathepsin L promoter just before the transcription initiation site [78].

1.2.4. Cystatin C—Cystatins are the group of inhibitors of lysosomal cysteine proteases, of which, cystatin C is the most prominent and tightly binding [28]. This 13 kD protein is translated with a 26 amino acid signal peptide that targets it for secretion approximately one hour after post-translational processing [79] and is produced by most nucleated cell types to keep high molar ratios in almost all bodily fluids to bind any free cathepsins that have escaped the endosomes/lysosomes. Cystatin C binds cathepsins in a tight, reversible fashion with an inhibition constant on the subnanomolar range [80]. Cystatin C has been found in normal human artery tissue sections [25, 26], but was absent in atherosclerotic sections, illustrating inverse regulation of cystatin C compared to the cysteine proteases it inhibits. Shear stress on endothelial cells alone is sufficient to inversely regulate cystatin C compared to cathepsins: unidirectional, vasoprotective shear stress increases endothelial cell mRNA, protein expression, and secretion of cystatin C, while oscillatory, pro-remodeling shear stress downregulate cystatin C expression by endothelial cells [81]. This inverse response for cystatin C to biomechanical stimuli compared to cathepsins, suggests this to be an important regulatory mechanism by which endothelial cells either maintain arterial wall structural integrity at sites of high unidirectional shear stress to prevent collagen and elastin degradation, or allow matrix degradation to remodel the collagen and elastin in the artery for plaque progression.

Secretion of cystatin C in cultured mouse peritoneal macrophages was reduced following treatment with pro-inflammatory lipopolysaccharide or IFN- γ [82]. Additional *in vitro* and *in vivo* studies utilizing IL-6, CpG oligodeoxynucleotides, and other inflammatory signals have provided additional evidence for an inverse relationship between inflammation and cystatin C expression and secretion in hematopoietic cells [83, 84]. In contrast, human gingival fibroblasts treated with periodontal pathogens and pro-inflammatory cytokines increased expression of cystatin C, suggesting cystatin C is differentially regulated in different cell types [85, 86].

2. Endothelial cells and cathepsins in cardiovascular diseases

The pathophysiological importance of cathepsins K, L, S and V in atherosclerosis has been demonstrated in double-knockout mice deficient in apolipoprotein E, which showed a reduction in the number and size of atherosclerotic lesions and in some cases decreased fragmentation of the elastic lamina. When cathepsin activity is pathologically increased within the arterial wall, either due to low, oscillatory shear stress or biochemical factors, vascular remodeling occurs due to degradation of the structural proteins that provide mechanical stability and stimuli (Figure 1). Cathepsin activity has been implicated in atherosclerosis, abdominal aortic aneurysms, and in heart valve disease, and their specific functions are described below. The biochemical and biomechanical stimuli and cardiovascular pathologies associated with cathepsins K, L, S and V are consolidated in Table 1.

2.1. Atherosclerosis

Atherosclerosis is the hardening of arteries due to lipid accumulation and oxidation, elastic lamina fragmentation promoting smooth muscle cell phenotype switching and migration into the neointimal space, foam cell accumulation and rupture, and a number of other

mechanisms that lead to hardened arteries, without the elastic recoil necessary to maintain the pressure wave of blood. Upon rupture, this plaque releases these accumulating toxins and apoptotic cells into the blood which causes a thrombotic event that can block passage of oxygenated blood [21]. If this were in a coronary artery, then this would be a heart attack. If in an artery supplying the brain, it would cause a stroke. As mentioned earlier, atherosclerosis occurs mainly at branches or bifurcations in the arterial tree where low, oscillatory shear stress occurs, and cathepsin upregulation at these regions are involved in the pathological tissue remodeling for these atherosclerotic plaques to develop. The most studied of these regions include the carotid artery bifurcation, the lesser curvature of the aorta, and the coronary arteries with the hemodynamics excellently reviewed by Ku [87].

Cathepsins are dually important in the context of cardiovascular disease because of their shear stress-dependent regulation. Mouse aortic endothelial cells increased cathepsin K, S, and L expression when exposed to oscillatory shear stress, while unidirectional shear stress inhibited gelatinase and elastase activity in a cathepsin-dependent manner [15, 16]. Cathepsin K levels in endothelium positively correlated with progression of atherosclerotic lesions in human studies of atherosclerosis [16]. Cathepsins K and S are significantly increased in atherosclerotic lesions compared to healthy vessels, [16, 43] and human atherosclerosis samples showed a positive correlation between atherosclerotic lesion development and cathepsin K levels in the endothelium [16]. Fibrous cap rupture can also be attributed to increases in proteolytic activity by cathepsins K and L as they can degrade or erode the extracellular matrix (ECM) destabilizing the plaque [26, 64]. Several *in vivo* studies have shown expression of cathepsin K by SMCs and macrophages and identified their roles in vascular remodeling [16, 88].

2.2. Abdominal aortic aneurysms (AAA)

Aneurysms are defined as a permanent dilatation of a blood vessel, and one that occurs in the infrarenal aorta is termed an abdominal aortic aneurysm. The abdominal aorta is subject to disturbed flows because of anatomical factors such as the diaphragm, the lumbar curvature, and low resistance in the renal arteries. These cause the formation of strong vortices, skewed flow, and reverse flow into the renal arteries, respectively [87]. Several human and animal studies have also demonstrated that abdominal aortic aneurysms occur in regions exposed to unstable flow conditions including flow reversal, low mean wall shear stress and high oscillatory shear index [89–93]. In contrast, relatively high levels of laminar shear stress were shown to reduce AAA progression in rat experimental models [94, 95]. Elastin and collagen destruction in this section of the aorta are characteristic of AAA with cathepsins implicated in human and animal studies [64]. Histological analysis revealed luminal endothelial cells of abdominal aortic aneurysms express cathepsins B, K and S [96]. Fragmentation of the elastic fibers in the medial layer is an initial step in AAA development, but loss of the structural support of types I and III collagen in the adventitia as they are degraded eventually causes rupture of the aneurysm, from which 65% of patients die [95].

2.3. Heart valve disease

The aortic valve experiences a complex hemodynamic environment depending on leaflet location facing the aorta or the ventricle, percent stenosis if stiffening or calcification has

occurred, or fusion if two of the three leaflets become stuck together. The hemodynamics change with the opening and closing of the valves during the cardiac cycle and ventricular contraction [97]. Valvular endothelial cells lining both faces of the leaflets respond differently to the shear stress [98], and cathepsins have been shown to be shear sensitive in this tissue [99]. Cathepsin L was regulated in porcine aortic valves by both shear stress and by cyclical pressure to simulate hypertension; hypertension caused a significant decrease in cathepsin L activity as did steady unidirectional shear stress, and immunohistochemical staining confirmed this regulation throughout the valve leaflet including the endothelium [17].

Stenotic aortic valves generate regions of pathologically high shear stress on the ventricular face of the leaflets, but are then subject to disturbed flow on the aortic face because of the jet streams caused by left ventricle contraction of the large volume of blood through the fixed, narrow opening [100]. Diseased heart valves contained higher mRNA levels for cathepsins S, K, and V compared to controls, and also increased cystatin C expression [101]. This direct relationship between the cathepsins and cystatin C differed from AAA and atherosclerosis where there was inverse regulation. Histological analysis revealed that the cathepsins and cystatins could be differently localized, however, which could allow uninhibited cathepsins to proteolyze tissue on regions of the valve [101].

Bicuspid aortic valve disease, a congenital disease where two of the aortic valve leaflets fuse together [101], results in altered shear stress from the normal trileaflet aortic valve [103]. Porcine valves subjected to wall shear stress consistent with bicuspid aortic valve disease displayed significantly increased fibrosa endothelial activation and increased production of cathepsins L (11.7 fold), and S (16.7 fold)[97]. Ventricularis and fibrosa of porcine aortic valve leaflets subjected to elevated fluid shear stress showed increased cytokine expression, while leaflets subjected to increased magnitude or abnormal frequency increased extracellular matrix degradation from cathepsins and matrix metalloproteinases [97, 102].

3. New frontiers: Cathepsin responses to sickle cell disease and HIV-mediated vasculopathy

Two other biochemical perturbations to the cardiovascular system are associated with accelerated cardiovascular disease: sickle cell disease and HIV infection. Both of which stimulate endothelial cells with new biochemical and inflammatory factors that, along with the disturbed flow present in the arterial tree, has exacerbated arterial wall remodeling and new studies implicate cysteine cathepsins in the elastin and collagen degradation to advance the disease.

3.1. Sickle cell disease vasculopathy

Sickle cell disease presents a new and unique challenge of biomechanical and biochemical regulation that converge at the endothelial cell, and recent studies by us have demonstrated the role of cathepsin activity in sickle cell associated arterial remodeling [99, 104]. The sickle point mutation induces changes to red blood cells (RBCs) that deforms and damages the membrane, and ultimately changes bulk flow properties in unexpected ways but also

alters the biochemical milieu to one of chronic inflammation [45, 46, 105]. During the major clinical trial STOP, Transcranial Doppler ultrasound was used to measure elevated time averaged mean blood flow velocity in the internal carotid artery, middle cerebral artery, or the anterior cerebral artery. If children with blood velocities greater than 200 cm/s were deemed at risk for stroke and placed on monthly blood transfusion regimens [106, 107]. The altered blood flow can be caused by stenoses in the arteries due to intimal hyperplasia, adhesions to the vessel wall by sticky, damaged red blood cells, trapped cell white blood cell/RBC aggregates or other mechanisms that can disturb the blood flow and may activate the shear stress sensitive cathepsins by endothelial cells.

Sickling of the RBCs occurs after deoxygenation causes hemolysis and release of hemoglobin into the plasma. The heme group generates reactive oxygen species and also scavenges nitric oxide (NO), which blocks flow-mediated vasodilation caused by endothelial cell production of NO under high shear stress[108]. Vaso-occlusive episodes occur in the postcapillary venules when the sickled RBCs block blood flow completely inducing a painful sickle crisis, accompanied by extreme pain, hemolysis, ischemia, and tissue damage. Sickling throughout the vascular tree induces chronic inflammation and damage, and as a consequence, there are increased numbers of circulating monocytes and concentrations of TNF α [105, 109, 110]. There is also accelerated elastin degradation in sickle cell disease with exacerbated fragmentation of the internal elastic lamina in the cerebral arteries [111–114]. Couple this with disturbed flow as indicated by the elevated velocities, and this opens the possibility of cathepsin involvement in sickle cell disease vasculopathy.

We have demonstrated recently that TNF α stimulation of human aortic endothelial cells increases monocyte adhesion and signals endothelial cell production of cathepsins K and V [115] under static conditions. More importantly, peripheral blood mononuclear cells isolated from the blood of people with sickle cell disease was shown to induce cathepsin K and V activity even without exogenous TNF α stimulation[116]. This illustrates that people living with sickle cell disease are subject to positive feedback where they have greater TNF α plasma concentrations, increased numbers of circulating monocytes, leading to increased adhesions to endothelium lining vessels, and induction of cathepsins K and V, the potent collagenase and elastase that can remodel the arterial wall for intimal hyperplasia and accelerated luminal narrowing. Altogether, this series of events could place children with sickle cell disease at risk for strokes.

TNF α and sickle cell disease mediated cathepsin K induction was shown to occur via JNK signaling; phosphorylation of JNK and c-jun was shown by Western blotting, and TNF α mediated induction of cathepsin K could be blocked with the JNK inhibitor SP600125 [117]. TNF α is known to also stimulate NF κ B activation and nuclear translocation for gene transcription and regulation. Studies have shown that activation of JNK via cytokine stimulation is accompanied by increased NF κ B nuclear translocation, and the AP-1 response is enhanced by the presence of NF κ B subunits [45, 46], even in endothelial cells. JNK phosphorylates c-jun which together with c-fos, forms the transcription factor AP-1. Additionally, NF κ B signaling has been shown to be increased in regions of pro-remodeling shear stress in mice, and *in vivo* inhibition of NF κ B protects against atherosclerotic lesion formation [118–120] (Figure 2). These may be other pathways by which sickle cell disease

activates cathepsin activity and how endothelial cell response to biomechanics and biochemistry mediate these actions.

3.2. HIV-mediated atherosclerosis

Over 65 million people worldwide have been infected with HIV-1 and the incidence continues to grow. As life expectancy of HIV-1 infected patients on antiretroviral therapy has significantly increased, cardiovascular disease as a comorbidity has become a significant problem. HIV+ populations are at increased risk of heart attacks [121–124] and higher prevalence of atherosclerotic lesions [125–127], and we have even demonstrated increased arterial stiffness due to the HIV proteins alone or due to the antiretrovirals [128, 129].

It is not definitive whether endothelial cells can become infected by HIV *in vivo*, but models using HIV-1 transgenic animals have shown that the viral proteins alone are enough to alter vascular function [130]. Of specific interest is HIV-1 Tat, a 14-kDa protein known as the transactivating factor, which performs regulatory functions and increases infectivity of the virus [131]. In HIV-1-positive patients, Tat concentrations in the plasma are between 2 – 40 ng/ml due to its secretion from infected T-cells and monocytes [132, 133]. We showed a synergism between HIV proteins and pro-atherogenic shear stress in our transgenic HIV mouse model that led to increased endothelial cell expression of cathepsin K, among the already expressed cathepsins V and S under pro-atherogenic shear stress [134] (Figure 3). This was done using an *in vivo* HIV-Tg mouse model. Endothelial-specific results were confirmed with an *in vitro* cone and plate bioreactor with human aortic endothelial cells stimulated with Tat protein. This was a direct demonstration of the integration of the biomechanical stimuli of shear stress and the biochemical stimuli of Tat protein to turn on cathepsin K in endothelial cells [135]. Immunohistochemical staining of isolated aortas from the HIV-transgenic mouse model showed strong cathepsin K staining in endothelial layers as well as medial layers suggesting that the endothelial cells were secreting cathepsin K basally into the wall for it to accumulate locally prior to degrading the elastic lamina. Cathepsins S and L were immunopositive as well, but cathepsin K showed the greatest response to HIV Tat. These results indicate a need to investigate endothelial cell responsive effects in people living with HIV and their increased risk of cardiovascular disease.

3.3. Cathepsin Cannibalism

With endothelial cells producing a number of different cathepsins in response to the biochemical and biomechanical stimuli discussed above, a comprehensive examination of their total impact on elastin and collagen degradation in the arterial wall must be considered to determine the true consequence. Cooperative or antagonistic activity between cathepsins during degradation of elastin and collagen must be elucidated to fully describe these systems, but also to appropriately dose and apply cathepsin inhibitors that have suffered during phase II clinical trials [54, 136, 137]. We recently demonstrated a novel mechanism by which cathepsin activity is additionally regulated: cathepsin cannibalism [138]. Cathepsins hydrolyzed each other as well as target matrix substrates, negating the assumption of inertness between proteases. Computational analysis in conjunction with experimental validation was necessary to parse the cleavage of cathepsin K by cathepsin S, but these relationships need not be limited to these two family members. By considering

cathepsin cannibalism in descriptions of the system of proteases induced during cardiovascular disease, sickle cell disease, or HIV-mediated atherosclerosis, whether by biomechanics or by biochemistry could challenge paradigms of enzyme-substrate catalytic reaction kinetics and complicate computational models of tissue remodeling.

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HIGHLIGHTS

- Endothelial cell cathepsins are regulated by biochemical and biomechanical stimuli
- Cathepsins are implicated in multiple cardiovascular pathologies
- Cathepsins are involved in HIV and sickle cell disease related CVD complications

Figure 1. Biochemical and biomechanical stimuli and signaling in endothelial cells

Cathepsin up or down-regulation is regulated by signaling from cytokines, growth factors, monocyte adhesion, and also shear stress and other biomechanical stimuli. The intracellular signaling proteins and transcription factors synergize or compete to control the transcription, translation, secretion, and activity of cathepsins K, L, S, and V extracellularly. Once outside of the cell, they are able to then degrade or remodel the elastic lamina and collagen layers in the artery wall.

Figure 2. Sickle cell disease alters the fluid dynamics and the inflammatory stimuli to elevate cathepsin activity

Cathepsins K and V have been shown to be elevated by endothelial cells when exposed to the chronic inflammation induced by sickled red blood cells, hemolysis, and damage caused by the sickle hemoglobin mutation. Elevated numbers of circulating monocytes, TNF α levels, and regions of disturbed flow and oscillatory shear stress converge on endothelial cell upregulation of cathepsins to accelerate elastin degradation in children promoting intimal thickening, lesion development, and strokes.

Figure 3. HIV proteins and antiretrovirals are additional biochemical stimuli that converge with biomechanics to induce accelerated cardiovascular remodeling

HIV infection increases risk of myocardial infarction and other cardiovascular diseases. The HIV protein Tat has been shown to induce cathepsin K activity in endothelial cells when they are exposed to the pro-atherogenic oscillatory shear stress. Antiretrovirals are also implicated in regulating cathepsin expression and/or activity, but the mechanisms of action are still to be determined (represented by the question marks in the image).

Table 1

Summary of biochemical and biomechanical stimuli that regulate cathepsin expression and activity in different cardiovascular diseases.

	Biochemical stimuli	Biomechanical stimuli	Type of CVD
Cathepsin K	IFN γ $\uparrow$, IL-1 β $\uparrow$, IL-6 $\uparrow$, IL-13 $\uparrow$, TGF β $\downarrow$, IL-10 $\downarrow$, TNF α $\uparrow$, VEGF $\uparrow$, JNK/c-jun, Akt, NF κ B, HIV-Tat $\uparrow$, monocyte adhesion $\uparrow$	Low, oscillatory SS $\uparrow$ High unidirectional SS $\downarrow$	Angiogenesis, Atherosclerosis, AAA, stenotic heart valve, sickle cell disease, HIV
Cathepsin L	VEGF, PDGF, promoter methylation $\downarrow$	Low, oscillatory SS $\uparrow$ High unidirectional SS $\downarrow$ Pressure $\uparrow$	Atherosclerosis, AAA, bicuspid heart valve
Cathepsin S	TNF α $\uparrow$, IFN γ $\uparrow$, IL-1 β $\uparrow$, VEGF $\uparrow$, bFGF $\uparrow$, PPAR γ	Low, oscillatory SS $\uparrow$ High unidirectional SS $\downarrow$	Atherosclerosis, AAA, angiogenesis, ischemia neovascularization, HIV, stenotic valves, bicuspid valves
Cathepsin V	TNF α $\uparrow$, JNK/c-jun, Akt, monocyte adhesion $\uparrow$	Low, oscillatory SS $\uparrow$ High unidirectional SS $\downarrow$	Atherosclerosis, Stenotic valves, sickle cell disease,