Chapter 2
Vascular Aging: Revealing the Role and Clinical Perspectives of the Urokinase System

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The average lifespan of humans is growing gradually, resulting in an increased percentage of people entering the 65 and older age group. It is expected that this age group will reach 20 % of the population by 2030. It is also predicted that more than 40 % of all deaths in this age group will result from cardiovascular diseases (CVD). Therapy costs will triple by 2030 (Heidenreich et al. 2011). Age is the most important determinant of vascular health. Conversely, a healthy cardiovascular system is of vital importance for an organism's longevity. More than 100 years ago, Sir William Osler (1849–1919) formulated accordingly: "You are as old as your arteries." Indeed, arterial health remains a valid predictor of disease risk and all-cause mortality. Understanding the nature and molecular mechanisms of age-related vascular dysfunction and its relation to CVD constitutes an important task for biomedical research aiming at developing new therapeutic strategies and improving the quality of life of the elderly population.

In this chapter, we will review the progress achieved in research deciphering the molecular mechanisms of vascular aging, address general and vascular disease-related functions of the urokinase-type plasminogen activator (uPA)/uPA receptor (uPAR) system, and finally give a short overview of therapeutic strategies being developed to target the urokinase system.

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2.1 Aging and Cardiovascular Diseases

Independently of other risk factors like hypertension, diabetes, and hypercholesterolemia, aging results in progressive morphological and functional changes in the vascular wall. The arterial wall is comprised of three layers: *tunica intima*, a single layer of endothelial cells lining the interior surface of the blood vessels; *tunica media*, circularly arranged vascular smooth muscle cells (VSMCs) embedded in VSMC-produced extracellular matrix (ECM), maintaining vascular tone; and *tunica adventitia*, connective tissue containing predominantly fibroblasts. Aging is characterized by changes in endothelium and VSMCs, leading to arterial wall thickening and increased stiffness along with exaggerated expression of inflammatory molecules and elevated uptake of plasma lipoproteins. These changes are clinically manifested by increased systolic pressure and represent a major risk factor for developing atherosclerosis, hypertension and stroke, and arterial fibrillation (North and Sinclair 2012).

Important functions of the endothelium include controlling vascular contraction/dilation, barrier function, and controlling blood clotting and inflammation. All of these change during aging. Endothelial dysfunction is considered as one of the main mechanisms of age-associated development of CVD. The hallmark of aged endothelium is diminished endothelium-dependent vessel vasodilatation. The availability of nitric oxide, the main endothelium-derived vasodilator, progressively decreases with age, whereas release of vasoconstriction factors increases along with secretion of pro-inflammatory molecules and enhancement of oxidative stress (El Assar et al. 2012).

Both endothelial cells and VSMCs are mitotic cells (Box 2.1) demonstrating proliferative response to injury to promote tissue repair. Mitotic cells having critically

Box 2.1 Mitotic and Postmitotic Cells

According to the classical view, cells of multicellular organisms are classified as terminally differentiated postmitotic cells, which cannot reenter the cell cycle and divide, and mitotic cells capable of proliferation. Depending on the proliferative capacity of the tissues, multicellular organisms are termed as simple or complex. After the development of the organism is completed, all non-germ cells of simple organisms (such as *Caenorhabditis elegans* and *Drosophila melanogaster*) are terminally differentiated postmitotic cells. Complex organisms such as mammals are composed of both postmitotic and mitotic cells. Mitotic cells are present in renewable tissues such as the skin, intestines, liver, kidney, and blood vessel wall, and enable renewal and repair. In addition, mitotic cells include the undifferentiated stem and progenitor cell populations.

The ability of a tissue to renew and repair allows complex organisms to achieve a significantly longer lifespan compared with simple organisms. However, mitotic cells are susceptible to malignant transformation and may undergo cellular senescence when challenged with carcinogenic genotoxic stimuli.

shortened telomeres because of replication exhaustion or challenged with extrinsic or intrinsic genotoxic stress may undergo cellular senescence—irreversible growth arrest. Senescent cells, however, remain metabolically active, show altered resistance to cell death (apoptosis) signals, and acquire a pro-inflammatory gene expression profile. Induction of cellular senescence is the main tumor-suppressor mechanism, which stops proliferation of incipient cancer cells. However, in aging organisms, senescence deteriorates tissue regeneration and repair, and promotes inflammation (Campisi and d'Adda di Fagagna 2007). This forms the main concept of the antagonistic pleiotropy hypothesis, first proposed by George C. Williams (1957) as an evolutionary explanation for aging. Senescent endothelial cells demonstrate decreased response to vascular injury. The integrity of the endothelial barrier becomes impaired, which in turn facilitates recruitment of inflammatory cells from the bloodstream and leads to VSMCs activation, migration, and proliferation (Wang and Bennett 2012). Extensive evidence also documents the presence of senescent VSMCs in aged vessel walls and within atherosclerotic plaques (Mahmoudi et al. 2008).

VSMCs comprise the medial layer of the blood vessel wall, and fulfill a variety of structural and physiological functions. During development, VSMCs produce ECM that gives the arterial wall the capacity to endure the pressure of circulating blood. Physiologically, the contractile activity of VSMCs generates blood pressure and regulates the vascular tone in response to mechanical and soluble factors. These cells express specialized compositions of contractile proteins, ion channels, and signaling molecules. This repertoire is unique in comparison to other cell lineages and serves as a marker of differentiated VSMCs. VSMCs are intrinsically involved in age-associated changes in the vasculature. With age VSMCs change from the physiological contractile phenotype, characterized by contractile activity maintaining vascular tone, towards the pathophysiological synthetic phenotype. Synthetic VSMCs are characterized by migration, proliferation, and release of inflammatory cytokines, as well as ECM synthesis. Progressive VSMC migration from the tunica media results in intima thickening, which leads to blood vessel lumen narrowing and creates a site of increased susceptibility to atherogenic factors even of low grade (Lacolley et al. 2012).

Various external and intrinsic transcriptional regulatory pathways cooperate to promote age-associated VSMC phenotypic changes. The balance between growth-promoting cytokines and growth factors [such as platelet-derived growth factor (PDGF), thrombin, fibroblast growth factor (FGF), and interleukin-1] and growth inhibitors/inducers of differentiation [such as transforming growth factor beta (TGFβ)] defines the transcriptional activity and the actual phenotype of VSMCs. One important transcription factor that dually regulates the VSMC phenotype is the serum response factor (SRF). Promoter-enhancer regions of most VSMC contractile genes contain multiple CArG and a TGFβ control element. Binding of SRF in complex with its main VSMC lineage cofactor myocardin (Box 2.2) to the CArG box activates the expression of contractile genes (Chen et al. 2002). On the contrary, when dissociated from myocardin and bound to the ETS domain-containing transcription factor, Elk-1, SRF activates the expression of immediate early genes and induces proliferation of VSMCs. In addition, as reviewed by Zheng et al. (2010), some reports, though contradictory, have shown the role of Kruppel-like factor 4 transcription factor in

Box 2.2 Myocardin Transcription Coactivator

Myocardin protein, recently discovered by Eric Olson's group (Wang et al. 2001), is a transcriptional coactivator of genes encoding smooth muscle-specific cytoskeletal and contractile proteins. Myocardin is the founding member of a protein family that includes myocardin itself and the two myocardin-related transcription factors, MRTF A and B. All of them show similar multi-domain organization that provides putative binding sites for several transcription factors and actin. The most important is the ternary complex that myocardin and MRTFs form with the MADS-box transcription factor, SRF, to synergistically activate transcription of contractile proteins. Vascular injury and other stimuli ultimately target myocardin/SRF complexes to modulate the VSMC phenotype.

Forced expression of myocardin in embryonic stem cells induces the expression of multiple contractile genes including SM-22α, SM-MHC, and SM-α-actin. Mice harboring a null mutation in the myocardin gene survive only to embryonic day (E)10.5 and exhibit obvious defects in the vasculature, including inhibition of smooth muscle cell differentiation. These data demonstrate that myocardin promotes VSMC differentiation and the contractile phenotype.

phenotypic modulation of VSMCs. Transcription pathways of age-dependent VSMC proliferation are also related to endothelial dysfunction. For example, diminished Jagged1 expression in aged endothelium has been shown to enhance VSMC proliferation and neointima formation after arterial injury (Wu et al. 2008).

In addition to growth factors, modified (phospho-) lipids and plasma lipoproteins are important factors regulating VSMC phenotypic modulation. Similar to PDGF-BB, oxidized phospholipids induce Elk-1 phosphorylation and binding to SRF, which results in inhibition of contractile genes' expression (Pidkovka et al. 2007; Yoshida et al. 2008). Outward radial convection of plasma lipoproteins and their retention by VSMC-secreted sulfated proteoglycans have also been shown to induce VSMC phenotypic modulation towards the synthetic and proliferative phenotype (Karagiannis et al. 2013; Padro et al. 2008). MicroRNA (miRNA) and reactive oxygen species (ROS) are also important regulators of the VSMC phenotype, affecting gene transcription, DNA damage, and expression of inflammatory genes (Davis-Dusenbery et al. 2011; Antoniades et al. 2009; Heistad et al. 2009).

Despite the progress achieved in VSMC physiology research, their significance in aging and vascular pathology remains to a high degree underestimated. The main role in initiation and progression of aging-associated diseases like atherosclerosis is attributed to inflammatory and endothelial cells. Some controversies still exist, and clear understanding of the underlying molecular mechanisms of VSMC phenotype regulation and senescence is far from being achieved. Recent reports, however, brought more attention to the functions and fate of VSMCs during atherogenesis. They also shed some light on a very complicated and controversial matter of lineage

tracing of particular cells within the plaque (Gomez and Owens 2012; Rong et al. 2003). In the early stage of plaque development, low-density lipoproteins (LDL) become trapped in the intima because of binding of apolipoprotein B100, the protein component of the LDL particles, to proteoglycans produced by VSMCs. Trapped lipoproteins are being modified and taken up at an enhanced rate by macrophages, resulting in the formation of macrophage foam cells, which contribute to the progression towards a more complicated lesion. More advanced atherosclerotic plaque includes migrated VSMCs, producing excessive ECM, and inflammatory cells such as macrophages, T lymphocytes, dendritic cells, and mast cells. Macrophage-derived foam cells eventually die by apoptosis, releasing their content into the plaque and further amplifying inflammation. Extracellularly located lipids and cell debris comprise a necrotic core surrounded by a fibrous cap of VSMCs, critically influencing the plaque's stability. VSMC death and degradation promote plaque rupture and may lead to thrombosis. Increased VSMC content has long been associated with plaque stability, and VSMC proliferation and ECM synthesis may promote plaque repair. Advanced plaques show multiple sites of plaque rupture and repair that ultimately lead, however, to vessel narrowing.

A recent report has shown that the role of VSMCs is not limited to structural maintenance of the plaques. VSMCs show active endocytic and phagocytic activities. They take up modified lipoproteins, and foam-like cells originating from VSMCs have been reported. In addition to lipoprotein uptake, VSMCs may engulf by phagocytosis apoptotic cells, crystals, and microparticles (Lacolley et al. 2012). VSMCs also propagate the inflammatory response by various mechanisms (Cole et al. 2010; Krug et al. 2010; Orr et al. 2010). Calcification of VSMCs leads to further arterial stiffness and clinical complications (Ellam and Chico 2012). Furthermore, essentially all these processes are influenced by VSMC senescence. Importantly, different activities of VSMCs induce expression of different markers that are not intrinsically expressed in VSMCs. Thus, endocytic activity endows VSMCs with expression of phagocytic markers like CD68 (Rong et al. 2003). On the contrary, cells other than the VSMC lineage express markers like smooth muscle α-actin, which is typically used to detect VSMCs (Gomez and Owens 2012). To summarize, recent reports have suggested that the role of VSMCs in aging and atherosclerosis is largely underestimated. Multiple mechanisms and signaling pathways define the phenotype and functional response of VSMCs. Deeper understanding of VSMC functions and regulation is important for developing effective therapeutic approaches.

2.2 uPA/uPAR System

Recent studies have revealed a profound interconnection between the fibrinolytic system, namely, the serine protease uPA and its specific multifunctional receptor (uPAR; Box 2.3), and the pathogenesis of CDV, inflammation, aging, and mortality. As reviewed by Binder et al. (2007), significant insight into the molecular basis of how uPA-/uPAR-directed cell behavior affects the pathogenesis of CVD has been gained.

Box 2.3 The uPA/uPAR Plasminogen Activator System

The serine protease uPA is the most effective physiological activator of plasminogen. uPA converts inactive proenzyme plasminogen to the active serine protease plasmin that in turn degrades fibrin polymers of blood clots into soluble degradation products.

Binding of uPA to its receptor (uPAR) enhances activation of pro-uPA into its active form, resulting in activation of plasminogen. uPAR is associated with the external surface of the cell plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Because uPA binds to uPAR and plasmin binds to multiple cellular receptors, the proteolysis is concentrated at the cell surface. In addition to fibrin, plasmin cleaves a broad spectrum of substrates. Plasminogen activators are involved in a wide range of physiological and pathophysiological processes associated with basement membrane and ECM turnover, for example, tissue remodeling and repair, tumor progression, and metastasis.

uPAR is a cell-surface GPI-anchored protein that can be shed from the cell membrane in a soluble form. Recent large-scale population studies identified soluble uPAR (suPAR) as a new independent inflammatory marker associated with the risk of CVD (Lyngbaek et al. 2012). These studies have shown that the circulating suPAR level is associated with subclinical organ damage, manifested as carotid atherosclerotic plaques, and thus may be even better for prediction of future CVD than the "classical" C-reactive protein (CRP) level, which mainly reflects inflammation associated with metabolic disturbances. uPAR expression in tissues is low under normal conditions. However, uPA/uPAR is drastically upregulated in numerous diseases, primarily those related to inflammation, vascular remodeling, and cancer (Binder et al. 2007; Pillay et al. 2006; Blasi and Carmeliet 2002).

Binding of uPA to its receptor is implicated in plasmin generation and extracellular proteolysis. In addition, the uPA/uPAR system also has a nonproteolytic role and induces various intracellular signaling pathways. Thus, uPAR realizes two important cellular functions: providing regulation of extracellular proteolytic cascades and serving as a signaling receptor to promote changes in cell functional behavior (Smith and Marshall 2010). uPAR-directed signaling can occur via uPA–uPAR binding or be uPA independent. As a GPI-anchored receptor lacking transmembrane and intracellular domains, uPAR associates with transmembrane proteins, such as integrins, tyrosine kinase receptors, and others, to initiate signal transduction. Multiple signaling cascades induced via these co-receptor cooperation have been identified over the last decade (Smith and Marshall 2010; Blasi and Carmeliet 2002). Although many advances have been made in the field, the mechanisms of uPAR signaling are still not completely clear and several controversies remain. At the level of cellular functions determining the cell fate in response to the microenvironment, uPAR-directed signaling is believed to regulate physiological and pathophysiological conditions, requiring changes in cell proliferation, migration, adhesion, and survival (Pillay et al. 2006). Because of these multifunctional properties, uPAR presents many opportunities to be used as a target for specific therapies in diverse human diseases.

2.3 uPAR in Vascular Diseases and Aging

Several reports have documented the presence of senescent VSMCs in the aging vessel wall and in atherosclerotic plaques. Senescent cells, though growth arrested, remain metabolically active and secrete multiple factors that may affect surrounding cells. Generally, the senescence-associated secretory phenotype (SASP), or the senescence-messaging secretome, promotes inflammation. Recently, large-scale analysis of SASP has been performed by Campisi and colleagues (Coppe et al. 2010). Three major families of factors are secreted by senescent cells, including soluble signaling factors (interleukins, chemokines, and growth factors), insoluble proteins/ECM components, and secreted proteases. The proteases include matrix metalloproteinases (MMPs), serine proteases, and regulators of plasminogen activation, including uPA and uPAR. Upregulated expression of the system has also been well documented in vascular remodeling and atherosclerosis (Binder et al. 2007). The main stream of research on the role of uPA/uPAR in vascular pathology is focused, however, on the expression and function of this system in adhesion/migration of inflammatory cells. In our research, we have identified several novel links between the urokinase system and the functional behavior of VSMCs during vascular remodeling and initiation/progression of atherosclerosis.

Over a decade ago, it has been reported by our group and others that uPA induces migration and proliferation of VSMCs (Dumler et al. 1998). As a GPI-anchored protein, uPAR needs to be associated with other transmembrane receptors to induce intracellular signaling. In our studies, we have identified PDGF receptor beta (PDGFR β) as a uPAR co-receptor in VSMCs (Kiyan et al. 2005). We have shown that uPA-activated uPAR associates with PDGFR β and induces phosphorylation and dimerization of the latter in the absence of its natural ligand PDGF, the main regulator of VSMC migration and proliferation. Phosphorylation of tyrosine residues in the PDGFR β cytoplasmic domain provides sites for interaction with multiple downstream signaling proteins. Furthermore, we have shown that uPA-/uPAR-induced activation of phosphatidylinositide 3-kinase (PI3K) and Rho proteins in VSMCs is mediated by their interactions with PDGFR β (Kiian et al. 2003; Kiyan et al. 2005).

Because uPAR/PDGFR β induces a VSMC proliferative and migratory response that requires phenotypic modulation of VSMCs, we have examined if and how uPAR interferes with the transcriptional activity of VSMCs and the expression of contractile proteins. We observed that uPAR expression correlates with the VSMC synthetic phenotype and that downregulation of uPAR by siRNA not only abolishes uPA-dependent events but also promotes VSMC differentiation towards the contractile phenotype (Kiyan et al. 2009). These observations correspond with in vivo and clinical data, showing increased expression of uPA/uPAR at sites of vascular remodeling and atherosclerosis, which contain phenotypically modulated VSMCs. Moreover, we have investigated the molecular mechanisms of VSMC phenotypic modulation in response to uPA. As mentioned above, most smooth muscle-specific genes characterizing the contractile phenotype contain common CArG elements in their promoter region. Binding of SRF in cooperation with the cofactor, myocardin, to these elements regulates expression of the corresponding genes. Using chromatin immunoprecipitation, we have shown that SRF/myocardin binding to promoters of contractile genes is

Box 2.4 The Ubiquitin Proteasome System

The ubiquitin proteasome system (UPS) includes ubiquitin ligation enzymes and proteasome particles. It is the major non-lysosomal pathway of protein degradation in eukaryotic cells. Nearly every cellular process is affected by the UPS. It performs regulatory functions by eliminating no-longer-needed proteins and quality control functions by degrading misfolded or damaged proteins.

Protein degradation by UPS is ATP dependent and a substrate-selective process. Substrate proteins are typically modified by energy-dependent covalent attachment of the small protein ubiquitin polymers via concerted action of ubiquitin ligation enzymes.

The 26S proteasome comprises two subcomplexes: the proteolytically active 20S core particle (CP) and the 19S regulatory particle. The latter is responsible for substrate recognition, removal of substrate polyubiquitin, unfolding, and translocation into the CP for degradation.

suppressed by uPA/uPAR signaling (Kiyan et al. 2012). Furthermore, myocardin is modified by ubiquitination, and the protein level of myocardin decreases after VSMC treatment with uPA, because of proteasomal degradation (Box 2.4).

The pathway includes internalization of membrane uPAR via the pinocytic amiloride-sensitive pathway and its nuclear translocation. In the cell nucleus, uPAR interacts directly with myocardin to induce its dissociation from SRF, resulting in decreased expression of contractile genes. Furthermore, myocardin translocates to proteasome-containing nuclear structures and undergoes degradation. Our experiments with expression of truncated forms of myocardin demonstrated that its N-terminal domain is required for binding uPAR. Thus, our new observations have shown that uPAR serves as a myocardin cofactor and directly interferes with the regulation of gene expression in VSMCs. Interestingly, nuclear localization and transcriptional activity of uPAR have also been reported in cancer cells (Asuthkar et al. 2012). uPA/uPAR provides a relatively rapid pathway for VSMC phenotypic modulation, because of myocardin degradation. In our study, we did not identify the nature of the enzyme conducting the myocardin ubiquitination. Recent evidence (Xie et al. 2009) has shown that the E3 ligase C-terminus of Hsc70-interacting protein (CHIP) ubiquitinates myocardin and represses myocardin-dependent gene expression and transcriptional activity.

Another interesting observation from our study was the intersection of the uPA/uPAR system and the UPS. The proteasomal system is the main pathway of protein degradation in eukaryotic cells. It is absolutely essential for maintaining cellular homeostasis by degrading damaged and dysfunctional proteins. Furthermore, the proteasome has an important regulatory function in events such as regulation of transcription, cell cycle, DNA repair, and apoptosis. In addition, inflammation and

regulation of oxidative stress are tightly controlled by the UPS. The system includes enzymes performing protein ubiquitination/deubiquitination and a 26S multisubunit proteasome complex that exerts proteolytic functions. A concerted action of E1, E2, and E3 ubiquitinating enzymes results in activation and conjugation of ubiquitin to a target protein. Protein ubiquitination determines if modified proteins undergo degradation via the 26S proteasome particle. Alternatively, ubiquitination may lead to protein functional alterations and/or regulate its intracellular localization. Inhibition of the proteasome leads to accumulation of misfolded and damaged proteins, and may result in cell death. In addition, the proteasome is essential for repairing DNA damage and cell cycle regulation. These features make the proteasome system an attractive target for developing anticancer therapy. Aging and senescence are generally associated with decreased proteasomal degradation and accumulation of dysfunctional proteins. Scientific progress made in recent years also confirmed the role of the UPS in atherosclerosis. In particular, the proteasome is implicated in promoting endothelial dysfunction, an initial stage of the disease (Herrmann et al. 2010). In advanced plaques, accumulation of ubiquitin conjugates and its correlation with apoptotic cell death suggests that proteasomal degradation is decreased. In uPAR-knockout mice, i.e., genetically engineered mice lacking expression of uPAR, we have observed that the proteasomal activity in aortic tissue is lower than that in wild-type mice (Kiyan et al. 2012). Thus, impaired proteasomal degradation of myocardin may explain the delayed vascular remodeling after injury, which we have shown in uPAR-knockout mice using the carotid artery ligation model.

In an attempt to explore additional relationships between uPAR and the UPS, we used a model of doxorubicin (Dox)-induced cell senescence. Senescence of mitotic cells may result from critical telomere shortening at the chromosome ends, because of replicative exhaustion, and is called “replicative” senescence. Alternatively, various genotoxic agents may cause the so-called “stress-induced premature” senescence. Multiple mechanisms are involved in cell senescence in aging and atherosclerosis. Moreover, they probably have cumulative effects on each cell type. Thus, both aged and plaque VSMCs and endothelial cells demonstrate telomere shortening. Additionally, oxidative stress may promote senescence by various mechanisms; epigenetic modifications have been shown not only as markers of senescence but also as playing a causative role. It has been reported recently that proteasome function is important for developing Dox-induced senescence in cardiomyocytes (Maejima et al. 2008). Dox is an anthracycline antibiotic, proven to be effective for cancer treatment. However, its application is strongly limited by severe cardiac toxicity. Recent research has shown that the mechanism underlying Dox toxicity involves induction of cellular senescence. We have tested the Dox effect on VSMCs and observed that Dox also induces senescence of VSMCs (Hodjat et al. 2013). VSMC treatment with Dox was accompanied by a boost of proteasome activity, most probably induced by DNA damage response mechanisms, which later resulted in cell senescence. Interestingly, uPAR-deficient cells failed to upregulate the proteasome activity and were protected against developing senescence. Among the proteins degraded by the proteasome in response to Dox is the telomere-binding factor-2 (TRF-2)—a component of the shelterin protein complex, protecting

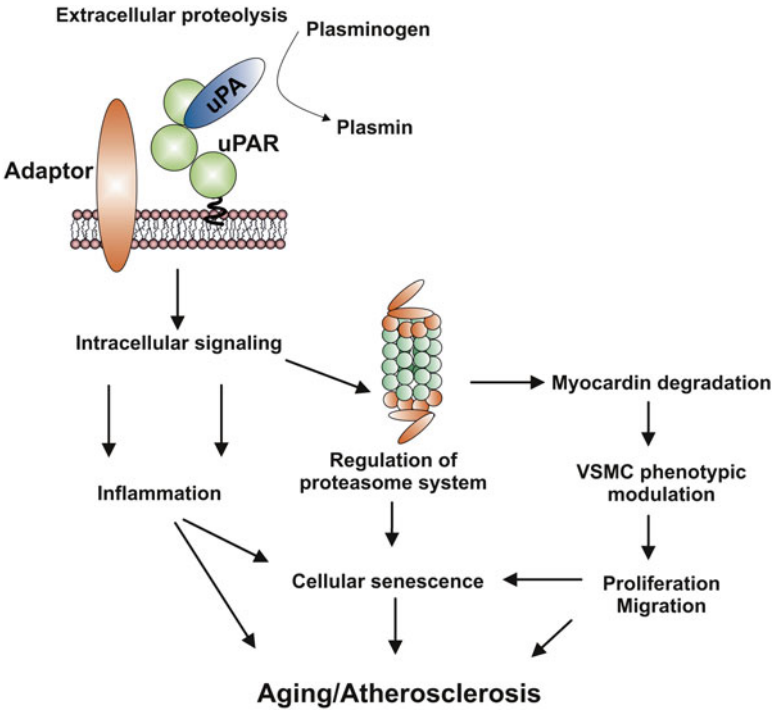

Fig. 2.1 The uPA/uPAR system regulates the physiology of VSMCs. In addition to mediating extracellular proteolysis, uPA/uPAR induces intracellular signaling that regulates the proteasome system. uPAR-regulated proteasome activity degrades myocardin and induces phenotypic modulation of VSMCs towards the synthetic state, accounting for protein turnover leading to cellular senescence

telomeres from being recognized as DNA breaks, leading to activation of the DNA damage pathway and cell cycle arrest. Proteasome degradation of TRF-2 was also impaired in the absence of uPAR.

Taken together, our data point to a new function of uPAR, which is directed at regulation of the proteasome system (Fig. 2.1). This may have crucial effects on cell physiology and survival. In acute response, uPAR serves as a myocardin cofactor, inhibiting its interaction with SRF, thus decreasing the expression of contractile genes. Absence of uPAR abolishes myocardin proteasomal degradation. In Dox-induced VSMC senescence, uPAR is essential for stress-induced activation of proteasomal activity and promotes degradation of TRF-2. Overall, the uPA/uPAR system seems to promote VSMC differentiation to the synthetic phenotype and plays deleterious roles in the progression of vascular diseases.

uPA has also been shown to promote oxidative stress in VSMCs and macrophages. Fuhrman and colleagues have shown that uPA activates the NADPH oxidase complex in macrophages, resulting in ROS production (Fuhrman et al. 2008). Our recent study defined an additional pathway of oxidative stress regulation by

uPA/uPAR. Paraoxonase 1 (PON1), a high-density lipoprotein (HDL)-associated lipolactonase, is the most studied member of the closely related PON family. Epidemiological and clinical studies unraveled a protective role of PON1 in atherosclerosis, but the mechanisms are just beginning to be understood. Studies performed on PON1-knockout mice have shown significant changes in the arterial wall, including increased oxidative stress, thrombogenicity, and increased leukocyte adhesion. HDL-associated PON1 regulates macrophage differentiation and has antioxidative and anti-inflammatory functions (Aharoni et al. 2013). PON1 is synthesized and released into the circulation by liver cells. Our study has shown that uPA/uPAR may regulate the expression of PON1 in the liver and thereby the availability of PON1 in the blood. Moreover, uPAR-dependent signaling relies on nuclear export of peroxisome proliferator-activated receptor γ (PPARγ) (Khateeb et al. 2012).

Thus, the urokinase system exerts its functions at different levels. It may regulate liver production of PON1, which is known to decrease oxidative stress in atherosclerotic lesions and attenuate the development of atherosclerosis. Furthermore, our research has shown that the urokinase system regulates VSMC modulation towards the synthetic phenotype, resulting in increased vascular remodeling and progression of atherosclerosis. uPA/uPAR induces a phenotypic modulation of VSMCs via proteasomal degradation of the transcription factor, myocardin, leading to increased migration and proliferation of VSMCs. The urokinase system's interference with the proteasomal system also has crucial importance for the induction of VSMC senescence, which may additionally influence the outcome of vascular remodeling and the fate of atherosclerotic plaques.

To summarize, our data show that expression of uPA and its receptor, uPAR, may be detrimental in promoting age- and atherosclerosis-associated morphological and pathophysiological changes in the vascular wall, both systemically and locally.

2.4 uPA/uPAR System Therapeutic Perspectives

A wide variety of functions exerted by the urokinase system in the vascular wall make it an attractive therapeutic target. Cleaved forms of uPAR, in particular suPAR, are found in biological fluids like plasma and urine. suPAR has recently been proven to serve as a marker and mediator of multiple inflammatory, cardiovascular, and kidney diseases. These findings explain the boosting interest in research and development of therapeutic approaches for targeting the uPA/uPAR system. Historically, uPAR has been considered as an attractive therapeutic target in the field of cancer treatment. Tumor tissues express high levels of uPAR, which has multiple roles associated with cancer progression and metastasis. Originally, therapeutic attempts aimed at preventing extracellular plasmin activation and proteolysis by interfering with uPA binding to uPAR. Peptide and small-molecule inhibitors were developed based on the structure of the uPAR growth factor domain to prevent uPA binding (Mazar et al. 2011). A panel of antibodies blocking uPA–uPAR binding has also been reported. However, in contrast to genetic knockdown experiments providing

strong evidence that decreased uPAR expression has robust antitumor effects, pharmacological inhibition of uPA/uPAR binding was minor (O'Halloran et al. 2013).

A number of novel proof-of-principle approaches targeting the uPA/uPAR system have been recently reported. Though uPAR is a GPI protein lacking a transmembrane domain, it may interact with a number of membrane proteins, such as growth factor receptors, integrins, and caveolin, as well as the ECM protein, vitronectin, resulting in pleiotropic activation of various intracellular signaling pathways. This has led to the hypothesis that targeting uPAR protein–protein interactions may be an effective therapeutic anti-uPAR tool. A number of peptide inhibitors have been developed for targeting uPAR–integrin interactions (Rabbani and Gladu 2002). Peptide inhibitors have attracted an enormous rise in interest as a new exciting therapeutic tool. At present, about 60 peptides are marketed worldwide, about 270 peptides are in clinical trials, and approximately 400 are in preclinical research phases. However, there has been little progress in targeting the uPAR–integrins interactions.

To conclude, despite the extensive accumulation of knowledge of uPA/uPAR biology and functions, this remarkably multifunctional system warrants further investigation as a promising diagnostic and therapeutic target, especially regarding vascular disease and aging.

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