Role of Transglutaminase 2 in vascular remodeling

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Citation for published version (APA):
van den Akker, H. H. O. (2011). Role of Transglutaminase 2 in vascular remodeling.
Role of Transglutaminase 2 in Vascular Remodeling

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Uitnodiging voor het bijwonen van de verdediging van mijn proefschrift

Role of Transglutaminase 2 in Vascular Remodeling

Dinsdag 17 mei 2011 om 14.00 uur
in de Agnietenkapel van de Universiteit van Amsterdam
Oudezijds Voorburgwal 231

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Akker_Omslag.indd   1
04-03-11   10:05
ROLE OF TRANSGLUTAMINASE 2
IN VASCULAR REMODELING
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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties ingestelde
commissie, in het openbaar te verdedigen in de Agnietenkapel
op dinsdag 17 mei 2011, te 14:00 uur
door Hieronymus Henricus Oscar (Jeroen) van den Akker
geboren te Utrecht
The research described in this thesis was supported by a grant of the Netherlands Heart Foundation (NHF-2005B080).
| Chapter | Title                                                                 | Page |
|---------|----------------------------------------------------------------------|------|
| 1       | General introduction                                                | 7    |
| 2       | Small artery remodeling: current concepts and questions              | 19   |
| 3       | Decomposition cross-correlation for analysis of collagen matrix deformation by single smooth muscle cells | 57   |
| 4       | Vascular smooth muscle cells remodel collagen matrices by long-distance action and anisotropic interaction | 75   |
| 5       | Transglutaminase 2 is secreted from smooth muscle cells by transamidation-dependent microparticle formation | 103  |
| 6       | The redox state of transglutaminase 2 controls arterial remodeling   | 133  |
| 7       | General discussion                                                   | 163  |
| Appendix | Summary                                                              | 178  |
|         | Nederlandse samenvatting                                             | 180  |
|         | Curriculum Vitae                                                     | 182  |
|         | Dankwoord                                                            | 184  |
Chapter 1

General Introduction

Jeroen van den Akker
General Introduction

The cardiovascular system carries blood throughout the body, providing cells with the required nutrients, oxygen and messengers, while removing waste products. The pressure required for this blood stream results from the pumping of the heart. Adequate local organ perfusion then depends on the resistance of small arteries and arterioles, which is governed by vessel diameter. In order to match changes in demand, arteries adjust their diameter rapidly by vasoconstriction and –dilation. This system, which is independent of humoral and neural pathways, is commonly referred to as autoregulation. However, when a change in flow or pressure persists for a period of several hours to days, vessels start to undergo structural changes in the organization of the existing wall components. Then at a time scale of 1 week to several months, remodeling continues by adapting the composition of the vessel wall through the breakdown and synthesis of specific matrix proteins. This cascade occurs among others during hypertension, flow restrictions and arteriogenesis, causing either outward or inward remodeling of arteries. In previous research, our group demonstrated that the enzyme “Transglutaminase 2” contributes to the first days of inward remodeling in small arteries. In particular, its activation, secretion and enzymatic activity are studied.

Small artery anatomy and inward vascular remodeling

The wall of small arteries (up to a diameter of ~200 μm) consists of three layers. The intima is composed of a continuous layer of endothelial cells that control vessel wall permeability, inflammatory responses and tone regulation. The subendothelial connective tissue, called the internal elastic lamina, separates the intima from the media. The media contains one or more layers of smooth muscle cells (SMCs) effectuating vasoconstriction and –relaxation, amongst others based on signals from the neighboring endothelium. In addition, SMCs are partly responsible for the balance between proteases and their inhibitors, and synthesize extracellular matrix molecules. In between the smooth muscle cells are thin layers of extracellular matrix fibers such as elastin, fibronectin and collagen. The outer layer of the vessel, referred to as adventitia, consists of a thick layer of connective tissue. In this layer, where cell density is relatively low, fibroblasts maintain a matrix of extracellular proteins providing mechanical stability to the vessel.

Inward remodeling of blood vessels is characterized by a reorganization of the existing wall components that provides mechanical stability to the vessel. This follows the Laplace relationship, aiming at a normalization of wall tension. In small arteries, this remodeling is frequently of eutrophic nature, meaning that the amount of wall material remains unchanged. Here, the turnover of proteins by proteolysis and synthesis is limited. Thus, while the vessel lumen decreases, the thickness of the vessel wall increases (Figure 1.1). In vivo, it is troublesome to
study the interactions between specific cell and matrix components, since they are difficult to isolate from their 3-D surroundings. Therefore, we developed several models that enabled us to look in detail at the role of the SMC in a matrix of collagen, which is one of the most important load-bearing element in the vessel wall (Chapter 3-4).

The ratio of the media thickness to the vessel lumen diameter can be used to characterize the amount of remodeling in a vessel. This media-to-lumen ratio was shown to correlate to the occurrence of cardiovascular events in hypertensive patients (Figure 1.2). The biology of small arteries, including inward remodeling, is reviewed in detail in Chapter 2 of this thesis.

![Figure 1.1: Schematic representation of vascular inward remodeling. Matrix elements (black) are stabilized by interfibrillar cross-links (blue); after a prolonged vasoconstriction, matrix fibrils are reorganized and new cross-links are synthesized. This so-called eutrophic remodeling is characterized by a thicker wall around a smaller lumen, while the amount of wall material remains the same.](image)

![Figure 1.2: Occurrence of cardiovascular events in patients with a media-to-lumen ratio of subcutaneous small arteries ≥0.11 (n=36, solid line) or <0.11 (n=92, dotted line). Mantel-Cox test and Breslow test between curves, P<0.00001 (figure adapted from Rizzoni et al.).](image)
Transglutaminase 2: a multi-functional enzyme

Transglutaminases constitute a family of enzymes characterized by their capability to cross-link proteins by a $N^\epsilon(\gamma$-glutamyl)lysine isopeptide bond, which is the result of a transamidation reaction\textsuperscript{11}. Additionally, TG2 catalyzes various other post-translational modifications of proteins including esterification and hydrolysis (Figure 1.3).
In addition to its catalytic actions, TG2 promotes cell adhesion\textsuperscript{12,13}, functions as a G-protein in cell signaling\textsuperscript{14}, aids in the regulation of cytoskeletal structure and cell contractility\textsuperscript{15,16} and is involved in apoptosis\textsuperscript{17}. While transamidation provides increased stability to a protein framework by cross-linking available existing substrates, TG2 is also involved in synthesis of new matrix components. At the transcriptional level, TG2 induces the activation of TGF\(\beta\)1 via NF-\(\kappa\)B, stimulating both the expression and deposition of matrix proteins such as fibronectin, collagen type I, III and IV\textsuperscript{18,19}. At the protein level, TG2 promotes the polymerization of fibronectin\textsuperscript{20}. In turn, the deposition of collagen, the major load-bearing element in the vascular wall, occurs in close interplay with fibronectin\textsuperscript{21}. Thus, indirectly TG2 controls polymerization of collagen as well. However, synthesis of new matrix components occurs at a longer time scale than required for the first phase of inward remodeling and will therefore not be regarded in this thesis.

![Image](image_url)

**Figure 1.3:** Overview of post-translational modifications of proteins catalyzed by Transglutaminases; disulfide isomerase activity is not shown in this overview. This thesis focuses mainly on TG2 cross-linking (a) two proteins are connected via a \(\text{N\textepsilon}(-\gamma\text{-glutamyl})\text{llysine isopeptide bridge between the deprotonated lysine donor residue of one protein (purple ellipse) and the acceptor glutamine residue of another (blue rectangle); (figure reprinted from Lorand et al.}^{11}).

![Image](image_url)

**Figure 1.4:** Conformation of TG2 is depended on binding by Ca\(^{2+}\) and GTP. Domains I-IV are coloured respectively in magenta, orange, blue and green; the regulatory loop between domain II and III is coloured red. The TG2 active site involved in cross-linking is obscured when GTP is bound, but calcium binding induces a conformational change that exposes the active site (figure adapted from Griffin et al.\textsuperscript{22}).
The activities of TG2 are regulated by several factors, including calcium, GTP, the redox potential and nitric oxide. The competitive binding of calcium and GTP determine the conformation that TG2 adopts, favoring respectively transamidation activity and GTPase function (Figure 1.4). In general calcium is high extracellularly, and GTP is high in the cytosol. Therefore, the action that TG2 fulfills strongly depends on its microenvironment. This stresses the importance of identification of the relevant cellular source of TG2, as well as the triggers causing active TG2 secretion.

**Transglutaminase 2 in the vasculature**

TG2 is expressed in many vascular cells such as endothelial cells, smooth muscle cells, fibroblasts, monocytes and macrophages. The largest pool resides in the cytosol, although a small fraction is present in the nucleus, at the cell membrane and in the extracellular matrix (Figure 1.5). The relative TG2 contribution from each cell type to vascular remodeling, as well the localization of TG2 activity, is largely unknown. For more information on the role of TG2 in the vasculature, the reader is referred to several reviews on the actions of TG2 in blood vessels.

*Figure 1.5: Immunohistochemical localization of TG2 protein and the specific TG2 cross-link in a preparation rat aortic tissue as prepared by Watts et al. Arrows point to the medial layer, where TG2 protein content is relatively high, but TG2 cross-linking is low and virtually absent in the extracellular matrix between SMCs.*
The signaling cascade leading to cellular release of TG2 and the secretion pathway itself have not yet been resolved. TG2 does not possess a signal sequence to the ER/Golgi, and is therefore believed to follow a non-classical secretory pathway. We investigated whether TG2 is released via the regulated pathway of microparticle formation (Chapter 5).

**Inward remodeling depends on Transglutaminase 2**

Under several physiological and pathological conditions, inward small artery remodeling was shown to depend on the enzyme Transglutaminase 2 (TG2). This was exemplified in a surgical model where the blood flow was selectively adapted by ligation of mouse mesenteric arteries. In wild-type mice, the ligated vessel experienced a low flow (LF) and remodeled to a smaller diameter within 2 days. Correspondingly, the adjacent vessel compensated by a higher flow (HF), which is associated with outward remodeling. However, when this model was conducted in a TG2 knock-out mouse, inward remodeling was completely absent (Figure 1.6).

Although these results clearly illustrate the importance of TG2 during inward remodeling, the exact mechanisms by which TG2 contributes had remained largely speculative, and form the topic of this PhD project.

*Figure 1.6 (next page): Schematic, simplified representation of the contribution of TG2 to inward remodeling in an experimental flow reduction model. The intestine is supplied with blood by several parallel branches of the superior mesenteric artery. If one of these vessels is ligated, it will experience a reduced blood flow while the neighboring vessel experiences an increased blood flow. A more distant vessel (“normal flow”) serves as control. In the wild-type mouse, this leads to outward remodeling of the high flow vessel and inward remodeling of the low flow vessel within 2 days. However, in a TG2 knock-out mouse, inward remodeling of the low flow vessel is absent (schematic representation of results in Bakker et al.)*
Figure 1.6: Schematic, simplified representation of the contribution of TG2 to inward remodeling in an experimental flow reduction model.
Outline of the Thesis

The review in Chapter 2 first presents several in vitro techniques that are widely used to study small arteries. Then the contribution of matrix proteins and smooth muscle cells to passive and active vessel mechanical properties are discussed. In addition, their role in vascular remodeling is explained, partly based on several conceptual simulation models. The remodeling of a matrix by cells can be studied in a simplified in vitro setup in order to focus specifically on cell-matrix interactions. In such a model, cell-cell processes can be included by adjusting the number of cells in a matrix. Chapter 3 presents an image analysis method to derive geometrical changes in a matrix resulting from displacements induced by one single cell. This method is applied in Chapter 4 to dynamically study the compaction of a collagen gel by a smooth muscle cell. In addition, an adaptation of this model is presented that focuses on networks of smooth muscle cells, collaborating to remodel tissue. We also investigated the contribution of TG2 in both models of matrix remodeling, because we previously showed that inward remodeling of small arteries depends on Transglutaminase 2\(^5\). Since the localization of TG2 largely determines its conformation as well as the substrates available for cross-linking, Chapter 5 studies the secretion of TG2 from smooth muscle cells. To this end, a green fluorescent TG2 protein (TG2/eGFP) was engineered and functionally tested. After transfection of the encoding DNA for TG2/eGFP into smooth muscle cells, the secretion of TG2 via microparticles was investigated both by immunofluorescent microscopy and flow cytometry. In Chapter 6, the regulators and localization of TG2 activity leading to inward vascular remodeling are studied. First, the relevant action of TG2 was established, as well as its dependence on known activators such as calcium, nitric oxide and redox balance. In addition, microarray data were analyzed to select candidate enzymes involved in activation of TG2. Moreover, transamidation substrates in the extracellular matrix of SMCs were analyzed because the medial layer displayed the highest TG2 activity.

In Chapter 7 (General Discussion), a framework is sketched that integrates our findings on the activation mechanisms of TG2 (Chapter 6), the subsequent secretion of TG2 from the cell (Chapter 5), the contribution of TG2 in matrix compaction as observed in inward remodeling (Chapter 4), and the cross-linking of extracellular substrates that stabilize the remodeled tissue (Chapter 6).
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Small artery remodeling: current concepts and questions

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Journal of Vascular Research
2010, 47: 183-202
Abstract

Blood flow regulation by small arteries and arterioles includes adaptation of both vascular tone and structure. It is becoming clear that tone and remodeling of resistance vessels are highly interrelated. Indeed, concepts pointing to continuous resistance artery adaptation and plasticity are emerging. The purpose of this review is to summarize such concepts and approaches related to vascular organization and remodeling, and to point out the missing links and possible directions for future research. We focus on the individual vessel level. Since several relevant studies are based on isolated vessels, we briefly re-iterate the available isobaric and isometric approaches. We further discuss the major elements of the small artery wall and their relation to the passive and active mechanical properties, as important determinants for vascular remodeling. The cytoskeletal elements and actin re-organization during remodeling are discussed, as well as the re-lengthening of smooth muscle cells during prolonged constriction. We then consider tone as major causal factors in remodeling and discuss the role of vessel wall inflammation. Finally we illustrate examples of current quantitative, integrative approaches of small artery mechanosensing and adaptation that may lead to a physiomics description of small artery adaptation in health and in diseases such as hypertension.
Introduction

Resistance vessels are the small arteries and arterioles that regulate local perfusion and organ resistance. These typically include vessels of around 200 micron or smaller in diameter. Flow regulation by these vessels occurs at time scales from seconds to weeks, involving adaptation of luminal diameter by both vascular tone and vascular structure. This occurs in response to a wide variety of local mechanical and biochemical stimuli as well as endocrine and neural influences. Flow regulation malfunctions in a variety of cardiovascular and metabolic pathologies. Examples include impaired endothelium-dependent dilation under oxidative stress and inward remodeling of resistance vessels in various hypertensive disorders. Furthermore, resistance vessels may adapt to the presence of flow-limiting stenoses in proximal vessels.

The notions that vascular caliber regulation involves multiple stimuli (e.g. wall tension, shear stress, metabolic factors) in a complex mechanical setting, widely diverging time scales and a distributed-resistance network spanning many generations raises several fundamental questions: How do blood vessels combine responses to pressure, flow, metabolic factors? In a remodeling vessel, how do functional characteristics such as the capacity for active tension generation change? If flow can be controlled by either tone or remodeling, what then determines their balance? I.e. how does a vessel ‘choose’ between deep tone and a wide structural caliber or shallow tone and a narrow caliber? Eventually, an integrative ‘physiomics’ approach could answer such questions. Such an approach would simultaneously consider the mechanical regime, various control loops (e.g. regulation based on wall tension and shear stress), different time domains (tone and remodeling), and the spatial organization of resistance vessels in networks, and ultimately would provide a detailed four-dimensional multi-scale approach of caliber regulation. Such an approach is not yet available, but might evolve from several useful initiatives. Accordingly, the purpose of this review is to summarize current concepts and approaches related to vascular organization and remodeling, and to point out the missing links and possible directions for future research.

We focus on the individual vessel level and on wall plasticity and eutrophic remodeling, i.e. in the absence of a change in wall cross-sectional area, ignoring proliferation and apoptosis in the vascular wall. Since several relevant studies are based on isolated vessels, we briefly re-iterate the classic experimental approaches, isometric wire-mounted and isobaric cannulated vessel segments. We further discuss the major elements of the small artery wall and their relation to the passive and active mechanical properties, as important determinants for vascular remodeling. We then consider cytoskeletal events and tone as major causal factors in remodeling and discuss the role of vessel wall inflammation. Finally we illustrate
examples of current quantitative, integrative approaches of small artery adaptation.

**Isolated small artery techniques**

The contractile properties of intact small arteries are generally studied by either of two methods\(^7\). Segments can be mounted in a wire myograph, where force development is measured at a certain, constant, internal circumference (isometric conditions). Alternatively, vessels are cannulated in a pressure myograph and diameter is measured while pressure is controlled (isobaric measurements).

**Wire myograph**

The wire myograph was first described by Mulvany and Halpern in 1976\(^8\), and was based on a method originally proposed by Bevan and Osher four years earlier\(^9\). This setup is now widely used in routine vascular physiology and pharmacology. In an isometric wire myograph, vessel segments are mounted as ring preparations on two wires, one of which is connected to a force transducer. The other wire is attached to a micrometer, thereby allowing precise control of vascular circumference. An equivalent radius \((r)\) can be calculated from this circumference, although it should be realized that there is substantial deformation from the normal circular shape. Tension (i.e. force per length) is recorded and based on the presumed parallel arrangement of SMC and extracellular matrix, active tension is calculated from the difference between recorded tension and the passive component obtained during full dilation. After mounting, the vessels are ‘normalized’, i.e. set to the optimal radius for active tension. While this would ideally involve establishment of the full active radius-tension relation, the practical solution is to base the normalization on the passive radius-tension relationship of the vessel\(^10,11\). First, the passive radius at an equivalent pressure of 100 mmHg \((r_{100})\) is determined from the interception of an exponential fit of the radius-tension relation and the Laplace relation \((T=P\cdot r)\) at 100 mmHg\(^11\). The distension is then usually set to \(0.9\cdot r_{100}\), since active force production of the vessel is postulated to be maximal at this strain, and kept constant during the experiment. This normalization is based on the frequently used rat mesenteric small arteries. It is not clear whether this would also reflect the optimum for force development in other vessels. For the bulk of studies, this is not relevant, as long as sufficient active tension can be recorded on top of a low passive tension. However, as we explain below, the active radius-tension relation is dynamic, while also the passive relationship changes during remodeling. Therefore, we would suggest including detailed registration of both relations in studies on remodeled vessels, but also in studies that address mechanosensing, or those that explicitly compare maximal active tensions between groups.
Pressurized vessels

In 1966, Burg et al. published a technique for the investigation of isolated, pressurized renal tubuli\textsuperscript{12}. This method, in which the wall is vacuum-clamped between two double-barreled pipettes, was adapted by the lab of Duling for arterioles\textsuperscript{13}, and still seems the approach of choice for cannulating the tiniest arterioles. This technique, as well as an alternative double-barreled technique\textsuperscript{14} require complex cannula construction, and a more general approach is the cannulation by two single-barreled pipettes and suturing\textsuperscript{15}. Pressures are generated by hydrostatic height, by roller pumps with pressure feedback, or by electric-pneumatic converters. Diameters are measured manually, using video calipers or off-line analysis of video images, or automatically, using a variety of diameter tracking algorithms\textsuperscript{16}.

An extensive ‘normalization’ protocol such as for wire-mounted vessels is not needed. Rather, the vessel is set to the (assumed) normal pressure, and the passive diameter is recorded, allowing for normalization of subsequent diameter recordings. Studies that include mechanosensing or remodeled vessels almost always include the pressure-diameter relation of the relaxed vessel in order to quantitate the mechanical properties and delineate the span of possible diameters. Axial stretch of cannulated vessels seems less standardized, and this issue seems somewhat ignored. \textit{In vivo}, vessels are under axial strain due to tethering to the surrounding tissue and the longitudinal stress resulting from the pressure. Vessels thus retract considerably upon isolation. Ideally, one should set the vessel back to its \textit{in vivo} length. Yet, this is difficult to determine, if at all constant. Usually, cannulated vessels are straightened at a standardized pressure. However, this remains a relatively coarse method. Setups including recording of the axial tension are available, possibly allowing better standardization.

There are many reasons for choosing either of the above techniques. Amongst these, the wires allow generally faster mounting and multiple parallel segments, are forgiving with respect to small side branches, and allow easier and much faster data collection. Moreover, the isometric protocols are relatively well standardized. The cannulas provide more realistic mechanical loading and allow the study of flow. Furthermore, spontaneous basal tone and myogenic responses are more easily induced in pressurized vessels. In general, while pharmacological and physiological studies might employ respectively the wires and cannulas, both techniques have shown to be useful for the understanding of tone control as well as vascular plasticity, as will be explained below.
Small artery matrix and passive mechanical properties

The organization of blood vessels in three layers (intima, media, adventitia) was described in detail by Rhodin in the Handbook of Physiology. Figure 2.1 provides TEM photographs of a mesenteric resistance vessel, showing these layers and their major components. The major components believed to determine the mechanical properties in passive vessels, and thereby the caliber of the vessel, are elastin and collagen. It leaves no doubt that remodeling requires reorganization of at least these components. We therefore discuss their structure, synthesis and embedding, cross-linking and degradation in relation to the mechanical properties of the passive vessel.

Elastin

In large vessels, the large amount of elastin (e.g. 111 mg/g wet weight in the rat carotid artery) functions to damp pressure pulsations. Elastin content decreases towards smaller vessels, but despite the ‘muscular’ appearance of the vessels is still abundant (e.g. 15 mg/g in small mesenteric arteries). This is reflected by the volume occupied by elastic matrix, around 50% in the aorta and 14% in the superior mesenteric arteries. In arterioles, elastin is restricted to the internal elastic lamina. This sheet-like structure is built up by intertwined elastic fibers. Based on electron microscopy, thickness of the internal elastic lamina is in the order of 1 µm. In somewhat larger resistance vessels, an external elastic media may still be present, as well as some elastic fibers between the SMC.

Elastin is produced mainly by smooth muscle cells, although also endothelial cells and adventitial fibroblasts are capable of synthesis of tropoelastin. Elastic fibers consist of a core of globular tropoelastin monomers joined by desmosine cross-links. The core of each fiber is surrounded by a sheet of unbranched microfibrils of 10-12 nm in diameter, containing fibrillins as well as an array of other macromolecules.

The incorporation of tropoelastin into elastic fibers is based on self-assembly and ordering (coacervation) and cross-linking. In vivo the coacervation process is guided by fibrillin. Thus, formation of elastic fibers and sheets starts with the assembly of fibrillin molecules at the cell surface due to transglutaminase induced cross-linking of fibrillin-1 monomers and microfibril-associated glycoproteins in the interbead filaments. These microfibrils form parallel bundles that may be stabilized at inter-microfibrillar regions and serve as scaffolds for deposition of tropoelastin. The resulting elastin is stabilized by lysyl oxidase-derived desmosine cross-links. This process is facilitated by fibulin-4 and -5.

Figure 2.1 (A) Transmission electron microscopic image of a rat mesenteric small artery fixated at 100 mmHg equivalent pressure. (B) Detail of the media and adventitia. EC = endothelial cell; IEL = internal elastic lamina; EEL = external elastic lamina; COL = collagen; SMC = smooth muscle cell; FIBR = fibroblast.
Elastin, once formed by cross-linking, is a remarkably stable protein in the absence of pathologies. Yet, maintenance is required to regulate the local amount of elastic fibers and their physical properties. In particular, remodeling of resistance vessels requires that also the internal elastic lamina is restructured, by a combination of incorporation of new tropoelastin molecules into the existing layer, degradation of part of the layer, and formation and degradation of intra- and intermolecular cross-links. We established a role for Transglutaminases in small artery eutrophic inward remodeling. While these enzymes are involved in the genesis of elastin fibers, there is currently no evidence that they cross-link mature elastin.

As the name indicates, elastic fibers can easily be distended, to around twice their resting length. Moreover, the incremental elastic modulus (i.e. the slope of the stress-strain relation) is essentially constant over a large part of this range, and is in the order of 600 kPa. The structural base for elastin elasticity is extremely complex and subject of ongoing discussions. Extension of the tropoelastin monomers forms the base of the elasticity of elastin. This extension is based on uncoiling of large dynamic hydrophobic regions. Upon stretch, tropoelastin becomes more ordered, or entropy becomes less, and indeed the elastic nature of elastin is of entropic origin. Hydration of tropoelastin and the presence of bulk water filling the space between the tropoelastin molecules is crucial; without water, elastin is brittle. Various tropoelastin isoforms exist due to alternative splicing. Fundamental elastic properties of these isoforms apart from resting length of the tropoelastin molecule are probably not very different if alternative splicing occurs in the hydrophobic regions, but in other regions this could affect the number of cross-linking sites and thereby affect the degree of cross-linking and the final architecture and stability of the elastin molecule.

**Collagen**

Like elastin, also collagen content decreases in smaller vessels (e.g. from 124 mg/g in carotid arteries to 67 mg/g in mesenteric vessels). Within small artery networks, collagen content decreases further towards the periphery, e.g. from 20 to 9% of the wall volume over the mesenteric bed. In small arteries, the non-fibrillar collagen IV forms the basement membrane, while collagen I and III are present in an irregular network of small fibrils in the media and in large amounts in the adventitia. Collagen organization and biomechanics in small arteries need more extensive investigation; Information below is therefore mainly derived from tendon, while some studies address collagen in the major arteries.

Collagen is assembled in a multistep-process with distinct steps at specific locations inside the cell and in the extracellular space. The base of the collagen hierarchical organization is formed by the single polypeptide, the central part of which folds into a tight, right-handed \( \alpha \)-helix. Three polypeptides form a left-handed triple helix with a pitch of around 10.4 nm. Collagen type I is a heterotrimer made up of two \( \alpha_1 \) and one \( \alpha_2 \) peptide; Collagen type III is a
homotrimer containing $\alpha_1$(III). In a so-called D staggered array, 280 nm long trimers are packed into microfibrils at an offset of 67 nm, generating the characteristic striation in fibrillar collagen seen in EM. A wide variety of models for microfibrillar organization has been described, most of them based on a thickness of 5 or more collagen trimers. In tendon collagen I, 5-molecule microfibrils are organized in a regular pattern, with individual collagen trimers traversing between microfibrils. This networked rope design would add to the strength of collagen. Many such microfibrils form fibrils. The fibrils finally form fibers and sheets via interfibrillar proteoglycans. The EM images in Figure 2.1 indicates that the ‘fibers’ in the media of this resistance vessel have only a few fibrils, while some fibrils can be seen in the adventitia that follow the same path and therefore could be considered to form a fiber. The final intra and intermolecular cross-linking within the ECM is mediated by lysyl oxidase.

Two proteolytic systems are responsible for the degradation of many of the ECM components including collagen. The fibrinolytic plasminogen activation system degrades laminin and fibronectin directly. Elastin and collagen are degraded by matrix metalloproteinases (MMPs), some of which can be activated by plasminogen. Possibly, a part of the collagen aggregates is internalized by fibroblasts after an initial extracellular proteolytic event. There, urokinase plasminogen activator receptor associated protein (uPARAP) may cause a further degradation of collagen, although this mechanism is still under debate.

The stress-strain curve of collagen could be based on deformation at many possible levels of integration, ranging from stretch of the individual triple helices to straightening of the complete fibrils, which are known to follow a wavy pattern in the vascular wall (Figure 2.1), and deformation of the interfibrillar connections. Understanding the organizational level at which deformation occurs could provide insight into the link between changes in molecular organization, such as cross-linking, and alterations of the pressure-diameter relation of the blood vessel. The basis for the deformation of collagen has been extensively studied in tendon. The stress-strain curve is characterized by a first region of low stress where macroscopic straightening occurs, followed by a ‘heel’ region where stiffness rapidly increases, where the lateral order of the collagen molecules increases, believed to be caused by a straightening of kinked molecules, and a linear part with high stiffness. Above ~5% strain, stiffness falls again due to irreversible changes. In the linear part, the axial D period (see above) increases with stress. Sasaki and Odajima found fibrillar strain to parallel tissue strain, and estimated a fibrillar stiffness of 430 MPa, but Puxkandl et al. observed that only 10-20% of the tendon strain is associated with strain of the fibrils for slow strain rates. The remaining strain may stem from deformation of the proteoglycan-rich matrix connecting the fibrils. In biaxially loaded bovine pericardium, Liao et al. observed that fibrillar strain only started to occur at 20% macroscopic strain, attributed to
straightening of the fibers, and above that accounted for only 32% of the strain, the remaining strain was attributed to inter-fibrillar slippage and heterogeneous straightening lengths of the fibrils. In similar experiments on porcine mitral valves, fibrillar strain only occurred at the end of the non-linear region of the stress-strain curve\textsuperscript{55}. The stiffness of the fibrils was estimated to be \( \sim 100 \) MPa, as compared to the macroscopic stiffness of 3.5 MPa. These differences were explained on the basis of orientation and alignment of the fibrils. Fibril strain in human aortic adventitia was only 1% for macroscopic strains of 16%; the curvilinear macroscopic stress-strain curve was explained by fiber straightening, fiber reorientation, and finally fiber strain\textsuperscript{56}.

The above findings leave little room for a role of collagen fibril extension in small artery mechanics at relevant blood pressures. Rather, fibrils are expected to remain at rather constant length and straighten towards a less wavy structure during vessel distension. While the waviness indicated in Figure 2.1 suggests room for straightening, possible intrafibrillar cross-linking and attachment to the ground substance may still provide substantial stiffness to these fibers. A further quantitation of collagen architecture in distended vessels will be needed to test whether this is indeed the case. In addition, local visco-elastic properties of collagen could be determined. A possible strategy is based on microrheology\textsuperscript{57} of beads bound to the collagen using antibodies.

**Matrix organization and mechanical properties of the passive wall**

Figure 2.2A depicts a schematic radius-tension relation of a small artery during maximal vasodilation, as would be measured in wire myography. The active curves in Figures 2.2B and 2.2C will be discussed below. Figure 2.2D ('tone=0') shows the passive characteristics in a cannulated vessel. Ignoring finite wall thickness and axial distension, passive curves recorded using isometric and isobaric methods are roughly equivalent, and these curves can be converted into each other via the law of Laplace\textsuperscript{58-63}. The passive vessels are characterized by an unloaded diameter and non-linear elasticity reflected by an increasing incremental elastic modulus at higher distensions. This non-linearity is reflected in the wire myograph-based curves and the stabilization of passive diameter at increasing pressures. Physiologically relevant matrix remodeling is reflected by a change of these curves. Thus, in Figure 2.2A, the dotted arrow and grey relation show an inwardly remodeled vessel.

The shape of the passive curves is generally believed to depend on the contribution of elastin and collagen\textsuperscript{13;64;65}, dominated at low strain by elastin, having a low elastic modulus (~0.4 MPa). At higher strains, the stiff (~100-1000 MPa) collagen fibers start to hook on, causing a rapid increase in stress. This 'hook on model' (Figure 2.3A) has originally been developed for large vessels\textsuperscript{66-68}. The organization of the mesenteric arterial wall was investigated by scanning electron microscopy in vessels fixed in either relaxed or contracted conditions. Collagen,
elastin and cells were selectively degraded in order to inspect the individual components at a more detailed level. This revealed a pericellular network consisting of irregular collagen fibrils in a network of fibrous elastin.\textsuperscript{39} Elastase increased the diameter of cannulated mesenteric vessels, especially at low pressures, but over the entire pressure range in mesenteric arteries of hypertensive rats.\textsuperscript{69}

There are concerns with the hook-on model and its relevance for resistance vessels. Thus, in this one-dimensional model, ongoing recruitment of only small fractions of the collagen fibers is needed to explain the gradual stiffening at higher distensions. Final recruitment at the highest pressures can be estimated to be

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**Figure 2.2:** Schematic drawing of radius-tension and pressure-radius relations. (A) Passive relation (solid brown). The dashed line indicates the 100 mmHg isobar on the basis of the Laplace relationship. The passive radius at 100 mmHg is indicated as $r_{100}$. Inward remodeling is reflected by a leftward shift of this curve (dotted brown curve and arrow). (B) Active radius-tension relation under full activation (solid green), demonstrating a maximum at 0.9$r_{100}$ (solid arrow). Smooth muscle cell reorganization would shift this curve (dotted green curve and arrow). (C) Total tension for tone = 0 (brown) to 1 (green); intermediate tone levels are indicated in grey. Intercepts with the dashed isobar indicate the radii at 100 mmHg for various tone levels. (D) Conversion of the relations in C to pressure-radius relations. Dash-dotted line indicates an increase in tone with pressure (myogenic response), required to stabilize the vascular radius.
5-6%\textsuperscript{70,71}. It seems not realistic that 95% of the collagen is not involved in the mechanics of the wall at all. Moreover, the fibers that are first recruited will be distended by substantial amounts by the time the vessel has reached its maximal diameter. This is at variance with the strain limit of 3-4% where collagen fibers are known to break\textsuperscript{72}. A final concern relates to remodeling: in a theoretical analysis we showed that outward remodeling in the hook-on model requires breakdown of nearly all of the collagen\textsuperscript{72}, followed by deposition of new collagen at larger hook-on diameters. In this transition, the vessels would be very vulnerable for mechanical overload. Moreover, there is no histological evidence for such massive degradation of collagen in outward remodeling. We formulated an alternative 1-dimensional conceptual model of vascular wall mechanics (Figure 2.3B) that is based on infinite stiffness of collagen fibers. In this model, elements are arranged in series. Each element has a linear elastin spring and a number of collagen strings, the shortest of which determines the maximal distension of that element. The three-parameter model (elastin stiffness and 2 parameters for distribution of collagen string length) could be fitted to experimental stress-strain curves, obtained on wire-mounted rat mesenteric small arteries. Moreover, the model predicts gradual outward remodeling following collagen degradation, and these predictions could be confirmed by collagenase experiments\textsuperscript{72}.

While the 1-dimensional models provide biomechanical concepts, they are clearly oversimplifying. More detailed 2D or 3D models and possibly finite element approaches will be required to quantitate the structural base of vascular biomechanics. Such work has mainly been performed for large vessels, but, provided sufficient anatomical information is available, could also be applied for the understanding of structural remodeling of resistance vessels. The models are generally based on constitutive equations that account for the biomechanical properties of relevant wall structures. The fundaments of such approaches can be found in the work of Fung\textsuperscript{32}. Most models treat the vessel wall as a structural continuum, in which relations are defined between normal and shear stresses and strains on the basis of a mathematical matrix of material properties such as Young’s and shear moduli.

Local stresses may remain in vessels under zero external load. Such residual stresses reduce the stress gradients across the vessel wall under normal load, providing a suitable mechanical environment for SMC contractile function\textsuperscript{73}. Residual stresses are of interest for remodeling, since on one hand they are likely to influence local cell behavior, and on the other hand local remodeling processes would underlies the development of residual stresses. Their study could thus provide further insight into vascular plasticity. Elastin appears to have an important role in stress distribution. Enzymatic digestion of elastin, but not collagen digestion or SMC destruction, was shown to reduce residual stress\textsuperscript{74}. The circumferential component of residual strain is characterized by the opening angle
Figure 2.3: The hook-on model (A) and serial elements model (B) both explain the non-linear passive radius-tension curve (C). In the hook-on model, the vessel is represented by a parallel arrangement of elastin and collagen springs. Increasing tension induces distension (grey area) and thereby a recruitment of collagen springs, raising the vessel stiffness. In the serial elements model, collagen is not a spring but a string that can bend but cannot be extended. Each element is an elastin spring in parallel to several of such strings. The vessel wall is represented by many of such elements in series. Upon ongoing tension, the elements become rigid one by one, also leading to a non-linear radius-tension relation. A and B are modified from 1, which also gives a detailed mathematical analysis and experimental test of both models.
following axial cutting of the vessel. This angle was shown to correlate well with the media-to-lumen ratio. In a biomechanical model, smooth muscle contraction is predicted to cause an increase in opening angle, while relaxation results in a decrease\textsuperscript{75}. In general, the opening angle decreases towards the periphery.

**Smooth muscle cells and mechanical properties of small arteries**

**Smooth muscle cells**

SMC content in the media increases with decreasing diameter, up to 85% in small arteries\textsuperscript{18}. Unlike in large vessels, SMCs in most small arteries and arterioles are aligned preferentially circumferentially, with a typical pitch angle smaller than 2°. This configuration has been suggested to provide an optimal resistance against vessel distension\textsuperscript{18,76-78}. The small angle would create a better overlap between cell tips, thereby creating a helical turn of SMCs in the vessel wall\textsuperscript{79}. Considering the current interest in vascular plasticity and re-lengthening of SMC during activation, the normal length of SMC along the resistance vessel tree in the various organs also becomes relevant. McGrath\textit{et al.}\textsuperscript{79} indicate lengths of around 100 micron, allowing the SMC in small arterioles to completely wrap the vessel lumen. Haas and Duling\textsuperscript{80} quantitated the dimensions of vascular cells in various microvascular beds and report SMC length of around 65 micron in dilated rat pial and hamster cheek pouch arterioles of 80-100 micron diameter. Miller\textit{et al.}\textsuperscript{81} report SMC length in rat intestinal arterioles to be 80-90 micron, essentially independent of vessel diameter in the range between 24 and 62 micron arterioles. A single SMC could thus cover the full circumference in small arterioles, but not larger resistance vessels. Although more complex shapes are found in branches and in the precapillary arterioles\textsuperscript{82;83}, it seems fair to generalize that small artery SMC are spindle-shaped, with a length of 60-100 micron that does not depend on the branching order, and a small pitch.

The dense focal adhesions at the membrane and cytosolic dense bodies may be considered to form the mechanical base of the smooth muscle cell. Various actin isoforms span these anchoring points, forming the actin cytoskeleton. Other actins interact with myosin during contractile force development, forming contractile elements analogue to sarcomeres\textsuperscript{84}, but with side-polar rather than bi-polar arrangement, allowing a larger range of shortening. Intermediate filaments surround these structures, while microtubules act as rigid though dynamic struts. We discuss the role of these elements in force development and maintenance of cell organization during activation and remodeling. In addition to studies on small arteries, we include some findings and concepts from non-vascular SMC that may be relevant for the resistance vessels too.
Stimulus-contraction coupling, contractile element signaling and interaction of actin and myosin during contractile activation of smooth muscle have been well reviewed. A common finding is that calcium-dependent activation is followed by calcium sensitization, i.e. myosin light chain phosphorylation at low intracellular calcium, and subsequent maintenance of force in the absence of myosin light chain phosphorylation, allowing the maintenance of intermediate and chronic force at low energy expenditure. Latch bridges, i.e. non-cycling actomyosin bonds in the absence of myosin light chain phosphorylation might explain the state of chronic tension and low energy expenditure of SMC. While it remains unclear whether such bridges exist in small arteries, the concept has recently been used for modeling small artery contraction. Alternatively, recent work on SMC makes clear that both myosin and actin fibers, as well as other intracellular fibers and cytoskeletal elements, form dynamic structures in SMC, whose dynamic organization may underlie adaptation to maintained activation and remodeling.

In a series of studies mainly on airway SMC, Seow and coworkers provide evidence that the contractile apparatus adapts to SMC load. Thus, new ‘sarcomeres’ are formed in series upon maintained lengthening of the cell, causing an extensive broadening of the active length-tension relation (see ). Rapid evanescence of myosin filaments and stabilization of the filaments by myosin light chain phosphorylation underlie this process. Whether such myosin polymerization also occurs in blood vessels, is unknown. Increased actin polymerization has been found during SMC contraction, and is indeed considered essential for such contraction. Recently, Chen et al. demonstrated that myosin phosphorylation triggers such polymerization in rat mesenteric small arteries, indicating that the polymerization concerns the ‘contractile element’ rather than ‘cytoskeletal’ actin. Such polymerization may alternatively reflect longer actin, more parallel actin, or the recruitment of cytoskeletal actin to the contractile apparatus. These possibilities remain to be investigated, e.g. on the basis of altered active force-length relationships.

This dynamic organization of the actin cytoskeleton seems to be of primary importance for maintained tone and vascular plasticity. Gunst and Zhang provide a paradigm for the regulation of smooth muscle cell contraction that could be valid for small arteries too: a tightly regulated polymerization of globular G-actin to fibrillar F-actin is required for tension generation of SMC. Such polymerization does not regulate the cross-bridge cycling, but rather forms an independent process that provides stabilization of the cytoskeleton. The polymerization seems to occur mainly in a submembranous area of the SMC, providing membrane rigidity and adaptation to local forces acting on the dense focal adhesions. It remains to be established whether polymerization occurs also at the actin fibers that interact with myosin. The focal adhesion junctions are not static structures. Rather, contractile stimulation recruits structural proteins such
as alpha-actinin and vinculin that connect actin filaments to these junctions. Both actin polymerization and focal adhesion junction remodeling may be locally controlled by stress. This way, the SMC can adapt its structure in order to optimally carry the forces resulting from activation and pressurization\textsuperscript{94}. Flavahan \textit{et al.} demonstrate that in mouse tail arterioles, actin polymerization occurs in the myogenic response but not in phenylephrine-induced constriction\textsuperscript{95}. At low pressure, F-actin staining was found at the cell periphery, while at high pressure, F-actin increased in the cell interior.

Intermediate filaments in vascular smooth muscle contain vimentin as the most prominent protein, while in smaller vessels also desmin is present\textsuperscript{96;97}. Vimentin is a substrate for Transglutaminases, which are possibly involved in the dimerization process\textsuperscript{98}. These filaments extend from the nucleus to the membrane, and also connect to the dense bodies. Vimentin filaments form a dynamic network whose organization is dependent on contractile stimuli. Thus, in airway SMC, serotonin induces a shift from a random network of curved fibers to a network of straight fibers along the long axis of the SMC in 5-15 min\textsuperscript{99;100}. Phosphorylation of Ser-56 occurs in response to contractile activation. This mediates intermediate filament disassembly\textsuperscript{99-102}, increasing disassembled fraction of vimentin from around 10 to 20\%\textsuperscript{99;102}. Vimentin depletion by antisense suppresses force development while signaling remains intact, underlining the requirement of these filaments for contraction\textsuperscript{102;103}. Likewise, in desmin -/- mice, small artery potential for active force development\textsuperscript{97} and phenylephrine-induced tone\textsuperscript{104} were impaired. As reviewed by Tang\textsuperscript{105}, the effect of the vimentin network on contractile properties may stem from several structural and regulatory aspects. The connection to the membrane at desmosomes and to cytoplasmic dense bodies, and thus to the actin fibers provides a base for force transmission. Vimentin organization may further guide the actin network and actin polymerization. Stimulus-induced depolymerization and reorganization of vimentin could thereby mediate the actin reorganization that seems so crucial for prolonged force generation. The vimentin cytoskeleton may also regulate the distribution of p130 Crk-associated substrate (CAS). The CAS family serves as docking station for integrin-mediated signaling\textsuperscript{106}, and translocation of CAS dissociated from vimentin may facilitate actin polymerization and force development\textsuperscript{100;107}. Vimentin may also translocate and activate Rho-kinase, which was shown to be involved in small artery basal tone maintenance\textsuperscript{108}. It remains to be established how critical these processes are in the small arteries. Similarly, the mechanisms by which desmin affects force in small arteries also need to be unraveled.

Microtubuli are made of tubulin dimers that nucleate at microtubule organizing centers such as the centrioles and basal bodies. They are well known to be crucial for mitosis, for dynamic positioning of organelles within cells\textsuperscript{109}, and for cell motility. Microtubule disruption increases force of SMC\textsuperscript{110}. A cell signaling
component is present\textsuperscript{111-113}, but increased force or shortening also occurs in maximally active SMC, providing evidence for their role in tensegrity, a model for mechanical balance of cells depending on actin elements act as prestretched fibers and the microtubule as rigid struts opposing the force generated by the actin filaments\textsuperscript{114}. On the other hand, microtubule destruction or stabilization did not affect the unloaded shortening velocity of permeabilized vascular SMC\textsuperscript{115} and intact coronary arteries\textsuperscript{116}. A role for microtubule in migration of vascular smooth muscle cells in neo-intima formation is well recognized and forms the therapeutic base of taxol-eluting stents\textsuperscript{117}. Considering that early eutrophic remodeling of small arteries involves increased overlap and therefore motility of SMC\textsuperscript{118}, it may well be that microtubules play a role in regulation of media architecture and vascular caliber. Clearly, this area needs more research.

Mechanical properties of the maximally active small artery wall

As was the case for the passive steady state mechanical properties, those of the small artery at full activation can be determined using isometric techniques. Figure 2.2B depicts a typical relation. Active tension is determined from subtracting the passive tension from total tension, assuming a parallel arrangement. Arteries display a gradual increase in maximal active tension at larger distension, generally reaching a peak at radii smaller than $r_{100}$, followed by a decline at further distensions. Remodeling of the SMC causes a change in this relation. The grey curve and dotted arrow in Figure 2.2B indicate remodeling towards a smaller optimal diameter.

Peak tension (i.e. force/length) is higher in larger vessels. This is mainly related to a thicker wall, such that active stress is more comparable between vessels of different caliber\textsuperscript{119;120}. We previously obtained isometric radius-pressure curves on cannulated small mesenteric arteries, using radius-driven feedback of the pressure\textsuperscript{121}. We found that the capacity for ‘active pressure’ generation is close to 200 mmHg for these vessels. Translating these curves into radius-tension curves on the basis of the Laplace law reveals a similar shape as found on the wires, indicating that the non-circular shape in wire-mounted preparations has little influence. A consequence of these relations is that at physiological and mildly higher pressures, fully active vessels remain almost closed.

The factors that determine the shape of the active radius-tension relation may include both the organization of the contractile and cytoskeletal elements, as discussed above, and the deformation of the vascular wall during constriction\textsuperscript{119;120}. In constricted vessels, the luminal surface of the vessel folds into ridges, consisting of not only the endothelial cells and internal elastic lamina, but also part of the SMC\textsuperscript{77}. This withdraws part of the SMC from the capacity for tangential force generation. This process of ridge formation has been suggested to depend on dense body organization and to be associated with reorientation of the myofilaments\textsuperscript{122}. Vessel mass luminal to the contractile filaments may furthermore
amplify the effect of contraction on diameter reduction, while steric hindrance between ridges may prevent full closure.

While small artery remodeling is commonly expressed in terms of a change in passive properties, the changes in maximal active radius-tension relations such as indicated in Figure 2.2B have hardly been studied. Yet, it is clear that this relation is tightly controlled. Thus, the optimum radius for active tension is linked to the passive radius. This link is found over many orders of vascular caliber, and apparently is maintained during growth and development. In addition, SMC length is fairly independent of vascular caliber (see above). Therefore, regulation of SMC length, in addition to the regulation of cytoskeletal organization, may underlie shifts in active radius-tension relations during remodeling.

**Small artery SMC and matrix remodeling**

Vascular remodeling reflects any change in vascular structure, including changes in lumen diameter, wall thickness, and wall composition in terms of cellular parameters and amount of extracellular matrix components. In addition, organization of these components may change, such that the functional or mechanical behavior of the vessel has changed. Remodeling is preferentially described in terms of shifts in mechanical characteristics of individual vessels. In addition, describing ‘remodeling’ (or altered modeling) between groups can be based on the wall-to-lumen ratio, or media-to-lumen ratio. This term allows the comparison of vessels from different individuals, irrespective of the anatomical location, size or branching order. To our knowledge, whether the wall-to-lumen ratio is constant over the arterial tree is a question that has not been extensively studied. Data in mice show that the wall-to-lumen ratio is similar in aorta, carotid and mesenteric arteries. However, data from Frobert et al. suggest that porcine coronary arteries show an increase in wall-to-lumen ratio with an increase in branching order. Similar findings have been made by Bevan et al. for human pial arteries. A concern of these studies is that different experimental conditions, such as varying pressure levels, were used. It is not obvious at which pressure, or pressures, these measurements should be made when vessels of different origin are compared. Some general recommendations regarding this issue have been made by Bund and Lee. While the wall-to-lumen ratio is undoubtedly increased in hypertension, it provides little information on the underlying process, which could be an increase in wall mass (hypertrophy or hyperplasia), a decrease in lumen diameter, or a combination of both. A useful graphical representation of the types of remodeling is given by Mulvany et al. where the authors define remodeling on the basis of lumen change (inward or outward) and wall cross sectional area (hypertrophic, hypotrophic or eutrophic). The contribution of each parameter depends on the particular model, but is dominated by eutrophic...
remodeling, i.e. a rearrangement of material around a smaller lumen without a change in wall cross-sectional area, in the case of essential hypertension. However, it should be stressed that SMC proliferation and apoptosis may form part of the remodeling of small arteries.

**Tone drives remodeling**

We found that chronic vasoconstriction in vitro results in the inward remodeling of small arteries. In addition, some arteries show outward remodeling in vitro in response to prolonged exposure to vasodilators. These observations have led to the suggestion that tone determines the direction of the remodeling response. This idea is in good agreement with results in hypertensive subjects, where vasodilator treatment, but not blood pressure reduction per se, corrects vascular structure. This is of importance, since an increased wall-to-lumen ratio is a predictor of cardiovascular events. In models of altered blood flow, changes in tone precede actual remodeling. These changes in tone appear to be an essential intermediary step in the remodeling process, since defective endothelial function and/or flow-induced dilation prevents flow-induced remodeling.

Based on the wall constituents described above and their dynamic structures, tone-remodeling coupling may occur by two pathways, one related to the cytoskeleton and the other to the ‘mold’ that is provided for newly formed extracellular matrix elements. Regarding the first pathway, in a recent review, Martinez-Lemus et al. argue that the boundaries between constriction and remodeling are blurred. They propose that the sequence of events from vasoconstriction, to intracellular reorganization of the cytoskeleton, to cellular repositioning and eventually, a change in the passive vessel diameter, should not be considered as separate events but rather as a continuum. These events rely on overlapping pathways and depend on the same structural elements, which form the cytoskeleton-integrin-extracellular matrix axis. This concept is based on experiments with isolated arteries that are contracted for various periods. Thus, following a 5-minute constriction, vessels fully relax to their original diameter. When constriction is maintained for 4 hrs, removal of the constrictor does not result in complete relaxation. The prolonged state of constriction is associated with increased overlap of the smooth muscle cells and re-lengthening of the SMC during the maintained vasoconstriction. The lack of full dilation would thus reflect a cytoskeletal brake on distension, and in early remodeling the cytoskeleton would thus take over this function from collagen. The repositioning of SMCs is suggested to redistribute wall stress to non-contractile vessel elements, thereby minimizing SMC energy expenditure. A re-lengthening of cells in constricted vessels would also cause a leftward shift of the active radius-tension curve, provided that the intracellular organization remains unaffected. We analyzed this relation in small arteries mounted in a wire-myograph setup, which were activated at either low or high distension with endothelin-1. Here we found that only vessels that are
activated at low distension indeed showed a shift in the active length-tension relationship towards a smaller diameter. Yet, from work by Gunst, Seow and others indicated above on non-vascular smooth muscle cells, it is highly likely that not only the cell length but also the organization of the contractile and cytoskeletal elements changes during such maintained vasoconstriction. Thus, during early remodeling, prior to matrix reorganization, there may be adaptation both of the SMC length\textsuperscript{118} and to the SMC length\textsuperscript{91}. Figure 2.4 provides a schematic drawing of such SMC plasticity. Following chronic vasoconstriction (Figures 2.4A and 2.4B), re-arrangement of the actomyosin elements would shift the active radius-tension curve leftwards, while reorganization of the cytoskeleton would prevent distension towards the original diameter (Figure 2.4C). Relengthening of the cells and increased overlap would induce similar macroscopic effects (Figure 2.4D).

Further work is needed to unravel the nature, balance and reversibility of these processes in early remodeling. It would furthermore be of interest to test whether the myogenic reactivity is shifted after prolonged constriction and re-lengthening of SMC.

An important consequence of the model proposed by Martinez-Lemus is that the traditional view of load-bearing structures under active and passive conditions needs to be reconsidered. Thus, under prolonged deep constriction part of the load is transferred away from active actomyosin cross-bridge cycling, and smooth muscle cells are able to reposition within the vessel wall. After subsequent relaxation, part of the load appears to be carried by cytoskeletal elements, rather than carried by elastin and collagen alone. The question rises whether cytoskeletal elements also limit distension of vessels that are not subjected to prolonged deep constriction. Experiments using cytochalasins indicate that this is not the case. Cytochalasins are widely used to study the cytoskeletal contribution to cell stiffness and motility. By binding to the growing ends of microfilaments, they block both assembly and disassembly of actin monomers\textsuperscript{140}. In our hand, cytochalasin D does not increase the passive diameter of freshly isolated vessels (unpublished).

Figure 2.4: Schematic representation of possible SMC plasticity and the consequences on vascular caliber. (A) left: a relaxed, pressurized vessel, consisting of spindle-shaped SMC (yellow) surrounded by distended matrix elements (black). Right: a single SMC, with dense bodies (black), a cytoskeleton (purple) and contractile elements (black actin, green myosin). (B) the same vessel in a deeply contracted state. Actomyosin interaction carries the tension, while matrix and subcortical cytoskeleton become unloaded. Capacity for tension generation becomes less due to reduced amount of effective myosin (green dashed lines). (C) and (D): alternative hypotheses for SMC plasticity during prolonged activation. In C, the reduction of number of actomyosin elements in series would induce a leftward shift of the active radius-tension curve. Reorganization of the cytoskeleton would prevent dilation to the original diameter upon SMC relaxation. In D, possible relengthening and rearrangement of SMC during prolonged activation is shown. Overlap of SMC in the wall increases, the active radius-tension curve would shift leftward, and the cytoskeleton would prevent dilation to the original diameter.
Cytochalasin B lowered the pressure for forced dilation of cerebral vessels at normal tone, but did not seem to change the passive diameter at high pressure. In desmin -/- mice, passive stresses as well as maximal active stresses were clearly reduced in second order mesenteric resistance vessels. Yet this might reflect altered development rather than a role for desmin in acute passive mechanisms. If indeed in an established structure the extracellular fibers determine the maximal diameter, and considering the link between diameter for optimal active tension and passive diameter, it follows that mechanisms exist that adapt the extracellular matrix to the contractile and cytoskeletal properties of the SMC.

Such a mechanism could reside in the ‘mold’ that is provided by the active vessel diameter (Figure 2.5). If newly formed matrix components are embedded in a constricted blood vessel, it can be envisioned that this would result in a reduction of the passive diameter. In the reverse case, when new matrix components such as collagen fibers are loosely placed in a vasodilated artery, the maximal diameter may ultimately increase. In this ‘mold’ hypothesis inward remodeling may even occur in the absence of newly formed material, when the existing matrix components are cross-linked by, for example, Transglutaminases. Data from our group and others have shown that members of this family of enzymes can induce small artery remodeling both in vitro and in vivo in hypertension and flow-induced remodeling. The role of Transglutaminases in remodeling was recently reviewed. The relationship between tone and remodeling may be further strengthened by the overlap in signaling pathways, which is evident from various studies. Thus, angiotensin II and endothelin-1 are known to induce proliferation and fibrosis in addition to inflammation and vasoconstriction, as reviewed by Intengan and Schiffrin. The vasodilator signaling of nitric oxide also overlaps with remodeling events, but diverges at the level of cGMP-dependent protein kinase type I.

Inflammation facilitates remodeling

A specific type of remodeling is the outgrowth of small pre-existing collateral arteries in the face of an arterial obstruction. This process is referred to as arteriogenesis and may be considered an extreme case of flow-induced remodeling. It is particularly relevant in the case of large artery stenosis which results from atherosclerotic lesions as it provides a natural bypass to alleviate ischemia. This is an area of intense research, which mainly focused on the stimulation of leukocyte recruitment to the area of vascular remodeling. These leukocytes, particularly monocytes, but also natural killer cells (a specific lymphocyte with regulatory as well as cytotoxic functions) and CD4+ T cells facilitate arteriogenesis, probably through the release of metalloproteinases and cytokines. We found that both inward- and outward remodeling in small mesenteric arteries of mice induced by altered blood flow depends on...
Figure 2.5: The ‘mold’ hypothesis linking vasoconstriction to inward remodeling. a: part of the relaxed vessel wall, showing a SMC (yellow), matrix fibers (black) and cross-links (blue). b: contraction unloads the existing matrix. C: Matrix turnover. A new matrix element (brown) is embedded that is relatively straight in the constricted vessel. In addition, new cross-links (orange) are formed between and within new and existing matrix. Some old elements have disappeared. D: upon SMC relaxation, the new elements and cross-links prevent dilation to the original diameter.
macrophages. A similar dependence on leukocytes is found in pregnancy induced vascular remodeling. In this case, natural killer cells play a crucial role in the dramatic outward remodeling of uterine arteries, which depends on interferon-γ release. In experimental hypertension, the inward remodeling of small arteries is dependent on proper macrophage function. Our current view is that the inward or outward direction of remodeling is linked to cytoskeletal reorganization and tone as indicated above, while inflammatory mechanisms would facilitate this process through degradation, cross-linking and rebuilding the extracellular matrix. Yet it is clear that this needs further research.

**Pressure-dependent remodeling**

While many stimuli and conditions influence regulation of small artery caliber, pressure plays a special role, since it determines the stresses and strains of the wall elements, it is a direct stimulus for SMC contraction, and since hypertension is one of the most relevant fields for remodeling research. The myogenic response, i.e. the increase in tone and reduction in diameter with pressure, was described by Bayliss in as early as 1902. Johnson pointed out that the myogenic response regulates total wall tension. This concept is still in use. VanBavel et al. and Buus et al. pointed out that total wall tension is influenced by vasoactive agents. Therefore, the myogenic response would not only regulate wall tension against changes in pressure, but also oppose vasoconstrictive influences under pressure-driven conditions (since the vasoconstriction causes a reduction of wall tension) while amplifying them under isometric conditions (since here the activation raises total wall tension). Signaling in the myogenic response has been well reviewed. The response invokes calcium signaling, but also heavily depends on the cytoskeleton and integrin signaling. Pressure-dependent remodeling may relate to such signaling. In addition the strength of the myogenic response may influence the nature of the remodeling. Thus, wall stress in hypertension can be normalized by inward eutrophic remodeling or hypertrophy. On the basis of the ‘mold’ hypothesis, the eutrophic inward remodeling in small arteries may be the consequence of the strong myogenic response known to exist in these vessels, while large vessels show both a limited myogenic response (i.e. increased diameter at increased pressure) and hypertrophic responses to hypertension. In support of this view, in hypertensive models with compromised myogenic responses, small vessels show hypertrophic remodeling.

**Quantitative approaches to vascular remodeling**

Functional and structural responses of small arteries to mechanical and metabolic stimuli are considered to serve homeostasis of tissue perfusion, wall stress and shear stress. Figure 2.6 indicates tone, plasticity and remodeling as a sequence of events in a possible control loop. Interaction schemes such as this can be refined
and extended to networks and metabolic variables. Yet the presence of multiple regulated variables, acute and structural effects, and the complexity of network architecture obscures a straightforward insight into the integrated behavior of the resistance vasculature. Several authors built simulation models of vascular regulation in order to obtain a better understanding or to derive new concepts for vascular regulation. To the best of our knowledge, no network-based models have been published that combine tone, remodeling, wall stress, shear stress and metabolic influences in an integrative approach. It is indeed questionable whether such an approach would be insightful at this moment. A more sensible strategy is to take this step by step. Here we suggest a definition of tone that can be used for modeling purposes and we highlight a number of model studies and concepts on diameter regulation of single segments that seem relevant for the progress towards an integrative approach in this area.

**How to quantify tone?**

‘Vascular tone’ is often defined as the degree of vasoconstriction, expressed as a percentage reduction in diameter. While this definition is fully justified for descriptive and statistical purposes and for the interpretation in terms of vascular resistance, a more fundamental definition may be based on the state of contractile activation of the SMC. Thus, assuming a single SMC compartment parallel to the matrix, tone can also be defined as the actual active tension divided by the maximal active tension at the same diameter. Several experimental and model studies have employed this definition. Figures 2.2C and 2.2D depict radius-tension and pressure-radius curves at various levels of tone according to this definition. As can be seen in Figure 2.2D, radius at any constant tone level increases rapidly with pressure. Vessels therefore need to increase tone with pressure (myogenic response) even to maintain a constant diameter, let alone to obtain a negative myogenic pressure-diameter slope (dash-dotted line in Figure 2.2D).

**Tone as a drive for remodeling**

The concept that tone drives remodeling was quantitatively analyzed by Jacobsen and co-workers. These authors built a simulation model based on a wall stress-driven myogenic response in combination with tone-dependent inward or outward eutrophic remodeling. While the authors made specific choices for passive and active radius-stress curves and dynamics of adaptation, the model is in essence a conceptual one, and the conclusions seem valid for a wide array of parameter choices. An increase in pressure resulted in activation and vasoconstriction, as discussed above, but at a longer time scale also induced inward remodeling. This again helps to restore wall stress and causes gradual reduction of tone during the remodeling response. In steady state, at higher pressure the vessel became inwardly remodeled with unchanged basal tone. This model was further tested by simulating vasodilator and vasoconstrictor influences,
Figure 2.6: Vascular tone as a central element in a sequence of interactions regulating local tissue perfusion and peripheral resistance.
from e.g. surrounding tissue and nerve endings. Continuous presence of a vasodilator caused outward remodeling, but tone was restored to original levels. Thus, by having an adaptive response of tissue structure driven by tone, vessels in this model are able to maintain a normal level of activation under a wide variety of chronic conditions. Tone, according to this model, could therefore be regarded as a long-term regulated variable. The exact myogenic responsiveness and the existence or not of a negative slope in the active pressure-diameter relation is not critical in this model. Thus, variations in the myogenic responsiveness, known to exist in the circulation, lead to differences in chronic tone but, possibly counter-intuitively, not to differences in passive vessel caliber.

The model of Jacobsen provides quite a useful approach in understanding integrated regulation of caliber. At the same time, it generates additional questions. The first question is what is actually being regulated in vessels in a network? The Jacobsen model considers regulation of wall stress as the drive for remodeling via tone. Other influences were considered to be simple, uncontrolled offsets in tone, eventually causing structural effects. This all occurs under the condition of controlled pressure, and indeed the authors pointed out that their model simulates an isolated, cannulated vessel under pressure control. However, one might also subject such a vessel to flow control. On the basis of both acute and chronic effects of flow, it has been argued that flow-dependent tone and remodeling act to regulate shear stress. One could construct a tone-remodeling model where tone depends on shear stress. A step increase in flow here would lead to increased shear stress, vasodilation, outward remodeling, reduction in shear stress and recovery of tone to its original level. Other vasoactive factors would lead to changed caliber with unaltered tone in steady state. Sensitivity to shear would influence the chronic level of tone, but not the caliber of the vessel. Such a model would thus be completely analogous to the Jacobsen model, but with pressure exchanged for flow. In a network, alterations in vascular tone and remodeling affect both pressure and flow, while also metabolic influences such as the local oxygen concentration might be considered to form regulated quantities. A necessary further step therefore is to build models combining acute regulation of both wall stress and shear stress with tone-remodeling coupling. These models should initially be analyzed in a setting with constant entrance and exit resistances. Once properly understood, such models could be incorporated in simulations of vascular networks.

**Modeling matrix rearrangement and turnover**

Jacobsen et al. incorporated tone-driven remodeling as a change over time of the unloaded passive diameter, without specification of turnover or rearrangement of individual fibers that underlines such change. A next step could be to include turnover of populations of fibers with dispersion of resting lengths. As an example, Gleason and Humphrey formulated conceptual models of large artery growth...
and remodeling in hypertension based on turnover of vascular elements. The model was based on the assumption that the individual wall elements deform together, but can turnover at different rates, based on the stresses acting on these elements. New elements would have resting lengths matched to the actual diameter of the vessel. Tone was included, but only as a way to maintain the actual diameter against changes in pressure. The authors concluded that stress-dependent turnover ensures normalization of wall stress, through increased wall mass. Differences in turnover rate between elastin, collagen and SMC would cause a stiffening of the vessel during hypertension. Such predictions reflect the hypertrophic rather than eutrophic remodeling of large vessels in hypertension. It needs to be established to what extent known differences in tone control between large and small arteries would affect predictions for remodeling based on such a turnover approach. As indicated above, a further concern in such turnover approaches is that the predictions rely heavily on the arrangement of collagen as parallel or serial elements. Clearly, more experimental evidence is needed for the rates of turnover and arrangement of existing and new fibers.

**Conclusions**

It is clear that the questions provided in the introduction with respect to integrated regulation of small artery caliber and flow cannot yet be answered. The bottle necks include the need for a better understanding of small artery matrix architecture, maintenance and mechanics, more extensive experimental data on SMC mechanics in remodeling vessels, and a better insight into regulation of SMC cell length and cytoskeletal architecture. We believe that there is much to learn from current progress in non-vascular SMC biophysical research. Considering the size and transparency of small arteries, the rapid progress in molecular and live imaging techniques should provide many new experimental approaches, allowing the bridging of cellular and matrix biophysics and vascular biomechanics. More detailed molecular information will remain needed, but simultaneously we should make sure to ‘see the physiological wood through the molecular trees’, and this requires a combined experimental and modeling approach.
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Decomposition cross-correlation for analysis of collagen matrix deformation by single smooth muscle cells

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Medical & Biological Engineering & Computing
2008, 46: 443-450
Abstract

Microvascular remodeling is known to depend on cellular interactions with matrix tissue. However, it is difficult to study the role of specific cells or matrix elements in an in vivo setting. The aim of this study is to develop an automated technique that can be employed to obtain and analyze local collagen matrix remodeling by single smooth muscle cells. We combined a motorized microscopic setup and time-lapse video microscopy with a new cross-correlation based image analysis algorithm to enable automated recording of cell-induced matrix reorganization. This method rendered 60-90 single cell studies per experiment, for which collagen deformation over time could be automatically derived. Thus, the current setup offers a tool to systematically study different components active in matrix remodeling.
Introduction

The extracellular matrix (ECM) provides a biophysical and biochemical environment for cell mechanical behavior. In turn, cellular interactions with the ECM resulting from adhesive, proteolytic and migratory activity govern continuous matrix reorganization. One such example occurs in eutrophic inward remodeling of small arteries. Here, the existing collagen matrix is rearranged around a smaller diameter. Such inward remodeling is a hallmark of many hypertensive disorders and has been shown to have predictive value for cardiovascular events.

In vitro setups of cell-seeded matrix scaffolds allow studying the specific components that are active in vascular remodeling. Especially, matrix reorganization can be monitored in the presence of specific cells, matrix elements, and blockers or markers of remodeling enzymes such as the matrix metalloproteases and Transglutaminases. Several approaches using multicellular preparations have been developed. Thus, free-floating collagen gels can be seeded with contractile cells, and the remodeling is monitored from the dimensions of these gels. In the so-called culture force monitor, collagen gels mixed with cells are allowed to polymerize between a fixed plate and a force transducer. Contractile force development is measured during subsequent culture of this preparation. However, interpretation of data derived from these multicellular approaches is troubled by variability in cellular mechanical activity, lack of information on cell density, and synergistic contractile effects. Therefore, microscopic observations on matrix reorganization by single cells may provide more detailed and fundamental information on the mechanisms of remodeling.

Several groups have studied the effect of single cell activity on local deformation of matrices. The most frequently used substrates are silicone rubber and polyacrylamide sheets. Local deformation is then monitored by tracing the texture, sometimes facilitated by using fluorescent beads or imprinted micropatterns. Although these inert scaffolds provide a geometrical framework to study processes like cell locomotion, they do not resemble the physiological ECM. This has been solved in some cases by coating the polyacrylamide membrane with a thin layer of collagen. However, remaining issues are the orientation of the collagen fibrils in the coating as compared to native collagen, and the inability of the cells to chemically modify (e.g. by cross-linking) the artificial sheets.

A possible way to overcome these issues is the use of collagen-based matrices. Cell-induced deformation patterns of these matrices and the degree of reversibility of such deformation provide information on physical remodeling and the underlying biochemical processes. Deformations have been assessed by manually tracing beads or landmarks in consecutive images. However, so far, only a few algorithms were developed for automatic detection of substrate
deformations. In particular, very few of such algorithms allow tracing in the absence of embedded beads\textsuperscript{25-27}, and more stable algorithms are required.

While single cell observations provide a more fundamental insight into matrix remodeling than macroscopic studies, a concern is efficiency. Time-lapsed video microscopy of single cells forms a labor-intensive and time-consuming experiment. Meaningful experiments require the comparison of large data sets, including different cell types, various matrix compositions, or the use of biochemical or molecular interventions. A fully automated, motorized microscopy setup is required that scans series of individual cells and their surrounding matrix. In addition, each individual cell should automatically be kept in focus during remodeling experiments, which take up 24 hrs or more. Finally, stable algorithms are needed for the automated analysis of geometrical reorganization with minimal user input.

The aim of this study is to develop an automated technique that can be employed to obtain and analyze local matrix remodeling by individual cells. The system that we present allows for monitoring of \~75 cells in parallel, using time-lapse video microscopy and computer-controlled stage positioning. In addition, we present and evaluate a new algorithm for automated detection of collagen matrix deformation around these cells.

**Methods**

Cell culturing and collagen matrix preparation
Smooth muscle cells, obtained from mesenteric small arteries, were cultured in Leibovitz medium with 10% (v/v) heat-inactivated fetal calf serum. Cells from passages 3 to 9 were used in experiments.

Matrix constructs were produced from calf skin collagen (MP Biomedicals) at a concentration of 1 mg/ml; pH was buffered by HEPES, and a mix of antibiotics (PSF and ciproxin) was added. Immediately after preparation at 4°C, the collagen mixture was poured into a 3.8 cm\textsuperscript{2} culture well and a 1.5-hrs polymerization period at 37°C was allowed. Then, SMCs were seeded at a concentration of about 1 cell per mm\textsuperscript{2} in the presence of 1 ml serum-free Leibovitz medium. The cells were maintained in an incubation chamber that was set to a temperature of 37°C throughout the experimental procedure. After a stabilization period of about 1 hr, cell-matrix interactions were monitored by microscopic imaging for a period of 24 hrs of spontaneous cell contraction.

Automated microscopic imaging
In order to enable simultaneous monitoring of cell-induced matrix remodeling at multiple locations, microscopy was combined with a motorized stage. Individual cells and their surrounding matrix were studied by phase-contrast microscopy (Olympus IMT-2 with 10x objective and 2.5x projection lens). Images were
captured by a Qimaging Retiga SRV camera. The calibration factor for these images (1392x1040 pixels) was 0.88 μm/pixel. The microscopic field of view was set by a motorized stage, controlled by custom written software (Matlab 7.0 with Image Acquisition Toolbox 2.0). After manually determining and storing a set of x,y,z-coordinates for about 60-90 appropriate cells, these positions were tracked through time at a 15-minute interval and images were captured by an automated procedure.

During the time-lapsed image acquisition, samples were kept in focus by means of implementation into the acquisition software of one of the general autofocus algorithms. Image contrast is optimal when a histogram of intensity values shows a broad distribution over all bins. This characteristic feature can be approximated by the standard deviation of pixel intensity values. For each image, contrast was enhanced by histogram equalization, and standard deviation was calculated. A normalized focus index (FI) was defined by dividing the standard deviation of the original image ($SD_{original}$) by the standard deviation of the contrast enhanced image ($SD_{enhanced}$):

$$FI = \frac{SD_{original}}{SD_{enhanced}}$$

For each cell, a series of images was captured at five different heights. The image was then defined to be in focus at the vertical level of maximal normalized focus. This height (z) was used as the central level when capturing the z-series in the next time step, thus allowing gradual vertical shift during the time-lapsed image acquisition. Finally, a stack of time-lapsed images was constructed for each xy-position and analyzed off-line.

**Gel dynamics analysis**

Cell-matrix interactions were quantified offline using in-house designed, automated image analysis software (Matlab 7.0 with Image Processing Toolbox 4.2). Matrix reorganization was assessed by calculation of the displacement field around a cell. This was achieved by performing a cross-correlation between each two successive images in an image stack; resolution of the displacement field was refined by correlation of subimages of decreasing size. This procedure is explained below and illustrated in Figure 3.1.

First, gross displacement was defined at the point of maximal correlation between two parent images $I_1$ (at $t = t_0$) and $I_2$ (at $t = t_0 + \Delta t$), using a correlation threshold of 0.5 and a maximum tested displacement of 11 pixels. Then, $I_1$ was decomposed into 4 equal-sized square subimages, for which another cross-correlation was performed. The position of the correlation subwindow of $I_2$ was refined using the displacement as calculated for the parent image, with a safety measure to prevent $I_2$ crossing the borders of $I_1$. This procedure of accurately positioning a correlation window not only reduced the possibility of an accidental
cross-correlation match between a subimage of $I_1$ and any random subimage of $I_2$, but also drastically reduced calculation time. Resolution of the displacement field was refined to the 5th decomposition stage in our experiments (Figure 3.1), using maximum tested displacements ($n_{cc}$) as indicated in Table 3.1. When insufficient correlation ($r<0.5$) was found in any small area, displacement field from the next larger images were used. The calculated displacements were then assigned to the corresponding subimage centers. Subsequently, a continuous displacement field was obtained by bicubic interpolation. Finally, matrix compaction for each stack was quantified by tracking circular areas centered around the cell (Figure 3.5).

![Figure 3.1: Graphical representation of parameters used in matrix deformation analysis. The image shows a single SMC in the center, surrounded with a collagen matrix of relatively smooth texture. The white box (768x768 pixels) indicates the area for the most coarse correlation analysis. This correlation between two successive images was applied over an area as indicated by the yellow box (960x960 pixels). The green boxes show the more refined cross-correlation windows (stages 2-5). Table 3.1 indicates the area expansion used for these correlation analyses.](image-url)
Validation

The method described above (decomposition CC) was validated on several test series against a straightforward cross-correlation analysis (direct CC), with settings according to decomposition stage 1.

The first test case consisted of an image of a collagen-embedded cell, which was artificially resized by 3%, thereby simulating matrix compaction. Secondly, increasing amounts of white noise were added to the resized image in order to test the stability of both correlation methods. Relative dispersion (RD), which is defined as standard deviation divided by mean, was used as a noise level index. Finally, image resizing was followed by a horizontal translation of 60 pixels for a low and high noise example (Table 3.2).

Table 3.2: Characteristics of validation images: CC was tested by addition of Gaussian white noise with mean 0.0 and increasing variance levels, in several cases the image was resized or translated.

| Index | Scaling (%) | Gaussian White Noise Variance | RD Image | RD Increase (%) | RD noise | Horizontal Shift (pixels) |
|-------|-------------|--------------------------------|----------|-----------------|-----------|--------------------------|
a     | 1           | 0                              | 0.25     | 0               |           | 0                        |
b     | 0.97        | 0                              | 0.251    | 0.5              | 0         | 0                        |
c     | 0.97        | 0.0005                         | 0.26     | 4.1              | 0.104     | 0                        |
d     | 0.97        | 0.001                          | 0.269    | 7.6              | 0.146     | 0                        |
e     | 0.97        | 0.002                          | 0.285    | 14.1             | 0.207     | 0                        |
f     | 0.97        | 0.003                          | 0.301    | 20.2             | 0.253     | 0                        |
g     | 0.97        | 0.004                          | 0.316    | 26.3             | 0.293     | 0                        |
h     | 0.97        | 0.005                          | 0.329    | 31.7             | 0.327     | 0                        |
i     | 0.97        | 0.0005                         | 0.259    | 4.1              | 0.108     | 60                       |
j     | 0.97        | 0.003                          | 0.303    | 20.2             | 0.265     | 60                       |

Relative Dispersion (RD) in an image is defined as standard deviation divided by mean, RD increase is expressed as RD value compared to case “a”, RD noise is calculated as ratio of noise variance and image mean.
Results

Parallel recording of matrix compaction movies
We were able to record on average 66 movies on collagen compaction by single cells in parallel at a time resolution of 15 minutes (n=10 experiments). Critical issues that limited this number were the need to avoid rapid acceleration and deceleration of the microscope stage, and the time-consuming autofocus algorithm. If cells were properly focused in the initialization stage, only a small fraction (< 5%) ran out of focus during 24-hour compaction experiments. Under the given incubation conditions, the majority of the cells adhered firmly to the collagen matrix and demonstrated little migratory activity. Typically, cell centroids remained within a 150 μm radius of their original XY position. Z position decreased slowly in many cases, reflecting compaction of the matrix in the vertical direction (data not shown).

Validation of the collagen compaction analysis
Analysis based on cross-correlation of images at a series of decomposition stages was compared with straightforward cross-correlation. This was performed on images simulating matrix compaction, subsequently followed by a challenge of increasing amounts of image noise and artificial translation.

Decomposition CC was more time-consuming than direct CC: respectively 84 and 54 seconds per image pair. This was due to a larger number of cross-correlations that have to be performed, and more complex data storage and lookup operations. For low noise levels (Table 3.2, case c and d), both analysis methods render 100% correct displacement vectors (Figure 3.2). When noise increased, the number of vectors in the smallest decomposition stage that had insufficient cross-correlation (r<0.5) rose. However, this effect was smaller in the decomposition CC versus direct CC. As an example, the percentage correct displacement vectors at 20.2% noise (Table 3.2, case f) was 77.7% in direct CC versus 89.5% in decomposition CC. Concurrently, the average displacement error at this noise level amounted to 2.1 and 0.7 pixels respectively.
Figure 3.3: Calculated area at a radial distance of 350 pixels after a simulated 3% compaction. Increasing displacement errors, caused by higher noise levels, resulted in a mismatch between calculated area and simulated compacted area (94% of original). The decomposition method showed stable results at larger relative dispersion values (Table 3.2, case c-h).

Figure 3.3 shows estimates for matrix compaction for these simulated deformations. The simulated compaction over a disc with 350 pixels radius was 6%. For noise levels up to 7.6% (Table 3.2, case d), such compaction was indeed found by both correlation methods. At higher noise levels, direct CC underestimated compaction much more severely as compared to decomposition CC. As an example, after a noise increase of 20.2%, decomposition CC estimated a compaction value of 5.9% as compared to only 4.1% for direct CC.

When an additional horizontal shift was imposed, both analysis methods correctly estimated compaction for 4.1% noise (Table 3.2, case i). For higher noise levels, decomposition CC succeeded in a correct area assessment. In contrast, at 20.2% noise and 60 pixels translation (Table 3.2, case j), direct CC predicted 1.2% rather than 6% compaction of the 350 pixels disc (Figure 3.4). This effect was not due to more incorrect displacement vectors, but could be attributed to a rise in average displacement error up to 14.6 pixels.
Collagen compaction by individual smooth muscle cells

Figure 3.5 shows an example of collagen matrix compaction by an individual smooth muscle cell. In this particular case, gross geometrical reorganization occurred in the first 6 hrs of the 22-hours observation period. Compaction magnitude decreases with distance, and there was a time delay of around 1.5 hrs before compaction visible at a radius of 97 µm (110 pixels) became apparent at a distance of 308 µm (350 pixels).
This study aimed at developing an automated technique for obtaining and analyzing matrix remodeling by individual cells. Emphasis was put on construction of an automatic, reliable algorithm for assessment of a detailed matrix displacement field. Especially, refinement of a cross-correlation based image analysis with a decomposition scheme was investigated. While ‘classic’ direct CC sufficed for pairs of images with high correlation and low noise, this was no longer the case when substantial matrix remodeling occurred within the time frame between two consecutive images. This resulted in failing of CC at spots of high geometrical reorganization. However, when using decomposition CC, the gross

![Image](image_url)

Figure 3.5: Typical example of collagen matrix compaction by individual smooth muscle cell as estimated by decomposition cross-correlation, obtained at a resolution of 1 frame per 15 minutes. (A) Top left: displacement field represented by vectors, refined in 5 decomposition stages. Vectors originating from a red asterisk were created at a larger correlation scale; for the smallest window, cross-correlation value at these positions was below threshold (0.5). Top right: visual representation of matrix compaction at a radial distance of 350 pixels, as estimated from a series of displacement fields, showing an initial (yellow) circle and its deformed (green) state. Bottom: estimation of compacted area after 22 hrs at distances of 110, 230 and 350 pixels (scaling: 0.88 μm/pixel).

Discussion

This study aimed at developing an automated technique for obtaining and analyzing matrix remodeling by individual cells. Emphasis was put on construction of an automatic, reliable algorithm for assessment of a detailed matrix displacement field. Especially, refinement of a cross-correlation based image analysis with a decomposition scheme was investigated. While ‘classic’ direct CC sufficed for pairs of images with high correlation and low noise, this was no longer the case when substantial matrix remodeling occurred within the time frame between two consecutive images. This resulted in failing of CC at spots of high geometrical reorganization. However, when using decomposition CC, the gross
displacements at these positions could be estimated by analysis of parent images with larger dimensions. This way, at a relative dispersion increase of 20.2% the average displacement error was lowered threefold in decomposition CC as compared to direct CC.

The method of refining displacement field accuracy with each decomposition step becomes progressively more important with larger displacements. Therefore we investigated the effect of a horizontal shift superimposed on a simulated compaction. Such shift occurred in our in-vitro experiments when a group of neighboring cells pulled strongly on the matrix adjacent to a cell of interest. The shift interfered with direct CC because zero deformation was assumed when no proper correlation could be found. The result was an irregular displacement field (Figure 3.4). When using decomposition CC, on the other hand, the gross displacement was already detected in the first decomposition stage. Displacement values were then adjusted when local compaction was detected in subsequent decomposition stages. Using this strategy, a lack of local correlation at the finest resolution resulted in only a minor displacement error, with an average of 0.9 as compared to 14.6 pixels for direct CC.

Both cross-correlation methods differ not only in stability of displacement field estimation, but in efficiency of calculation time as well. Direct CC requires a large search area, i.e. the subwindows of I2 have to extend considerably beyond the boundaries of their corresponding I1 subwindow in order to perform a meaningful correlation. On the other hand, with decomposition CC ncc can decrease with each stage (Table 3.1), since each subwindow of I2 is repositioned according to the preliminary displacement as calculated for its parent window. Due to the increased number of calculations and data manipulations that have to be performed, analysis time was still about 50% larger for decomposition CC at the settings as stated in Table 3.1.

We chose a correlation threshold of 0.5 for both techniques, as well as cross-correlation windows as indicated in (Table 3.1). These values were empirically determined as an optimum in the trade-off between calculation time, false positive displacements and overlooking local deformations. Clearly, these choices depend on the contrast and texture of the images and the nature of the deformation, and will need to be optimized for specific future experiments.

Cell traction is frequently assessed by quantification of deformations in a flexible substratum. Both 2D and 3D approaches have been used, resulting in different cellular morphologies8;28;29. While the latter seems more physiological, interpretation of the 3D experiments is more complex27. 2D substrates offer more straightforward tools for analyzing mechanical behavior of single cells. These materials can be enriched with fluorescent microbeads to increase image contrast. In order to achieve a high resolution in the vicinity of cells, which can change their morphology rapidly, manual tracking of specific landmarks was employed in
several studies\textsuperscript{13,15,18}. However, this labor-intensive method inherently limits the number of cells under study. In several studies particle tracking was performed on individual microbeads\textsuperscript{21}. In this case, resolution depends on particle distribution, which is typically far from homogeneous in collagen substrates\textsuperscript{26}.

Several nested cross-correlation methods have been developed\textsuperscript{27}. All of these algorithms are based on empirical determination of square size, distance for pattern search, and normalized CC threshold. In order to spatially limit the search region, a relative translational shift has been derived from image registration\textsuperscript{25,26}. Several schemes have been implemented to increase displacement field resolution in a step-by-step manner. In a single refinement cycle, Dembo and colleagues\textsuperscript{22} decreased the subwindow size if significant displacement was observed. On the other hand, window size can be increased if correspondence failure occurs at high resolution\textsuperscript{26}. The algorithm developed by Wang and coworkers\textsuperscript{25} resembled our decomposition CC to a large extent. However, these authors aimed at construction of traction fields with a smooth nature by incorporation of filtering procedures at several stages.

Geometrical matrix reorganization provides a qualitative index of the traction forces present in the underlying material. The actual forces can be derived from a displacement field series using material properties of the ECM construct. However, for collagen scaffolds these are highly heterogenous. Frequently, local stiffness is estimated by either microneedles\textsuperscript{13,14,17,30} or optical tweezers\textsuperscript{31-33}. Traction can then be calculated by application of stress-strain relationships, including appropriate boundary conditions\textsuperscript{24,34-38}. However, this translation to quantitative traction forces lies beyond the scope of this article.

In conclusion, we presented integral methodology for the study of matrix remodeling. These techniques allow systematic screening of the role of matrix components such as collagen, elastin, fibronectin and laminin. Likewise, the function of stationary cells like smooth muscle cells, fibroblasts, and osteoblasts can be investigated. Our method can be applied under a wide variety of other experimental conditions. The single requirement for the image quality is a sufficiently high contrast in the material under investigation, without the need for laborious and potentially interfering micropatterning. Furthermore, our graphical user interface enables a flexible tuning of parameters such as number of decompositions, size of correlation window, search area and cross-correlation threshold. Using our motorized microscopy stage it is possible to patch individual images together in order to create one large field of view for the study of motile cells such as keratocytes. Finally, the method can be extended with fluorescence imaging of specific cell structures, cytokines, hormones and enzymes\textsuperscript{33}. 
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Vascular smooth muscle cells remodel collagen matrices by long-distance action and anisotropic interaction

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Submitted for publication 2010
Abstract

Rationale: While matrix remodeling plays a key role in vascular physiology and pathology, the underlying mechanisms have remained incompletely understood.

Objective: We studied the remodeling of collagen matrices by individual vascular smooth muscle cells (SMCs), clusters and monolayers. In addition, we focused on the contribution of Transglutaminase 2 (TG2), which plays an important role in the remodeling of small arteries.

Methods & Results: Single SMCs displaced fibers in collagen matrices at distances up to at least 300 μm in the course of 8-12 hours. The matrix was locally compacted up to about 200 μm. This exceeds the distance over which cellular protrusions are active, implicating the involvement of secreted enzymes such as TG2. Reversibility of compaction upon cytoskeletal disruption was significantly higher for TG2 knock-out cells. This difference increased with distance, suggesting a lack of TG2 cross-linking here as compared to wild-type cells. At increasing cell density, cells cooperated to establish compaction. In a ring-shaped collagen matrix, this resulted in preferential displacement in the radial direction, perpendicular to the cellular long axis. This process was unaffected by inhibition of TG2 cross-linking.

Conclusions: These results show that SMCs are capable of matrix remodeling by prolonged, gradual compaction along their short axis. This process could add to the 3-D organization and remodeling of blood vessels based on the orientation and contraction of SMCs.

Keywords:
matrix, compaction, Transglutaminase, enzymatic cross-linking, migration
Introduction

Arterial structure is normally well matched to the functional needs. Thus, presence and organization of elastin and collagen fibers ensures the non-linear stress-strain relation required for a stable diameter under pressure, and matrix strength is sufficient to withstand pressure in the order of 1000 mmHg, providing a 10-fold safety range against acute rupture. Peak force development of the vascular smooth muscle cells occurs at typically ~90% of the distended passive diameter at 100 mmHg, and allows constriction against ~200 mmHg. Such properties hold over many orders of arterial branching, indicating that mechanisms exist for their maintenance during development and outgrowth of the arteries. These mechanisms of structural control are crucial not only for development, but also for vascular inward remodeling in response to a wide variety of physiological and pathological stimuli. Yet, they are only partly identified.

The in vitro or in vivo study of SMC cell-matrix and cell-cell interaction during arterial remodeling could identify fundamental mechanisms of tissue organization. Examples of such studies in small arteries address the cell relengthening and reorganization and the activity of Transglutaminases in the vascular wall during early inward remodeling. Still, the complex composition and architecture of even the isolated small artery has provided quite a challenge for such approaches. As an example, the passive diameter of arteries at high operating pressures is believed to be dominated by organization of the collagen backbone. Yet, it is not clear whether adventitial or medial collagen is relevant, and which cell type is effectuating the organization (fibroblasts, SMCs, invading leukocytes). Moreover, it is not clear whether the stiffening in early inward remodeling indeed reflects modification of the collagen backbone. Alternatively, such remodeling could be effectuated by other matrix and cytoskeletal fibers.

An additive strategy is the study of cell-cell and cell matrix interaction in well-defined artificial systems, with specific cells and matrix elements. Such models include the compaction of collagen matrices by SMCs. A single SMC is able to locally remodel the collagen fibril organization by microscopic movement of cell protrusions. This process is independent of rapid contractions, as evidenced by the lack of effect of myosin light chain kinase and protein tyrosine kinase inhibitors. We previously developed technology for the study of tractional forces exerted by a single smooth muscle cell on the underlying substratum. Collagen compaction has also been studied at the macroscopic level, where the area of a disc of collagen densely seeded with SMCs is monitored over time. However, these studies generally provide little mechanistic insight, and it is not clear whether such remodeling represents a mere summation of single cell behavior, or reflects properties emerging in clusters and monolayers of SMCs. Such synergistic effects could be based on physical restriction of protrusion movement or coordinated
cooperation between cells that are in contact with each other, thereby providing a much more efficient and powerful way to remodel tissue as compared to single cell compaction.

Here we study remodeling of collagen matrices by vascular SMCs in models of increasing complexity, ranging from single cells to polarized monolayers. We quantitate the dynamics, reversibility and spatial extent of remodeling, and demonstrate a transition from isotropic collagen compaction by single cells to highly anisotropic compaction by pairs of cells and in monolayers, where remodeling occurs predominantly in a direction perpendicular to the cell axis. In addition, considering the crucial role of Transglutaminase 2 in vascular inward remodeling, we tested the involvement of this pleiotropic enzyme in the remodeling of these model systems, aiming especially at its cross-linking function.

Materials & Methods

Expression Vectors
The IMAGE clone 3256943 (GenBank: BC016492) for mouse Transglutaminase 2 (TG2) was obtained from imaGenes (Berlin, Germany). Using standard molecular biology techniques, nucleotides 67-2150 were cloned into pEGFP-N1 (Clontech 632469), generating a vector that expresses TG2 with a C-terminal fluorescent tag (TG2/eGFP). This cDNA sequence was verified by DNA sequencing.

Cell Culture and Transfection
Small artery smooth muscle cells were obtained by the explant method from mesenteric small arteries of mice (WT and TG2 knock-out having a mixed Bl6/SVJ background). After 2-3 weeks of initial growth in L-15 medium with 20% (v/v) heat-inactivated fetal calf serum (HI-FCS; Invitrogen), cells were trypsinized, suspended in L-15 medium with 10% (v/v) HI-FCS, and seeded in 25 cm² plastic culture flasks (seeding density: 3200 cells / cm²). Typically, cells achieved confluence within 3 days and then cell number was constant for one week. Cells from passages 3 to 9 were suspended in L-15 without serum and used for experiments.

A smooth muscle cell line (MOVAS, ATCC CRL-2797) was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum (Gibco) and a mix of antibiotic-antimycotic (Gibco). If transfection with TG2/eGFP was required, cells were seeded at about 60% confluency in 12-well plates. After 24 hrs, cells were transected using Lipofectamine LTX (Invitrogen, 15338), according to the manufacturer’s protocol. Per well, 1000 ng DNA, 2.5 μl Lipofectamine LTX and 1.0 μl PLUS reagent were used.

Immunofluorescent Staining of Contractile Markers
The phenotype of smooth muscle cells under normal culturing conditions was studied by immunofluorescent staining of several contractile markers. MOVAS,
Explant wild-type or TG2 knock-out cells were trypsinized and reseeded in microscopic culture chambers (BD Falcon 354102, untreated glass). After 24 hrs, cells were washed with warm PBS and fixated with formaline (20 min on ice). Cells were permeabilized with 0.05% Triton X-100 and blocked with 3% BSA/5% goat serum. Samples were then incubated for 1 hr at room temperature with mouse monoclonal antibodies against either α-actin (DAKO MO851, 1:500), calponin (Sigma C2687, 1:1000) or myosin heavy chain (Abcam Ab-683, 1:400). Subsequently, anti-mouse Cy3 (Brunschwig 115-165-166, 1:300) was used as secondary antibody, and slides were mounted in Vectashield/DAPI (Vector Laboratories H-1500). In addition, rabbit monoclonal anti-mouse smoothelin (gift from Guillaume van Eys, Maastricht University, 1:1000) followed by anti-rabbit Cy3 (Brunschwig 111-165-144, 1:300) were employed. Cells were then visualized using a Leica confocal microscope (TCS SP2).

**Preparation of Collagen Matrix**

Soluble calf skin collagen was purchased from MP Biomedicals. Collagen was dissolved in acetic acid 0.2 M; collagen solution was neutralized by titrating with NaOH 2M in the presence of HEPES buffer and then water was added to reach the desired collagen concentration. The collagen concentration used in compaction experiments was 1 g/l. Gels were obtained by polymerization for 1.5 hrs at 37 °C. After polymerization, the gel was repeatedly washed with either L-15 or DMEM in order to bring the ionic composition, pH and osmolarity of the gel to that of the culture medium.

**Microscopic Compaction of Collagen Matrix**

Tissue remodeling by a reorganization of the collagen architecture was studied using smooth muscle cells that were sparsely seeded on a collagen gel. Experiments were performed in plastic cell culture wells (surface 3.8 cm²) coated with a 300 μm thick collagen layer (1 g/l) and filled with L-15 culture medium. The contribution of TG2 to microscopic compaction was established using cells from either WT or TG2 knock-out (KO) cultures. Cells were resuspended in L-15 + HI-FCS (10%) to inactivate trypsin and washed with L-15 after adhesion to the collagen substrate to achieve a final suspension with less than 0.5 % (v/v) HI-FCS. We aimed to obtain one cell per microscopic field; this resulted in an optimal seeding density of ~ 2 cells / mm². Seeded cells were allowed 20 minutes to attach to the collagen matrix. After seeding, wells were incubated at 37 °C in a transparent incubator on the microscope stage. Cells were allowed to compact the gel for 24 hours and then cytochalasin D (final concentration 10^{-6} M) was added to disrupt cytoskeleton and assess reversibility of compaction.

The setup used for microscopic imaging of individual cells was described elsewhere. Time-lapsed video recordings for each position were manually screened off-line for image quality and cell activity. Series of images (stacks) having good optical quality and active cells were analyzed using a nested cross-
correlation algorithm as previously described\(^{13}\). The cross correlation relied on the ability to recognize the pattern of individual spots in the collagen, which had a fibrous texture, in successive images. From the derived displacement fields we calculated the area change (\(\Delta A\)) of circles with various initial radii (\(r = 96, 131, 166, 201, 236, 271\) and \(306 \, \mu m\)), centered around the center of the cell. Average radial matrix displacement (\(\Delta r\)) corresponding to each initial radius (\(r, t = 0\)) was calculated using the formula:

\[
\Delta r = r, t = 0 - \frac{\pi \cdot (r, t = 0)^2 - \Delta A}{\pi}
\]

Strain within the gel was calculated based on the initial radii and maximum displacements corresponding to each radius using the following formula (e.g. for strain between 100 and 200 micrometer from the cell center, as visualized in Figure 4.2):

\[
\varepsilon = \frac{\Delta r_{200} - \Delta r_{100}}{r_{200, t = 0} - r_{100, t = 0}}
\]

**Macroscopic Compaction of Collagen Matrix**

Tissue remodeling during organization of the cellular and intercellular architecture and proliferation towards a monolayer was tested using a circular collagen matrix, allowing subsequent mechanical testing. An in-house fabricated Teflon mold (15 mm diameter) was placed in the center of a 6-well plate with ultra-low binding coating. Then 2 ml of collagen solution was added around the mold, the gel was allowed to polymerize and repeatedly washed with L-15. Immediately after seeding MOVAS cells, the gel was detached from the edges of the well and the central mold was removed. The contribution of TG2 cross-linking to macroscopic compaction was assessed using the active site inhibitor L682777\(^{16-18}\) (Zedira, T101: 5 \(\mu mol/L\), also known as R283).

Macroscopic compaction was quantified based on reduction of the outer perimeter of the gel, which was imaged after 6 and 24 hrs. The underlying cellular processes during global compaction were studied using time-lapsed microscopy\(^{13}\). In order to limit gel movement during automated horizontal movement between different locations in the matrix, the amount of medium was decreased as compared to the macroscopic observations. We defined three distinct phases of 100-140 minutes during which images were taken at a 3-minute interval. In the first period, starting about 2.5 hrs after cell seeding, SMCs developed a preferential alignment. After 8 hrs, the initial formation of cellular networks was investigated. One day after cell seeding, cell-matrix interactions of a confluent layer of SMCs were studied. During the latter 2 phases, compaction was calculated locally in both the radial and circumferential direction. First, an independent observer picked cell pairs that were either aligned parallel or head-to-tail in the first image of a time series. Another observer measured the distance between paired cell centroids at
the beginning and end of each time series. Local compaction was then calculated as the ratio between the change in distance divided by the initial distance.

About 72 hrs after cell seeding, each gel was mechanically tested in a wire myograph (Danish Myo Technology). For this, the gel was placed around two clamp screws such that force development was just above threshold. Then, the gel was strained about 35% over a period of 7 minutes and several parameters were calculated to characterize the visco-elastic properties of the gels. These were the slope of the linear part of the stress-strain curve during the gradual stretch and the stretch-relaxation behavior, quantified as the percentage of the peak force that remained in steady state (taken at 15 minutes) and the time required to drop to 50% of this steady state force.

Subcellular localization of TG2 in the collagen matrix used for macroscopic compaction was performed using MOVAS cells transfected with TG2/eGFP. About 24 hrs after seeding the cells on the collagen gel, samples were formalin-fixated and viewed with confocal microscopy as described above.

**Intracellular Localization of TG2 in Migrating Cells**

MOVAS cells were seeded onto chambered coverglass (Lab-Tek II, Nunc 155379) coated with fibronectin (75 μl of 10 μg/ml) and transfected with TG2/eGFP 24 hrs later. After another 24 hrs, they were imaged with a Leica inverted fluorescence microscope using a 63x oil objective. For each cell, typically 1 auto-focused phase contrast and 9-11 fluorescence images at different cell heights (step size: 400 nm) were captured. Vertical stacks were processed with Huygens Pro deconvolution software (Scientific Volume Imaging, The Netherlands) to enhance image contrast.

**Statistical analyses**

Results are given as average +/- SEM. One way ANOVA with Bonferroni correction was used to assess statistical significance, unless otherwise specified. Significance level was set at p = 0.05. Statistical analysis was performed using SPSS 16.0 for Windows.

**Results**

**Cultured smooth muscle cells exhibit a synthetic phenotype**

We characterized the phenotype of the MOVAS, WT and KO SMCs used for seeding onto the collagen matrices (Figure 4.1). Explant SMCs were typically more spread compared to MOVAS cells. All cells displayed α-actin immunostaining with evenly distributed intensity along the length of the fibers that is characteristic for synthetic SMCs. All three cell types stained positively for calponin, which was distributed throughout the cytosol with an elevated concentration near the cell periphery. Myosin heavy chain staining was very weak and lacked fiber organization. If present, this signal appeared randomly distributed with a slight
preference for cellular protrusions. Smoothelin immunostaining was weak in all cell types as well, and was mainly observed in the more elongated cells in a punctate pattern. Together, all these markers indicate that the vast majority of SMCs used in these experiments exhibited a synthetic phenotype\textsuperscript{21-23}.

Figure 4.1: Phenotypical characterization of smooth muscle cells (see next page).
Figure 4.1: Phenotypical characterization of smooth muscle cells. The MOVAS cell line and explant cells obtained from mesenteric small arteries (WT and KO) were stained for α-actin, myosin heavy chain, calponin and smoothelin, followed by cy-3 secondary antibody (red); nuclei are shown in blue.

Figure 4.2: Typical compaction pattern by a single cell. (A) Images acquired after reaching 10% and 90% of the total compaction. Cell edge is depicted in blue, displacement of matrix situated initially on a circle at 300 µm from the center of the cell is followed in time (green line). (B) Average displacement in time of a point situated initially at 300 µm from the center of the cell. Cellular contractile forces are lost upon administration of cytochalasin D, as indicated by ‘cyt D’. (C) Mean displacement of matrix originally situated at three distances: 100, 200 and 300 µm (yellow circles in A). Strain is given by a gray scale; white depicts expansion and black depicts compression.

Figure 4.3: Matrix compaction by individual SMCs from wild-type (WT) and TG2 knock-out (KO) mesenteric arteries. (A) The maximal displacement was not statistically different at any measured distance for WT or KO cells. (B) Both WT and KO SMCs compacted the matrix at a distance up to 200 µm; between 200 and 300 µm, the matrix expanded locally, while this area moved to the cell center as well. The horizontal line indicates a level of zero strain. (C) The time required to achieve the initial 10% of the maximal compaction was not statistically different between WT and KO cells, but at distances >200 µm the compaction was significantly faster for wild-type SMCs. (D) When cytochalasin D was added after 24 hrs of compaction, the matrix expanded locally due to the loss of cellular contractile forces. The amount of relaxation was significantly lower in WT cells.

# WT vs. KO: P < 0.05 (panel B)
* different from zero: P < 0.05 (panels C & D)

Figure 4.4: Experimental overview of macroscopic compaction of a collagen matrix. (A) A ring-shaped collagen gel was casted using a Teflon mold in an ultra-low binding multiwell plate; the pictures display the gel at the start and after 6 hrs of cell-induced compaction. (B, C) Macroscopic compaction, quantified as the inner or outer perimeter of the gel, was not dependent on the TG2 active site inhibitor L682777: n = 9 for both groups.
Figure 4.2: Typical compaction pattern by a single cell.
Figure 4.3: Matrix compaction by individual WT and TG2 KO SMCs.
Figure 4.4: Experimental overview of macroscopic compaction of a collagen matrix.
A single SMC compacts large areas of collagen matrix: role of TG2

Figure 4.2A shows a typical example of matrix compaction by a single SMC, imaged at the moment of 10% and 90% of the maximal compaction. The nested cross-correlation algorithm could be used reliably to calculate the displacement field at a distance larger than 100 μm from the cell center. Matrix displacement was not analyzed closer to the cell due to artifacts resulting from cell locomotion. The largest virtual circle that was tracked (300 μm) displayed a strong, continuous inward displacement over a period of about 8 hrs, after a short initial lag phase. Cytochalasin D, administered after 24 hrs, partly reversed gel compaction (Figure 4.2B), indicating a cytoskeletal contribution to compaction. The matrix was inwardly displaced up to the tested distance of 300 μm. Between 100 and 200 μm the gel in this example was locally compacted. However, between 200 and 300 μm the matrix expanded locally, as indicated by the positive strain values in Figure 4.2C.

The degree and speed of matrix remodeling was studied for 36 wild-type versus 52 TG2 knock-out SMCs, divided over 5 experiments. These data are summarized in Figure 4.3. Significant matrix displacement occurred for all tested ranges. We could not track displacement at distances beyond 300 micron from the cell, but an extrapolation of the data in Figure 4.3A indicates that displacement is likely to occur over much larger ranges. A significant inward displacement occurred at all tested radii in both WT and KO mice. Maximum radial displacement for WT and KO cells was 30.1 ± 4.2 versus 34.3 ± 2.6 μm occurring at a distance of 200 μm from the cell center (Figure 4.3A). Gel displacement was not statistically different for WT and KO cells at any distance. Compaction was maximal closest to the cell for both WT and KO cells, and was significant up to 114 resp. 149 μm for WT and KO cells (Figure 4.3B). At 184 μm, the gel was merely displaced towards the cell, without a change in radial strain. At distances larger than 254 μm, inward matrix transport coincided with significant expansion (Figure 4.3B). WT and KO cells started their compaction around the same time, as indicated by the almost identical period required to reach 10% of the maximal compaction. WT cells reached 90% of their maximal compaction about 2-3 hrs faster than KO cells, which was significant at a distance ≥ 236 μm. However, while KO cells compact longer, this is accompanied by a slightly larger maximal displacement (Figure 4.3C). Reversibility of compaction upon administration of cytochalasin D was significant for both WT and KO cells. This reversibility was significantly larger in KO cells for all radii except the smallest one and became larger at increasing distances from the cell center for both WT and KO cells (Figure 4.3D).
Collagen gel compaction by networks of SMCs occurs perpendicular to the SMC long axis

Figure 4.4A illustrates the compaction of a ring-shaped collagen gel by smooth muscle cells. Within 6 hrs after cell seeding, the outer layer of the gel curled up and folded over the medial layer of the gel. By this time, the outer perimeter had shrunk to 58.5 ± 3.4% of its initial perimeter. Reduction of the inner perimeter was significantly slower (P < 0.01), to 86.5 ± 3.1% after 6 hrs. In the next 18 hrs, the outer perimeter decreased to 28.5 ± 1.3%, while the inner perimeter shrunk to 40.3 ± 3.3% of the initial size (inner vs. outer perimeter: P < 0.01). After both 6 and 24 hrs, the decrease in inner and outer gel perimeter was unaffected by the TG2 cross-linking inhibitor (Figure 4.4B-C, P = N.S.). After 24 hrs, macroscopic compaction had reached steady state. Mechanical tests in a wire myograph after 72 hrs of compaction revealed no effect of blocking TG2. Thus, the slope during the straining protocol, the force drop during stress relaxation and the time required for this force drop were not affected by L682777 (Figure 4.5, P = N.S. for all parameters).

A

B

C

|          | Slope (μN/s) | Fend/Fmax (%) | T50 (s) |
|----------|--------------|---------------|---------|
| control  | 9.4 ± 2.4    | 42.4 ± 2.6    | 70.1 ± 8.2 |
| L682777  | 10.9 ± 1.4   | 40.9 ± 1.6    | 70.9 ± 3.8 |
| N.S.     | N.S.         | N.S.          |         |

Figure 4.5: Mechanical characterization of collagen gels (see next page).
Figure 4.6 shows the cellular orientation during three key phases of gel compaction. These image series could only be obtained by lowering the culture medium level. This reduced the compaction rate as compared to the macroscopic data in Figure 4.4. In addition, the gels maintained a disc shape rather than transforming towards a doughnut shape. About 2.5 hrs after seeding, cells were still round and started to align in arrays. One hundred minutes later, these cells had partially elongated and their alignment approached the direction of the gel boundary (Figure 4.6A, near inner perimeter). After 8 hrs, cell density was still low and alignment had proceeded parallel to the gel boundary. About 140 minutes later, compaction had occurred most notably in the radial direction of the gel, perpendicular to the cells (Figure 4.6B, near inner perimeter). After 26 hrs, the gel was overgrown by a confluent layer of SMCs, but still exhibited macroscopic compaction over a period of 100 minutes (Figure 4.6C, near outer perimeter). At low cell density, such as observed in Figure 4.5B, compaction was 28 ± 7% and 8 ± 5% in the radial respectively circumferential direction. At high cell density, this amounted to 8 ± 1% respectively 1 ± 1%. Thus, during the continuous process of cell and matrix organization, local compaction was significantly larger in the radial direction as compared to circumferentially (Figure 4.6D). In effect, circumferential gel compaction was virtually absent after 24 hrs.
Figure 4.6: Typical cellular orientation during different phases of matrix remodeling as observed in the ring-shaped collagen gel model (see next page).
TG2 distribution during cell locomotion and matrix compaction

In order to further assess the role of TG2 in cellular locomotion, elongation and matrix remodeling we imaged sub-cellular distribution of TG2/eGFP. Since compaction at low cell density is believed to result from repeated movement of cell protrusions, we studied MOVAS cells during random migration over fibronectin. In initial static experiments using SMCs seeded on a fibronectin coating, TG2/eGFP was distributed throughout the cytosol with an elevated peri-nuclear density. TG2/eGFP was absent in the nucleus and in non-identified organelles. During random migration, TG2 translocated to the leading cell edge, where it appeared in the tips of the cellular protrusions (indicated by arrows in Figure 4.7). This redistribution of TG2 was a dynamic process, taking place at a time-scale of several minutes.

The localization of TG2 in a compaction model of high cell density was studied using a fixated collagen gel preparation. Figure 4.8 shows the sub-cellular distribution of fluorescent TG2 in SMCs after 24 hrs of gel compaction. Similar to cells grown on fibronectin, all cells demonstrated exclusion of the fluorescence from the nucleus. Close to the outer perimeter, where macroscopic compaction peaked, TG2/eGFP remained distributed randomly throughout the cytosol.
Figure 4.7: Localization of TG2 in migrating smooth muscle cells. Shown is a typical example of a MOVAS cell transfected with TG2/eGFP. In addition to a bright perinuclear eGFP signal, TG2/eGFP along the leading edge (indicated by arrows) in migrating over a fibronectin coating. The cell moves from right to left and displays TG2/eGFP along the leading edge (indicated by arrows) in addition to a bright perinuclear eGFP signal.
This work was initiated by the current lack of understanding of cellular and matrix rearrangement in eutrophic inward remodeling of small arteries, which occurs in hypertension, under low flow, or in the continuous presence of a range of vasoconstrictors. We envisioned that a separation of the relevant processes (e.g. contraction, locomotion, compaction) in space and time would allow a more detailed observation and generate hypotheses for further testing at the intact vessel level. We appreciate that the current matrix experiments do not resemble vascular remodeling in several respects, but we do identify some key processes of possible relevance. These include the very large range of action of remodeling by single cells, the highly anisotropic nature of matrix remodeling, and the involvement of Transglutaminases. In addition, the current work is of relevance for vascular tissue engineering.

Figure 4.8: Localization of fluorescent TG2 in collagen gel after about 24 hrs of macroscopic compaction. The outer rim of the gel had curled up and folded over the central gel area (indicated by a yellow dot in the side view and a yellow dotted line in the top view). During sample preparation for confocal microscopy, these layers are compressed, but could still be discriminated by refocusing. At this outer layer, cells are elongated along the direction of the gel boundary; this was not associated with preferential sub-cellular distribution of TG2/eGFP. Scalebar = 100 μm
Matrix remodeling by an individual SMC
The concentric matrix remodeling by single cells disclose two simultaneously occurring remodeling processes: matrix displacement and radial compaction at a distance from the cells. Matrix displacement by single cells was a continuous process, occurring at a relatively constant speed after an initial lag phase of around 2-3 hours, resulting in concentric inward remodeling of the matrix by ~25 μm. This continuous and large deformation cannot be established by simple contraction of an attached cell, but rather reflects continuous reorganization of matrix fibrils in the area immediately surrounding the cell by dynamic movements of cell protrusions. Strikingly, displacement extended over at least 300 μm and probably much larger distances. It leaves little doubt that this distance is dictated by the density and length distribution of the collagen fibers and the frictional forces associated with their movement. Displacement leveled off after ~10 hours. This could be due to the counterbalancing of cell-derived traction by the elasticity of the matrix, possibly in combination with inhibitory effects of the increased local collagen density on cellular dynamics.

If only the dynamic cell protrusions were involved in matrix displacement, one would expect only positive radial strain over the full matrix, such that displacement becomes gradually less towards the gel periphery. However, strain was clearly negative up to 200 μm distance from the cell center (Figure 4.3B), well beyond the reach of the cellular protrusions. This distant compaction can therefore not be effectuated by direct cell-matrix interaction or membrane-bound enzymes, pointing at a role for secreted enzymes. We considered the role of TG2 by comparing distant compaction between WT and KO cells. Those results provide a complex picture. On the one hand, distant compaction was still present in the KO cells. On the other hand, cytoskeletal disruption revealed a clear difference in elastic recoil at distant locations but not in the area surrounding the cell. This would be in accordance with less cross-linking activity at distant sites for the KO cells. Clearly, understanding the role of TG2 and other cross-linking enzymes, including lysyl oxidases, needs further research.

Recoil upon cytoskeletal disruption was limited, even in the KO cells. It has been shown that collagen gels can be permanently compacted in the absence of cells in a purely mechanical fashion by means of externally applied forces. If this deformation is large enough, it will induce structural anisotropy by aligning collagen fibers, which proved to be irreversible as a result of non-covalent chemical interactions. Likewise, the low reversibility in the current experiments might relate to such permanent compaction by the cells, independent of covalent cross-linking.
Matrix remodeling by clusters and monolayers of SMCs

The matrix remodeling by single cells predicts a potential for coordinated remodeling by clusters of SMCs. The collagen ring experiments indeed revealed such coordinated remodeling at various stages between seeding and final remodeling by monolayers. Shortly after seeding, cells aligned in arrays, without making physical contact (Figure 4.6A-B). These arrays were aligned circumferentially and were most notable near the gel perimeter, which can be regarded as a free surface. It was previously shown that parallel to a free surface, cells interact elastically to form strings. These aligned cells started matrix remodeling as seen for the single cells, but also became gradually elongated. This cell alignment and polarization could be due either to preferential distribution along collagen fibers or result from sensing the traction stresses exerted by neighboring cells. Of relevance for both cases, cells were previously shown to strengthen focal adhesions and cytoskeleton organization in the direction of the largest effective stiffness. Cell alignment is believed to be an important step, since gel compaction was previously shown to be a cooperative effect resulting from mechanical interaction between cells. Indeed, local and global compaction are known to depend non-linearly on cell number. This synergistic effect may arise from the fact that contractile activity along a particular direction renders the matrix stiffer, thus prompting neighboring cells to further pull along it.

An unexpected finding in the current study was that cells, once polarized to a more elongated shape, displaced their surrounding matrix predominantly perpendicular to the cell axis, coinciding with radial compaction of the matrix (Figure 4.6D). This anisotropy became increasingly dominant during the process, such that in the final monolayer of elongated cells, transversal matrix compaction was absent while radial compaction continued. This could be a property of the matrix as well as the cells. Concerning the role of the matrix, isotropic forces exerted by individual cells close to a boundary would produce a compaction that is highest in the direction perpendicular to the free surface, where stiffness is low. Indeed, radial compaction was high both at the inner and outer perimeter of the collagen gel. Thus, compaction seemed to propagate from the edges of the matrix into the bulk, as previously reported. The macroscopic consequence of this anisotropic microscopic behavior was that the outer perimeter of the collagen disk compacted much faster than the inner rim (Figure 4.4). Concerning the role of the cells, the full lack of circumferential compaction upon reaching a monolayer remains surprising. While circumferential matrix resistance may be substantial, many cells can combine forces in a tug of war along this direction. Apparently, the force per cell exerted on the collagen is indeed low in this direction. Simultaneously, radial compaction continued even after a monolayer had developed. We could not reliably image the 3D structure in these areas, but
possibly part of this compaction reflects transition of the cells towards a multi-
layered 3D structure. Alternatively, apoptosis may have occurred.

**Role of Transglutaminase 2**

While TG2 is known to be involved in vascular inward remodeling and stiffening\(^\text{14}\), its mechanism of action remains ill-defined. We previously showed that compaction of a collagen matrix by coronary artery SMCs was enhanced by stimulation with exogenous TG2. This could be blocked by the TG2 competitive substrate cadaverine\(^\text{4}\), indicating a role for transamidation. Besides cross-linking of matrix elements, fibronectin binding, cytoskeletal effects and signaling are among the mechanisms that may be involved, affecting matrix stability, cell locomotion, and cell contraction\(^\text{33}\). In low-to-medium cell density gels, compaction results from fibrillar reorganizations\(^\text{5;7;10}\). In turn, fibril movement is correlated with cell locomotion\(^\text{34}\), and cell locomotion is mediated by TG2\(^\text{16}\). TG2 is known to mediate cell adhesion and migration independent of its cross-linking activity, as shown by the equal capability in stimulating cell migration for wild-type as compared to cross-linking mutant TG2\(^\text{16;35-37}\). The current TG2/eGFP experiments indeed demonstrate that TG2 translocates to the leading edge of migrating SMCs (Figure 4.7). However, WT and KO cells displaced matrix with roughly comparable dynamics, despite the reported decrease in migratory behavior for myofibroblasts from this TG2 knock-out mouse\(^\text{38}\). The KO mice have a mild phenotype, and we previously demonstrated that during inward vascular remodeling under low flow, compensation occurs by other Transglutaminases\(^\text{39}\). Whether similar compensation has occurred in the current experiments, awaits to be determined.

The role of matrix cross-linking activity by Transglutaminases was investigated using an analysis of distant compaction in WT and KO cells, as well as by active site inhibition in the multi-cellular models. As indicated above, distant compaction remained but was more reversible in the KO cells. The active site inhibitor had no effect on macroscopic compaction in the multicellular experiments (Figure 4.4). In particular, mechanical properties were identical, indicating no difference in cross-linking during the compaction period. Together, the current results do not clearly identify possible roles for TG2 in collagen matrix remodeling.

**Implications for small artery inward remodeling**

There are obviously clear differences between the current experiments and the inward remodeling of blood vessels. The currently used SMCs had a distinct synthetic and proliferative phenotype at the moment of seeding onto the matrices. We did not monitor phenotypic changes during matrix remodeling, but the continuous proliferation towards a monolayer indicates that the cells remained synthetic during most of the remodeling process. SMCs in blood vessels are in a differentiated, contractile phenotype\(^\text{40}\). However, when matrix reorganization and synthesis is required, SMCs undergo a transient phenotypic modulation to a
synthetic phenotype\textsuperscript{21,23}. This dedifferentiation was also observed in mesenteric arteries while they underwent inward remodeling\textsuperscript{41,42}, although the capacity for vasoconstriction seems largely unaffected here\textsuperscript{43}. The current matrix stiffness was much lower than that of blood vessels, and the current model was essentially 2-dimensional. Despite these differences, some possible implications emerge that may warrant future studies.

First, the anisotropic compaction would allow SMCs to organize into dense circular bundles with very little collagen between the SMCs, and maintain this organization with a relatively low tendency for inward remodeling. Compacting forces along the vessel length, also acting perpendicular to the SMCs, would provide a mechanism for the well-known axial stretched state of \textit{in vivo} blood vessels.

Second, the current models were mechanically unloaded during matrix remodeling. One might therefore question the relevance for vascular remodeling under physiological pressure. Yet, vascular matrix and SMCs are generally considered to be organized in a parallel fashion, such that total wall tension is the sum of an active and passive component. During vasoconstriction, SMCs carry the tension and effectively unload the matrix. Under this condition, dedifferentiation of only a few SMCs to a synthetic phenotype would allow inward remodeling, based on the long ranging effect of individual cells. Moreover, shape of such cells were shown to deviate from the spindle shape at low pitch of contractile SMCs in small vessels\textsuperscript{44}. Considering that matrix remodeling occurs perpendicular to the cell axis, this would introduce a radial component, acting on a relatively unloaded matrix. This is speculative, but it would be an explanation for the relation seen between vasoconstriction and inward remodeling.

In summary, we studied collagen reorganization by vascular SMCs. Remodeling by individual SMCs was isotropic and characterized by both local displacement and distant compaction. In clusters and monolayers, cells elongated and compacted ring-shaped collagen scaffolds in primarily the radial direction. TG2 did not affect the amount and rate of matrix displacement, but limited the reversibility of matrix compaction. Moreover, TG2-induced cross-linking was too low to affect the overall mechanical properties of these collagen matrices. The current approach identifies possible mechanisms for vascular organization and remodeling.
Acknowledgements

The help from Judith de Vos with immunofluorescent labeling was greatly appreciated. Ron Hoebe and Jan Stap assisted with time-lapsed fluorescence microscopy and subsequent image analysis.

Sources of Funding

This work is supported by the Netherlands Heart Foundation (grant NHS.2005.B080 to Jeroen van den Akker).
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Transglutaminase 2 is secreted from smooth muscle cells by transamidation-dependent microparticle formation

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Submitted for publication 2011
Abstract

Transglutaminase 2 (TG2) is a pleiotropic enzyme involved in both intra- and extracellular processes. In the extracellular matrix, TG2 stabilizes the matrix by both covalent cross-linking and disulfide isomerase activity. These functions become especially apparent during matrix remodeling as seen in wound healing, tumor development and vascular remodeling. However, TG2 lacks the signal sequence for a classical secretory mechanism, and the cellular mechanism of TG2 secretion is currently unknown. We developed a green fluorescent TG2 fusion protein to study the hypothesis that TG2 is secreted via microparticles. Characterization of TG2/eGFP, using HEK/293T cells with a low endogenous TG2 expression, showed that cross-linking activity and fibronectin binding were unaffected. Transfection of TG2/eGFP into smooth muscle cells resulted in the formation of microparticles (MPs) enriched in TG2, as detected both by immunofluorescent microscopy and flow cytometry. The fraction of TG2-positive MPs was significantly lower for cross-linking deficient mutants of TG2, implicating a functional role for TG2 in the formation of MPs. In conclusion, TG2 is secreted from the cell via microparticles through a process regulated by TG2 cross-linking.
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2

Introduction

Transglutaminases (TGs) constitute a family of enzymes involved in post-translational modification of proteins through transamidation\(^1\);\(^2\). In addition to these covalent structural changes, the different subtypes of TGs all exhibit very specific cellular functions\(^3\);\(^4\), which depend strongly on subcellular localization\(^5\). Intracellularly, TG2 plays a role in signaling by acting as a G-protein\(^6\) and in cytoskeleton organization, partly regulated by interaction with membrane-bound heparan sulfate proteoglycans\(^7\);\(^9\). Extracellularly, TG2 mediates cell adhesion in cooperation with fibronectin and integrins\(^10\);\(^12\) and supports the polymerization of fibronectin and collagen\(^13\);\(^15\). These TG2 effects have been linked to a variety of (patho)physiological conditions\(^3\);\(^5\);\(^6\). As an example, we previously showed that TG2 plays a key role in vascular inward remodeling, a process occurring in hypertension and hypoperfusion\(^16\);\(^18\).

In many cell types, the main pool of TG2 is cytosolic under physiological conditions\(^3\);\(^4\). Cross-linking of matrix proteins, as observed in tissue remodeling, requires transportation of TG2 to the cell membrane and subsequently secretion into the extracellular space. However, TG2 does not possess a signal sequence to the ER/Golgi, and is therefore believed to follow a non-classical secretory pathway\(^2\);\(^15\);\(^19\);\(^21\). Several prerequisites for cellular secretion have been established. Thus, TG2 translocation requires the active site cysteine, an intact N-terminal \(\beta\)-sandwich domain and a non-proline cis-peptide bond at tyrosine-274, which is near the active site\(^4\);\(^8\);\(^20\);\(^22\). Sequestration of TG2 into the pericellular matrix is mediated by fibronectin (FN) binding\(^21\);\(^23\). Indeed, after truncation of the FN-binding site at the N-terminus, TG2 is absent from the cell surface\(^21\) or greatly lowered in cell-conditioned media\(^24\). In addition, immunostainings reveal that membrane trafficking is often observed at sites where \(\beta_1\) integrins or heparan sulfate proteoglycans are present\(^9\);\(^23\). Possibly, post-translational modification through N-acetylation serves as a secretion signal\(^25\). However, despite this information, the exact process of TG2 release remains unclear.

A possible route for externalization of TG2 is the formation of microparticles (MP). MPs are released membrane vesicles that are actively involved in normal physiology and numerous diseases\(^26\). MPs can contain cytoplasmic components and surface proteins from the cell they originate from. Carrying these components, MPs can affect the local environment such as the atherosclerotic plaque, or act as a long-distance messenger when secreted into the bloodstream\(^27\);\(^28\). MPs are released during specific cell activation such as seen with thrombin for platelets, or after cell apoptosis. Calcium plays a crucial role in release, initiating the rapid loss of membrane phospholipids asymmetry and dissociation of actin from membrane glycoproteins\(^26\);\(^28\). MPs originating from platelets, erythrocytes, leukocytes and endothelial cells have been related to
vascular function, remodeling, angiogenesis, haemostasis, thrombosis and cardiovascular diseases such as atherosclerosis and collagen vascular disorders. More recently, smooth muscle cells were identified as a source of MPs, which seem to be active mainly within the vascular wall.

In this study, we investigated the hypothesis that cellular secretion of TG2 occurs via microparticles. In order to track TG2 in MPs, we developed fluorescently tagged TG2 proteins with intact cross-linking activity and fibronectin binding. Secretion of MPs enriched in TG2 was studied in smooth muscle cells (SMCs), using time-lapsed fluorescence microscopy and flow cytometry. We found not only that TG2 is indeed secreted from SMCs via MPs, but also that release of MPs depends on cross-linking activity of TG2.

**Materials & Methods**

**Expression Vectors**

The IMAGE clone 3256943 (GenBank: BC016492) for mouse TG2 was obtained from imaGenes (Berlin, Germany). Using standard molecular biology techniques, nucleotides 67-2153, encompassing the complete TG2 open reading frame, were cloned into pCMV-SPORT6 to obtain a non-tagged TG2 expression vector. Alternatively, nucleotides 67-2150 were cloned into pEGFP-N1 (Clontech 632469) or pmCherry-N1 (Clontech 632523), generating vectors that express TG2 with a C-terminal fluorescent tag.

Two different point mutations were used to inhibit Transglutaminase activity. Mutating the active site cysteine (C277S) was already frequently employed to abolish TG2 activity. This mutation is accompanied by greatly impaired GTP binding, thereby changing the intracellular conformation of TG2. Mutation of the tryptophan residue bridging the hydrophobic tunnel next to the active site, W241A, was reported to completely abolish TG activity as well. With this mutation, GTP binding is retained, suggesting that TG2 conformation is only adapted very locally near the active site. Transamidation defective mutants of TG2/eGFP were created with the QuikChange XL-Site directed mutagenesis kit (Agilent Technologies, 200516) according to the manufacturer’s protocol. Oligonucleotide primers used for creating mutant TG2-C277S/eGFP were 5’-gaagtacggcgagctgctgggtgtttgcag-’3 and 5’-ctgcaaacacccagactgccggtacttc-’3. For mutant TG2-W241A/eGFP, 5’-gtgcttctgggcggcggcaacaacatggagca-’3 and 5’-ccatagttgtgctcggcggcggcagaagcac-’3, were employed. All cDNA sequences were verified by DNA sequencing.

In several experiments, pEGFP-N1 and pmCherry-N1 were used as transfection controls.
Cell Culture and Transfection

HEK/293T cells (ATCC CRL-11268) and smooth muscle cells (MOVAS, ATCC CRL-2797) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum (Gibco) and a mix of antibiotic-antimycotic (Gibco). One day before transfection, cells were seeded at about 60% confluency in 12-well plates. HEK/293T cells were transfected using Effectene (Qiagen), according to the manufacturer’s instructions. The ratio of DNA to Effectene Reagent used was 1 μg to 20 μl. Smooth muscle cells were transfected using Lipofectamine LTX (Invitrogen, 15338), according to the manufacturer’s protocol. Per well, 1 μg DNA, 2.5 μl Lipofectamine LTX and 1.0 μl PLUS reagent were used.

Cells were lysed in 0.01 M TRIS, 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100 and 1 tablet of protease inhibitors (Roche 11873580001), dissolved in a total volume of 50 ml. After a sonication period of 10 seconds, lysis continued for 10 min on ice and samples were centrifuged 15 min at 18,890 g at 4°C. The protein concentration in the supernatant was then determined using the Bradford assay, and samples were stored at -80°C until further usage.

Western Blot

Protein samples (40-50 μg) were boiled for 5 minutes, and separated on a 7% SDS-polyacrylamide gel. The proteins were subsequently blotted on an Immobilon-P Transfer membrane (Milipore) in a tank system. After o/n blocking with a 1:1 solution Odyssey Blocking Buffer (LI-COR, 927-40000) and PBS, blots were probed with rabbit polyclonal TG2 Ab-4 (Neomarkers RB-060-P) at 1:1000. Donkey anti-rabbit InfraRed IRDye 680 was used as secondary antibody at 1:15.000. As a loading control, β-actin was detected using mouse monoclonal antibody A1978 (Sigma) at a 1:1000 dilution for and IRDye 800 CW donkey anti mouse (Westburg) at 1:15.000. Finally, antibodies against eGFP (ClonTech 632375, Living Colors GFP monoclonal) and mCherry (ClonTech 632393, Living Colors DsRed monoclonal) were used to confirm the appropriate size of fluorescently-tagged TG2. All antibodies were incubated for 1 hr at room temperature. Blot signal was measured using an Odyssey infrared detector, and protein molecular weight was estimated using Odyssey Prestained Molecular Weight marker (LI-COR, 928-40000).

TG2 Activity Assay

TG2 activity was measured using a colorimetric microassay (Covalab). Samples, together with biotin-cadaverine as amine donor, were added to a 96-well plate to which an amine acceptor was covalently coupled. TG2, which is activated with calcium and dithiothreitol, then cross-links donor and acceptor. In the second step, biotin is linked to streptavidin-labelled peroxidase. In turn, peroxidase activity is revealed using H$_2$O$_2$ and tetramethyl benzidine. Finally, absorbance is read at 450 nm. The relative activity of TG2 in cellular lysates was compared to absorbance values measured for different concentrations of TG2 isolated from guinea pig liver (Sigma T5398).
Immunostaining of TG2 and Fibronectin

For the immune-fluorescent detection of TG2 and fibronectin, cells were trypsinized and reseeded in microscopic culture chambers (BD Falcon 354102, untreated glass). After 24 hrs, cells were washed with warm PBS and fixed with formalin (20 min on ice). Cells were permeabilized with 0.05% Triton X-100 and blocked with 3% BSA/5% goat serum. Samples were then incubated for 1 hr at room temperature with either a rabbit polyclonal TG2 antibody Ab-4 (Neomarkers RB-060-P, 1:10) or with rabbit polyclonal fibronectin Ab-23750 (Abcam, 1:400). Subsequently, anti-rabbit Cy3 (Brunschwig 111-165-144, 1:200 respectively 1:300) was used as secondary antibody, and slides were mounted in Vectashield/DAPI (Vector Laboratories H-1500).

For both the TG2 and fibronectin immunostaining, eGFP-positive cells were randomly selected before the red Cy3 signal was visualized using a Leica confocal microscope (TCS SP2). These two fluorescence images were obtained in sequential mode, in which the blue excitation for eGFP was shut off during recording of the red Cy3 image. This was done in order to prevent any possible contribution of eGFP to the red signal. We established that such cross-talk was indeed absent for the used confocal settings. In order to quantify the degree of colocalization between two 12-bit images, the Pearson correlation coefficient was calculated for each pair of images using Matlab software, excluding all background pixels. Subsequently, the correlation coefficients were averaged over a number of cells.

Cellular Localization of TG2

Both intra- and extracellular TG2 localization were studied in detail using cells incubated either on glass, fibronectin or collagen type I substrates. A fibronectin coating was made by 1-hr incubation at 37°C of 75 µl of 10 µg/ml fibronectin solution per compartment of the microscopic culture chamber. Collagen type I (MP Biomedicals 160084, bovine skin) was dissolved in acetic acid at 4°C. Then, the pH was elevated with a mixture of 1M HEPES-NaOH and 2M NaOH, 120 µl of this collagen solution (1 mg/ml) was poured into a microscopic chamber and allowed to polymerize for 1 hr at 37°C. Cells were transfected with TG2/eGFP or control eGFP and reseeded into microscopic chamber slides.

For confocal microscopy, HEK/293T cells were incubated for 24 hrs and formaline fixed as described above. For quantification of extracellular TG2, eGFP-positive cells were randomly selected. Images of these cells were taken at a 40x-magnification and all eGFP-positive extracellular spots were counted in a 200-µm perimeter of the cell nucleus. Differences in the number of vesicles when seeded on glass, fibronectin and collagen type I were tested for statistical significance using a 1-way ANOVA with Bonferroni posthoc test.

Time-lapsed imaging was performed using a CO₂ and temperature controlled setup described in detail elsewhere45,46. In short, MOVAS SMCs were
seeded onto fibronectin-coated, chambered coverglass (Lab-Tek II, Nunc 155379) and imaged with a Leica inverted fluorescence microscope using a 63x oil objective. A motorized stage enabled imaging of a large set of cells, with typically 1 auto-focused phase contrast and 9-11 fluorescence images at different cell heights (step size: 400 nm). Vertical stacks were processed with Huygens Pro deconvolution software (Scientific Volume Imaging, The Netherlands) to enhance image contrast.

**Flow Cytometry**

The typical diameter of MPs is between 100 nm and 1 μm. Consequently, the size of MPs is in the order of and even below the resolution of optical microscopy. Furthermore, the fluorescence intensity of individual MPs approaches the detection limit of fluorescence confocal microscopy. Therefore, a quantitative assessment of TG2-dependent MP release and secretion of TG2 by MP was made using flow cytometry. The size of SMC-derived MPs was then estimated by comparing the light scattering intensity with beads of known size. The scattering intensity depends on size, shape and refractive index of a particle. The refractive index of vesicles is unknown, but is likely to be in the order of 1.4. Therefore, we selected silica beads with a diameter of 500, 1000 and 5000 nm (Kisker Biotech) and a refractive index of approximately 1.46 for the wavelength of 488 nm used.

About 24 hrs after the start of the transfection protocol, cells were washed and 1 ml of fresh medium was added. Secretion of microparticles was stimulated with either 10% FBS for 24 hrs or the calcium ionophore A23187 (5 μM) in serum-free medium for 2 hrs. Culture supernatant was then collected and cell debris was removed by centrifuging for 15 min at 200 g at 4°C. The top 700 μl supernatant was then snap-frozen in liquid nitrogen and stored at -80°C until further use.

At the day of measurement, samples were thawed in ice water and centrifuged for 60 min at 18,890 g at 20°C. The top 650 μl supernatant was discarded and the pellet was resuspended in 200 μl 0.22 μm filtered PBS. Samples were centrifuged again at 18,890 g for 30 min and 215 μl supernatant was discarded. Then 10.0 μl of the resuspended microparticle pellet was diluted in 80 μl filtered PBS with 2.5 mM CaCl₂. This was incubated for 15 min together with 10 μl APC-tagged Annexin-V (Caltag Laboratories, AnnexinV05, prediluted 40x) that binds to phosphatidylserine groups exposed on the surface of MPs. Specificity of Annexin V binding was checked by incubating MPs in calcium-free PBS supplemented with 0.32% citrate.

Samples were analyzed using a Calibur flow cytometer (Becton Dickinson). Forward scatter (FSC), side scatter (SSC) and fluorescence were set in a logarithmic scale. Events were first gated using the FSC and SSC signal and MPs were identified as described previously. Subsequently, the APC signal from Annexin-V, thresholded using the negative control, was used to confirm the selected MPs. Transfected TG2 was identified in these particles using the eGFP
signal, for which thresholding was set using a non-transfected control sample. Differences in MP fraction positive for the transfected protein and MP fluorescence intensities were tested with a 1-way ANOVA with Bonferroni Post Hoc test.

Results

Transamidation activity is unaffected in fluorescently tagged TG2
Upon transfection, 95-99% of the HEK/293T cell population expressed the transfected TG2. Expression of TG2 with or without fluorescent tags (eGFP or mCherry) was not significantly different, as determined from quantification of western blots (Supplemental Figure 5.1 and Supplemental Table 5.2). Non-modified TG2 showed a strong band at 80 kDa while TG2/eGFP was present at the expected size of 110 kDa. However, in addition to the 110 kDa band, about half of the TG2/mCherry fusion product was observed at 90 kDa. These data are presented and discussed in detail in the Online Supplement. Because of this partial degradation of TG2/mCherry, all subsequent imaging experiments below were carried out using TG2/eGFP.

Transglutaminase activity of cell lysates from TG2 transfectants was measured in vitro, using incorporation of biotin-cadaverine. Activity as expressed per microgram total protein was not statistically different between fluorescently-tagged and untagged TG2 (Supplemental Table 5.2). Background activity was zero in non-transfected or eGFP transfected HEK/293T cells. In lysates from HEK/293T cells transfected with TG2/eGFP containing one of the inactivating mutations C277S or W241A, transamination activity was absent as well.

Immunostaining confirms localization of TG2 for TG2/eGFP
In order to assess if the fluorescent signal indeed represented TG2, transfected HEK/293T cells were immunostained for TG2 (Figure 5.1A-D). This revealed a strong colocalization between eGFP and the Cy3-labeled TG2 antibody, which was quantified by a Pearson correlation coefficient of 0.78 ± 0.04 (P<0.01) for a representative selection of 12 cells (Figure 5.1E-F). Omission of the primary TG2 antibody resulted in virtual absence of immunostaining and lack of colocalization with eGFP (r = -0.13 ± 0.13, P = N.S., data not shown).

Immunostaining confirms interaction of TG2/eGFP with fibronectin
The fibronectin binding site of TG2 is known to mediate the secretion of TG2. The binding between TG2 and fibronectin was studied by immunostaining HEK/293T cells transfected with TG2/eGFP. Figure 5.2 shows a representative example, where 2 of the 4 cells were expressing TG2/eGFP. As can be seen, fibronectin staining was only visible in the cells expressing TG2. Indeed, TG2 strongly colocalized with fibronectin, with a Pearson correlation coefficient of 0.69 ± 0.04 (P<0.01) for a collection of 16 transfected cells.
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2

Figure 5.1: Colocalization of TG2/eGFP and immunostaining for TG2 (see page 113).
Figure 5.2: Immunostaining of fibronectin for HEK/293T cells transfected with Tc2/eGFP and reseeded on glass (see next page).
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2

Sub-cellular localization of TG2 is substrate-dependent in HEK/293T cells
Subcellular localization of TG2/eGFP was first studied on HEK/293T cells cultured on different substrates. When cells were seeded on glass (Figure 5.2) or fibronectin (Supplemental Figure 5.2), TG2 was distributed homogeneously throughout the perinuclear space. Seeding on collagen type I resulted in differential localization of TG2 between the base and top of the cell. At the adherent side, eGFP fluorescence revealed an elongated network of TG2 bound to fibrils of unknown origin (Figure 5.3C). At the top side, TG2/eGFP appeared more concentrated as distinct spots (Figure 5.3B). Although we could not discern whether these vesicles were localized intra- or extracellularly, the largest spots often appeared at the cell membrane, where they possibly are in the process of externalization (Figure 5.3A). Under high excitation intensity, TG2/eGFP vesicles were also observed at distances up to 200 μm from the cell membrane (Figure 5.3D-E). We believe that these large vesicles consist of aggregates of microparticles, since at high magnification multiple green centers could be discerned within one spot (insert in Fig. 5.3E). For TG2/eGFP expressing cells seeded on glass or fibronectin, these extracellular vesicles were virtually absent (see Table 5.1). This substrate-dependent release of the fluorescent construct was not found in control experiments where only eGFP was transfected in cells seeded on collagen.
Figure 5.3: Subcellular localization of fluorescent TG2 particles on collagen type I (see next page).
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2

As was the case for the HEK/293T cells, in transfected MOVAS cells TG2/eGFP accumulated in vesicles that were found especially at the boundary of the cell (Figure 5.4, open arrows). These spots often disappeared suddenly within minutes after their appearance. Occasionally, such a spot was observed to transform into a large membrane vesicle that was released from the intact cell (Figure 5.4, closed arrows).

SMCs did not produce MPs when serum was withdrawn from the culture medium for a short period of time. Thus, after 2 hours serum deprivation, MPs were virtually undetectable. In the presence of serum, however, SMCs continuously produced MPs, as measured by flow cytometry. By comparing the FSC-SSC signal of beads of known sizes and a similar refractive index, the size of SMC-derived MPs was estimated to be between 500 and 1000 nm (Supplemental Figure 5.3). Figure 5.5A-D shows an example of MP production by TG2/eGFP transfected cells after 24 hours of serum stimulation. In Figure 5.5A, MPs and other small fragments were separated from cell debris based on low forward and side scattering. This fraction was then gated according to annexin-V labeling to positively identify microparticles (5.5B). The non-specific binding of annexin-V in a citrate buffer that lacked calcium amounted to only 0.5%. The annexin-V positive fraction was then analyzed for eGFP intensity (5.5C). 5D correlates intensity of annexin-V staining with eGFP fluorescence, and demonstrates that 96-98% of the eGFP-positive hits were MPs.

Figure 5.3: Subcellular localization of fluorescent TG2 particles on collagen type I. HEK/293T cells were transfected with TG2/eGFP and reseeded on a coating of collagen type I. At the adherent side, TG2 appeared partly as a green meshwork (panel C). Along the upper cell surface, bright eGFP spots were visible throughout the perinuclear space (panel B). Panel A shows an example of a typical top view, with concentrated TG2/eGFP particles both inside and outside the cell boundaries. Panels D-E illustrate extracellular TG2: a large cluster of TG2 particles (indicated by a white arrow) was observed at a distance of 100-200 μm from the cell. Examples in panels A-D were taken from different images.

Table 5.1: Number of extracellular fluorescent TG2 particles on different substrates.

| Substrate         | Extracellular fluorescent TG2 particles (average number per cell) |
|-------------------|---------------------------------------------------------------|
|                   | TG2_eGFP         | eGFP                    |
| plastic           | 0.50 ± 0.17      | not determined          |
| fibronectin       | 0.47 ± 0.27      | not determined          |
| collagen type I   | 14.0 ± 2.81\(^a\) | 0.75 ± 0.36             |

\(^a\) P<0.01 (collagen type I vs. glass or fibronectin)

HEK/293T cells were transfected with TG2/eGFP and reseeded on untreated glass, a coating of fibronectin or collagen type I. Extracellular particles are given in average per cell ± standard error of the mean.

**Smooth muscle cells secrete microparticles enriched in TG2**

As was the case for the HEK/293T cells, in transfected MOVAS cells TG2/eGFP accumulated in vesicles that were found especially at the boundary of the cell (Figure 5.4, open arrows). These spots often disappeared suddenly within minutes after their appearance. Occasionally, such a spot was observed to transform into a large membrane vesicle that was released from the intact cell (Figure 5.4, closed arrows).

SMCs did not produce MPs when serum was withdrawn from the culture medium for a short period of time. Thus, after 2 hours serum deprivation, MPs were virtually undetectable. In the presence of serum, however, SMCs continuously produced MPs, as measured by flow cytometry. By comparing the FSC-SSC signal of beads of known sizes and a similar refractive index, the size of SMC-derived MPs was estimated to be between 500 and 1000 nm (Supplemental Figure 5.3). Figure 5.5A-D shows an example of MP production by TG2/eGFP transfected cells after 24 hours of serum stimulation. In Figure 5.5A, MPs and other small fragments were separated from cell debris based on low forward and side scattering. This fraction was then gated according to annexin-V labeling to positively identify microparticles (5.5B). The non-specific binding of annexin-V in a citrate buffer that lacked calcium amounted to only 0.5%. The annexin-V positive fraction was then analyzed for eGFP intensity (5.5C). 5D correlates intensity of annexin-V staining with eGFP fluorescence, and demonstrates that 96-98% of the eGFP-positive hits were MPs.

**Table 5.1: Number of extracellular fluorescent TG2 particles on different substrates.**

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| plastic       | 0.50 ± 0.17                                                     |
| fibronectin   | 0.47 ± 0.27                                                     |
| collagen type | 14.0 ± 2.81\(^a\)                                                |

\(^a\) P<0.01 (collagen type I vs. glass or fibronectin)

**HEK/293T cells were transfected with TG2/eGFP and reseeded on untreated glass, a coating of fibronectin or collagen type I. Extracellular particles are given in average per cell ± standard error of the mean.**
Figure 5.4: Intracellular formation and secretion of microparticles enriched in TG2 (see next page).
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2

Figure 5.4: Intracellular formation and secretion of microparticles enriched in TG2. Smooth muscle cells were seeded on a coating of fibronectin and transfected with TG2/eGFP. The top panel shows the phase contrast series, the lower panel the fluorescence images after deconvolution of a vertical image stack. Microparticles are visible near the cell periphery at all time points, as indicated by open arrows. The closed arrow points to a microparticle that is assembled and subsequently released into the extracellular matrix.

Figure 5.5E and F show average numbers and fluorescence intensities of events classified as MP according to Figure 5.5A-B. Transfection with TG2/eGFP resulted in a strong concentration of TG2 into microparticles in the presence of serum (5.5E, middle panel). While typically 16-18% of the cells were successfully transfected with TG2/eGFP, 42.4 ± 2.8% (SEM) of all microparticles were positive for TG2/eGFP. In contrast, transfection with control eGFP resulted in 15.9 ± 1.4% eGFP-positive MPs, despite the higher transfection efficiency (~25%) for eGFP as compared to TG2/eGFP. The high number of TG2-enriched MPs depended on the TG2 cross-linking activity, since in both TG2 mutants the percentage of eGFP-positive MPs was similar to the control eGFP group. In agreement with the elevated eGFP-positive fraction for TG2/eGFP, this group also displayed significantly higher fluorescence intensity when averaged per MP (Figure 5.5E, bottom panel). We considered that the higher eGFP signal in TG2/eGFP transfected MP might simply be related to the formation of larger MPs. However, Annexin-V intensity, as a marker of MP surface area, was virtually identical in MPs derived from TG2/eGFP-transfected cells as compared to cells transfected with eGFP, TG2-C277S/eGFP and TG2-W241A/eGFP, even though these values were slightly higher than those from non-transfected cells (Figure 5.5E, top panel). In addition, when flow cytometry was performed at the level of the intact cell, fluorescence was about 4-5 times higher for eGFP, as compared to both TG2/eGFP and its two mutants. Thus, in order to override this effect, the higher fluorescence of TG2/eGFP in MPs must be due to a strong mechanism of concentrating TG2 into microparticles.

This role of TG2 in MP formation during serum stimulation could be mimicked by stimulation using the calcium ionophore A23187 for 2 hrs. This resulted in a fraction of 31.5 ± 5.0% of the identified MPs that were positive for TG2/eGFP. Both TG2 mutants were present in a smaller fraction of the MPs (TG2-W241A/eGFP: 18.2 ± 3.2%, P=0.067 vs. TG2/eGFP; TG2-C277S: 14.8 ± 3.9%, P<0.05). Control eGFP was identified in only 1.4 ± 0.2% (Figure 5F, middle panel). The elevated fraction for TG2/eGFP as compared to the TG2 mutants was accompanied by a higher eGFP signal, although this did not reach statistical significance (5F, bottom panel). MP size, as estimated by Annexin-V intensity, was not different over all groups (Figure 5.5F, top panel).
Figure 5.5: Secretion of TG2 via microparticles, quantified using flow cytometry. Microparticles were identified first by FSC and SSC characteristics (region indicated with ‘SF’ in panel A) and subsequently by annexin-V/APC signal (B); eGFP was thresholded using non-transfected control cells (C). There was no difference in MP size between the various eGFP-positive proteins, as estimated by average intensity of annexin-V/APC. MPs were particularly enriched in TG2/eGFP compared to eGFP, and both TG2 cross-linking mutants, both upon stimulation with serum (E) or calcium ionophore A23187 (F). Average eGFP fluorescence was elevated for TG2/eGFP for both stimuli, which reached significance for the serum group (E-F). P-values are given with respect to TG2/eGFP, unless indicated otherwise.

For panels E-F: see next page.
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2

**E**  FCS, 24 hrs

**F**  A23187, 2 hrs

- **E**
  - N.S.
  - Average intensity (A.U.)
  - Percentage of Ann-V positive MPs (%)
  - Average intensity (A.U.)

- **F**
  - N.S.
  - Average intensity (A.U.)
  - Percentage of Ann-V positive MPs (%)
  - Average intensity (A.U.)

- **Groups**
  - non-transfected
  - eGFP
  - TG2/eGFP
  - TG2-W241A/eGFP
  - TG2-C277S/eGFP
  - non-transfected
  - eGFP
  - TG2/eGFP
  - TG2-W241A/eGFP
  - TG2-C277S/eGFP
Discussion

This study showed that TG2 is secreted from smooth muscle cells via microparticles (MPs). Moreover, we demonstrated that release of MPs depends on the cross-linking activity of TG2. Here, we address how the major tool for these experiments, fluorescently tagged TG2, was developed and validated. We then discuss the potential mechanisms for TG2-dependent MP formation, and depict the relevance of the current findings for cell-matrix interaction, in particular in the context of vascular remodeling and disease.

We generated an expression vector encoding a fluorescently tagged TG2 protein in order to study TG2 subcellular localization and secretion. A few papers report the use of fluorescent Transglutaminase, but to the best of our knowledge these tagged proteins have not been used for localization of TG2 in particular micro-environments. Zainelli et al. developed a N-terminal TG2 fusion product, which was employed to identify TG2-substrates in Huntington disease in vitro. Recently, Jeong et al. described the preparation of GFP-traceable viruses containing TG2. Here, the GFP signal was used to monitor TG2 expression level. Factor XII, well-known for its role in blood coagulation, is another member of the Transglutaminase family. A recent paper by Jayo et al. describes cloning of the active subunit of FXIII into a GFP vector. The green cell protrusions containing FXIII that were detected, were postulated to be cell protrusions active in motility via RhoA.

We used HEK/293T cells for initial functional characterization of eGFP-tagged TG2, because these cells have a very low endogenous TG2 expression and are readily transfected. Since it is clear that fluorescent tagging may affect functional properties of enzymes, we addressed attention to two aspects of TG2 function. First, we established that the eGFP-tagged TG2 retains its characteristic cross-linking activity at a level comparable to that of the wild-type, while this was absent from both TG2 mutants. A second critical property of TG2 is its fibronectin binding site, since this is indispensable for TG2 secretion. Fibronectin binding is accomplished by the N-terminal amino acids 1-73 and 88-106 in TG2. We therefore chose to fluorescently tag TG2 at the C-terminus, which is mainly involved in receptor signaling via adreno-receptors. Indeed, it has previously been shown that fusion of the TG2 C-terminus to the reporter enzyme β-galactosidase had no effect on interactions between TG2 and FN. Another reason to prefer C-terminal tagging, was that N-acetylation has been proposed as a possible mechanism for TG2 externalization. Our eGFP-tagged TG2 showed a strong colocalization with fibronectin (Figure 5.2), suggesting that TG2 binding to fibronectin had remained intact. We observed a rather uniform distribution of fibronectin throughout the cytoplasm. Since we permeabilized the cells for immunostaining, this may represent either membrane-bound fibronectin or an
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2

An intracellular pool of this protein, originating either from cellular synthesis or uptake from the serum used for culturing. These results indicate that the N-terminus of our TG2/eGFP construct has retained its characteristic fibronectin binding site, which is critical for TG2 secretion. A next step in the interaction between TG2 and FN is fibronectin polymerization into networks. We could not detect whether such networks developed, mainly because of the strong signal coming from what we believe is monomeric fibronectin. Since fibrillogenesis was not the main issue of the current study, this effect of the various TG2 constructs was not further studied.

Using the functionally intact TG2/eGFP, we visualized the sub-cellular distribution of TG2 first in HEK/293T cells, and subsequently in SMC. Vesicles, and especially vesicular aggregates up to several micrometers in size, became evident when TG2/eGFP transfected cells (SMC or HEK) were cultured in a micro-environment of collagen type I in the presence of serum. Using HEK/293T cells transfected with TG2/eGFP and grown on fibronectin or glass, these structures were rarely observed by confocal microscopy. However, live-imaging or flow cytometry measurements showed that MPs were produced irrespective of the substratum used during SMC culturing. Therefore, the 3-D collagen matrix used with HEK/293T cells seems to have functioned to entrap MPs, resulting in an elevated number of observed MPs as compared to fibronectin and glass in our histological preparations. It remains to be tested whether collagen fibers could also bind the MP, or further stimulate the release of MP by interaction with the native cells.

Our choice for serum and the calcium ionophore A23187 as stimuli for the induction of MPs needs explanation. It is clear that the exact serum component(s) causing release has not been identified, while the calcium ionophore is an extremely strong stimulus with little relevance for pathological conditions. Yet, it was not our purpose to unravel signaling leading to MP release in specific conditions, but rather to test the involvement of TG2 in final release, irrespective of upstream events. We therefore used both serum and the calcium ionophore as robust stimuli. Our finding that serum stimulation enhances MP generation is in agreement with the activity of MP-derived tissue factor that is virtually absent in quiescent cultures of SMCs, ECs, fibroblasts and macrophages. For the calcium ionophore, several studies employed a 10 μM concentration for 10 minutes only. We preferred to prolong the incubation time to 2 hrs while lowering the dose to 5 μM, since this is known to activate intracellular TG2, and in our hands greatly increased the accumulated number of microparticles. However, we realize that part of the MP pool measured after stimulation with A23187 may originate from apoptotic cells, even though microscopic observations revealed a low level of apoptosis in confluent SMC cultures under these conditions.
We based our conclusion that TG2 is released via microparticles, in a cross-linking dependent manner, on the comparison of MPs from SMCs transfected with TG2/eGFP to control eGFP and two TG2 cross-linking mutants. Unfortunately, we could not confirm these experiments on non-transfected cells, since the available antibodies against mouse TG2 appeared not to be suited for flow cytometric measurements. This also prevented flow cytometric testing for TG2 release during pathological conditions such as vascular remodeling. However, western blotting on cell lysates showed that the eGFP signal could indeed be used to track TG2.

We propose the following hypothesis for secretion of TG2 from vascular cells. After cell activation, TG2 is translocated to the cell membrane. We speculate that this occurs preferably at sites of α5β1 and ανβ3-integrins, which were frequently shown to colocalize with concentrated spots of TG28,20. Interestingly, these integrins were also identified at the surface of SMC-derived MPs involved in tissue factor activation34. In addition, heparan sulfate proteoglycans such as syndecan-4 may function to recruit TG2 from the cytosol to these membrane sites23. Based on the enrichment of MP with mutant TG2 (Figure 5.5F), we believe that TG2 cross-linking is not required for this translocation. Indeed, a recent paper by Antonyak et al. showed that after transfection of HeLa cells with wild-type TG2 or mutant TG2 defective in transamidation or GTP-binding, all isoforms could be triggered to translocate to the cell membrane upon stimulation with EGF55. Then after transportation to the cell membrane, cross-linking activity is required for release of these TG2-enriched MPs. This would require a high calcium level that is known to be locally present during the development of MPs26,28. In support of this theory, upon transfection of 3T3 fibroblasts with TG2 or C277S mutant, both forms were detected on the cell surface, but only wild-type TG2 was detected in the ECM and cell culture supernatant22. Possibly, TG2 cross-linking activity is required to link cytoskeletal elements that together form the structural basis for the MP. This is similar to the setting of lung cancer cells challenged by mechanical damage, where TG2 was shown to promote membrane resealing56. When pulmonary artery SMCs were stimulated with serotonin, this induced transamidation of proteins, which could be detected both in cells and their culture supernatant. Three of the four major TG2 substrates were found to be non-muscle myosin heavy chain, filamin B and plakin57, which are involved in stabilization of the cytoskeletal network to the cell membrane58. Correspondingly, Factor FXIII may play the role in platelets that TG2 fulfills in SMCs. When stimulated with thrombin or calcium ionophore, which are known to trigger MP generation26, FXIII is transported to the platelet periphery within 1 minute59. After isolation from the cytoskeletal fraction, filamin and vinculin were cross-linked into multimeric complexes, which could be prevented by preincubation with the Transglutaminase inhibitor iodoacetamide.
Thus, Transglutaminases clearly have a high affinity for cytoskeletal elements that may function as building blocks for microparticles.

In summary, we developed a green fluorescent TG2 protein to study the translocation and secretion during vascular remodeling. Using HEK/293T cells, we confirmed that protein cross-linking and fibronectin binding of TG2 were unaffected by the eGFP tag. In smooth muscle cells, TG2/eGFP was translocated to sites of vesiculation near the cell periphery upon stimulation with a calcium ionophore or a non-identified serum component. The cross-linking function of TG2 was then required for secretion of microparticles. This may provide the mechanism for the activation, translocation and extracellular activity of TG2 as seen in tissue remodeling and specifically vascular remodeling.

Acknowledgements

The help from Judith the Vos with immunofluorescent labeling was greatly appreciated. We also thank Eric A.J. Reits for donating pEGFP and pmCherry. Ron Hoebe and Jan Stap assisted with time-lapsed fluorescence microscopy and subsequent image analysis. Anita Grootemaat was very helpful with the preparation and flow cytometry measurements of microparticles.
Supplement

Construction of Fluorescent TG2 expression vectors

The mouse TG2 encoding IMAGE clone 3256943 (GenBank: BC016492) in the expression vector pCMV-SPORT6 was obtained from imaGenes (Berlin, Germany). In order to remove the stop codon, nt 1655-2101 were amplified (Taq PCR core kit, Qiagen) using primers 1 and 2 (Supplemental Table 5.1). The resulting PCR fragment (447 bp) was cloned into pGEM-Teasy (Promega) and verified by nucleotide sequence analysis using BigDye Terminator v3.1 (Applied Biosystems), and primers 3-4. This amplified TG2 PCR fragment was then used to replace the 3’ end of TG2 in pCMV-Sport6 using the restriction enzymes NotI (New England Biolabs) and BglII (Roche). The complete TG2 open reading frame without stop-codon was subsequently amplified by touchdown PCR (Advantage GC-2 PCR kit, Clontech) starting at 70°C, with 20 steps of 0.5°C using primers 16 and 17. After 3’-T tailing, the PCR fragment was cloned into pGEM-Teasy and verified by nucleotide sequencing using primers 3-8 and 10-14. The TG2 open reading frame without stop-codon fragment was excised from pGEM-Teasy using restriction enzyme XmaI and EcoRI-HF (New England Biolabs) and cloned into either pEGFP-N1 or pmCherry-N1 (Clontech 632469 resp. 632523), generating vectors that express TG2 with a C-terminal fluorescent tag under control of a CMV promoter. Finally, the desired sequence was confirmed using primers 5-9, 11-15 and 18-19.

Supplemental Table 5.1: Primers used for construction of fluorescent TG2.

| #  | Primer Name          | Sequence 5’ → 3’ |
|----|---------------------|-----------------|
| 1  | Tgm2m-for           | TCC CAC TTC GAA TCC TCT ACG A |
| 2  | Tgm2m-nostop-Smal-Mlu-rev | ACG CTT CCC GGG CCG CGA TGA TAA CAT T |
| 3  | M13-rev             | CAG GAA ACA GCT ATG AC |
| 4  | M13-fwd             | CTG GCC GTC GTT TTA C |
| 5  | Tgm2-cDNA-Fwd1      | AAC AGG ACA ATG TCC TCT |
| 6  | Tgm2-cDNA-Fwd2      | CTG CCA TGA TGA CCA GG |
| 7  | Tgm2-cDNA-Fwd3      | AGA AGG GCC AAG GGA CA |
| 8  | Tgm2-cDNA-Fwd4      | AAC GAC ACC TCG GAG AC |
| 9  | Tgm2-cDNA-Fwd5      | GGA GCA GAA GTG TGT GG |
| 10 | Tgm2-cDNA-Rev1      | TTC TGA AGA GGC CCC AGG CA |
| 11 | Tgm2-cDNA-Rev2      | GGA CCC GGA TCT GTA TT |
| 12 | Tgm2-cDNA-Rev3      | GCC TTG GTG AAG ACT TC |
| 13 | Tgm2-cDNA-Rev4      | TTG TTG CTC TCC AGC TCC CC |
| 14 | Tgm2-cDNA-Rev5      | TAT CCA GGA TTC CAT CC |
| 15 | Tgm2-cDNA-Rev6      | AAC GCT AGG CTG TCC AC |
| 16 | Tgm2/nostop-cDNA-For | GAA TTC GCC GCC GCT CTG AGC TGT CGC CGC TA |
| 17 | Tgm2/nostop-cDNA-Rev | CCC GGG TGG CCG GGC CGA TGA TAA CAT T |
| 18 | EGFP-N-Fwd          | CGT GTA CGG TGG GAG GTC TA |
| 19 | EGFP-C-Rev          | GTT GTG GTT GTA CAA AAC TCA |
Partial Degradation of TG2/mCherry
We tested the functional characteristics of TG2 tagged with either eGFP or mCherry, since the latter would provide a better signal-to-noise ratio in tissues with a high green autofluorescence level. Moreover, GFP was previously reported not be a substrate for transamidation, excluding the participation of our fusion protein as a substrate in transamidation. Expression of TG2 with or without fluorescent tags was not significantly different, as determined from quantification of western blots (Supplemental Table 5.2). Non-modified TG2 showed a strong band at the expected size of 80 kDa. However, when either eGFP or mCherry was tagged at the C-terminus of TG2, in addition to the expected band at 110 kDa, a second band at about 90 kDa was observed (Supplemental Figure 5.1). The lower band was particularly apparent in the TG2/mCherry fusion product, where it constituted about half of the protein recognized by the TG2 antibody (Supplemental Table 5.2). In cells expressing TG2/eGFP on the other hand, the 90 kDa band contributed only about 10%. When western blots were probed with antibodies against eGFP or mCherry, both the 90 and 110 kDa bands were recognized (data not shown), excluding the possibility of cleavage of the complete fluorescent tag from the TG2 fusion protein. All subsequent imaging experiments below were carried out using TG2/eGFP. At the moment, this difference between these closely related fluorescing proteins cannot be explained. Therefore, all localization experiments were conducted using TG2/eGFP. Although about half of the TG2/mCherry protein was partially degraded, this did not lead to a diminished in vitro transamidation activity (Supplemental Table 5.2).

**Supplemental Table 5.2: Quantification of TG2 western blot and transamidation activity assay.**

| Sample              | Western Blot (n = 3) | Transglutaminase Activity (μUnits/μgram) |
|---------------------|----------------------|------------------------------------------|
|                     | 80 kDa (intensity)   | 90 kDa (intensity) | 110 kDa (intensity) | Total (intensity) |
| control             | <1                   | -              | -                 | <1               | <10         |
| TG2                 | 35 ± 6.4             | -              | -                 | 35 ± 6.4         | 654 ± 212   |
| mCherry             | <1                   | -              | -                 | <1               | <10         |
| TG2/mCherry         | -                    | 16 ± 4         | 12 ± 4.4          | 28 ± 8.4         | 602 ± 268   |
| eGFP                | <1                   | -              | -                 | <1               | <10         |
| TG2/eGFP            | -                    | 2.7 ± 1.1      | 24 ± 7.1          | 26 ± 8.1         | 660 ± 292   |

Supplemental Figure 5.1 (see next page): Example of western blot for determination of TG2 protein content. HEK/293T cells were transfected with full-length TG2, TG2 fused to eGFP or mCherry, or only these fluorescent proteins. Two different proteins were visualized simultaneously using 2 antibodies in the infrared spectrum: TG2 is shown in red, green represents respectively the loading control protein β-actin, mCherry and eGFP. Non-tagged TG2 shows a distinct band at 80 kDa; fluorescently-tagged TG2 appears as expected around 110 kDa, but has an additional band at ~90 kDa.
Supplemental Figure 5.1: Example of western blot for determination of TG2 protein content.
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2.

Supplemental Figure 5.2: Subcellular localization of fluorescent TG2 on fibronectin. HEK/293T cells were transfected with TG2/eGFP and reseeded on a coating of fibronectin. TG2 appears distributed randomly throughout the cytosol, extracellular TG2/eGFP particles are absent.
Supplemental Figure 5.3: Flow cytometry measurements of FSC and SSC characteristics for beads of known sizes. Silica beads of 500, 1000 and 5000 nm were used, PMT settings are identical to those used for measurement of SMC-derived microparticles (Figure 5.5). The gated region indicated by 'R1', including the majority of the 500 and 1000 nm beads, corresponds to the MP settings.
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2

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Chapter 6

The redox state of Transglutaminase 2 controls arterial remodeling

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Submitted for publication 2010
Abstract

Rationale: While inward remodeling of small arteries in response to low blood flow, hypertension, and chronic vasoconstriction depends on type 2 Transglutaminase (TG2), the mechanisms of action have remained unresolved.

Objective: We studied the regulation of TG2 activity, its (sub) cellular localization, substrates, and its specific mode of action during small artery inward remodeling.

Methods & Results: Inward remodeling of isolated mouse mesenteric arteries by exogenous TG2 required the presence of a reducing agent. The effect of TG2 depended on its cross-linking activity, as indicated by the lack of effect of mutant TG2. The cell-permeable reducing agent DTT, but not the cell-impermeable reducing agent TCEP, induced translocation of endogenous TG2 and high membrane-bound Transglutaminase activity. This coincided with inward remodeling, characterized by a stiffening of the artery. The remodeling could be inhibited by a TG2 inhibitor and by the nitric oxide donor, SNAP. Using a pull-down assay and mass spectrometry, 21 proteins were identified as TG2 cross-linking substrates, including fibronectin, collagen and nidogen. Inward remodeling induced by low blood flow was associated with the upregulation of several antioxidant proteins, notably glutathione-S-transferase, and selenoprotein P.

Conclusions: These results show that a reduced state induces smooth muscle membrane-bound TG2 activity. Inward remodeling results from the cross-linking of vicinal matrix proteins, causing a stiffening of the arterial wall.

Keywords:
Transglutaminase, smooth muscle cell, redox, S-nitrosylation, vascular remodeling

Non-standard Abbreviations and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| DTT          | dithiothreitol |
| GST          | glutathione S-transferase |
| NO           | nitric oxide |
| SMC          | smooth muscle cell |
| SNAP         | S-Nitroso-N-acetyl-DL-penicillamine |
| TCEP         | tris(2-carboxyethyl)phosphine hydrochloride |
| TG2          | Transglutaminase 2 |
Introduction

Small arteries represent the main site of resistance in the vascular system, and as such, have a large impact on tissue perfusion and blood pressure. Inward remodeling of small arteries occurs after a reduction in blood flow, but is also associated with high blood pressure\(^1-^3\). It is a hallmark of essential hypertension and a strong predictor of cardiovascular events\(^4\). Previous work from our group showed that Transglutaminases, in particular type 2 Transglutaminase (TG2), play a crucial role in the inward remodeling of small arteries after reduced blood flow and hypertension \textit{in vivo}, and chronic vasoconstriction \textit{in vitro}\(^5-^11\). The mechanism by which TG2 contributes to vascular remodeling however, remains poorly understood.

One reason for the elusive role of TG2 in remodeling may relate to its wide range of actions. Its best known function is the stabilization of matrix proteins through the formation of a specific cross-link, the \(\mathrm{N}^{\epsilon}\text{(\textgamma-glutamyl})\text{lysine isopeptide bond, which is the result of a transamidation reaction}^1^2\). This reaction is regulated by several factors including calcium, GTP, nitric oxide and the redox potential\(^1^3\);\(^1^4\). The isopeptide bond provides mechanical strength and resistance to proteolytic degradation in tissues. Recent work from Santhanam et al\(^1^5\) showed that the cross-linking action of TG2 is highly relevant for human cardiovascular pathology. Thus, these authors showed that the formation of cross-links by TG2 relates to the stiffening of large arteries that is associated with aging. Besides the formation of isopeptide bonds within and between proteins\(^1^2\), TG2 promotes cell adhesion via its binding to fibronectin, integrin \(\alpha_5\beta_1\) and heparan sulfate proteoglycans\(^1^6\);\(^1^7\). In addition, TG2 acts as protein disulfide isomerase\(^1^8\), functions as a G-protein\(^1^9\) and aids in the regulation of cytoskeletal structure and cell contractility\(^2^0\);\(^2^2\).

Of great potential relevance for cardiovascular pathology, Stamnaes et al.\(^2^3\) recently showed that TG2 contains a cysteine residue that acts as a redox sensor, which could regulate the cross-linking activity of TG2 in the extracellular environment. In the present study we investigated the activation, (sub) cellular localization, and substrates of TG2 during the inward remodeling of small arteries. Besides the regulation of TG2 by calcium and nitric oxide, we focused on the redox state of TG2 using reducing agents with different properties regarding cellular permeability. We report that TG2 is translocated to the surface of smooth muscle cells (SMCs) upon intracellular reduction. In a reduced state TG2 acts as a membrane-bound cross-linking enzyme in SMC, which coincides with inward remodeling. Using mass spectrometry, we identified several matrix proteins as TG2 substrates. Taken together, these data elucidate the regulation, secretion and substrates of TG2 in the process of vascular remodeling.
Methods

Mice and vessel isolation
Four months old male C57Bl/6 mice (Harlan) were anesthetized using isoflurane and sacrificed by cervical dislocation. Then, the abdomen was opened and the mesentery was excised and placed in cold MOPS buffer. First and second order arteries were isolated from the mesenteric vasculature. Arteries were cut in equal-sized pieces where one segment was randomly assigned as control and the other segments subjected to various interventions. This approach allowed for pair-wise statistics and drastically decreased variability which results from anatomical variation in vessel caliber. All protocols consisted of a 24-hour incubation period at 37 °C, where the vessels were placed in 100 μL buffer containing Leibovitz medium with 10% fetal bovine serum (Gibco), a mix of antibiotic-antimycotic solution (Gibco), and additional compounds, depending on the specific protocol. All experiments were approved by the local committee for animal experiments.

Pressure myograph and remodeling
To determine remodeling of the arteries, segments were cannulated in a pressure myograph system after the incubation period and inner diameters were recorded as described previously. After checking for leaks, a passive pressure-diameter relationship was determined in calcium-free MOPS buffer, supplemented with papaverine (0.1 mmol/L) to rule out influences of vasomotor tone.

Exogenous recombinant TG2
In this set of experiments, vessels were exposed to recombinant human TG2 (Zedira, T002) or cross-linking deficient C277S-TG2 (Zedira, T018), with or without the membrane-impermeable reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 1 mmol/L). Control experiments were included where segments only were exposed to TCEP. In all experiments, recombinant TG2 was administered at 50 μg/mL.

Activation of endogenous TG2
In this set of experiments, vessels were incubated with calcium ionophore A23187 (Sigma, C7522: 1 μmol/L). Alternatively, vessels received 2 mmol/L DTT (Sigma, 43816), which is membrane-permeable. In the latter experiment, TG2 activity was blocked using either the TG2 active-site inhibitor L682777 (Zedira, T101: 10 μmol/L, also known as R283) or the NO donor SNAP (Sigma, N3398: 1 mmol/L).

The effect of DTT on vessel viability was assessed in a separate set of vessels. Here, the contractile response to the thromboxane/prostaglandin agonist U46619 (Sigma, D8174: 1 μmol/L) was measured after a 24-hour incubation with 2 mmol/L DTT.
Localization of TG2 activity
In vessels stimulated with calcium ionophore or DTT, TG2 activity was visualized using the pseudo-substrate cadaverine, linked to either FITC (AnaSpec, 81504; 100 μmol/L) or AlexaFluor594 (Invitrogen, A-30678; 10 μmol/L). In experiments where SNAP was used to inhibit TG2 activity, cadaverine was added >30 min after SNAP. SNAP was used at 10⁻⁴ mol/L with calcium ionophore and at a concentration of 10⁻³ mol/L with DTT. Vessels were fixed with formalin, mounted on glass slides using Vectashield/DAPI (Vector Laboratories H-1500) and imaged on a confocal microscope (Leica TCS SP2). TG2 activity was quantified by spatial integration of FITC or AlexaFluor594 signal in ImageJ. Data were corrected for vessel size and depicted in arbitrary units.

Immunostaining of TG2
The effect of DTT on the translocation of TG2 was assessed by immunofluorescent staining of extracellular TG2 on cultured mouse smooth muscle cells (MOVAS, ATCC CRL-2797). Cells were grown in microscopic culture chambers (BD Falcon 354102, untreated glass) that were coated with fibronectin. After a culture period of 24 hours in DMEM with 10% FCS, 0.1 mmol/L DTT was added for 2 hrs. Then cells were washed 3 times with warm PBS and fixed with cold formalin. After blocking with BSA/goat serum, the non-permeabilized cells were stained with a rabbit polyclonal TG2 antibody Ab-4 (Neomarkers RB-060-P, 1:10; 1 hr at room temperature) followed by anti-rabbit Cy3 (Brunschildwig 111-165-144, 1:200; 1 hr at room temperature) as secondary antibody, and slides were mounted in Vectashield/DAPI (Vector Laboratories H-1500).

Substrates of Transglutaminase in smooth muscle cells
The substrates for transamidation catalyzed by TG2 were determined using a mouse smooth muscle cell line (MOVAS, ATCC CRL-2797). Cells were cultured in DMEM with 10% FCS for 96 hours in T75 flasks. Then either BPA (biotinylated pentylamine, Invitrogen A1594, 1 mmol/L) or Q-peptide (Biotin-GQEPVR, synthesized using standard Fmoc-based solid phase peptide synthesis, 0.25 mmol/L) were added to function as lysine and glutamine donors respectively, while a control group was left without competitive substrate. All groups received 0.1 mmol/L DTT to increase the amount of active TG2 in the extracellular matrix. After a 24 hrs incubation period, the cultures were washed 3 times with warm PBS to remove non-bound BPA and Q-peptide. Then the lysates were collected in a 1% SDS solution and boiled for 5 minutes at 95°C to denature the proteins. For each group, the material from 2 T75 flasks was pooled and stored at -20°C until further use.

Lysates were sonicated (5 times 30 seconds at room temperature) and dialysed against 1% SDS (3 times 500 mL for 1 hour at room temperature) to further decrease the amount of non-bound BPA and Q-peptide. Then lysates were centrifuged at 15.700 g for 10 minutes at room temperature and 13 mL buffer...
containing 100 mmol/L NaCl, 50 mmol/L Tris HCl pH 7.5, 1 mmol/L EDTA, 0.5% NP40 was added to 1.2 mL supernatant. Streptavidin-Agarose (50 µL, Sigma S1638) was added and the mixture was rotated end-over-end at room temperature for 20 hours. The agarose-beads were washed with 100 mmol/L NaCl, 50 mmol/L Tris HCl pH 7.5, 1 mmol/L EDTA, 0.05% NP40 (3 times 5 minutes end-over-end at room temperature) and taken up in 2x sample buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.06% bromophenol blue and 0.5 mol/L Tris HCl pH 6.8).

For Western blot analysis 2% of each pull-down was loaded on a 12% SDS-PAGE gel and after blotting stained with IRdye 800CW Streptavidin (Li-Cor), rabbit polyclonal anti-Fibronectin (GIBCO 1A0540) and rabbit polyclonal nidogen-1 (Immun Diagnostik AP1003.1).

For MS analysis 20% of each pull-down was loaded on a 12% SDS-PAGE gel and stained with colloidal Coomassie Brilliant Blue. The biotin containing region (~30 kDa and up) from each Coomassie stained lane was sliced into 16 equal parts. Proteins in the slice were reduced with DTT, alkylated with iodoacetamide and digested with trypsin using the Proteineer DP digestion robot (Bruker, Bremen, Germany), adapted in house to accommodate larger gel pieces. The tryptic peptides were extracted from the gel, lyophilized, dissolved in 95/3/0.1 v/v/v water/acetonitril/formic acid and subsequently analyzed by online nanoHPLC MS/MS using a 1100 HPLC system (Agilent Technologies), as previously described. Peptides were trapped at 10 µL/min on a 15-mm column (100-µm ID; ReproSil-Pur C18-AQ, 3 µm, Dr. Maisch GmBH) and eluted to a 200 mm column (50-µm ID; ReproSil-Pur C18-AQ, 3 µm) at 150 nL/min. All columns were packed in house. The column was developed with a 120-min gradient from 0 to 30% acetonitrile in 0.1% formic acid. The end of the nanocolumn was drawn to a tip (ID ~5 µm), from which the eluent was sprayed into a 7-tesla LTQ-FT Ultra mass spectrometer (Thermo Electron). The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of 3,000,000. The two most intense ions were then isolated for accurate mass measurements by a selected ion monitoring scan in FT-ICR with a resolution of 50,000 at a target accumulation value of 50,000. Selected ions were fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. In a post-analysis process, raw data were first converted to peak lists using Bioworks Browser software v 3.2 (Thermo Electron), then submitted to the SwissProt database version 51.6 using Mascot v. 2.2.04 (www.matrixscience.com) for protein identification and finally sorted and compared using Scaffold software version 3.0.1 (www.proteomesoftware.com). Mascot searches were with 2 ppm and 0.8 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme. Scaffold filtered for identified proteins.
with at least 2 peptides with 95% confidence. Collision-induced dissociation spectra were also manually inspected. Common contaminants were removed manually from the list.

**Regulators of redox balance during inward remodeling**
The expression of redox regulating enzymes in inwardly remodeling vessels was investigated using a microarray approach published previously by Wesselman et al.\(^3\) In short, flow-modifying surgery was performed on rat first-order mesenteric arteries. This leads to a flow reduction in ligated vessels to approximately 10% of control, and a doubling of flow in adjacent high flow vessels. Animals were sacrificed after 1, 2, or 4 days and for each time point vessels from 4 animals were pooled. cDNA from either low or high flow vessels linked to a Cy5 probe was hybridized onto 2 different microarrays in the presence of Cy3-labeled cDNA from control vessels. Up- or down regulation of a number of redox regulating enzymes with decreased flow was then normalized to control values. If genes were present more than once in the 2 arrays employed, their values were averaged.

**Statistics**
Data are shown as mean ± SEM. For all measurements of P,d-curves, differences in diameter between groups were tested at each pressure level using a paired T-test with Bonferroni correction when appropriate. For quantification of fluorescence, 3 images were averaged per vessel. In all figures, P-values smaller than 0.05 resp. 0.01 are indicated by single or double symbols (e.g. * and **).

**Results**

**Vascular remodeling by recombinant TG2 requires a reducing agent**
Small mesenteric arteries were incubated for 24 hours under various conditions. After the incubation period, vascular remodeling of these vessels was determined by cannulation and recording of a passive pressure-diameter (P,d) relationship. Incubation of mesenteric arteries with exogenous recombinant TG2 had no effect on the P,d-curve as compared to untreated control arteries (Figure 6.1A). Administration of 1 mmol/L TCEP, a cell-impermeable reducing agent, also did not affect the P,d curve (Figure 6.1B). However, when TG2 was added together with TCEP, vessel diameter was significantly reduced at higher pressure levels (Figure 6.1C). When the catalytically inactive TG2 mutant Cys-277 was used instead of recombinant TG2, inward remodeling was again absent (Figure 6.1C). These data therefore show that inward remodeling by TG2 depends on its cross-linking action and requires the presence of a reducing agent.
Figure 6.1: Microvascular remodeling is controlled by transglutaminase induced cross-linking after activation with reducing agent TCEP. (A) Exogenous TG2 had no effect on vessel properties in the absence of a reducing agent. (B) Cell-impermeable TCEP did not induce remodeling by itself. (C) When exogenous TG2 was combined with TCEP, inward remodeling was observed. This effect was absent with recombinant TG2 defective in cross-linking. Data were averaged over 6 vessels obtained from 3 mice.

** TCEP vs. TG2+TCEP:
A cell-permeable reducing agent activates endogenous TG2
In order to test if endogenous TG2 could be activated by a cell-permeable reducing agent, vessels were incubated with dithiothreitol (DTT). As indicated by FITC-cadaverine incorporation, DTT induced a profound increase in TG2 activity in the vessel wall (Figure 6.2A). This activity was completely abolished in vessels incubated with the TG2 inhibitor L682777 (Figure 6.2A-B). The activation of endogenous TG2 with DTT caused a highly significant inward remodeling, which was almost completely prevented by L682777 (Figure 6.2C). The effect of endogenous TG2 on vascular remodeling was similar to that of exogenous TG2, with a reduction in vessel diameter at higher distending pressures only (Figure 6.2C).

In subsequent experiments we tested if remodeling induced by DTT could also be counteracted by nitric oxide. Transglutaminase activity was strongly reduced by the NO donor SNAP, as indicated by the incorporation of Alexa Fluor-594/cadaverine (Figure 6.3A-B). This was paralleled by an almost complete inhibition of the remodeling (Figure 6.3C). Hence, these results show that a shift of the intracellular redox balance to a more reduced state activates a TG2-dependent inward remodeling and, in addition, this remodeling can be inhibited by nitric oxide (NO).

Calcium ionophore A23187 stimulates TG2 activity but does not induce inward remodeling
Calcium triggers a conformational change in TG2, providing access of substrates to TG2’s active site. Normally, under physiological conditions the level of intracellular calcium is too low to induce cross-linking activity by TG2. However, after exposure to vasoconstrictor substances, or under pathological conditions, the level of intracellular calcium may rise considerably. To test the role of calcium we analyzed the effect of the calcium ionophore A23187 on TG2 activity and arterial remodeling. Administration of the ionophore increased TG2 activity in the vessel wall about 3-fold, as demonstrated by the incorporation of FITC-cadaverine (Figure 6.4A-B). This calcium-induced intracellular activity could be inhibited by 1 mmol/L of the NO donor SNAP (Figure 6.4A-B). Despite the increase in TG2 activity, the calcium ionophore did not induce small artery inward remodeling, as reflected by an unchanged P-d curve after incubation of cannulated arteries with 1 μmol/L A23187 (Figure 6.4C).
Figure 6.2: Activation of endogenous TG2 by the cell-permeable reducing agent DTT causes inward remodeling via crosslinking. (A-B) Exposure to DTT induces TG2 activity in the medial layer of the vessel, as shown by the incorporation of FITC cadaverine. (C) TG2 activation with DTT induces inward remodeling, as indicated by a downward shift of the P,d-curve. This was blocked with a site-specific TG2 inhibitor (L682777). Data were averaged over 6 vessels obtained from 3 mice, with 3 images per vessel; scalebar = 75 μm.

* non-stimulated vs. DTT: P<0.05, ** P<0.01
# DTT vs. L682777+DTT: P<0.05, ## P<0.01
Figure 6.3: DTT-induced activation of TG2 can be counteracted with the NO donor SNAP. (A-B) Incorporation of AlexaFluor594/cadaverine was significantly inhibited with SNAP. (C) SNAP abolished the inward remodeling induced by DTT. Data were averaged over 6 vessels obtained from 3 mice, with 3 images per vessel;

scalebar = 75 μm

* non-stimulated vs. DTT: P<0.05, ** P<0.01
# DTT vs. SNAP+DTT: P<0.05, ## P<0.01
Figure 6.4: Incubation with calcium ionophore (A23187) induces transglutaminase activity without remodeling. (A-B) Exposure to A23187 stimulates the incorporation of FITC cadaverine. TG2 activity can be inhibited by the NO donor SNAP. (C) A23187 does not induce inward remodeling. Data were averaged over 6 vessels obtained from 3 mice, with 3 images per vessel; scalebar = 75 μm
* non-stimulated vs. A23187: P<0.05
# A23187 vs. A23187+SNAP: P<0.05
Localization of TG2 activity is stimulus-dependent
While activation of intracellular TG2 with DTT caused significant inward remodeling, this was absent for activation by the calcium ionophore A23187. We tested whether this difference is related to localization of TG2 activity. In the adventitia, TG2 activity was virtually absent (Supplemental Video 6.1). The incorporation of FITC-cadaverine around endothelial cells was also relatively low. In contrast, TG2 activity following stimulation with A23187 or DTT was prominent in the medial layer. The subcellular staining pattern in the SMCs was strongly dependent on the stimulus for TG2 activation. Following stimulation with the calcium ionophore, TG2 activity appeared throughout the cytosol, while cellular boundaries remained clearly visible (Figure 6.5). On the other hand, DTT induced TG2 activity at the cell membrane, producing a mirror image of the activation pattern with A23187. In addition to the TG2 activity at the membrane, patches of high TG2 activity were observed at the interface of the endothelium and smooth muscle layers (Supplemental Video 6.1). Thus, a clear difference was observed in the localization of TG2 activity with A23187 and DTT.

Figure 6.5: Differential activation of transglutaminase when stimulated with either DTT or A23187. (A) when vessels were incubated with the cell-permeable reducing agent DTT, FITC cadaverine was cross-linked at the cellular membrane of smooth muscle cells (B) when vessels were incubated with the calcium ionophore A23187, FITC cadaverine appeared throughout the cytosol.
Intracellular reduction increases TG2 protein at the cell surface

The confocal images showed a membrane-bound TG2 activity upon stimulation with DTT, but did not provide sufficient resolution to determine whether the activity is intra- or extracellular. We therefore studied the presence of TG2 on non-permeabilized smooth muscle cells with immunocytochemistry. Cultured SMCs were stimulated with a low dose of DTT and stained for extracellular TG2. This revealed a strong increase in the level of extracellular TG2 as compared to untreated control cells (Figure 6.6). Hence, these data suggest that a reduced state triggers translocation of TG2 from the cytosol to the cell surface.

**Figure 6.6:** Incubation of cultured smooth muscle cells with DTT (0.1 mmol/L) increases the amount of extracellular TG2 (shown in red), as visualized by immunostaining of non-permeabilized cells. Fluorescence intensity was integrated per cell and averaged over 35-37 cells per group; scalebar = 20 μm. **non-stimulated vs. DTT: P<0.01**
Table 6.1: TG2 substrates in cultured smooth muscle cells

| Extracellular Matrix | control | BPA | Q-peptide | known substrate |
|----------------------|---------|-----|-----------|-----------------|
| fibronectin precursor| 63      | 47  | yes       |                 |
| collagen alpha-1 chain precursor| 4 | 1  | yes       |                 |
| fibulin-2 precursor   | 2       |     | no        |                 |
| nidogen-1 precursor   | 2       |     | yes       |                 |

| Cytoskeleton & Cell Membrane | control | BPA | Q-peptide | known substrate |
|-----------------------------|---------|-----|-----------|-----------------|
| vimentin                    | 6       | 14  | 13        | yes             |
| actin                       | 3       | 14  | 12        | yes             |
| tubulin alpha-1             | 1       | 7   | 5         | no              |
| tubulin beta-5              | 7       | 3   | yes       |                 |
| moesin                      | 4       | 2   | no        |                 |
| WD repeat protein 1         | 2       |     | no        |                 |
| annexin A1                  | 3       |     | no        |                 |

| Cell Metabolism | control | BPA | Q-peptide | known substrate |
|-----------------|---------|-----|-----------|-----------------|
| alpha-enolase   | 1       | 10  | 2         | yes             |
| L-lactate dehydrogenase| 2 | 10| 6 | no |
| pyruvate kinase | 9       |     | 2         | no              |
| GAPDH           | 4       | 4   | 6         | no              |
| aldehyde dehydrogenase, mitochondrial| 2| 1 | no |
| voltage-dependent anion-selective channel | 2 | 1 | no |
| transketolase   | 2       |     | no        |                 |

| DNA, RNA & Protein Synthesis | control | BPA | Q-peptide | known substrate |
|------------------------------|---------|-----|-----------|-----------------|
| elongation factor 1          | 7       | 4   | yes       |                 |
| histone H1.2                 | 5       |     | yes       |                 |
| THO complex                  | 4       | 3   | 4         | no              |
| initiation factor 4A-1       | 1       | 3   | 3         | no              |
| ribosomal protein 53-A       | 2       | 3   | no        |                 |
| elongation factor 2          | 1       | 3   | no        |                 |

| Various | control | BPA | Q-peptide | known substrate |
|---------|---------|-----|-----------|-----------------|
| heat shock protein 90        | 7       | 4   | yes       |                 |
| guanine nucleotide-binding protein| 5 | 3 | no |
| serum albumin precursor      | 1       | 4   | no        |                 |
| multifunctional protein ADE2 | 2       |     | no        |                 |

1 Database used: http://genomics.dote.hu/wiki/index.php/Category:Tissue_transglutaminase

Overview of TG2 substrates in culture of smooth muscle cells. BPA and Q-peptide were used as lysine respectively glutamine donor, background was measured in a control sample without biotinylated substrate. Values represent the number of unique peptides found for each protein and give an indication of the abundance of the protein in the sample.
Chapter 6

Substrates of TG2
As TG2 activity at the smooth muscle cell membrane was associated with inward remodeling, we next investigated the extracellular substrates of TG2 using cultured SMCs that were stimulated with DTT. Labeling of TG2 substrates with the lysine donor biotinylated pentylamine (BPA) or a specifically designed Q-peptide (glutamine donor) was followed by a pull down assay and mass spectrometry. This revealed a number of proteins as substrate for TG2 (Table 6.1). Fibronectin was the major extracellular substrate, cross-linked both to BPA and Q-peptide. In addition, BPA identified the ECM components collagen, fibulin-2 and nidogen-1 as glutamine donors for transamidation. Both fibronectin and nidogen-1 were subsequently confirmed as TG2 substrates in western blots (Supplemental Figure 6.3). Furthermore, a number of cytoskeletal and other intracellular proteins were identified.

Regulators of redox balance during in vivo remodeling
The activation of TG2 by DTT proved to be a strong stimulus for inward remodeling, but represents an artificial means of manipulating the redox state. Therefore, the expression of several enzymes capable of reducing thiol groups on proteins was studied in vessels remodeling in vivo. The mRNA expression was assessed in vessels stimulated to remodel inwardly by reducing blood flow in vivo. In arteries undergoing inward remodeling, selenoprotein P upregulation peaked after 2 days (Table 6.2). Glutathione transferase was already increased 80% after 24 hrs, followed by a gradual decline of expression. In outward remodeling vessels on the other hand, these enzymes were downregulated after 1 and 2 days (data not shown).

Table 6.2: Changes in mRNA of redox regulating enzymes during inward remodeling

| Overview of changes in mRNA expression of redox regulating enzymes in vessels stimulated to remodel inwardly in vivo, expressed as percentage compared to control vessels. For each time point, per condition vessels from 4 animals were pooled. Some genes were present several times on the microarrays. | Day repetitions |
|-----------------|-----------------|
| Glutathione Reductase | 40 30 10 | 1 |
| Glutathione Synthetase | 10 7 30 | 3 |
| Glutathione Transferase T1 | 80 50 -20 | 2 |
| Macrophage Migration Inhibitory Factor | 57 13 70 | 3 |
| Protein Disulphide Isomerase | 0 -10 20 | 1 |
| Selenoprotein P | 28 174 62 | 5 |
| Thioredoxin | -30 -70 0 | 1 |
| Thioredoxin Reductase | 20 20 20 | 1 |
| Xanthine Dehydrogenase | 73 90 47 | 3 |
Discussion

Vascular remodeling of small arteries after reduced blood flow, hypertension, and exposure to vasoconstrictors depends on TG2. In large arteries TG2 is involved in vascular calcification, and atherosclerotic plaque development and stability. Recently, also large artery stiffening associated with aging was shown to be dependent on TG2. While many studies have contributed to the understanding of the regulation and functions of TG2, the actual role of TG2 in vascular remodeling remained largely unknown. In our previous work we identified a strong relationship between vascular tone and remodeling. We reported that persistent vasoconstriction induces inward remodeling in several types of arteries. This remodeling could be inhibited or reversed by vasodilator compounds such as the calcium channel inhibitors verapamil or amlodipine. As constriction and dilation mechanisms act partly through modulation of intracellular calcium levels, we herein tested the hypothesis that elevation of intracellular calcium triggers TG2 activity and remodeling. The calcium ionophore indeed increased intracellular Transglutaminase cross-linking activity, but remodeling was completely absent. Thus, although intracellular proteins might have been cross-linked, this did not affect vessel caliber. These results suggested that other mechanisms of TG2 activation than elevation of intracellular calcium operate during vascular remodeling.

We found that the redox state of TG2 is a critical determinant in small artery remodeling. DTT induced a strong inward remodeling response, which was inhibited by the TG2 inhibitor. The data on remodeling by exogenous recombinant TG2 underline the need for a reduced environment. As the cell-impermeable reducing agent TCEP did not change blood vessel diameter, the remodeling most likely stems from activation of an intracellular source of TG2. Since Transglutaminase activity associated with remodeling was located at the surface of smooth muscle cells, we inferred that upon intracellular reduction, TG2 is excreted but remains bound to the cell membrane. This was substantiated by specific staining of extracellular TG2, using cultured SMCs. Similar mechanisms may exist in endothelial cells. We speculate that after translocation to the cell membrane, TG2 activity is fully uncovered by the high extracellular calcium concentration.

The reversible formation of disulfide bridges, S-nitrosylation and S-glutathiolation of key cysteines are increasingly recognized as crucial mediators of protein activation and function. TG2 is a good example of such regulation. It contains 18 sulfhydryl residues which can potentially be oxidized to form disulfide bridges, thereby impairing TG2 function. The active site cysteine (Cys-277) was shown not to be easily prone to form disulfide bridges, probably due to the low accessibility of the active cysteine. However, disulfide bonds formed
using cysteine residues from other parts of TG2 have been shown to strongly reduce cross-linking activity as well\textsuperscript{46,49,50}. Recently, a crucial cysteine pair at amino acids 370-371, controlled by Cys-230, was identified\textsuperscript{23}. It has been hypothesized that these disulfide bridges impede the calcium-triggered conformational change that is required for activation\textsuperscript{23,46}. Thus, a reduced state is necessary for TG2 to allow its cross-linking action. Generally speaking, the intracellular compartment is relatively reduced, whereas the extracellular environment is more oxidized\textsuperscript{51}. More detailed studies have revealed that the redox balance is further controlled at the subcellular level\textsuperscript{51-53}. In addition, the redox state of proteins can be individually regulated\textsuperscript{54}. The cell-permeable reductant DTT that we used is known to activate in situ transamidation without affecting other TG2 functions\textsuperscript{55}. We recognize that DTT is a non-specific reducing agent that might exert toxic effects. However, incubation with DTT did not affect vessel viability, assessed by its ability to contract (Supplemental Figure 6.2). Hence, DTT did not have an overt detrimental effect on the used ex vivo vessels. In addition, the concentration of DTT that was used appears relatively high, but one needs to keep in mind that intracellular reducing compounds such as glutathione are present in the millimolar range\textsuperscript{14}.

The observation that a reduced state of TG2 is essential for its cross-linking activity, seems to contradict a report that TG2 is activated by oxidative stress via reactive oxygen species (ROS)\textsuperscript{56}. However, UV irradiation or administration of exogenous H\textsubscript{2}O\textsubscript{2} did not potentiate in vitro TG activity in a large number of cell types\textsuperscript{14,57}. Therefore, TG2 activity in response to ROS may be a secondary effect, possibly linked to calcium leakage over the damaged cell membrane or increased TG2 expression in apoptosis-prone cells\textsuperscript{14,28,58}. Indeed, cytosolic ROS were reported to trigger an increase in cytosolic calcium\textsuperscript{51}. In addition, generation of ROS was reported to inhibit TG2 degradation\textsuperscript{59}.

In addition to disulfide formation, TG2 cross-linking activity can be regulated by nitrosylation of cysteines\textsuperscript{47}. Here, the redox balance plays an important role as well: cationic nitric oxide causes S-nitrosylation, but anionic NO leads to formation of a disulfide bridge\textsuperscript{31}. In the present study, we showed that activation of TG2 within the vessel wall, either by reduction or elevated intracellular calcium is inhibited by NO. The inhibition of TG2 activity fully prevented inward remodeling. As the nitric oxide level falls in several physiological and pathological conditions, such as low blood flow, hypertension and aging, this may be an important determinant in Transglutaminase activity in vivo. Indeed, we previously showed that inhibition of NO synthesis results in TG2 dependent inward remodeling\textsuperscript{1,60}. In further support, recent work by Santhanam et al.\textsuperscript{15} showed that vascular stiffening associated with aging depends on TG2 activity and a reduction in nitric oxide levels.
The substrates of TG2 in small artery remodeling have not been previously studied. We identified fibronectin as both a lysine and glutamine donor (Table 6.1). Another relevant extracellular substrate is fibulin-2, which is active in tissue remodeling by cross-linking several elements in the pericellular matrix. The confocal images showed high TG2 activity at the interface between smooth muscle cells and endothelial cells (Supplemental Video 6.1). This layer, the internal elastic lamina, contains laminin-nidogen complexes and collagen type IV, which are both known substrates for TG2. We confirmed nidogen-1 as a substrate for SMC-derived TG2 using BPA. Regarding the collagen family, the alpha-1 chain of collagen type I was detected as TG2 substrate. Thus, although we identified a number of proteins that are cross-linked and known to determine extracellular matrix properties, the actual substrate(s) responsible for remodeling remains to be determined. Also, the relevance of the intracellular substrates, including cytoskeletal proteins and glycolytic enzymes, warrants further investigation.

An important question is how reduction of specific proteins such as TG2 is achieved in vivo. We addressed this question by studying mRNA expression of redox-related proteins using a micro-array approach. Here we found several reducing enzymes to be quickly upregulated in vessels undergoing inward remodeling (Table 6.2). Glutathione transferase (GST), which catalyzes the conjugation of reduced glutathione to substrate proteins, was strongly upregulated after 1 and 2 days, together with glutathione reductase. Interestingly, a broad protein interaction study identified GST as the 2nd most important interaction partner of immobilized TG2, a finding confirmed in several pathological conditions. Thioredoxin is expressed amongst others in endothelial and SMCs, was detected in plasma and is involved in protein denitrosylation. Surprisingly, thioredoxin was downregulated in inward remodeling vessels, although its corresponding reductase was slightly upregulated at all time points. On the other hand, the expression of selenoprotein P, which is structurally closely related to thioredoxin reductase, was elevated 174% after 2 days. Although protein disulfide isomerase was reported to act as reductase at the cell membrane of SMCs, its expression level was unchanged. Macrophage migration inhibitory factor, which plays a role during inflammation but also in redox regulation at the cell membrane, was upregulated at all time points. Taken together, the upregulation of a panel of reducing enzymes was identified in vessels in the process of inward remodeling. These enzymes could provide a more reduced state necessary for TG2 cross-linking activity.

In summary, using the Cys-277 mutant of TG2 and an active-site inhibitor, we showed that the cross-linking action of TG2 is necessary to induce inward remodeling of small arteries. Importantly, TG2 needs to be kept in a reduced state to fulfill its action. Within the vessel wall, we found that smooth muscle cells respond to intracellular reduction with a strong increase in TG2 activity at the cell
membrane. This localized Transglutaminase activity was associated with inward remodeling. Of physiological and pathological relevance, TG2 activity and remodeling could be inhibited by addition of a NO-donor. Using mass spectrometry, we identified a number of proteins as substrates for TG2 that could be responsible for the change in vessel caliber. Finally, we found several reducing enzymes that were strongly upregulated in vessels undergoing inward remodeling induced by low blood flow. These enzymes could help to provide a more reduced state during inward remodeling in vivo.

**Funding**
This work is supported by the Netherlands Heart Foundation (grant NHS.2005.B080 to Jeroen van den Akker and NHS.2001.T038 to Hanke L. Matlung).
Redox state of Transglutaminase 2 controls arterial remodeling

Transglutaminase

exogenous TG2

endogenous TG2

TG2 cross-linking defective TG2

cell-impermeable reductor (TCEP)

cell-permeable reductor (DTT)

Calcium ionophore (A23187)

TG2 secretion (immunostaining)

TG2 inhibitor (L682777)

NO donor (SNAP)

Extracellular TG2 activity

Intracellular TG2 activity

(Incorporation of fluorescent cadaverine)

No Yes No No Yes Yes No

microvascular inward remodeling

(Pressure-diameter curve)
Supplemental Figure 6.1 (see page 153): Overview of experimental results, summarizing the role of TG2 during microvascular remodeling. Exogenous, recombinant TG2 required a reducing agent to induce inward remodeling, which was accomplished by cross-linking. Only a cell-permeable reducing agent activates a pool of endogenous TG2 that can induce inward remodeling. Intracellular TG2 translocates to the cell surface and subsequently cross-links a number of proteins. This can be prevented with a site-specific inhibitor of TG2 or an NO donor. Exposure to calcium ionophore increases intracellular transglutaminase activity, which also can be counteracted with SNAP. In this case, however, inward remodeling is absent.

Supplemental Figure 6.2: Treatment with DTT did not significantly alter the vessel response to the thromboxane analogue U46619. After incubating vessels for 24 hrs with 2 mmol/L DTT, maximal contraction to U46619 was unchanged. Data are expressed as percentage of vessel diameter at full relaxation.
Supplemental Figure 6.3 (see page 155): Fibronectin and nidogen-1 are substrates for TG2 in DTT treated smooth muscle cells. TG2 substrates were labeled with BPA or Q-peptide and subsequently purified by streptavidin pull down. Control cells were treated with DTT only. (A) Coomassie staining of the total lysates and pull downs (* indicates streptavidin band). (B) Western blot stained with antibodies against fibronectin and nidogen-1 show specific labeling of both proteins with the lysine donor BPA. For fibronectin an additional high molecular weight product can be observed which is labeled with both the lysine and the glutamine donor substrate.
Supplemental Video 6.1: Visualization of TG2 activity by incorporation of AlexaFluor594/cadaverine over different layers of the blood vessel. TG2 activity is low in the intima and adventitia. Smooth muscle cells in the medial layer, aligned perpendicular to the vessel long axis, display strong membrane-bound TG2 activity. (shown are representative snapshots of the video at the intimal, medial and adventitial layer)

scalebar = 75 μm
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Redox state of Transglutaminase 2 controls arterial remodeling

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Chapter 7

General Discussion

Jeroen van den Akker
General Discussion

Although we previously showed that Transglutaminase 2 (TG2) mediates inward remodeling of small arteries, the underlying mechanisms remained elusive. In this thesis, we investigated which of the functions of this pleiotropic enzyme is involved, and how its activity is regulated. In addition, we studied which cell type provides the source of TG2, how TG2 is distributed over intra- and extracellular compartments and which TG2 enzymatic function is responsible for remodeling.

Overview of TG2 in inward vascular remodeling

Figure 7.1 summarizes the major findings on TG2-mediated small artery remodeling, as presented in this thesis. Remodeling starts when smooth muscle cells establish active vasoconstriction for a period of at least several hours. During this period, cell-mediated compaction may displace matrix proteins in an initial process of remodeling that may still be reversible upon vasodilation (Chapter 4). However, if vasoconstriction is maintained, gradual stabilization by transamidation of ECM substrates would occur as a result of the presence and activity of extracellular TG2. Such activity requires a reduction of cytosolic TG2, which triggers translocation to the cell membrane. Reduction and translocation may be facilitated by a relative lack of NO, as occurs under low flow and in hypertension and aging. Extracellular calcium then activates membrane-bound TG2 leading to transamidation of matrix proteins close to the cell membrane (Chapter 6). Such membrane-bound activity would be orchestrated by a complex of TG2, fibronectin and integrins, leading to cross-linking as well as movement of local matrix fibers. Alternatively, transamidation activity at the cell membrane aids in the formation of microparticles (Chapter 5). Possibly, MP secretion occurs preferentially at sites where certain integrins and membrane-bound fibronectin are present. TG2-enriched MPs may function to cross-link the more mature ECM substrates that are not in close proximity to the cell membrane, or act as messenger for other processes leading to cross-linking or synthesis of matrix proteins. In the next sections, the components in this schematic overview are discussed in detail.
General Discussion

Matrix compaction may support inward vascular remodeling

Inward vascular remodeling may be facilitated by cell-mediated compaction of matrix fibers. In previous research, our group had shown that macroscopic compaction of a collagen gel by SMCs was stimulated by exogenous TG2, and could be inhibited by the competitive TG2 substrate cadaverine. Similarly, Eftekhari and colleagues used cystamine to inhibit spontaneous gel compaction. However, the required effective concentration of TG2 and its competitive substrates was quite high. Thus, it remained questionable whether TG2 is indeed required for SMC-mediated tissue compaction. Therefore, in Chapter 4 we tested the hypothesis that SMCs are able to inwardly remodel tissue by TG2-mediated compaction. We showed that single cells displace matrix over a distance of at least 300 μm, but probably much more. We attribute this to cyclic movement of cell protrusions. One could have expected intracellular TG2 effects to play a role in such cellular dynamics and therefore in the matrix displacement. Thus, in Chapter 6, we
identified several cytoskeletal elements (actin, vimentin, both alpha and beta tubulin, and moesin) as cross-linking substrates in MOVAS cells. Moreover, TG2/eGFP constructs localized to the cell protrusions. However, the rate of compaction was not different between WT and TG2 KO cells. It should be said that this comparison was only made under very basic conditions, using synthetic cells in the absence of extrinsic stimuli or mechanical forces. In vessels, both vasoconstrictors and wall tension are known to increase intracellular calcium, possibly activating intracellular TG2 and changing protrusion dynamics and matrix displacement rate. Another possible explanation for the limited difference in matrix compaction may be that knock-out of TG2 is known to induce significant compensation mechanisms by other members of the Transglutaminase family.6

Matrix compaction by pairs and monolayers of cells unexpectedly occurred preferentially perpendicular to the SMC long axis. In Chapter 4 we indicated that, extrapolated to the intact vessel, this would maintain a tightly packed matrix and the development of axial stress while limiting the tendency for inward remodeling, since the latter requires a component of compaction along the SMC long axis. Clearly, this view needs further work.

Altogether, there is little evidence for a role of TG2 in matrix displacement resulting from cycling SMC protrusions. However, in chapter 4 it also became clear that matrix is not only displaced but also actually compacted at a distance from the cell. Moreover, this distant compaction was more reversible in matrix seeded with TG2 KO cells. Thus, these experiments provide evidence for stabilization and local compaction of collagen by released TG2.

**TG2 contributes to remodeling by transamidation**
We previously proposed in our “mold hypothesis” that TG2 contributes to remodeling by cross-linking. Thus, during a period of prolonged vasoconstriction, cross-linking of existing ECM components and synthesis of new elements would provide mechanical stabilization of the vessel at a smaller diameter. Using recombinant TG2 with a mutated active site, we confirmed that isolated vessels could be fixed at a certain diameter by transamidation. We then showed that the in vitro remodeling of vessels by endogenous TG2 was abolished by an active site inhibitor (Chapter 6).

Such experiments using mutated enzymes and active site inhibitors leave little doubt that indeed of all the possible actions of TG2, cross-linking is critical. However, remodeling requires actual displacement and not only cross-linking of the matrix. As indicated above, cyclic movement of cellular protrusions could be involved here. Another possibility, supported by the above distant compaction, is that transamidation of matrix proteins by itself induces matrix shrinkage. When polymers come into close proximity during deformation of a network, cross-linking can lead to local build-up of internal stress. This can cause a mutual pulling
of polymers to each other, as was demonstrated for the cross-linking of actin by filamin. In turn, these deformations may initiate cross-linking of other branches that approached each other. Together, these results support our “mold hypothesis” during inward remodeling where cross-linking is, at least in part, mediated by TG2.

Transamidation activity is controlled by redox balance
As any protein rich in sulfhydryl groups, TG2 is known to be sensitive to chemical modifications such as disulfide bridging and S-nitrosylation. Indeed, exogenous TG2 required a reducing agent, which reverses these sulfhydryl modifications, for sufficient activity leading to remodeling. Considering the fact that no remodeling occurred when this cell-impermeable reducing agent was used in the absence of exogenous TG2, we derived that the pool of extracellular TG2 must be extremely small. However, reduction of the intracellular pool did trigger TG2-dependent remodeling (Chapter 6). Although cells in the vascular wall are traditionally believed to possess a reducing intracellular environment, it has been shown that the redox balance is actually controlled at a subcellular level, and within a subcellular compartment the redox state of proteins can be individually regulated. We believe that the reduction required for TG2 activity must be regulated by a reducing enzyme with high binding affinity for TG2, possibly within a specific subcellular compartment. Indeed, when we incubated remodeled vessels with fluorescein maleimide, there was no obvious difference in the amount of extracellular sulfhydryl groups between control, inward and outward remodeled vessels (pilot, data not shown). In Chapter 6, we identified several reducing enzymes that are likely candidates to be involved in TG2 activation.

Nitric Oxide affects redox regulation of TG2 activity
Nitric Oxide (NO) is an important signaling molecule in the cardiovascular system. The release of endothelium-derived NO, which causes vasodilation, depends directly on the vascular shear stress level. In addition, nitric oxide in conjunction with ROS plays a key role in the activity regulation of redox-sensitive proteins. Lai et al. showed that TG2 activity can be inhibited by S-nitrosylation of only 1 or 2 out of the possible 18 cysteine groups in TG2. In addition to direct NO-effects, S-nitrosylation renders TG2 more sensitive to GTP inhibition.

In several papers, our group showed that NO plays an important role in the regulation of the direction of remodeling, possibly by inhibition of TG2 activity. In a cannulated vessel model, flow caused a NO-mediated dilation, which inhibited inward remodeling. However, after application of L-NNA, which blocks NO production, these vessels remodeled inwardly. Oppositely, the NO donor nitroprusside inhibited inward remodeling induced by exogenous TG2. In a subsequent paper, a similar NO synthase inhibitor was administered in a
hypertension model. In WT mice, this led to inward remodeling, which was impaired in TG2 KO mice. In Chapter 4 we demonstrated that application of the NO-donor SNAP inhibited the incorporation of a fluorescent TG2 substrate both after intra- and extracellular TG2 activation. Moreover, SNAP fully inhibited the TG2-dependent inward remodeling as induced by the reducing agent DTT. Similarly, Santhanam et al.\textsuperscript{18} administered the NOS-inhibitor L-NAME using an osmotic minipump, and observed increased TG2-mediated cross-linking.

S-nitrosylation/denitrosylation provides a dynamic system of TG2 (in)activation. Interestingly, NO production is impaired in several conditions where TG2 activity is high, such as low blood flow, hypertension, aging and atherosclerosis\textsuperscript{13}. Considering the high sensitivity to S-nitrosylation, it is well possible that even under conditions of low NO production, an active process of denitrosylation is required to induce TG2 activity. This may be accomplished by the enzymes involved in redox balance regulation, as discussed above.

**TG2 activity is mainly present in the medial ECM**

In Chapter 6, we demonstrated that inward remodeling of isolated vessels was associated with TG2 activity in the medial layer, as visualized by incorporation of a fluorescent TG2 substrate. This required the presence of a cell-permeable reducing agent. Incorporation of fluorescent cadaverine occurred especially at the smooth muscle cell membrane and was also elevated in a patchy pattern at the internal elastic lamina. While it may seem that such membrane-bound TG2 activity is much stronger than any activity of (MP-based) released TG2, the latter would lead to a rather diffuse staining that might not have been appreciated in the confocal studies that we performed.

A large array of potential extracellular targets is present near the SMC membrane. However, the pull-down assay, based on cultured SMCs under low reductive stimulation, revealed a small number of specific targets. Fibronectin, which is deposited in the early phase of matrix synthesis, was abundantly cross-linked by TG2\textsuperscript{19}. In addition, substrates such as collagen type I, fibulin-2 and nidogen-1 were identified. Possibly, association of TG2 with specific integrins adds to the specificity for which ECM substrates in the ECM are cross-linked\textsuperscript{4}.

In addition to the cross-linking activity of membrane-bound TG2, there is evidence that this enzyme is involved in fibronectin and collagen fibrillogenesis, a process that occurs at the cell surface for many ECM proteins\textsuperscript{20;21}. The TG2 active site mutant is equally potent as wild-type TG2 in this process, demonstrating that other actions than cross-linking are involved here\textsuperscript{22}. Since membrane-bound TG2 remains associated with fibronectin after polymerization, the fibronectin binding site seems to play a dominant role in this process\textsuperscript{22}. In this thesis we have not further investigated this process, but there may well exist an intimate relation
between formation of new fibrils and cross-linking of more mature ones. Such processes at the cell membrane deserve future attention.

In contrast to the high activity that we observed in specifically the media, several studies revealed a relatively low immunostaining for TG2-specific cross-links in this layer\textsuperscript{5;6;23;24}. This suggests that if transamidation in the media is indeed occurring during inward remodeling, this must be a transient mechanism of tissue stabilization. Possibly, a mechanism exists to break down the TG2 cross-link in order for tissue to be able to continuously adapt to changing mechanical demands. Interestingly, transamidation cross-links can be cleaved by TG2 or FXIII itself\textsuperscript{25}. However, so far the in vivo activators for TG2-catalyzed hydrolysis remain unknown. Contrary to a possible dynamic turnover of TG2 cross-links in the media, it was recently shown that over time TG2 cross-links accumulate in the adventitia (Figure 7.2)\textsuperscript{18}. With aging, the NO supply to adventitial TG2 becomes partly insufficient, causing transamidation and thereby vessel stiffening. These results together present a picture where TG2 affects short-term remodeling by transamidation in the media, while long-term remodeling appears predominantly in the adventitia.

![Transglutaminase 2 and Cross-link](image)

*Figure 7.2: Immunohistochemical localization of TG2 protein and the specific TG2 cross-link in mouse aortic tissue as prepared by Santhanam et al.\textsuperscript{18}: TG-specific cross-links are higher in old compared to young rat aorta, expression is unchanged.*
TG2 is transported to the ECM via microparticles

The passive diameter of blood vessels at higher pressures is believed to be dominated by mature collagen type I and III fibers. A large fraction of these mature fibers are not in close contact with the cell membrane. Their cross-linking and remodeling thus seem to depend on secreted rather than membrane-bound TG2. Our collagen type I gel experiments have indicated that distant compaction indeed can occur. Since TG2 does not possess a signal sequence to the ER/Golgi, TG2 secretion must be alternatively regulated. We hypothesized that TG2 secretion occurs via the formation of microparticles (MPs).

Currently, little is known about the mechanisms of MP formation and secretion. In a recent review Burnier et al. discriminated between 2 mechanisms of MP formation. Ectosomes are formed via outward blebbing of the cell membrane. Proteins that were originally at the inside of the cell membrane may end up at the outside of the MP after flipping of membrane phospholipids. Alternatively, endosomes result from inward blebbing, which causes intracellular membrane proteins to end up at the outside of the MP. Multiple endosomes are then collected in a multi-vesicular body before they are released from the cell as exosomes. Considering the large size of MPs (or multi-vesicular bodies!) enriched in TG2/eGFP that we observed by immunofluorescent microscopy (Chapter 5), this would speculatively plead for a secretion of TG2 via the endosome-exosome pathway.

In addition to being transported by MPs, TG2 may play an active role in MP formation. Indeed, in Chapter 5 we demonstrated that transamidation activity stimulated MP formation. Cytoskeletal elements undoubtedly play a role in MP formation, notably via the endosome/exosome mechanism, and as indicated above many of these elements are targets of TG2. We tested whether reducing agents activate MP formation via activation of TG2. However, stimulation of TG2/eGFP-transfected smooth muscle cells with the reducing agent DTT did not increase the fraction of eGFP-positive microparticles, nor did it increase the absolute number of MPs (pilot, data not shown). These preliminary data suggest that reduction of intracellular TG2 by itself is not sufficient to stimulate TG2 release via increased MP production. Possibly, the release into the matrix depends on interaction with extracellular fibronectin, since it is previously shown that the fibronectin-binding site in TG2 is indispensible for TG2 secretion. This would also help to explain the high level of cross-linking that we observed in fibronectin (Chapter 6). Moreover, secretion may occur in conjunction with integrins \( \alpha_5\beta_1 \) and \( \alpha_v\beta_3 \) that frequently colocalize with concentrated spots of TG2.

Altogether, TG2 appears to be secreted into the extracellular space via microparticles. We suggest that an initial reduction step is required for translocation to the cell membrane and cross-linking activity for formation of MPs. MPs may transport TG2 over long distances, but also increase stability of
extracellular TG2, which is a known target for proteases such as MMP-2. Based on the current data, we speculate that transamidation of maturated extracellular substrates occurs via MPs enriched in TG2. This may be the case not only for proteins in the media, but for the adventitia as well, considering the massive presence of collagen and sparse cellular density.

**Unresolved issues and recommendations for future direction**

The work in this thesis addressed the role of TG2 in inward vascular remodeling, focusing on issues such as the cellular secretion and extracellular activity. Thus, the role of TG2 in vascular events such as atherosclerosis, vessel permeability and angiogenesis was not considered. In addition, other factors influencing remodeling, such as the facilitation by inflammation, are beyond the scope of this thesis. This section discusses several issues that remained unresolved and includes some recommendations for future research.

Since inward remodeling is dependent on both vessel tone and TG2, the large pool of TG2 in smooth muscle cells is likely to be involved. We previously showed that prolonged vasoconstriction, but not passive collapse to a small diameter, is necessary to induce inward remodeling. An attractive possibility is that tone activates TG2, either by affecting the redox state, the secretion of MPs or otherwise. The other way around, it has been speculated that TG2 directly or indirectly stimulates the level of tone. TG2 is known to play a role in the signal transduction pathway activated by α1-adrenoreceptors. However, when we tested α1-adrenergic signaling in mesenteric arteries by measuring the contractile response to phenylephrine, this showed no differences between vessels obtained from wild-type or TG2 KO mice. Therefore, we preliminary concluded that TG2 does not mediate inward remodeling of these small arteries by stimulating α1-adrenergic signaling. In addition, TG2 can stimulate vasoconstriction through the RhoA/ROCK-2 cascade. TG2 can trigger RhoA signaling either via transamidation to serotonin or various other polyamines, or via integrin clustering when cells adhere to substrates like FN via membrane-bound TG2. Indeed, maximal constriction of aortic vessel segments upon either high potassium or serotonin stimulation was significantly inhibited both by cystamine and dansylcadaverine. In addition, angiotensin-II mediated constriction of mesenteric arterioles was inhibited by these TG2 competitive substrates. Therefore, the question whether TG2 actively contributes to vessel tone remains to be determined. This is especially of relevance since our group previously showed that Rho kinase was required to maintain a basal tone level in isolated mesenteric arteries.

In addition to a role for TG2 in SMC contractility, it needs to be tested whether contractile SMCs are able to compact matrices perpendicular to their long axis orientation, just as the synthetic SMCs in Chapter 4. TG2 may play a more
pronounced role in compaction in these contractile cells, because they might accommodate the required calcium influx for transamidation of cell-protrusion elements. These experiments should also be performed in models that more closely resemble the vessel wall.

Although we showed that reduction of TG2 is a critical step for activation (Chapter 6), the nature of the group to be reduced remains to be determined. This can be a thiol modified by either disulfide bridging, S-nitrosylation or S-glutathiolation. Immuno electron microscopy of thiols and TG2 could form an approach here. In addition, the physiological enzyme responsible for reduction of TG2 deserves further attention. Here, the list of candidates retrieved from our gene array approach can function as a starting point.

The in vivo substrates for transamidation need to be identified further. In particular, our pull-down results need to be confirmed at the vessel level during in vivo or in vitro remodeling. The single cell compaction setup presented in Chapters 3-4, combined with fluorescent TG2, could then be used to study the interaction of TG2 with such substrates. Moreover, the local mechanical effect of transamidation of matrix proteins can be assessed with active microrheology using our optical tweezer setup. In such a system, a particle is manipulated at the nano scale with an optical trap. The visco-elastic properties of the surrounding matrix are then derived from the dynamic displacements nearby trapped particles using laser interferometry.

While the concept of tissue stabilization by transamidation is widely accepted, it remains to be determined how these cross-links can be broken down in vivo. This is indispensable in order to achieve a dynamic system that retains its capability for tissue remodeling over time.

Further insight into the mechanism of TG2 secretion is critical, since knowledge on the nature of this pathway facilitates research on the triggers for TG2 activation and externalization. Chapter 5 showed that after transfection of TG2/eGFP into SMCs, the eGFP signal could be detected in microparticles. However, the undisputable presence of TG2 in these MPs remains to be proven. Unfortunately, preliminary attempts with antibodies in both western blots and flow cytometry were unsuccessful. An indication for an active contribution of TG2 in MP formation can be obtained by comparing the MPs secreted from WT and TG2 KO erythrocytes, where compensation by other TG family members is limited. In addition, application of cell-permeable inhibitors of transamidation is expected to decrease the number of secreted MPs. Similarly, MP formation is likely to be inhibited upon increasing the level of intracellular NO, thereby providing a possible physiological switch. If TG2 is indeed secreted via MPs, the question remains whether this TG2 is inactive inside the MP, or active at the MP membrane. This should be tested in vitro under conditions that leave the MP integrity intact.
Clinical Implications

Since TG2 is an ubiquitously expressed enzyme, a simple inhibition of its cross-linking activity in an attempt to improve small artery caliber in hypertension and other cardiovascular diseases will not be without side effects. This is especially the case since most, if not all, current active site inhibitors also act on the coagulation Factor XIII due to the high sequence homology with TG2$^{47}$. In addition, TG2 is not necessarily a ‘bad’ enzyme for the vascular system. Our studies point at a role in the maintenance of a normal organization, while also plaque stability may depend on transamidation$^{48}$. Any attempt for the therapeutic interference with TG2-related processes therefore needs to attack specific cells and conditions. The inhibiting role of NO on transamidation would provide a starting point$^{18}$. In addition, interfering with specific redox reactions and with MP formation could have therapeutic potential. However, clearly, much more basal research will be needed for this step can be taken.

We studied very fundamental processes related to matrix organization in the context of small artery remodeling. It seems very likely that such processes are of relevance for a wide range of other cardiovascular and non-cardiovascular pathologies. Thus, the redox dependence, microparticle formation, membrane bound and distal effects that we described may be involved in the role of TG2 in vessel stiffening associated with aging, stabilization of atherosclerotic plaques or vascular injury, and calcification. Moreover, extracellular transamidation affects a number of other pathologies, among which are several types of cancer and neurodegenerative diseases$^{47}$.

Conclusions

We found Transglutaminase 2 to be an intriguing enzyme, whose role in vascular remodeling is only beginning to emerge. This enzyme is not easily giving away its secrets, and the current thesis can only be a small step. The new mechanisms that we identified warrant further research and should, in the future, provide new therapeutic tools for hypertension and ischemic diseases, as well as for a range of other pathologies.
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Appendix
Summary

Adequate local organ perfusion depends on the diameter of small arteries and arterioles. During several pathological conditions, such as hypertension and flow reduction, vessels remodel inwardly. This change in vessel caliber increases the resistance for blood flow. As a result, upstream pressure rises and the flow capacity for downstream tissue decreases. We previously showed that inward remodeling depends on the enzyme Transglutaminase 2 (TG2). The aim of this thesis was to elucidate the activation, localization and activity of TG2 in vascular remodeling.

The first Chapter introduces several basic concepts in vascular anatomy and inward remodeling. Then the key characteristics of TG2, as well as its role in the vasculature, are presented. In addition, the clinical relevance of inward remodeling is illustrated.

Chapter 2 reviews in detail how the individual components of the vessel wall together determine both the passive and active mechanical properties. Then the role of tone, pressure and inflammation in small artery remodeling are discussed. Several in vitro techniques, which are employed frequently in Chapter 6, are explained.

The third Chapter presents an image analysis method that enables the assessment of 2-D deformations in a biological tissue. Based on the type of images used in Chapter 4, we demonstrate that this method performs better as regards to matrix shrinking/expansion, large displacements and artificial noise as compared to standard cross-correlation techniques. Using this technique, we show in Chapter 4 that individual smooth muscle cells (SMCs) induce local matrix compaction up to a distance of 200 μm. TG2 did not enhance compaction, but limited the reversal of compaction upon cytoskeletal disruption. This was especially evident at a distance between 250-300 μm, suggesting that TG2 was secreted from the cells into the matrix. At increasing cell density, cells cooperated to establish compaction. In a ring-shaped collagen matrix, this resulted in preferential displacement in the radial direction, perpendicular to the cellular long axis.

Chapter 5 investigates the hypothesis that TG2 is secreted from SMCs via microparticles (MPs). We could detect the fluorescence of TG2/eGFP in MPs isolated from SMC cultures. Using two site mutations in TG2, we showed that this strongly depended on TG2 cross-linking, indicating a possible functional role for TG2 in the formation of MPs. We believe that TG2-enriched MPs may be important in the cross-linking of matrix proteins as observed in Chapters 4 and 6.

The sixth Chapter studies the localization and activation of TG2 that is required for inward remodeling. We demonstrate that cross-linking of the extracellular matrix results from a reduction or denitrosylation of intracellular TG2. This was investigated in vitro with a non-specific reducing agent. We selected
several candidate enzymes, from a microarray database obtained from remodeled vessels, that could function as physiological redox activator of TG2. In addition, mass spectrometry identified a few TG2 substrates that may be subjected to cross-linking within the medial vessel layer, as seen in inward remodeling.

Chapter 7, the General Discussion, puts the findings of this thesis together in a coherent view. Moreover, the implications of these findings for small artery remodeling are discussed. Several important questions that remain to be answered are given, as well as recommendations for future investigations on the role of TG2 in the vasculature.
**Samenvatting**

De lokale doorbloeding van weefsel en organen hangt af van de diameter van de kleine arteriën en arteriolen. Bij verschillende pathologiën, zoals een verhoogde bloeddruk of verminderde doorbloeding, remodelleren vaten inwaarts naar een kleinere diameter. Hierbij neemt de weerstand voor de doorbloeding toe. Als gevolg hiervan stijgt de druk stroomopwaarts, terwijl de capaciteit voor doorbloeding van weefsel stroomafwaarts daalt. In eerder onderzoek hebben we reeds aangetoond dat inwaartse remodellering afhangt van het enzyme Transglutaminase 2 (TG2). Het doel van dit proefschrift was om meer inzicht te verkrijgen in de activatie, lokalisatie en activiteit van TG2 tijdens vasculaire remodellering.

Het eerste hoofdstuk introduceert verschillende basale concepten in vasculaire anatomie en inwaartse remodellering. Ook worden de belangrijkste eigenschappen van TG2, alsmede de rol van TG2 in het vasculaire systeem, besproken. Bovendien wordt de klinische relevantie van inwaartse remodellering toegelicht.

Hoofdstuk 2 behandelt in detail hoe de individuele componenten van de vaatwand samen de passieve en actieve mechanische vaateigenschappen bepalen. Vervolgens wordt de rol van tonus, druk en inflammatie tijdens de remodellering van kleine vaten besproken. Verscheidene in vitro technieken, die met name in hoofdstuk 6 veelvuldig worden toegepast, worden hier nader toegelicht.

Het derde hoofdstuk beschrijft een beeldanalyse methode die gebruikt kan worden om de 2-dimensionale verplaatsingsvelden in biologisch weefsel te bepalen. Aan de hand van beelden zoals later gebruikt in Hoofdstuk 4 tonen we aan dat deze methode significant beter werkt dan standaard cross-correlatie technieken als het gaat om lokale krimp of rek in de matrix, grote verplaatsingen en kunstmatig toegevoegde ruis. Deze techniek wordt vervolgens in Hoofdstuk 4 gebruikt om te laten zien dat individuele gladdespierscellen lokale krimp in een collageen matrix kunnen veroorzaken tot op een afstand van 200 μm. TG2 heeft geen actieve bijdrage aan deze krimp fase, maar beperkt wel het terugveren van de matrix nadat het cellulaire cytoskelet vernietigd is. Dit effect was het sterkst zichtbaar op een afstand van 250-300 μm, wat erop duidt dat TG2 door cellen wordt uitgescheiden, waarna het actief is in de extracellulaire matrix. Als we het aantal spiercellen in een dergelijke matrix verhogen, werken de cellen in synergie samen aan matrix compactie. In een ring-vormige matrix resulteert dit in een verplaatsing met een radiale voorkeursrichting, loodrecht op de lange as van deze cellen.

Hoofdstuk 5 bestudeert de hypothese dat gladde spiercellen TG2 uitscheiden via micropartikels. Als spiercellen TG2 produceren waaraan een fluorescerende groep is toegevoegd, kan dit signaal worden gemeten in de
micropartikels die deze cellen produceren. Met behulp van twee verschillende mutaties in het TG2 gen, hebben we aangetoond dat dit sterk afhankelijk is van de cross-linking activiteit van TG2. Dit zou erop kunnen duiden dat TG2 een actieve rol speelt in de productie van deze micropartikels. Wij geloven dat zulke micropartikels bijdragen aan de cross-linking van eiwitten in de extracellulaire matrix (Hoofdstuk 4 en 6).

Het zesde hoofdstuk is gericht op de lokalisatie en activiteit van TG2 tijdens inwaartse remodellering van intakte bloedvaten. We laten hier zien dat reductie of denitrosylatie van intracellulair TG2 leidt tot cross-linking van de extracellulaire matrix. Hiervoor is in vitro een niet-fysiologische, chemische stimulus gebruikt. Vervolgens hebben we uit een gen-expressie database, verkregen van geremodelleerde vaten, verschillende kandidaat enzymen geselecteerd die wel als fysiologische activator van TG2 zouden kunnen optreden. Hiernaast hebben we met behulp van zogenaamde massa spectrometry een aantal substraten voor TG2 cross-linking geïdentificeerd die van belang kunnen zijn voor vasculaire remodellering.

Hoofdstuk 7, de Algemene Discussie, plaatst de bevindingen van dit proefschrift in een samenhangende context. Bovendien worden de implicaties hiervan voor de remodellering van kleine vaten bediscussieerd. Verschillende belangrijke vragen die onbeantwoord blijven worden kort toegelicht. Vervolgens wordt afgesloten met enkele aanbevelingen voor vervolgonderzoek naar de rol van TG2 in het vasculaire systeem.
Curriculum Vitae

Jeroen van den Akker werd op 15 augustus 1980 te Utrecht geboren. In 1998 behaalde hij zijn VWO diploma aan het Utrecht Stedelijk Gymnasium. Aansluitend studeerde hij Biomedische Technologie aan de Technische Universiteit Eindhoven. Onderdeel hiervan vormde zijn stage aan de University of California te San Diego, waar hij onderzoek deed naar de electromechanische activatie van het hart. Zijn afstudeerwerk betrof de realisatie en validatie van een experimentele perfusie opstelling waarin een vers geïsoleerd hart in conditie gehouden kan worden voor het testen van cardiovasculaire therapieën en apparaten. Zijn MSc diploma werd behaald met het predikaat “hoge waardering”. Vervolgens zette hij zijn academische carrière voort aan de afdeling Biomedical Engineering and Physics van het Academisch Medisch Centrum te Amsterdam. Dit promotie-project, gefinancierd door de Nederlandse Hartstichting, richtte zich op de rol van het enzym Transglutaminase 2 tijdens de remodellering van kleine bloedvaten. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Naast zijn wetenschappelijke opleiding en carrière is Jeroen actief geweest als denksporter (dammen). Dit leidde onder andere tot de mondiale jeugdtitel, het landskampioenschap voor teams, een geslaagde wereldrecordpoging simultaan-dammen en de 12e plaats op het WK algemeen.
Dankwoord

Elke promotie is een beproeving. Vol goede moed start men aan een wetenschappelijke uitdaging. Maar op enig moment blijkt dan immer dat er meer onbekenden dan vergelijkingen zijn. Hier wil ik graag de constanten bedanken die hebben bijgedragen aan de oplossing (dit proefschrift).

Allereerst mijn promotor, Ed van Bavel: je bent een onuitputtelijke bron van goede, en soms minder goede, ideeën. Biologische paden, experimentele opstellingen en mathematische modellen. Er was altijd meer mogelijk dan haalbaar (zie stelling 10). In dit onbegrensde speelveld leerde je mij om een balans te vinden tussen creatief, kritisch, eigenwijs, analytisch en pragmatisch. Kortom: je maakte een echte wetenschapper van me!

Ten tweede mijn copromotor, Erik Bakker: de rol van copromotor is je op het lijf geschreven. Op jouw kenmerkende, bescheiden wijze kon je laaiend enthousiast raken over vasculaire experimenten. Je wist me te interesseren voor 2 disciplines die ik niet voor mogelijk had gehouden: celbiologie en hardlopen. Beide werden steeds belangrijker naarmate mijn promotie vorderde. In het bijzonder in hoofdstuk 6 is je praktische bijdrage van groot belang geweest.

Hanke: vier jaar lang was jij mijn onmisbare kamergenote. Natuurlijk was transglutaminase onze verbindende factor en substraat van eindeloze discussies. Je luisterend oor en praktische oplossingen waren echter minstens even waardevol. De alledaagse gezelligheid en onze onderonsjes zal ik zeker gaan missen. De keuze om elkaar als paranimf bij te staan, behoefde niet eens hardop uitgesproken te worden.

Adrian: als mijn voorganger zul je jouw geest kunnen herkennen in mijn werk. Hoewel sommige van onze opvattingen uit onze gezamenlijke periode inmiddels achterhaald (b)lijken, heb je mij in korte tijd veel geleerd. De studenten die ik tijdens mijn promotietijd mocht begeleiden (Aad, Maurits, Babette, Guus en Roy) wil ik hartelijk danken voor hun enthousiasme en hulp aan mijn project.

De dames van de Carvas groep hebben mijn leven verrijkt. De praktische hulp van onze charmante analisten had ik niet kunnen missen. Judith, moeder van de groep: soms zeer streng, soms erg meelevend! Angela: het was geen makkelijk avontuur, wel zeer leerzaam! Cristina: nos rendez-vous ont été instructif aussi bien que agréable. Ook de overige Carvas mensen wil ik danken voor waardevolle discussies en gezelligheid tijdens lunches, meetings, congressen en labuitjes.

Een van de gedachten achter de integratie van het lasercentrum met de Medische Fysica was meer interdisciplinaire kruisbestuiving. Ik ben er dan ook trots op dat de samenwerking met Edwin leidde tot Hoofdstuk 5 van dit proefschrift. Bovendien bracht Edwin’s opgewekte verschijning ons menig gratis muffin en brownie. Roy: de dagelijkse koffiemomenten waren tijdens de laatste maanden mijn dagelijkse oplaadmoment, terwijl de zondagse hardlooppessies in het
Amsterdamse Bos alle stress hielpen vergeten. Ze zeggen wel dat in de marathon de mannen van de jongens worden gescheiden, en wie ben ik om dat te ontkennen? Het hoofd van Martijn doet je denken aan een kind: vrolijk en ondeugend. Je was altijd bereid om mee te denken, en op zijn minst in staat om het probleem te relativeren. Nienke, buurvrouw, ‘t was immer een genoegen om jou tegen het lijf te lopen. Gezelligheid kent geen tijd, en dat blijft zo. Als Barbara kwam binnenwaaien, was er spontaan tijd voor een kletsmomentje. Ook denk ik met veel genoegen terug aan verschillende feestjes en etentjes, waar de belangrijke zaken des levens werden besproken. Annemarie bracht altijd leven in de brouwerij, met platte praat of mooie muziek. Ik denk dat ons zichtbare geploeter, slechts onderbroken door een snelle hap in de AMC kantine, een wederzijdse steun was de laatste maanden. Het was dan ook een passend einde om samen een afscheidsborrel te geven. Ook de overige collega’s van L0 wil ik natuurlijk danken voor een geweldige tijd in het AMC. Hierbij mag Jetty niet onvermeld blijven: altijd paraat met raad en daad! Dank ook aan Jelmer en Robert voor de technische ondersteuning, o.a. bij onze LED-aventuren. Daarnaast heb ik met veel genoegen samengewerkt met collega wetenschappers van binnen en buiten het AMC: Gijs Afink en Carrie Ris-Stalpers (AMC/reproductieve biologie), Rienk Nieuwland, Anita Böing en Anita Grootemaat (AMC/laboratorium voor experimentele & klinische chemie), Jan van Marle, Jan Stap, Ron Hoebe en Eric Reits (AMC/Celbiologie & histologie), Wilbert Boelens en Remon van Geel (Radbout Universiteit Nijmegen) en Jo de Mey (Universiteit Maastricht).

Robert: ik ben vereerd dat je als mijn paranimf wilt optreden. Het feit dat jouw verhuizing naar Chicago onze vriendschap niet in de weg is gaan staan, zegt eigenlijk al genoeg. Max: de trouwe vriend waarop ik altijd kan rekenen. Ik kijk er naar uit om onze (sportieve) avonturen in binnen- en buitenland nog lang door te zetten. Aan al mijn overige vrienden en vriendinnen: om jullie bescheidenheid niet in verlegenheid te brengen, zal ik geen namen noemen. Ik ben bijzonder blij met de persoonlijke band met ieder van jullie.

Het is een cliché om je (schoon)ouders en familie te bedanken voor hun onvoorwaardelijke steun. Dank jullie wel dat jullie me zo af en toe weer met beide benenjes op de grond weten te zetten.

Corianne, moppie: jij bent met afstand het mooiste resultaat uit mijn promotietijd! Het vooruitzicht op onze reis door het Midden-Oosten was een uitstekende motivator voor mijn zware, laatste loodjes. Het vooruitzicht op een leven lang samen is onbeschrijfelijk.
