Inflammation and transglutaminases in vascular remodeling and atherosclerosis
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ROLE OF TRANSGLUTAMINASES IN CUFF-INDUCED Atherosclerotic Lesion Formation in Femoral Arteries of ApoE3 Leiden Mice

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Background
Transglutaminases play an important role in vascular remodeling, calcification, cell adhesion and endothelial barrier function. In this study we investigate the influence of combined inhibition of both tissue type transglutaminase (TG2) and the plasma transglutaminase FXIIIA on early lesion development.

Methods
A cuff was placed around the femoral arteries of ApoE3Leiden mice while fed a Western type diet to induce atherosclerotic lesion development. An osmotic minipump was placed in the intraperitoneal cavity containing an irreversible inhibitor of TG2 and FXIIIA activity ((1,3,4,5-Tetramethyl-2-[(2-oxopropyl)thio] imidazolium chloride, Zedira). Atherosclerotic lesion composition was analyzed using immunohistochemistry and RT-PCR.

Results
Inhibition of transglutaminases did not influence lesion size or geometric remodeling of the vessels. However, systemic transglutaminase inhibition resulted in 41% less macrophage infiltrate in the media of the vessels. Additional in-vitro experiments on HL60 cells confirmed a decreased migratory response during transglutaminase inhibition.

Conclusion
Inhibition of TG2 and FXIIIA during early development of lesions reduced the macrophage content in the media of atherosclerotic vessels, while not affecting lesion size or geometric remodeling.
INTRODUCTION

Atherosclerosis is a multi-factorial disease in which endothelial damage, inflammation, lipid accumulation and thrombotic events play a role. Recent work shows that transglutaminases are involved in several processes relevant for atherosclerosis [1-3]. Transglutaminases form a class of enzymes with pleiotropic function that consists of 9 known members [4]. Type 1 transglutaminase (TG1), type 2 or tissue-type transglutaminase (TG2) and Factor XIII A (FXIII A) are expressed in the vessel wall [5]. TG1 is associated with endothelial barrier function [6]. TG2 is more widely expressed and particularly abundant in endothelial cells. Its expression is elevated in endothelial cells exposed to turbulent shear stress [7], or after stimulation with thrombin [8]. Monocytes/macrophages express both TG2 and FXIII A. Data show that these transglutaminases influence macrophage adhesion [9], motility [10,11], phagocytosis [2] and the cross-linking of angiotensin II receptors on monocytes [1]. Transplantation of TG2 knock-out bone marrow revealed that macrophage TG2 is involved in atherosclerotic lesion formation in LDLR knock-out mice [2]. Furthermore, lack of TG2 influences atherosclerotic plaque development in a ApoE-/- model [3]. In a later stage of atherosclerosis, transglutaminases TG2 and FXIII A are associated with calcified areas within human carotid atherosclerotic plaques [12].

Based on the studies mentioned above, we hypothesized that transglutaminases play a role in atherosclerotic lesion development in ApoE3Leiden mice. More specifically, we speculate that the cross-linking activity of transglutaminases stabilizes the atherosclerotic lesion and reduce its development. We previously reported that TG2 knock-out mice display impaired vascular remodeling [13-15]. However, TG2 knock-out mice were found to partially overcome the absence of TG2 by a compensatory action of FXIII A [14]. Others also found that TG2 knock-out mice have a normal musculo-skeletal phenotype due to a compensatory role of FXIII A [16]. In the present study we therefore used a combined inhibitor of TG2 and FXIII A, and studied its effect on gene expression, lesion size and composition in a model of cuff-induced lesion formation in ApoE3Leiden mice.
MATERIALS AND METHODS

**Mice and diet**

Male ApoE3Leiden [17] mice were used in the experiments at the age of 12-15 weeks. Mice were fed standard chow until the start of the experiment. From that moment on they were fed a high fat diet containing 1% cholesterol (4021.36, Arie Blok BV, the Netherlands). The high fat diet was given for 3 weeks prior to the surgical procedure and continued until the end of the experimental period (a subsequent 2 weeks). All experiments were approved by the local committee for animal experiments.

**Experimental procedures**

Three weeks after the start of the high-fat diet mice were anesthetized with a mixture of Midazolam (Roche, 5mg/kg), Medetomidine (Orion, 0.5 mg/kg) and Fentanyl (Janssen, 0.05 mg/kg), given by i.p. injection. The left and right femoral artery were dissected from the surrounding tissue, and injured through a series of pinches with a fine forceps. After this, non-constrictive polyethylene cuffs were placed around the femoral arteries (Portex, 0.4 mm inner diameter; 0.8 mm outer diameter, 2 mm length) [18]. In n=6 mice additionally to the cuff placement an osmotic mini-pump was placed in the intraperitoneal cavity (Alzet, model 1004) containing 0.1 M L682.777 (1,3,4,5-Tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride, Zedira) dissolved in 100 µL PBS containing 9% DMSO), a specific inhibitor of both TG2 and FXIIIA [19].

**Measurement of in-vitro and in-situ TG-activity**

To determine the inhibitory effect of the transglutaminase blocker in-vitro, femoral arteries of C57BL/6 mice were incubated with Alexa Fluor-cadaverine. Femoral arteries (n=4) were incubated in Leibovitz culture medium (Gibco) containing 10% fetal calf serum and 2mM dithiothreitol in the absence and presence of TG inhibitor (10^{-5}, 10^{-6} and 10^{-7} M) and Alexa Fluor cadaverine (10^{-4} M) for 24 hours at 37°C. Incorporation of Alexa Fluor-cadaverine was visualized using confocal microscopy (Leica) at 594 nm. For every artery, at least three separate fields of view were
analyzed. ImageJ was used to quantify the intensity of the incorporated Alexa Fluor-cadaverine, relative to the area of the vessel. Nuclei were stained with DAPI (Vectashield mounting medium, VECTOR Laboratories).

The effect of the blocker on transglutaminase activity in-vitro was measured using a commercially available colorimetric assay [20] (Covalab). In this assay, biotin-cadaverine was incorporated into immobilized substrate on the bottom of 96-wells plate by guinea-pig transglutaminase (gpTG) for 1 hour at 37 °C in the presence or absence of transglutaminase inhibitor. After incubation, wells were washed three times with TBS buffer (pH 7.5) containing 0.1% Tween. Wells were then incubated with streptavidin-labelled peroxidase, which was revealed using H₂O₂ as HRP substrate and tetramethyl benzidine as chromogen. Reaction was stopped by adding H₂SO₄ and the absorbance was read at 450 nm on a microplate reader [20].

The stability of the inhibitor was tested by comparing the inhibitory effect of fresh inhibitor (10⁻⁵ M) versus inhibitor (10⁻⁵ M) that was incubated at 37 °C for 9 days. This showed excellent stability of the compound (78% and 80% TG inhibition when using freshly prepared TG inhibitor and TG inhibitor that was incubated at 37 °C for 9 days respectively).

To investigate the inhibitory effect of the transglutaminase blocker during the in-vivo experiments, unfixed cryostat sections of liver from control mice (n=6) and mice that received the inhibitor (n=5) were incubated with 10⁻⁴ M FITC-cadaverine (AnaSpec) for 1 hour at 37°C in the presence of 10 mM CaCl₂. Incubation with 20 mM EDTA was used as negative control to establish background signal. Incorporation of FITC-cadaverine was visualized using confocal microscopy (Leica) at 496 nm. For every section, two separate fields of view were analyzed. To quantify the FITC-cadaverine incorporation, Matlab software (The Mathworks) was used to determine the median intensity of the FITC signal above background for each section.

**Endothelial function**

To determine endothelial function, segments of the femoral artery of C57BL/6 mice (n=4) were isolated and mounted in a wire myograph (Danish Myo Technology). Arteries were pre-contracted with 30 mM potassium, after which a dose-response relationship was determined for methacholine (10⁻⁹ M – 10⁻⁵ M). Arterial segments were then incubated with the TG inhibitor (10⁻⁵ M) for 1 hour at 37°C after which responses to potassium and methacholine were repeated in the presence of the TG inhibitor (10⁻⁵ M).
Plasma cholesterol and triglyceride levels

Blood samples were taken under general anesthesia from the vena cava at the time of euthanization. Total plasma cholesterol and triglycerides was determined enzymatically using a commercially available kit (bioMerieux).

Immunohistochemical analysis of lesions

Two weeks after cuff placement, the animals were sacrificed. From each animal the right femoral artery was dissected, fixed in 4% formalin and embedded in paraffin for immunohistochemistry. Equally spaced serial cross-sections (5 μm thick, every 200 μm) were stained with hematoxylin and eosin, picrosirius red to detect collagen, for TG2 (TG2-ab-4 1:10, Labvision), α-actin to detect smooth muscle cells (1A4 1:500, Dako) and FXIIIA (FXIIIA Ab-2, Labvision). Cross-sections were also analyzed for the appearance of macrophages (Mac3, concentration 1:30, Pharmingen) and the product of transglutaminase activity (Ne(y-L-glutamyl)-L-lysine isopeptide cross-link, concentration 1:250, Covalab). Sections were de-paraffinized followed by antigen retrieval by boiling sections in 0.01 M citrate buffer. Primary antibodies were incubated for 1 hour at room temperature (Mac3, 1A4) or overnight at 4°C (TG2, FXIIIA, cross-link) followed by 30 minutes incubation at room temperature with the appropriate HRP conjugated secondary antibodies. Antibodies were visualized with DAB for Mac-3 and 1A4 staining (Sigma Aldrich) or AEC for TG2, FXIIIA and cross-link staining (Sigma Aldrich). Sections were counterstained with hematoxylin. Omission of primary antibodies was used as negative control. BioPix IQ software (BioPix) was used for quantification of positive staining.

RT-PCR

After sacrifice the left femoral artery was dissected and immerged in Tri-reagent (Sigma Aldrich) and stored in -80 °C for RNA extraction. cDNAs were subsequently synthesized using an Omniscript reverse transcriptase kit (Qiagen). Quantitative real-time PCR was performed in iQTM SYBR® Green Supermix buffer (Invitrogen) using a MyIQ (Biorad) thermal cycler. Primers for mouse CD68 were designed using Beacon software. Primer sequence CD68: FW: 5’- GGACTACATGGCGGTGGAATAC -3’ and RV: 5’-GAGAGCAGGTCAGGTGAACAG-3’.
Gene expression of 84 atherosclerosis related genes was measured in pooled samples of the cuffed femoral arteries using a RT-PCR gene array (SABiosciences). This array contains genes involved in several processes relevant for atherosclerotic development such as blood coagulation, cell growth and proliferation and cell-adhesion (see Supplemental Table 1 for gene table). Gene array data were analyzed using the accompanying software (SABiosciences).

The mRNA expression of TG2 and FXIIIA was determined in human promyelocytic leukemia cells (HL60). Cells were washed with ice-cold PBS before administration of Tri-reagent for RNA extraction. cDNA was synthesized using a Transcriptor first strand cDNA synthesis kit (Roche). Primers for human P0-ribosomal protein (RPLP0), TG2 and FXIIIA were acquired from SABiosciences.

**In-vitro migration assay**

HL60 cells were cultured in IMDM culture medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Gibco). HL60 cells were differentiated into macrophages by culturing the cells in the presence of phorbol 12-myristate 13-acetate (PMA, concentration 10^{-8} M, Sigma) for the duration of the experiment. Migration assays were performed using Transwell Boyden chambers (Corning) with a polycarbonate filter (8 μm pores) placed between the upper and lower chamber. HL60 cells (2.5*10^4 cells in culture medium supplemented with 10^{-8} M PMA) were placed in the upper chamber and allowed to migrate to the lower chamber containing culture medium with angiotensin II (10^{-8} M) and PMA (10^{-8} M). To block transglutaminase activity, the transglutaminase inhibitor (10^{-5}M) was added to the upper chamber. After 24 hours, images in five separate fields per well were made from migrated cells adhering to the lower chamber. Cells were counted automatically with ImageJ software.

**Statistical analysis**

Data are expressed as mean ± SEM and are compared by Student’s t-test or one-way ANOVA followed by Dunnett’s post-hoc test with SPSS version 16.0. Differences were considered statistically significant at p<0.05.
RESULTS

Mice

The effect of combined inhibition of FXIIIA and TG2 on atherosclerotic lesion development was investigated using a cuff model in ApoE3Leiden mice, which were fed a high-fat Western type diet [18]. Table 5-1 shows the characteristics of the control mice and mice receiving the transglutaminase inhibitor. Body weight at the start as well as at the end of the experiment did not differ between control mice and mice receiving the transglutaminase inhibitor. After two weeks of cuff placement, plasma cholesterol levels and triglyceride levels were equally high in both control mice and mice receiving the TG inhibitor (see Table 5-1).

Table 5-1: Body weight before and after receiving the Western type diet, plasma cholesterol levels and plasma triglyceride levels in control mice and mice that received the TG inhibitor. Data represent mean ± SEM, n=6 per group.

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The Western type diet combined with cuff placement around the femoral artery lead to the formation of lesions. These lesions were characterized by high smooth muscle cell content, whereas the media of the cuffed vessels was virtually absent of smooth muscle cells (Figure 5-1B). In comparison, the media of normal femoral arteries stained abundantly for smooth muscle cells as detected by 1A4 antibody (Figure 5-1A). No significant differences were found in the smooth muscle cell content of the lesions in control mice compared to mice receiving the TG inhibitor (516 ± 96 μm² and 476 ± 153 μm² respectively). Macrophages, as detected by Mac-3 antibody, were virtually absent in normal vessels (not shown). In cuffed arteries, macrophages were sparse in the atherosclerotic lesions (Figure 5-1B), but abundantly present in the media. The amount of collagen in the lesions, as characterized by Picrosirius Red staining (Figure 5-1B), did not differ between control mice and mice receiving the TG inhibitor (690 ± 166 μm² and 582 ± 181 μm² respectively). Negative controls of the different stainings are shown in Figure S5.
Transglutaminases in cuff-induced atherosclerotic lesions

The presence of TG2, FXIIIA and the cross-link was studied using immunohistochemistry on sections of both normal mouse femoral arteries and cuffed femoral arteries. In normal femoral arteries, TG2 staining is seen most profoundly in endothelial cells, but positive staining is observed also in the adventitia and media of the vessel (Figure 5-1A). These images cannot be used to differentiate between intra- and extracellular TG2. Staining for FXIIIA is seen in the adventitia of the vessel, possibly reflecting the presence of macrophages. Also residues of blood in the vessel lumen stain positive for FXIIIA (Figure 5-1A). The cross-link stained most strongly in the perivascular area, while a more diffuse staining pattern is seen.
Figure 5-2: A) Typical examples of Alexa Fluor-cadaverine incorporation (red) in mouse femoral arteries in the absence and presence of the TG inhibitor. Nuclei are stained blue. Scale bar represents 75 μm. B) Quantification of Alexa Fluor-cadaverine incorporation. * = statistically significant, p<0.01. C) Typical example of confocal images of FITC-cadaverine incorporation in liver cryosections from control mice (n=6) in the presence of 10 mM CaCl₂ or 20 mM EDTA and mice that received the TG-inhibitor (n=5) in the presence of 10 mM CaCl₂ or 20 mM EDTA. Scale bar represents 75 μm. D) Quantification of FITC-cadaverine incorporation in liver cryosections. Data represent mean ± SEM; * = statistically significant, p<0.04. E) Endothelial function as measured by responses to methacholine in femoral arteries in the absence and presence of the TG inhibitor (10⁻⁵ M).
in the media (Figure 5-1A). Induction of the atherosclerosis model results in dilation of the vessel (Figure 5-1B). In cuffed femoral arteries, TG2 positive staining is seen in endothelial cells and both media and adventitia (Figure 5-1B). FXIIIA stains positive in single cells in the newly formed intima of the vessel and media (Figure S5-1). The cross-link is abundantly present, mostly in the media of the vessel (Figure 5-1B) and co-localizes with TG2 staining patterns.

Use of transglutaminase inhibitor

In the present study, the activity of both TG2 and FXIIIA was inhibited by the use of the TG inhibitor L682.777 (Zedira). While this inhibitor was originally designed to inhibit FXIIIA, the incorporation of biotin-cadaverine shows an effective inhibition (73%, p<0.01) of gpTG (Sigma) by the inhibitor (Figure S5-2).

To determine the effect of the inhibitor on transglutaminase activity in murine femoral arteries in vitro, vessels were incubated with Alexa Fluor-cadaverine in the absence and presence of the TG inhibitor (10^-7, 10^-6, 10^-5 M) for 24 hours. Figure 5-2A shows typical examples, and Figure 5-2B the quantification of Alexa Fluor-cadaverine incorporation in femoral arteries. A concentration-dependent inhibition of Alexa Fluor-cadaverine incorporation is seen in the femoral arteries. The TG inhibitor shows a 78% inhibition of TG activity at a concentration of 10^-6 M as determined by Alexa Fluor-cadaverine incorporation in the femoral arteries (p<0.01).

To determine the effect of the transglutaminase inhibitor on transglutaminase activity in vivo, liver cryosections were incubated with FITC-cadaverine. Figures 5-2C shows typical examples of transglutaminase induced FITC-cadaverine incorporation in livers of respectively control mice and mice that received the inhibitor. Incubation of the cryosections with FITC-cadaverine in the presence of EDTA was used to determine the background signal (Figure 5-2C). Quantification of the FITC-cadaverine incorporation showed a 32% reduction of transglutaminase activity in livers of mice that received the inhibitor compared with control mice (Figure 5-2D, p < 0.05).

To determine local inhibition at the site of lesion formation, we quantified the staining for the cross-link in the neo-intima and media of the cuffed vessels. This showed no difference between control mice and mice that received the inhibitor. Mean cross-link positive staining of the intima was 290 ± 75 μm^2 (control) vs. 562 ± 173 μm^2 (inhibitor), mean cross-link positive staining of the media was 1557 ± 161 μm^2 (control) vs. 2379 ± 422 μm^2 (inhibitor).

To investigate the effect of the TG inhibitor on endothelial function, we tested the response to methacholine in segments of femoral arteries. In order to test the
effect of the TG inhibitor on NO-dependent dilation only, vessel segments were precontracted with 30 mM potassium to eliminate EDHF-induced relaxation. Figure 5-2E shows the response of the vessels to methacholine. The TG inhibitor had no effect on the NO-dependent relaxation of the femoral arteries compared to control mice.

**Effect of transglutaminase inhibition on global expression of atherosclerosis-related genes**

A PCR-array was used to investigate the influence of transglutaminase inhibition on the mRNA expression of 84 atherosclerosis related genes (SABiosciences), including several adhesion molecules and cytokines. Data are given in Supplemental Table 1, threshold was set at 2 times up- or downregulation. Compared with control mice, the mRNA of 3 genes was down-regulated in mice receiving the inhibitor: cadherin 5 (-2.13 fold), selectin (-2.03 fold) and serine peptidase inhibitor 1 (-2.45 fold). The

![Graphs and images](image-url)  
Figure 5-3: A) Images of representative cross-sections of cuffed femoral arteries stained with HE. Dashed lines represent the internal and external elastic lamina; scale bar represents 50 μm. B) shows intimal lesion formation in control vessels and vessels from mice receiving the TG inhibitor. C) Circumferences of lumen, internal elastic lamina (IEL) and external elastic lamina (EEL). D) shows media to lumen ratio of cuffed femoral arteries of control mice and mice receiving the inhibitor. N.s. = not significant.
mRNA of serine peptidase inhibitor 2 was 2.31 times up-regulated in mice receiving the inhibitor. None of the cytokines was up- or downregulated.

**Transglutaminase inhibition does not influence lesion size**

Lesion size was analyzed in cross-sections stained with hematoxylin and eosin (Figure 5-3A). Inhibition of FXIIIa and TG2 did not lead to significant differences in lesion size compared to control mice (control: 2186 ± 377, TG inhibitor: 2215 ± 805 μm², Figure 5-3B). Media areas were comparable in both groups, 9614 ± 743 μm² in control versus 8896 ± 431 μm² in TG inhibitor vessels. Circumferences of lumen, internal elastic lamina and external elastic lamina were not significantly different between control mice and mice receiving the transglutaminase inhibitor (Figure 5-4).

**Figure 5-4:** A) Images of representative cross-sections of cuffed vessels stained for Mac-3 to detect macrophages. B) Macrophage area in the media for control vessels and vessels that received the TG inhibitor. Scale bar represents 50 μm. * = statistically significant, p<0.02. C) Relative expression of TG2 and FXIIIa mRNA compared with RPLP0 in unstimulated HL60 cells and cells stimulated with PMA for 24 hours. D) Migration of PMA-stimulated HL60 cells through a Transwell membrane for 24 hours; control cells and cells treated with 10⁻⁵ M TG inhibitor.
TG inhibition influences macrophage content of the vessel wall

RT-PCR was used to quantify the mRNA levels of CD68, reflecting the total amount of macrophages in the cuffed vessels. Expression of CD68 showed a tendency to decrease in mice that received the inhibitor (0.27 ± 0.04 in control mice compared to 0.19 ± 0.05 in mice receiving the transglutaminase inhibitor), but this did not reach statistical significance (p=0.25). To further analyze macrophage content in the different layers of vessel wall, immunohistochemical staining for macrophages using a Mac3 antibody (see Figure 5-4A for representative images) was performed. This revealed no significant difference in macrophage content in the newly formed intima of the vessels (157 ± 46 μm² (control) vs 190 ± 53 μm² (TG inhibitor)). However, macrophage content in the media, the most abundant source of macrophages, of vessels treated with the TG inhibitor was decreased by 41% compared with controls (p = 0.02, see Figure 5-4B).

Transglutaminases affect macrophage migration

As a reduced media content of macrophages suggests a role for transglutaminase in monocyte/macrophage migration, we further studied macrophages in vitro. HL60 cells were stimulated with PMA to differentiate towards a macrophage phenotype. PMA stimulation for 24 hours resulted in a large upregulation of TG2 mRNA (p=0.03, see Figure 5-4C). FXIIIA was also expressed in HL60 cells without PMA stimulation, but its expression was not significantly changed during differentiation into macrophages (see Figure 5-4C). To further investigate the effect of transglutaminases in macrophage migration, a migration assay with PMA-differentiated HL60 cells was used. Migration of PMA-differentiated HL60 cells through the transwell membrane in the presence of the transglutaminase inhibitor (10⁻⁵ M) was decreased by 13% compared with control (p<0.01, Figure 5-4D).

DISCUSSION

In this study we investigated the role of transglutaminases in a model of cuff-induced lesion formation in ApoE3 Leiden mice, which develop accelerated atherosclerosis
when fed a high-fat diet [18]. In this model an irreversible active-site inhibitor was used which inhibits both TG2 and FXIIIA [19,21]. We chose a combined TG2 and FXIIIA inhibitor for previous studies have shown that FXIIIA can compensate for the deficiency of TG2 [14,16]. Both transglutaminases are associated with vessel wall remodeling [14] and have been suggested to play a role in the stabilization of atherosclerotic lesions [8,22,23]. Experimental work in mouse models however, showed that inhibition of either transglutaminase lead to opposing effects on lesion size [1,2]. Thus, lack of TG2 in bone-marrow derived cells resulted in larger lesions in LDLr−/− mice [2]. Inhibition of either FXIIIA or deficiency of TG2 in a ApoE−/− model led to smaller lesion sizes [1,3]. Recently, a study by Williams et al. reported no influence of TG2 on plaque composition and size in the ApoE−/−TG2−/− model [24]. Some of the differences in these studies may relate to the particular model that is used. However, also the redundancy among transglutaminases may play a role here. In the present study, we found that the simultaneous inhibition of TG2 and FXIIIA did not influence lesion size or geometric remodeling of the cuffed vessels. We did however find a reduced macrophage content in the media with transglutaminase inhibition.

**Model and inhibitor used**

Placement of a cuff around the femoral artery is a widely used model leading to the development of atherosclerotic lesions. We chose this particular model of cuff-induced lesion formation because lesions develop relatively fast, in the course of several weeks [18]. This allows the use of osmotic minipumps to deliver the transglutaminase inhibitor. Both in vitro assay measurements using gpTG, and in situ measurements in liver cross-sections of control mice and mice receiving the inhibitor showed significant inhibition of transglutaminases. The effectiveness of the inhibitor was also shown on isolated femoral arteries in vitro using Alexa Fluor cadaverine. However, local inhibition of the transglutaminase activity within the lesion of cuffed arteries could not be demonstrated with immunostaining for the cross-link. We speculate that the cause of this may be two-fold. First, the cross-link is already present in healthy, untreated vessels (Figure 5-1A). Second, additional activation of transglutaminases may occur during the surgery and the deliberate injury of the vessel in this model. While in preliminary experiments we found that this injury is necessary to initiate lesion formation, this injury might lead to immediate local cell damage and coagulation, inducing transglutaminase and FXIIIA activity [25]. This would result in a rapid formation of cross-links in the vessel wall, when the systemic concentration of the inhibitor is not yet optimal.
Furthermore, we did not monitor the in vivo mouse plasma concentrations of the blocker, because of the very complex nature of such analyses. However, the effects on both liver TG activity and vascular macrophage accumulation make clear that effective concentrations of the inhibitor were obtained. Based on the in-vitro experiments on labeled cadaverine incorporation, we estimate the concentration to have been in the order of 1-10 µM. Release kinetics of the minipumps reveal that this concentration should have been reached within a few hours. Clearly, there is a need for determining the pharmacokinetics of these TG blockers.

**Macrophage recruitment and transglutaminases**

An important result of the present study is that systemic blocking of TG2 and FXIIIA lead to less macrophage infiltrate in the media of the cuffed vessels. The additional in vitro studies that we performed on HL60 cells showed that inhibition of transglutaminases imposed a mild inhibition of leukocyte migration, providing a possible explanation for the reduced macrophage infiltrate in the vessel media of the inhibitor-treated mice. These data are in good agreement with several in vitro studies on migration of fibroblasts, monocytes and dendritic cells [9-11] as well as in vivo studies of other research groups, which have shown a decreased leukocyte recruitment in the healing myocardial infarct in FXIII−/− mice [26] and less leukocyte infiltrate in atherosclerotic lesions when inhibiting FXIIIA[1]. Development of atherosclerosis is a process that involves the production of reactive oxygen species (ROS) mainly in the adventitia of the cuffed vessels [18,27,28]. Macrophages are the dominant source of ROS in this process, and the reduced macrophage infiltration is expected to reflect a similarly reduced production rate of ROS. It is unknown whether, in addition to their effect on macrophage infiltration, Tgases are also relevant for ROS signaling in macrophages, and this clearly awaits future work.

**Effect of transglutaminase inhibition on atherosclerotic-related gene expression**

Results of the PCR array showed a limited number of genes that were altered in lesions of mice that received the TG inhibitor; only 4 genes out of 84 showed a change in mRNA expression by TG inhibitor treatment. These affected genes belong to the family of cell adhesion molecules and serine peptidase inhibitors, also known as plasminogen activator inhibitors. Cell adhesion molecules are associated with barrier function of endothelial cells [29]. Although transglutaminases, in particular
TG1, are thought to play a protective role in endothelial barrier function [6], no direct effect of transglutaminases on cell adhesion molecules has been described in literature. In relation to serine peptidase inhibitors, transglutaminase is known to influence retinol-induced serine peptidase inhibitor 1 production, which could be blocked by the use of the TG inhibitor also used in this study [30]. Furthermore, serine peptidase inhibitor 2, which is secreted by monocytes/macrophages is known to be a substrate for transglutaminases [31].

In conclusion, we found that combined TG2 and FXIIIA inhibition resulted in reduced macrophage content in the media of atherosclerotic vessels. This observation was consistent with an inhibition of macrophage migration in vitro by the transglutaminase inhibitor. While transglutaminases may play a more prominent role in later stages of atherosclerosis, when calcification of the plaque develops [12], we observed no difference in lesion size in this model of early developing atherosclerosis.

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REFERENCES


Figure S5-1: Negative controls of FXIIIA, CL, TG2 and 1A4 staining. Positive control of FXIIIA showing positive staining in the neo-intima. Scalebar = 25μm

Figure S5-2: In-vitro transglutaminase activity in the absence and presence of the TG inhibitor (0.5*10^{-5} M).
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