TIMP3 is reduced in atherosclerotic plaques from subjects with type 2 diabetes and increased by SirT1

Running Title: SirT1 regulates Timp3 expression

Marina Cardellini, MD*; Rossella Menghini, PhD,*; Eugenio Martelli, MD†; Viviana Casagrande, BS*; Arianna Marino, BS‡; Stefano Rizza, MD/PhD‡; Ottavia Porzio, MD*; Alessandro Mauriello, MD‡; Anna Solini, MD§; Arnaldo Ippoliti, MD‡; Renato Lauro, MD*; Franco Folli, MD§ and Massimo Federici, MD*#

From Departments of *Internal Medicine, †Surgery, ‡Biopathology and Diagnostic Imaging, University of Rome “Tor Vergata”, Rome, Italy; §Department of Internal Medicine, University of Pisa; ¶Division of Diabetes, Department of Medicine, University of Texas Health Science Center, San Antonio, Texas;

^Drs. Cardellini and Menghini equally contributed

#Address correspondence to:
Massimo Federici, MD
E-mail: federicm@uniroma2.it

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Objective: Atherosclerosis is accelerated in patients with Type 2 Diabetes Mellitus (DM2) by unknown mechanisms. We identified Tissue Inhibitor of Metalloproteinase 3 (TIMP3), the endogenous inhibitor of A Disintegrin and Metalloprotease Domain 17 (ADAM17) and other Matrix MetalloProteinase (MMP), as a gene modifier for insulin resistance and vascular inflammation in mice. Here we tested its association with atherosclerosis in patients with type 2 diabetes mellitus (DM2) and identified Siruin 1 (SirT1) as a major regulator of TIMP3 expression.

Research design and methods: We investigated ADAM10/17/MMP9, TIMP1/2/3/4 expression levels in human carotid atherosclerotic plaques (n=60) from subjects with and without diabetes. Human Vascular Smooth Muscle cells exposed to several metabolic stimuli were used to identify regulators of Timp3 expression. SirT1 small interference RNA (siRNA), cDNA and TIMP3 promoter gene reporter were used to study SirT1 dependent regulation of TIMP3.

Results: Here, we show that in human carotid atherosclerotic plaques TIMP3 was significantly reduced in subjects with DM2 leading to ADAM17 and MMP9 overactivity. Reduced expression of TIMP3 was associated in vivo to SirT1 levels. In smooth muscle cells, inhibition of SirT1 activity and levels reduced TIMP3 expression, while SirT1 overexpression increased TIMP3 promoter activity.

Conclusions: in atherosclerotic plaques from subjects with Type 2 diabetes the deregulation of ADAM17 and MMP9 activities is related to inadequate expression of TIMP3 via SirT1. Studies in vascular cells confirmed the role of SirT1 in tuning TIMP3 expression.
Diabetes mellitus is characterized by accelerated atherosclerosis although molecular mechanisms explaining this phenomenon are still undefined (1,2). We and others have shown that chronic hyperglycemia increases MMP and ADAMs activities providing a potential clue to atherosclerotic plaque progression, as confirmed by studies using vasculature from subjects with diabetes (3-5). Increased MMPs and ADAMs activities may be linked also to unbalanced expression of endogenous inhibitors, called Tissue Inhibitor of Metalloproteinase (TIMP) 1-4 (4). We identified the deficiency of TIMP3 as a link between insulin resistance and vascular inflammation (6-8). Recently, Paigen and coworkers found Timp3 gene among Quantitative Trait Locis (QTLs) associated with diabetes and dyslipidemia, identifying a mutation causing lower gene expression in diabetic mice (9). Moreover, Timp3 is among the few genes downregulated in a microarray analysis of pericytes treated with glycated oxidized Low-density-Lipoproteins (LDL) (10). Since TIMP3 uniquely among TIMPs retains the ability to inhibit shedding enzymes such as ADAM17, which are involved in inflammatory processes (11), we hypothesized down-regulation of TIMP3 as an hallmark for atherosclerosis in diabetic patients. We tested this hypothesis in atherosclerotic plaques from subjects with different degrees of glucose tolerance, linking TIMP3 expression to activity of deacetylase SirT1. SirT1 is a deacetylase localized at nuclear levels acting as transcriptional regulator either on histones or on transcription factors such as Forkhead box O1 (FoxO1), Liver X Receptor (LXR), p53 and transcriptional cofactors such as Peroxisome proliferator-activated receptor Gamma Coactivator 1α (PGC1α) (12). Recently, it has been suggested that loss of SirT1 activity may be associated to metabolic diseases such as type 2 diabetes and atherosclerosis (13). Several laboratories have shown that SirT1 gain of function either by genetic manipulation or through ligand activation may protect from insulin resistance associated to obesity and from atherosclerosis in experimental disease models (14,15).

However, little is known on SirT1 activity in human subjects affected by atherosclerosis and diabetes. Our data reveal a new potential role for Timp3 and SirT1 in the atherosclerosis process in patients with diabetes.

METHODS

Subjects. Sixty atherosclerotic plaques, from Normal Glucose Tolerant (NGT, n=37) subjects or Type 2 Diabetes mellitus (DM2, n=23) patients [according to medical records or Oral Glucose Tolerance Test (OGTT)] subjected to carotid endarterectomy for symptomatic disease at Policlinico Tor Vergata University Hospital, were included in the study. Patients characteristics and treatments are described in table 1. The study was approved by the ethics commitee and patients gave their informed consent for the use of atherosclerotic material for research use. All procedures were performed according to the declaration of Helsinki.

Histological Analysis Carotid plaques were removed en bloc during surgery to preserve plaque structure entirely. For histology, surgical samples were fixed for 24 h in 10% buffered formalin immediately upon removal. After decalcification, specimens were sectioned transversely every 5 mm and paraaffin embedded. Hematoxylin-eosin was performed for morphologic study (4). Immunohistochemistry was performed on serial 3-µm thick sections cut from paraaffin blocks of carotid plaques using the following antibodies; (a) polyclonal rabbit anti-human...
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TIMP3 antibody (Calbiochem,Ca), (b) monoclonal anti-CD68 (human macrophage) antibody (DAKO, Denmark) and (c) monoclonal anti-alpha smooth muscle actin antibody (Europa Ventana Medical System, S.A., France). The primary antibodies were detected with avidin-biotin-peroxidase complexes (DAKo). All antibodies were employed with positive and negative controls.

**Real-time quantitative RT-PCR analysis.** Frozen plaque samples were homogenized using a polytron homogenizer. Total RNA and Single strand cDNA were obtained as described (7). Real-time PCR RNA expression analysis of ADAM17, ADAM10, TIMP3, TIMP1, TIMP2, TIMP4, MMP9, SirT1 (primers available upon request) was performed with ABI PRISM 7000 System (Applied Biosystems, Foster City, CA) and normalized to 18S rRNA. Each reaction was carried out in duplicate and analysis performed by $2^{-\Delta\Delta Ct}$ method as described (8).

**ADAM17 and MMP9 activities.** Proteins were extracted as previously described (4, 6-8). ADAM17 activity was determined by the SensoLyte™ 520 ADAM17 Activity Assay kit Fluorimetric (AnaSpec, San Jose CA). MMP9 activity was measured by the Amersham MMP-9 Biotrack Activity Assay System (GE Healthcare UK) according to manufacturer’s instructions. Active MMP-9 was detected through activation of the modified pro-detection enzyme and subsequent cleavage of its chromogenic peptide substrate. The resultant colour was read at 450 nm in a microplate spectrophotometer (Victor 1420).

**LDL preparation, Cell culture, western blots:** LDL preparation, Cell culture, western blots are described in detail in the online appendix.

**Timp3 promoter regulation assay:** For Timp3 promoter regulation assay CASMC were transfected with 2 µg of Human SirT1 cDNA clone (RG218134, Origene), 10 ng of renilla and Timp3 Gene Promoter Reporter Vector (LR1034, Panomics) or 2 µg of TransLucent Control Vector using primary smooth muscle cells Nucleofector solution with program A-033 (AMAXA). The Luciferase assay was performed with Dual-Luciferase Reporter Assay System (Promega) according to manufacturer’s instructions.

**Statistical Analysis.** Statistical analysis was performed using Student’s t Test, one-way ANOVA and Pearson’s correlation coefficient (r) on SPSS software program version 13.0 for Windows. Data are expressed as means ± SD. P value <0.05 was considered statistically significant.

**RESULTS**

Our data show that in atherosclerotic plaques, among ADAM10/17, MMP9 and TIMP1/2/3/4, only TIMP3 expression was lower in DM2 compared with NGT (Figure 1A,B). Western blot assay confirmed the significant decrease of TIMP3 in patients with DM2 (Figure 1C). Immunohistochemistry completed the link between TIMP3 downregulation and diabetes (Figure 1D and 1G, for non DM2 and DM2 patients, respectively). Analysis of consecutive sections with anti-CD68 for macrophages and anti-alpha smooth muscle actin for smooth muscle cells, suggested that both cell types are associated with TIMP3 expression in NGT patients (Figure 1E,F for NGT and Figure 1H,J for DM2 patients; negative control for antibodies in Supplementary Figure 1 which is available at [http://diabetes.diabetes.org](http://diabetes.diabetes.org)). To verify that the reduction of TIMP3 in atherosclerotic plaques resulted in increased ADAM17 and MMP9 activity we used a fluorimetric assay. We found that both ADAM17 and MMP9 activities were higher in DM2 than in NGT (Figure 1K,I).

Analysis of clinical characteristics (Table 1) showed that TIMP3 expression negatively correlates with LDL Cholesterol (R=-0.29,
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p<0.03) and HbA1C (R=-0.31, p<0.02), but not with sex, age or pharmacological treatment.

To identify metabolic factors reducing TIMP3 expression, CASMCs were treated with high glucose (20 mM), mannitol (osmotic control, 20mM), hyperinsulinemia (10^{-7} M), LDL, oxidized LDL, glycated LDL (100ug/ml). Since previous data suggested that LXR regulates TIMP3 expression (17), we used LXR agonists such as T0901317, 22-s and 22-r hydroxycholesterol and GW3965. Since LXR is regulated by SirT1 deacetylase (12), we also used SirT1 inhibitor Sirtinol. Interestingly, we found that among the various treatments only high glucose and Sirtinol significantly reduced TIMP3 expression in CASMC (Figure 2A).

Treatment of CASMC with Sirtinol determined an increased metalloprotease activity as measured by ADAM17 activity assay (Figure 2B). Sirtinol and HG significantly reduced TIMP3 expression also in Human Umbilical Vein Endothelial Cells (HUVEC) and monocyctic THP1 cells (Figure 2C). Analysis of CASMC, HUVEC and THP1 revealed that HG significantly reduced SirT1 expression (Figure 2D) in CASMC and THP1 with a trend also in HUVEC. SirT1 expression was significantly reduced in atherosclerotic plaques from patients with DM2 compared to NGT subjects (Figure 2E), and we observed that TIMP3 expression positively correlated with SirT1 expression (R=0.4; p<0.03; Figure 2F), although NGT and DM2 patients were mixed and therefore diabetes may represent a confounding factor.

To confirm a direct role of SirT1 in regulating TIMP3 expression, we used a small interference RNA (siRNA) approach. Knockdown of SirT1 in CASMC resulted in marked reduction of TIMP3 expression but not Timp1/2/4 and ADAM10/17/MMP9 (Fig. 3A); a similar effect was observed in HUVEC and THP1 cells (Fig. 3B). To substantiate SirT1 effects on TIMP3, CASMC were cotransfected with human SirT1 cDNA and Timp3 promoter luciferase reporter vector, confirming that SirT1 positively modulates TIMP3 expression (Fig. 3C). Finally, in CASMC, HUVEC cells and THP1 we found that SirT1 overexpression prevented the reduction of TIMP3 expression determined by HG (Figure 3D).

DISCUSSION

We recently observed that TIMP3 deficiency is necessary to develop fatty streaks characterized by macrophage infiltrate, using the Insulin Receptor heterozygous mouse model fed a diet rich in lipids (8). The relevance of TIMP3 is demonstrated by the reverse phenotype caused by TACE deficiency in the same mouse model, suggesting that loss of TIMP3 may favor the development of atherosclerotic lesions (8). While other studies on models such as ApoE and LDL Receptor knockout are necessary to fully characterize the role of TIMP3 in the progression of atherosclerosis under diabetic conditions, here we analyzed its role in human atherosclerosis accompanied by diabetes. Our results suggest that subjects with diabetes exhibit reduction of TIMP3, increased activity of ADAM17 and MMP9, possibly because of the more intense oxidative stress caused by hyperglycemia, a known activator of both the enzymes in a PKC-dependent manner (3,4,18,19). Therefore, our data suggest that metabolic-dependent reduction in TIMP3 expression may increase the activity of inflammatory and proteolytic enzymes, which play a role in atherothrombosis (20,21). Previous studies showed that TIMP3 expression was increased in extracts from atheroma compared to non atherosclerotic tissue in non diabetic subjects (22). In view of our results, TIMP3 reduction is emerging as a specific factor in the atherosclerosis process of patients with diabetes. Loss of TIMP3 may lead to increased TNF-alpha and EGFR signalling,
potentially increasing the inflammatory burden inside the atherosclerotic plaque (6-8). Moreover, loss of TIMP3 increases MMP9 activity in atherosclerotic plaques, a feature known to be increased in vasculature from subjects with Type 2 Diabetes (5) and may potentially affect plaque stability in the long term.

The role of TIMPs in diabetic atherothrombosis is still undefined. Recent data in animal models supported a role for imbalanced MMP/TIMP ratio favouring increase degradation of extracellular matrix that may promote progression of atherosclerosis (23).

Factors regulating TIMPs expression in atherosclerotic plaques are undefined, although previous data suggested a role for growth factors such as TGF-β and PDGF (23). However, the role and regulation of TIMP3 in diabetic vascular disease has been thus far unexplored. Our data suggested that TIMP3 is negatively associated to HbA1c and LDL cholesterol levels. Exposure of CASMC to different stimuli linked to gluco- and lipotoxicity revealed that both high glucose or inhibition of the deacetylase SirT1 led to reduced TIMP3 expression and activity. SirT1 is emerging as a master of integrated metabolic response to nutrients availability (12-15, 24). Data from ApoE knockout mice suggest that overexpression of SirT1 from endothelial cells may defend against atherosclerosis progression, although the basic mechanisms remained undefined. Our results, via knockdown of SirT1 or its overexpression, confirmed a role for this deacetylase in the modulation of TIMP3 expression in vascular smooth muscle cells and especially monocyte/macrophage SirT1 controls gene expression through deacetylation of histones and transcription factors; one or both of the two mechanisms may be involved in regulating TIMP3 expression. Previous studies using T0901317 compound, a LXR agonist, suggested that LXR transcription factors regulate TIMP3 expression (17). In our cell systems we did not observe an effect of T0901317. However, this may depend from several factors including different experimental models and culture conditions. Moreover, SirT1 is a positive regulator of LXR as well as other transcription factors potentially involved in TIMP3 expression such as FoxO1 (25). Because SirT1 overexpression is able to rescue TIMP3 expression in the presence of gluotoxicity, it is possible that SirT1 affects events linked to de-repression of TIMP3 promoter via transcription factors such as FoxO1 or histone deacylation.

To our knowledge, this is the first gene/mecanism linked to SirT1 identified in the context of diabetes and atherosclerosis diseases using human vascular specimens. Therefore, our results provide further support for the protective role played by SirT1 against metabolic diseases.

In conclusions, we observed that atherosclerotic plaques from subjects with impaired glucose metabolism are characterized by TIMP3 deficiency and increased metalloproteases activity. In vitro studies and clinical correlations suggested that SirT1 regulates TIMP3 expression, which is emerging as a specific factor for the atherosclerosis process in diabetes.

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Table 1. Clinical data of patients subjected to carotid endarterectomy

|                                  | NGT    | DM2    |
|----------------------------------|--------|--------|
| n                                | 37     | 23     |
| Sex (Men/Women)                  | 25/12  | 14/9   |
| Age (years)                      | 71.0±6.6 | 70.6±8.9 |
| BMI (kg/m²)                      | 25.3±3.9 | 24.1±4.8 |
| Hypertension (Yes/No)            | 29/8   | 20/3   |
| Anti-hypertensive drugs (Yes/No) | 23/14  | 15/8   |
| Antiaggregant drugs (Yes/No)     | 35/2   | 22/1   |
| Statins (Yes/No)                 | 25/12  | 15/8   |
| Total Cholesterol (mg/dl)        | 188.9±31.0 | 192.35±36.86 |
| HDL Cholesterol (mg/dl)          | 45.32±10.2 | 43.3±9.6  |
| LDL Cholesterol (mg/dl)          | 115.7±24.6 | 122.18±28.1 |
| Triglycerides (mg/dl)            | 125.69±62.26 | 139.13±72.04 |
| HbA1c (%)                        | 5.6±0.2 | 7.2±1.6* |
| Fasting plasma glucose (mg/dl)   | 89.7±12.0 | 126.9±51*  |
| Fasting plasma insulin (µU/ml)   | 7.38±5.59 | 12.96±3.51  |
| Oral agents/Insulin treatment    | -      | 15/8   |

* p<0.001 by Student’s t test.
Figure Legends

Figure 1. TIMP3 is reduced in atherosclerotic plaques of subjects with diabetes. (A) ADAM10, ADAM17, MMP9 and (B) TIMPs expression in NGT (n=37) and DM2 subjects (n=23), *** p<0.001 by 1-way Anova; C) Western blot using extracellular matrix extracts from representative NGT (n=2) and DM2 (n=4) patients. D-J) Immunohistochemistry confirmed that TIMP3 is reduced in DM2 (n=8) versus NGT (n=8) subjects; one representative image is shown for TIMP3, anti-α-smooth muscle actin and CD68 for NGT (D-F, 4x) and DM2 subjects (G-J, 4x). K) ADAM17 activity measured by a fluorimetric assay and l) MMP9 activity measured by a fluorimetric assay are increased in DM2 subjects (n=23) compared to NGT (n=37); ***p<0.001 by Student’s t test for both.

Figure 2. Effects of diabetes on TIMP3 expression in vascular cells (A) TIMP3 expression in CASMC treated with different metabolic stimuli [HG, High Glucose 20 mM; Man, Mannitol 20 mM; Ins, Insulin 10^{-7} M; Low Density Lipoprotein, LDL 100 µg/ml; Oxidized LDL, OxLDL 100 µg/ml; Glycated LDL, GlyLDL 100 µg/ml; LXR agonists (T0901317, T09 5 µM; GW3965, GW 3 µM; R-hydroxycholesterol, RH 10 µM; 22-S-hydroxycholesterol, SH 10 µM; SirT1 inhibitor Sirtinol 50 µM]; n=4 for all experiments; *p<0.05 by Student’s t test versus Control (CT). (B) Sirtinol increased ADAM17 activity in CASMC; n=4 for all experiments; ***p<0.001 by Student’s t test. (C) TIMP3 expression in HUVEC and THP1 treated with Sirtinol and High Glucose (HG, 20 mM); n=4 for all experiments; *p<0.05 by Student’s t test versus Control (CT). (C) SirT1 expression is reduced in CASMC, HUVEC and THP1 treated with High Glucose (HG, 20 mM) compared with Control (CT); n=4 for all experiments; *p<0.05 by Student’s t test; (E) SirT1 levels are decreased in DM2 compared with NGT patients, *p<0.05 by Student’s t test; (F) SirT1 correlates with TIMP3 in atherosclerotic plaques from NGT (n=37) and DM2 (n=23) subjects.

Figure 3. Regulation of TIMP3 expression in CASMC. (A) SirT1 knockdown decreased TIMP3 expression but not TIMP1/2/4 and ADAM10/ADAM17/MMP9 in CASMC; n=4 for all experiments; ***p<0.001 by Student’s t test versus control. (B) SirT1 knockdown decreased TIMP3 expression in HUVEC and THP1; n=4 for all experiments; ***p<0.001 by Student’s t test. (C) SirT1 cDNA overexpression increased Timp3 promoter activity; n=4 for all experiments; **p<0.01 by one-way ANOVA. (D) SirT1 overexpression increased and prevented loss of TIMP3 expression caused by High Glucose (HG, 20 mM) in CASMC, HUVEC and THP1; n=4 for all experiments; *p<0.05 by Student’s t test.
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A

B

C

NGT

DM2

NGT

DM2

D

E

F

G

H

J

K

I

TIMP3

α-smooth muscle actin

CD68

Adam17 activity (RFL)

MMP-9 activity (ng/ml)
SirT1 regulates Timp3 expression
SirT1 regulates Timp3 expression