Association of MMP-9 and TIMP1 Genes Expression With Pro-inflammatory Cytokines in Patients With Coronary Artery Disease

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Abstract

Background

Coronary artery disease (CAD) is the main cause of death and is characterized by atherosclerosis in coronary arteries. Since inflammation plays an essential role in progression and development of atherosclerosis, we investigated the effects of interaction MMPs, TIMP and cytokines in the pathogenesis of atherosclerosis.

Method and results

The present study consisted of 132 Iranian individuals underwent coronary angiography, 65 patients with CAD and 67 controls. The Matrix metalloproteinase 9 (MMP-9), tumor necrosis factor alpha (TNF- α), Interleukin 6 (IL-6) and vitamin D levels were measured by ELISA technique. The genes expression of MMP-9 and tissue inhibitor of metalloproteinases 1 (TIMP1) were estimated by Real time PCR assay. A considerable increase in both levels and PBMC gene expression of MMP-9, serum levels of IL-6 and TNF- α were found in CAD patients compared with controls. While a significant decrease was detected in vitamin D levels of CAD patients in comparison with controls. A considerable direct correlation was found between MMP-9 levels and MMP-9 and TIMP1 genes expression in CAD patients. MMP-9 levels positively correlated with LDL-C and in CAD. Correlation between TIMP1 gene expression and IL-6 levels was also negatively significant. Moreover, there were positive correlations between MMP-9 levels and IL-6 and TNF- α serum levels in CAD patients.

Conclusion

This study showed that the interaction between MMPs, TIMP and cytokines could play a role in the pathogenesis of atherosclerosis.

Introduction

Coronary artery disease (CAD) is a leading cause of morbidity and mortality globally and is characterized by atherosclerosis in coronary arteries. Lifestyle, genetic and environmental factors play role in CAD pathogenesis [1–3]. In addition to the established risk factors for CAD and atherosclerosis (diabetes, dyslipidemia, hypertension and obesity), biological factors could potentially be involved in the genesis and progression of vascular atherosclerosis. Among these, cytokines levels and balance between metalloproteinases and tissue inhibitor of metalloproteinases (TIMPs) play a remarkable role in the pathogenesis of atherosclerosis [4]. The immune system also plays a key role in this process and regulation of inflammatory pathways that could be a possible therapeutic approach to drug targets in atherosclerosis [5]. Although predisposition of severity and mechanisms that are involved in the development of cardiovascular and atherosclerosis diseases, are not comprehended completely. Matrix metalloproteinases (MMPs) are responsible for the degradation of adhesion proteins and extracellular matrix, and belong to a large family of zinc-dependent, extracellular endopeptidases MMP-9 (gelatinase

B) is a 92 kDa gelatinase (type IV collagenase) enzyme which represents the largest and most complex member of this family [6]. MMP-9 activity is inhibited by TIMP under normal conditions, especially by TIMP1, it can bind to the active form and precursor form of MMP-9 [7].

It has been shown that MMPs could facilitate the vascular remodeling and cardiovascular complications [6]. MMPs are key zinc-dependent proteolytic enzymes in the transformation of the ECM in pathophysiological conditions such as inflammatory [6]. Disintegration of ECM components by matrix metalloproteinases leads to decrease size of the plaque and increases its instability. Immune-inflammatory cells infiltration is increased by MMPs through base membrane disruption that provide a condition for angiogenesis via stimulating the endothelial cells proliferation that results in the plaque growth and its increased sensitivity to rupture [5]. Circulating levels of MMP-9 were remarkably elevated in coronary artery disease patients with and without T2DM compared to their controls [6]. It has been reported that polymorphism of MMP 9 related to hypertension in CAD patients. MMP-9 gene-expression and TIMP1 levels elevated in patients with a history of myocardial infarction. TIMP1 levels increase also was found in diabetics [8]. The imbalance between extracellular matrix synthesis and degradation leads to increase in atherosclerosis and stenosis in the coronary arteries. The effect for most MMPs, is the rupture of the coronary plaque by degrading the vascular extracellular matrix components [9]. The results of a follow-up study on 337 type 1 diabetic patients revealed that increase of levels MMP-2 was related with cardiovascular disease (CVD) and enhancement of MMPs 1, 2 and 3 were main cause of mortality [10].

Maintenances of the suitable functional active sites of Metalloproteinases depend on sufficient calcium and zinc ions. An in vitro study showed that progressive degradation of collagen fibrils in the hybrid layer of dentin bonds was a result of optimum MMPs activity in presence of calcium and zinc ions. They also showed that MMPs are inactive in absence of zinc [11]. Animal model study on interaction between Cytokines and matrix metalloproteinases identified that although MMPs have a considerable influence on myocardium, but cytokines have ability to regulate the transcription and synthesis of various MMPs through some transcription factors that are affected in with cytokine signaling pathway [12].

Studies have suggested a substantial role for vitamin D in CAD etiology and it has been shown vitamin D levels are lower in CAD patients than controls. Vitamin D exerts has several favorable activities in protecting from atherosclerosis such as inflammation, regulation of vascular cell growth, regulation of cytokine production, migration and differentiation, modulation of immune response, and fibrotic pathways, all of which play a critical role in atherosclerotic development and progression. In the heart vitamin D with anti-hypertrophic effect could inhibit the foam cells formation and regulate the ECM turnover i.e. modulating the MMPs and TIMPs expression and calcium influx and cardiac contractility [13]. Negative associations of vitamin D with MMP-9 and TIMP1 were reported in Chronic Obstructive Pulmonary Disease (COPD) patients. Polymorphonuclear leukocytes of COPD patients released significantly more MMP-9 than healthy controls [7]. A remarkable increase in MMP-2 and MMP-9 levels, and lower serum concentration of vitamin D were reported in patients with neuroinflammatory disease [14]. In present study, we tried to determine the circulating levels of inflammatory markers of TNF- α , IL-6

and vitamin D, Zn²⁺, Ca²⁺, MMP-9 levels and MMP-9 and TIMP1 genes expression in peripheral blood mononuclear cells (PBMCs) of 67 patients with CAD and 65 controls, as well as the correlation between them.

Material And Methods

Population

The resent case-control study was conducted on 134 Iranian subject who performed coronary angiography at Hazrat-e-Rasoul Hospital, Iran University of Medical Sciences. 65 patients with CAD and 67 healthy controls recruited in this study between April 2016 and February 2017. The diagnosis of CAD was according to coronary angiography. A cardiologist evaluated all the angiograms and the presence of more than 50% stenosis in at least one major coronary artery categorized as CAD [15]. In addition, participants with less than 25% stenosis in all the coronary artery were considered as healthy controls.

Participants with history of cancer, diabetes, renal failure, liver diseases, and any other chronic diseases and those taking immunosuppressive, steroids medications, and anti-inflammatory during last six months were excluded from the study. Also, subjects who had unstable angina, carotid plaque and any history of cardiovascular disease, including cerebrovascular, acute coronary syndrome, and peripheral artery disease were excluded from control group. Medical history, demographic data and medications consumption of the studied subjects were collected through a questionnaire. Basic anthropometric data including systolic and diastolic blood pressure, weight, and height were measured. Ethics approval was obtained from the Research Ethics Committee of Iran University of Medical Sciences and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki (IR. IUMS.REC1395.9221184201).

Biochemical parameter measurements

Fifteen mL of venous blood were collected after an overnight fasting (8 to 12 hours) and stored at -80°C. Total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) and fasting blood glucose (FBG) levels were measured, using commercially available kits (Pars Azmon kit, Iran).

PBMCs isolation and RNA-Extraction

Ten mL EDTA-containing whole blood tubes was used for PBMCs isolation, by Ficoll-Hypaque density-gradient centrifugation as previously described [16]. Total RNA was extracted from the cell lysates, using the miRNeas mini kit (QIAGEN, USA) according to manufacturer's instructions. The purity and concentration of extracted RNA were evaluated via 1.5% agarose gel electrophoresis (Biorad, US) and the nanodrop spectrophotometer (thermo-scientific, US).

cDNA synthesis and Quantitation of mRNA

Gene expressions analysis was performed using real time PCR. In the first step, 1 µg total RNA were converted to cDNA, by Revert Aid First cDNA Synthesis Kit (Takara, Japan). Real time PCR was run in an ABI-Step One (Applied Biosystems, USA) system, using RealQ Plus 2x Master Mix Green (Amplicon, Denmark). GAPDH was used as internal control. Specific primers used to amplify genes are listed as follows: GAPDH: F: 5' CCC CTT CAT TGA CCT CAA CTA C 3', R: 5' TGGT GAC AAG TTC CCG TCT C 3'; MMP - 9, F: 5' CCT GGG CAG ATT CCA CT, R: 5' CCA AGT GTT CCG AGT AGT TTT GGA 3; TIMP1, F: 5' ACT GCA GGA TGG ACT CTT GCA 3, R: 5' TTT CGA AGC CTT GGA GGA GCT 3'.

Statistical Analysis

SPSS version 20 was used for the statistical analysis of the data. Continuous variables were shown as mean ± standard deviation and tested by Student t-test or Mann–Whitney U according to normality test. Categorical data tested using chi-square test and represented using frequency and percentage. Spearman's correlation test was applied for the correlation between continuous variables. ROC was applied to test the diagnostic ability of the desirable variables. A p value less than 0.05 considered as statistically significant.

Results

Anthropometric and biochemical measurement

As it shown in Table 1 there were no remarkable differences between CAD and control group in terms of BMI, age, sex. Moreover, SBP and DBP indicated no considerable change between the groups. Furthermore, serum concentrations of FBS, TG, LDL-C and TC indicated no considerable change between the groups, while HDL-C considerably lower in CAD compared to controls. Furthermore, Zn^{+2} had no significant difference between the groups, while Ca^{+2} decreased in compared with controls.

Table 1
Anthropometric and laboratory profiles of study population

	Control (n = 67)	CAD (n = 65)	P
Age (Years)	58.78 ± 8.41	60.16 ± 7.64	0.328
Sex (male)	33	36	0.481
BMI (kg/m ²)	26.50 ± 4.31	27.68 ± 3.13	0.075
SBP (mmHg)	129.09 ± 18.46	131.4 ± 30.61	0.550
DBP (mmHg)	81.75 ± 12.47	81.82 ± 16.26	0.978
FBS (mg/dL)	92.45 ± 10.73	94.95 ± 14.35	0.257
TG (mg/dL)	132.48 ± 51.02	138.55 ± 43.45	0.463
TC (mg/dL)	135.83 ± 28.87	138.18 ± 30.98	0.654
LDL-C (mg/dL)	70.06 ± 30.95	73.36 ± 23.51	0.492
HDL-C (mg/dL)	44.35 ± 8.86	38.88 ± 9.9	0.001
Zn ⁺² (µg/dL)	13.14 ± 1.39	13.51 ± 1.72	0.188
Ca ⁺² (mg/dL)	9.76 ± 1.55	8.98 ± 2.29	0.025

Serum levels of cytokines, MMP9 and Vitamin D, and gene expression of MMP9 and TIMP1

Serum levels of TNF-α (Fig. 1a) and IL-6 (Fig. 1b) elevated significantly in CAD patients compared to controls. However, Vitamin D levels diminished in CAD compared to controls (Fig. 1c). MMP-9 gene expression (Fig. 1d) and serum levels (Fig. 1e) were found to be higher in CAD in comparison to controls, while there was no considerable change in terms of TIMP1 between the groups (Fig. 1f).

Moreover, ROC curve analysis for PBMC expression of MMP-9 gene indicated a good ability to distinguish between CAD and controls (area under curve = 0.763, 95% CI (0.682, 0.845), p value = 0.001) (Fig. 2).

As it shown in Table 2 Pearson correlation analysis in controls showed a positive significant correlation ($P < 0.05$) between MMP-9 Levels and MMP-9 gene expression. There was a positive correlation ($P < 0.05$) between BMI and TIMP1 gene expression level. A positive correlation ($P < 0.05$) was found between MMP-9 and IL-6 levels

Table 2
Pearson analysis of correlation between variables in controls

	TIMP1 gene expression	MMP-9 Levels	MMP-9 gene expression
TIMP1 gene expression	1	0.034	0.231
MMP-9 Levels	0.034	1	0.289*
MMP-9 gene expression	0.231	0.289*	1
Age (years)	0.146	-0.042	-0.036
BMI (m ² /Kg weight)	0.248*	-0.064	-0.065
SBP (mmHg)	0.074	-0.081	-0.200
DBP (mmHg)	0.109	-0.080	-0.081
FBS (mg/dl)	-0.079	-0.203	-0.084
TG (mg/dl)	0.087	0.154	0.060
TC (mg/dl)	-0.178	0.009	-0.240
LDL-C (mg/dl)	-0.145	-0.054	-0.174
HDL-C (mg/dl)	-0.073	-0.036	-0.050
Vitamin D	0.121	-0.001	0.151
TNF- α (pg/mL)	-0.102	0.068	-0.003
IL-6 (Pg/mL)	-0.114	0.261*	-0.019
Zn ⁺² (μ g/dL)	0.105	-0.082	0.064
Ca ⁺² (mg/dl)	-0.051	-0.001	-0.115
*p < 0.05 and **p < 0.01			

Pearson analysis for correlation between variables in CAD patients are presented in Table 3. Remarkable positive correlations were detected between TIMP1 gene expression and both MMP-9 serum levels and PBMC MMP-9 genes expression (P = 0.049 and P = 0.001 respectively). A considerable negative correlation (P < 0.01) was identified between BMI and TIMP1 and MMP genes expression. Relations between MMP-9 levels and both LDL-C levels and DBP were positive significantly (P = 0.023 and P = 0.040 respectively). TNF- α positively correlated with MMP-9 levels (P = 0.003). there was a positive correlation between IL-6 levels and MMP-9 Levels (P = 0.007).

Table 3
Pearson analysis for correlation between variables in CAD patients

	TIMP1 gene expression	MMP-9 Levels	MMP-9 gene expression
TIMP1 gene expression	1	0.306*	0.411**
MMP-9 Levels	0.306*	1	0.288*
MMP-9 gene expression	0.411**	0.288*	1
Age (years)	-0.159	0.125	0.097
BMI (m ² /Kg weight)	-0.324**	-0.120	-0.287*
SBP(mmHg)	-0.065	0.177	-0.127
DBP (mmHg)	-0.092	0.292*	-0.132
FBS (mg/dl)	0.064	-0.145	-0.096
TG (mg/dl)	-0.020	0.118	-0.045
TC(mg/dl)	0.157	0.186	-0.137
LDL-C(mg/dl)	0.107	0.254*	-0.129
HDL-C (mg/dl)	-0.051	-0.062	-0.058
Vitamin D (IU)	0.174	-0.088	0.101
TNF- α (pg/mL)	0.215	0.572**	0.072
IL-6 (pg/mL)	0.225	0.543**	0.107
Zn ²⁺ (μ g/dL)	0.099	-0.128	0.069
Ca ²⁺ (mg/dl)	0.150	-0.043	0.104
*p < 0.05 and **p < 0.01			

Discussion

Present study revealed a remarkable increase of both MMP-9 levels and PBMC MMP-9 gene expression in CAD patients as compared with controls. A positive correlation was also found between MMP-9 levels and PBMC MMP-9 gene expression of CAD patients as well. Our findings are in agreement with the findings of studies which reported elevated serum levels of MMP-1, MMP-9, are in patients with CAD [9, 17]. TIMP1 is expected to be lower in CAD patients, but contrary to the results of some studies, our results did not show a significant difference between TIMP1 gene expression of CAD patients and controls. Although a positive correlation was found between TIMP1 gene expression and both serum and gene expression levels of MMP-9 in our studied CAD patients. This positive association could be attributed to a

compensatory mechanism to neutralize the increase of gene expression and serum concentration of MMP-9. The result of a study on patients who had atrial fibrillation, the myocardial IMP1 protein level is increased and in the failing heart of patients with dilated cardiomyopathy or ischemic cardiomyopathy, IMP1 and IMP3 levels were declined [18]. In concordant with our result, the result of a study that conducted by Asli et al., showed no difference between TIMP1 of ST elevation myocardial infarction and non-ST elevation myocardial infarction patients [9]. Therefore, these some discrepancy in how IMP levels are changed in patients with various types of heart disease, could be related to the type of disease, the region of where the specimen was collected, stage of disease and the ethnicity of the studied population. As it shown in our results a significant correlation was identified between LDL-C and MMP-9 serum levels in CAD group, while this relation was not found in the controls. In a study on mice MMP-9 ^{-/-}, Samuel et al., showed that mice MMP-9 ^{-/-} could not reduce the expression of sterol regulatory element binding protein 2 pathway genes in the liver, which controls the synthesis and uptake of cholesterol. They also observed that in MMP-9 ^{-/-} mice plasma secreted phospholipase A2 activity was increased. Moreover, they showed that MMP-9 modulates cholesterol metabolism through axis of MMP-9–plasma secreted phospholipase A2 that regulates transcriptional responses to dietary cholesterol in the liver. They suggested that MMP system dysregulation lead to metabolic disorder, which result in atherosclerosis pathogenesis [19]. In a study by Xiao-Ping et al, the influence of ox-LDL on MMP-9 expression was investigated and the results demonstrated that ox-LDL increase MMP-9 expression and reducing expression of TIMP1 in macrophages. They also suggested that, ox-LDL may play a role in macrophage-mediated matrix cleavage in the atherosclerotic plaques, which increase the risk of plaque rupture and/or vascular remodeling [20]. A positive relation was found between MMP-9 levels and DBP of our studied CAD patients. Eugenia et al. showed that hypertension-induced vascular remodeling is result of alteration in structure and composition of the ECM that is mediated by MMPs/TIMPs balance deficiency. On the contrary, the inhibition of MMPs could attenuate the arterial remodeling in animal models of atherosclerosis and hypertension, as well as the arterial remodeling of humans [21]. Conversely, the results of a study by Wiktor et al. on patients with stable CAD and 15 healthy subjects showed that concentration of, MMP-9 indicated no considerable difference between subjects with and without hypertension [22]. DBP of our CAD patients showed a significant positive correlation with MM-9 levels, whereas there was no correlation was shown for systolic blood pressure. A study by Yasmin et al. showed that increased circulating elastases, in particular MMP-9, might play a role in the process of arterial stiffening and systolic hypertension [22].

MMPs expression is related with the inflammation and oxidative stress, and play a critical role in atherosclerosis development and plaque rupture. MMPs production and activation are stimulated by oxidative stress that is involved in cardiovascular disease incidence. Atherosclerotic plaque evolution accompanied with increase of MMP-9 levels as well. Increment of MMP-9 plasma concentration has been associated with hypertension has been. Human atherosclerotic plaques are associated with increased TIMP1 levels [23]. While, in patients with aortic stenosis, myocardial TIMP1 is reported to decrease in both mRNA and protein levels [24]. The discrepancy could be related to the type of disease, stage of disease, region of sampling and the ethnic background of the population [25]. As it shown in the results,

the vitamin D levels of CAD patient decreased significantly than controls. In agreement with our results, an analysis of NHANES III 1988–1994, showed that vitamin D deficiency was associated with CVD. The results of several meta-analysis studies demonstrated a negative relation between vitamin D levels and risk of cardiovascular disease and they also show that low vitamin D levels increase risk of ischemic heart disease and early death. While high levels of vitamin D could be a favorable factor for desirable lipid profile and less metabolic syndrome occurrence. It has been suggested that vitamin D deficiency increase risk of high blood pressure through renin-angiotensin system. Moreover, Vitamin D deficiency is associated with increase of the E-selectin and vascular cell adhesion molecules that could induce the atheroma plaque formation. Vitamin D deficiency is involved in oxidative stress and inflammation formation that have an impressive role in atherosclerosis of the intima. Atherosclerosis is induced by vitamin D deficiency through repression of vascular smooth muscle cell proliferation.

MMPs, and their inhibitors alteration have been also reported in obese patients. However, controversy exists due to a multitude of studies showing elevated levels of MMP-2, MMP-9 and TIMP-1 in obese individuals, whereas other studies reporting no differences in subjects with obesity [33]. Our results showed a significant negative correlation between BMI and both PBMC TIMP-1 gene expression and serum level of MMP of CAD patients and a positive significant correlation between BMI and PBMC TIMP-1 gene expression of controls. Although there was no significant between BMI of CAD patients and controls. A study by Barbara et al. showed that MMP-9 and TIMP-1 concentrations were higher in obese children than in the control group. They also showed relations between MMP-9 and TIMP-1 with BMI [33]. In a study by Carla et al. showed that overweight individuals had lower TIMP-1 levels and higher MMP-9 and MMP-2 in saliva comparing to normal weight subjects [34].

Conclusion

Taking together, our results show MMP9 and cytokines levels could have a role in the process of atherosclerosis. Although literature data suggest a role for MMPs activation in cardiovascular diseases, prospective data are needed to evaluate whether interventions aiming at modification of MMPs activity can reduce atherosclerosis.

Declarations

Data availability

All data collected and analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

There is no conflict of interest for authors to declare in this study.

Availability of data and material

The data that support the findings of this study are available from corresponding authors, [J.H, N.M.], upon reasonable request.

Code availability

Not applicable

Author Contribution

RA and NM: Methodology. SB, TK and TA: Performed experiments. RF: Data analysis. MM and TG: Investigation and visualization. SF: Supervision and revised final manuscript and confirmed; All authors read and approved final manuscript

Ethics approval

The study was approved by the Ethics committee of Iran University of Medical Sciences (IR.IUMS.REC1395.9221184201).

Consent to participate

Informed written consent was signed by all studied participants.

Consent for publication

Not applicable

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Figures

Figure 1

Serum levels of cytokines, MMP9 and Vitamin D, and gene expression of MMP9 and TIMP1 in studied groups. Fig.1 a) Serum levels of TNF- α were higher in CAD patients (12.39 ± 5.25) compared to controls (9.27 ± 3.96 , $p < 0.001$). b) Serum IL-6 was found to be higher in CAD (7.48 ± 5.25) in comparison to controls (5.11 ± 3.25 , $p < 0.001$). c) Circulating Vitamin D indicated lower levels in CAD (29.58 ± 22.03) compared to controls (44.26 ± 22.02). d). PBMC gene expression of MMP-9 decreased in CAD (1.11 ± 0.17) compared to control group (1 ± 0.14 , $p < 0.001$). e) MMP-9 serum levels were found to be increased in CAD (1143 ± 357.3) .** $P < 0.01$

Figure 2

ROC curve analysis for MMP-9 gene expression level of CAD patients