

Neutrophil serine proteases and their endogenous inhibitors in coronary artery ectasia patients

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ABSTRACT

Objective: Proteolytic enzymes possibly contribute to coronary artery ectasia (CAE). This study aimed to determine whether neutrophils, neutrophil serine proteases (NSPs), and their endogenous inhibitors participated in the pathological process of CAE.

Methods: The study consisted of 30 patients with CAE, 30 patients with coronary artery disease (CAD), and 29 subjects with normal coronary arteries (Control). The following circulating items were measured: the main NSPs, including human neutrophil elastase (HNE), cathepsin G (CG), and proteinase 3 (PR3); soluble elastin (sElastin), which was a degradation product of elastin fibres; NSP inhibitors such as α 1-protease inhibitor (α 1-PI), α 2-macroglobulin (α 2-MG), secretory leucoprotease inhibitor (SLPI), and elafin; as well as two neutrophil activation markers (myeloperoxidase and lactoferrin) and three classic neutrophil activators [tumor necrosis factor- α (TNF- α), interleukin-8 (IL-8), and bacterial endotoxin].

Results: The levels of HNE, CG, and sElastin were elevated in the CAE group. The levels of α 1-PI and α 2-MG were also significantly increased in the CAE group. The levels of myeloperoxidase and lactoferrin were higher in the CAE group. The levels of TNF- α , IL-8, and endotoxin were unchanged in the CAE group compared with those in the CAD group.

Conclusion: Neutrophils may participate in the process of vessel extracellular matrix destruction and coronary ectasia by releasing NSPs in a non-classical manner. (*Anatol J Cardiol* 2016; 16: 23-8)

Keywords: coronary artery ectasia (CAE), neutrophil serine proteases (NSPs), human neutrophil elastase (HNE), soluble elastin (sElastin), extracellular matrix (ECM)

Introduction

Coronary artery ectasia (CAE) was defined as the inappropriate dilatation of a coronary artery, with the luminal diameter 1.5 or more times wider than that of adjacent normal segments (1). More than 50% of CAE patients had obstructive coronary artery disease (CAD) (2). To date, the pathogenesis of CAE has remained elusive. The pathological manifestations in CAE were characterized by an extensive destruction of musculoelastic elements, particularly elastin fibers which were dominant components of the extracellular matrix (ECM) of the coronary wall (3, 4). Proteolytic enzymes may play vital roles in such destruction processes because they generally serve as terminal effectors mediating tissue destruction, and most attention has been focused on two groups of enzymes: the serine proteinases (5, 6) and matrix metalloproteinases (MMPs). Neutrophil serine proteases (NSPs), a subfamily of serine proteinases, is well known to principally damage ECM components, particularly elastin fibres (7, 8). NSPs were mainly

released from activated neutrophils (9-11). The blood neutrophil to lymphocyte ratio was reported to be higher in CAE populations (12-14), thus raising the question that whether neutrophils and NSPs participated in the coronary ectasia process.

The principal NSPs were human neutrophil elastase (HNE), proteinase 3 (PR3), and cathepsin G (CG) (15). They were mainly released from azurophilic granules of neutrophils, while neutrophils were exposed to various cytokines and chemo attractants such as tumor necrosis factor- α (TNF- α), interleukin-8 (IL-8), C5a, and lipopolysaccharides (LPS) (10, 11). The main endogenous inhibitors of NSPs included α 2-macroglobulin (α 2-MG), α 1-protease inhibitor (α 1-PI), secretory leucoprotease inhibitor (SLPI), and elafin (7, 11, 16). The imbalance between NSPs and their inhibitors may lead to the damages of ECM proteins and contribute to CAE.

The main aim of this study was to detect the profile of the NSP system and to comprehensively evaluate the neutrophil activation status in the CAE population by measuring the follow-

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ing parameters: 1) NSPs and their endogenous inhibitors; 2) the elastin fiber degradation marker, i.e., soluble elastin (sElastin); 3) two types of neutrophil activation markers, i.e., myeloperoxidase (17) and lactoferrin; (18, 19) and 4) three classic neutrophil activators, i.e., TNF- α , IL-8, and bacterial endotoxin containing LPS. To the best of our knowledge, this is the first study about the NSP system and neutrophil activation in the CAE population, and our results would be useful clues for further studies.

Methods

Patient population and design

This study has been designed in a cross-sectional and prospective observational manner.

In total, 1239 patients underwent coronary angiograms from October 2013 to July 2014 at the cardiac catheterisation centre of our Hospital. Among these patients, 42 (3.39%) of them had CAE and 30 of them without conditions listed in the inclusion criteria were included into the CAE group. During the same period, we randomly selected 30 patients with angiographically documented CAD and 29 subjects with relatively normal coronary arteries (named Control). The three groups were balanced with respect to age, gender, and other baseline characteristics. This study was approved by the local Ethics Committee and was in accordance with the Declaration of Helsinki. Informed consent was obtained from all the study participants.

Inclusion criteria

Each angiogram was interpreted by two independent cardiologists. CAE was defined as an ectatic artery diameter exceeding 1.5 times that of adjacent normal segments (3). CAD was defined as 50% or more stenosis in one or more major coronary arteries (20). Subjects without abnormal coronary arteries were used as controls.

Exclusion criteria

The exclusion criteria were acute coronary syndrome, cardiomyopathy, valvular heart disease, heart failure, aneurysm in other vessels, collagen tissue diseases, vasculitis, syphilis, chronic obstructive lung disease, pulmonary hypertension, early menopause, organic hepatic diseases, renal failure, known malignancy, local or systemic infection, previous history of infection (<3 months), other inflammatory diseases, and any medications that could potentially interfere with the measurement of these markers.

Medical records and blood samples

Most of the data in this study were extracted from the medical records in our hospital. The blood samples were collected immediately after the coronary angiography, and serum and plasma samples were separated within 6 h and maintained at -80°C.

The enzyme-linked immunosorbent assay (ELISA) was used for the detection of circulating items related to the NSP system and neutrophil activation.

The ELISA kits for HNE, α 1-PI, SLPI, elafin, lactoferrin, and sElastin were purchased from Elabscience (Elabscience Biotechnology Co., Ltd, Wuhan, China). The ELISA kits for PR3

and CG were purchased from Cusabio (Cusabio life science Inc., Wuhan, China), the ELISA kits for α 2-MG and myeloperoxidase were purchased from Boster (Boster Biological Engineering Co., Ltd, Wuhan, China), and the ELISA kit for IL-8 and TNF- α were purchased from Neobioscience (Neobioscience Technology Co., Ltd, Beijing, China). Quantitative determinations of the above items were performed using sandwich ELISA kits according to the manufacturer's instructions.

Bacterial endotoxin test by the gel clot method

A bacterial endotoxin test kit (TIANDZ Inc., Beijing, China) was used to determine whether the samples were endotoxin free. This gel clot method uses components found in the blood of the blue horseshoe crab that forms a gel-like clot when incubated in the presence of endotoxins. Briefly, the plasma samples were incubated with the horseshoe crab reagent at the same time as that of a standard series of the control standard endotoxin, which was used as a positive control, and endotoxin-free water, which was used as a negative control. After the incubation period, the tubes containing the controls and the plasma samples were evaluated to determine the presence or absence of the gel clot (i.e., the presence/absence of endotoxins).

Statistical analysis

Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) ver. 17.00 package software. General descriptive characteristics are presented as the mean \pm standard deviation (SD). Categorical data were presented as percentages (%). Gender and risk factors were compared by the chi-square test. Continuous numeric data were tested with a one-way analysis of variance or the Kruskal-Wallis test. The least significant difference (LSD) method, i.e., the Fisher's test was used for multiple comparisons between the groups. The lowest level of significance was accepted as $p < 0.05$.

Results

The baseline characteristics of the CAE, CAD, and Control groups are given in Table 1. The three groups were similar with respect to age, gender, presence of hypertension, blood pressure, fasting glucose, lipid profile, and other cardiovascular risk factors, except a family history of CAD. The blood cell subtype counts, hs-CRP, hepatic functions, and renal functions were also balanced among the three groups.

1) Two of the three types of circulating NSPs were elevated in the CAE group.

As shown in Table 2, both HNE and CG were significantly higher in the CAE group than in the CAD and Control groups ($p^1 < 0.05$, $p^2 < 0.05$). However, there was no difference in PR3 among the three groups ($p > 0.05$).

2) The elastin fiber degradation maker, sElastin, was increased in the CAE group.

Table 1. Clinical characteristics of the study patients

Variables	CAE (n=30)	CAD (n=30)	Control (n=29)	P
Age, years	62.80±10.17	60.07±9.21	63.96±9.59	0.286
Gender, male/female	20/10 (66.67%)	21/9 (70.00%)	15/14 (51.72%)	0.304
Diabetes mellitus	11/19 (36.67%)	13/17 (43.33%)	10/19 (33.33%)	0.765
Glucose, mmol/L	6.12±1.56	6.21±1.73	5.96±1.29	0.957
Hypertension	24/6 (80.00%)	21/9 (70.00%)	20/9 (68.97%)	0.570
Systolic blood pressure, mm Hg	132.57±17.96	132.13±17.07	129.86±16.77	0.813
Diastolic blood pressure, mm Hg	79.33±12.14	74.93±14.56	75.55±11.32	0.357
Heart rate, per minute	71.37±10.06	70.83±9.02	72.86±11.63	0.736
Family history of CAD	7/23 (23.33%)	12/18 (40.00%)	3/26 (10.34%)	0.030
Family history of type 2 diabetes mellitus	2/28 (6.67%)	6/24 (20.00%)	3/26 (10.34%)	0.269
Smoking	10/20 (33.33%)	15/15 (50.00%)	9/20 (31.03%)	0.259
Alcohol consumption	6/24 (20.00%)	10/20 (33.33%)	9/20 (31.03%)	0.471
TC, mmol/L	1.66±1.22	1.73±0.84	1.50±1.05	0.696
HDL cholesterol, mmol/L	1.08±0.31	1.08±0.39	1.15±0.32	0.270
LDL cholesterol, mmol/L	2.39±0.80	2.50±0.87	2.31±0.78	0.701
TG, mmol/L	1.66±1.22	1.73±0.84	1.50±1.05	0.139
Body mass index, kg/m ²	26.45±3.83	25.51±3.77	25.16±2.98	0.387
Hs-CRP, mg/L	2.90±3.60	2.25±2.24	2.16±2.61	0.542
Leukocytes, 10 ³ /μL	6.26±1.26	6.47±1.62	6.36±1.37	0.852
Neutrophils	3.74±1.00	3.99±1.28	4.04±1.23	0.560
Lymphocytes	1.90±0.60	1.85±0.58	1.74±0.52	0.523
Monocytes	0.39±0.14	0.39±0.13	0.37±0.12	0.765
Neutrophil /lymphocyte ratio	2.16±0.87	2.27±0.73	2.60±1.30	0.545
ALT, U/L	26.07±20.76	28.70±27.03	26.10±14.23	0.844
BUN, mmol/L	6.11±1.73	5.50±1.93	5.75±1.60	0.246
Creatinine, μmol/L	85.67±29.64	78.87±18.50	73.69±19.53	0.310
Ectatic vessels of CAE				
LM	3/27 (10.00%)			
LAD	11/19 (36.67%)			
LCX	18/12 (60.00%)			
RCA	20/10 (66.67%)			

P values for comparison among and between the groups. The significance level was 0.05. CAD - coronary artery disease; CAE - coronary artery ectasia; Control - control group; HDL-c - high-density lipoprotein cholesterol; LAD - left anterior descending coronary artery; LCX - left circumflex coronary artery; LDL-c - low-density lipoprotein cholesterol; LM - left main coronary artery; RCA - right coronary artery; TC - total cholesterol; TG - triglyceride

As shown in Table 2, the plasma level of sElastin was significantly higher in the CAE group than in the CAD ($p^1 < 0.05$) and Control groups ($p^2 < 0.05$).

3) The profile of the NSP endogenous inhibitors was changed in the CAE group.

According to Table 3, the levels of α 2-MG and α 1-PI increased significantly in the CAE group compared with those of the other two groups ($p^1 < 0.05$, $p^2 < 0.05$). However, the levels of SLPI and elafin were the same in the three groups ($p > 0.05$).

4) Neutrophil activation makers were higher in the CAE population.

As shown in Table 4, the levels of both myeloperoxidase and

lactoferrin were significantly higher in the CAE group than in the CAD and Control groups ($p^1 < 0.05$, $p^2 < 0.05$).

5) The levels of neutrophil activators did not change in the CAE group.

As shown in Table 4, the levels of both IL-8 and TNF- α in the CAE group was similar to that of the CAD group, and none of the subjects from the three groups were bacterial endotoxin positive.

Discussion

This study mainly evaluated NSPs and their endogenous inhibitors as well as the neutrophil activation condition in CAE

Table 2. The three NSP and elastin fibre degradation production

Variables	CAE (n=30)	CAD (n=30)	Control (n=29)	P	P ¹	P ²	P ³
HNE, ng/mL	34.86±10.17	26.66±4.24	26.22±4.23	0.000	0.000	0.000	0.387
CG, pg/mL	659.82±262.33	544.08±132.38	507.59±180.17	0.031	0.027	0.016	0.184
PR3, ng/mL	1.30±0.33	1.22±0.19	1.24±0.20	0.376	0.574	0.927	0.628
sElastin, ng/mL	22.67±11.71	13.86±5.46	10.13±1.03	0.000	0.001	0.000	0.380

P values for comparison among groups and comparison between groups using the LSD method: *P*¹, CAE group vs. CAD group; *P*², CAE group vs. Control group; *P*³, CAD group vs. Control group. The significance level was 0.05. CAD - coronary artery disease; CAE - coronary artery ectasia; Control - control group; CG - cathepsin G; HNE - human neutrophil elastase; PR3 - proteinase 3; sElastin - soluble elastin

Table 3. The main circulating NSP inhibitors

Variables	CAE (n=30)	CAD (n=30)	Control (n=29)	P	P ¹	P ²	P ³
α1-PI, ng/mL	423.22±178.61	207.70±37.08	193.32±36.42	0.000	0.000	0.000	0.610
α2-MG, mg/mL	5.28±2.30	3.99±1.65	3.31±1.47	0.001	0.034	0.000	0.075
SLPI, pg/mL	329.15±264.25	406.40±337.48	289.52±97.95	0.202	0.101	0.561	0.116
Elafin, pg/mL	170.00±163.02	197.80±143.11	156.64±99.71	0.510	0.510	0.491	0.198

P values for comparison among groups and comparison between groups using the LSD method: *P*¹, CAE group vs. CAD group; *P*², CAE group vs. Control group; *P*³, CAD group vs. Control group. The significance level was 0.05. α1-PI - α1-protease inhibitor; α2-MG - α2-macroglobulin; CAD - coronary artery disease; CAE - coronary artery ectasia; Control - control group; SLPI - secretory leucoprotease inhibitor

Table 4. Two activation markers for neutrophils and three classic neutrophil activators

Variables	CAE (n=30)	CAD (n=30)	Control (n=29)	P	P ¹	P ²	P ³
MPO, ng/mL	5.25±3.86	1.38±0.52	1.89±1.07	0.000	0.000	0.000	0.291
LTF, ng/mL	182.92±103.75	92.01±44.52	79.92±38.98	0.000	0.000	0.000	0.504
IL-8, pg/mL	42.46±4.48	41.77±2.12	40.70±4.47	0.071	0.712	0.051	0.450
TNF-α, pg/mL	48.18±8.34	47.78±7.42	46.40±6.98	0.039	0.657	0.030	0.026
Endotoxin positive rate	0/30 (0.00%)	0/30 (0.00%)	0/29 (0.00%)				

P values for comparison among groups and comparison between groups using the LSD method: *P*¹, CAE group vs. CAD group; *P*², CAE group vs. Control group; *P*³, CAD group vs. Control group. The significance level was 0.05. CAD - coronary artery disease; CAE - coronary artery ectasia; Control - control group; IL-8 - interleukin-8; LTF - lactoferrin; MPO - myeloperoxidase; TNF-α - tumor necrosis factor-α

patients. The CAD group was enrolled in this study because most of the CAE patients had obstructive CAD (2). In the present study, 90% of the CAE patients had CAD. The baseline characteristics were balanced among the three groups, except the family history of CAD. This study failed to duplicate the results of previous studies (12, 13), which found that the neutrophil to lymphocyte ratio was upregulated. This may be due to the limited sample size in the present study.

The three NSPs (HNE, PR3, and CG) were major components of neutrophil azurophilic granules (16, 21). In addition to their bacterial defense function, NSPs had an important role in the ECM destruction process (6, 11, 22). Elastin fibers were the main targets of extracellular NSPs. This study showed that the circulating concentrations of HNE and GC were increased in the CAE group. Unlike HNE and CG, which could be released to extracellular sites, PR3 was constitutively expressed on the membranes of neutrophils and that could explain why circulating PR3 was not changed in this study (11). At the same time, the circulating sElastin, a degradation product of elastin fibers, was also higher in CAE patients, thereby indicating that the

degradation of elastin fibers may be due to the exposure to the increased NSPs. Elastin fibres were the dominant ECM proteins in the coronary media, constituting up to 50% of the vessel's dry weight (23, 24) and supporting the elasticity and tensile strength of the vessels (7, 8, 25). Because of a lack of de novo synthesis of elastin in adults (25), the chronic degradation of elastin fibers was irreversible and may eventually lead to coronary ectasia.

During the same period, four types of NSPs inhibitors, including α1-PI, α2-MG, SLPI, and elafin, were detected in plasma. α1-PI was the most abundant serpin present in human blood, synthesized primarily by hepatocytes (11). It was an irreversible inhibitor of HNE, PR3, CG, and other proteinases (11, 26, 27). α2-MG was a polyvalent homotetrameric inhibitor, which inhibited all classes of proteases (11, 26, 27). SLPI belonged to the chelonianin family and was expressed by epithelial cells, macrophages, and neutrophils. It could reversibly inhibit HNE and CG but not PR3 (11, 27). Elafin was found in the bronchial secretions and skin. It could inhibit HNE and PR3 but not GC (11, 26, 27). The results showed that α1-PI and α2-MG

increased in the CAE group, whereas SLPI and elafin were unchanged, possibly because the vessels were not the main distribution sites for SLPI and elafin. The increases of the above two NSPs inhibitors seemed to play the role of restricting the activity of NSPs and leading coronary ectasia into a chronic process.

In the present study, the NSPs were most likely released from activated neutrophils because two neutrophil activation makers, myeloperoxidase and lactoferrin, (17, 18) were elevated in the CAE group. Briefly, myeloperoxidase, the most abundant granule enzyme, was released into the phagosome or the extracellular space upon neutrophil activation (6, 17, 28). Lactoferrin was a non-heme iron-binding glycoprotein, which was also mainly found in neutrophil-specific granules (6, 18, 19). This finding indicated that the simultaneous increase of neutrophil activation makers and NSPs was most likely due to a single reason, i.e., the neutrophils were activated.

CAE was considered as a chronic systemic inflammatory disease because of the disorder of a set of inflammatory mediators in circulation and the infiltration of inflammatory cells, including neutrophils, in the walls of coronary arteries in ectasia (1, 4, 29). In addition to participating in acute inflammatory, activated neutrophils could induce tissue damage and regulate inflammatory responses by releasing NSPs, deriving oxidative metabolism, and producing a set of pro-inflammatory (7, 30). Excessive or dysregulated neutrophil responses together with inadequate repair contributed to persisting tissue damage that underlay many chronic inflammatory diseases, including rheumatoid arthritis, pulmonary emphysema, asthma, and so on (9-11, 21). In the present study, the neutrophils in the CAE population seemed to be continuously activated in a special manner because this study failed to find any difference in the three classic neutrophil activators (IL-8, TNF- α , and bacterial endotoxin containing LPS) between the CAE and CAD groups. Briefly, the neutrophils and NSPs may play vital roles in the pathological process of CAE by releasing NSPs in a non-classical manner. Because of the scarcity of published data with respect to neutrophils and CAE, the present study provides important clues for future in-depth studies of CAE.

Study limitations

The blood neutrophil samples were not collected because of the experimental design at the beginning. All the experimental data were collected from the analyses of blood samples. The changes in the serum markers may not always match the changes in the coronary arteries. However, CAE was considered a chronic systemic disease (29), and the three groups had distinctive features in the coronary walls; thus, those circulating markers were supposed to be able to reflect the changes in coronary arteries to some degree.

Conclusion

This study showed that the imbalance between circulating NSPs and their inhibitors did exist in the CAE population, indicating that the NSPs may participate in vessel ECM destruction process and contribute to coronary ectasia. At the same time, the circulating concentration of the neutrophil activation markers were also higher, suggesting that the NSPs were mainly released from activated neutrophils. Finally, the classic neutrophil activators in the CAE group were not different from the CAD group, thereby indicating an unidentified mechanism of neutrophil activation. In summary, neutrophils and NSPs may play important roles in the pathological process of coronary ectasia.

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