New Markers in Atherosclerosis: Thrombospondin-2 (THBS-2) and Leukocyte Cell-Derived Chemotaxin-2 (LECT-2); An Immunohistochemical Study

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Current research investigating the role of THBS2 and LECT-2 in atherogenesis is very limited. Therefore, we designed this study to demonstrate the role of THBS-2 and LECT-2 in atherosclerosis at the tissue level in fresh specimens.

Material/Methods: A total of 32 patients who underwent coronary bypass surgery were enrolled. Aortic wall punch biopsies were obtained at the site of proximal aortosaphenous bypass graft anastomosis. A specimen of left internal mammarian artery (LiMA) was taken from the segment just proximal to its anastomosis. The aortic tissue is representive of the atherosclerotic tisue, and LiMA tissue is representative of the non-atherosclerotic area. The specimens were painted with CD68 for macrophage, and THBS-2 and LECT-2 antibodies for immunohistochemical staining.

Results: Aortic THBS-2 levels were significantly lower, whereas aortic LECT-2 levels were significantly higher when compare to LiMA (14.4±9.9 (5–30) and 36.9±13.0 (5–60) p: 0.0001 and 20.3±15.0 (5–60) and 20.8±13,8 (10–30) p: 0.0001, respectively). CD68+ and monocyte level correlated significantly with AHA atherosclerosis grade (p=0.01, r=0.45 and p=0.001, r=0.56, Spearman's test). CD68+ level correlated significantly with LECT-2 levels in atherosclerotic aortic tissue (p=0.026, r=0.392, Spearman's test), whereas aortic TSBN-2 levels were not.

Conclusions: The present study has taken the first steps to highlight new markers in atherosclerosis by using immunohistochemical method. The study results suggest that the tissue levels of THBS2 and LECT-2 may correlate with the stage of atherosclerosis.

MeSH Keywords: Aorta • Atherosclerosis • Chemotaxis, Leukocyte • Immunohistochemistry • Thrombospondins

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Background

Atherosclerosis is a chronic low-grade inflammatory disease, in which inflammatory mediators and cells play crucial roles, however, several mechanisms underlying the pathogenesis of atherosclerosis remain unexplained.

Inflammatory cell activation is critical to the onset of clinical events in atherosclerosis. Monocytes are key players in the atherosclerotic process as monocyte accumulation is considered to be the first step in this process [1]. There is some evidence in the current literature suggesting that thrombospondin-2 (THBS-2) plays an important role in atherosclerosis by regulating cell-to-matrix interaction, neovascularization, and extracelular matrix (ECM) proliferation [2–4]. Moreover, leukocyte cell-derived chemotaxin-2 (LECT-2) is a hepatokine that is related to insulin resistance and obesity; and acts as a chemoattractant for leukocytes [5,6]. Current research investigating the role of THBS2 and LECT-2 in atherogenesis is very limited [7]. Therefore, we designed this study to demonstrate the role of THBS-2 and LECT-2 in atherosclerosis at the tissue level in fresh specimens.

Material and Methods

Patients and tissue sampling

A total of 32 patients who underwent coronary bypass surgery were enrolled in this study. Aortic wall punch biopsies were obtained at the site of proximal aortosaphenous bypass graft anastomosis. A specimen of left internal mammarian artery (LiMA) was taken from the segment just proximal to its anastomosis. The aortic tissue is representative of the atherosclerotic tisue and LiMA tissue is representative of the non-atherosclerotic area.

This study was approved by the local ethics committee. Informed consent was obtained from each patient.

Blood sampling

Hemoglobin and WBC count were measured as part of the automated complete blood count (CBC) using a Sysmex XT-1800i (USA) hematology analyzer. Patients with a WBC count >12,000 cells/μL or <4,000 cells/μL, and body temperatures greater than 38°C were excluded from the study to eliminate co-esisting subclinical inflammatory states.

Immunohistochemisty and histopathology

Aortic tissue with visible atherosclerotic lesion was selected, sampled, and submitted for histological evaluation according to American Heart Association (AHA) histological criteria [8]. During histological classification, lesions were designated by Roman numerals representing lesion grade. Clinically silent atherosclerotic plaques were included in this study, while ruptures and healed plaques (type VI) were not included.

In the present study, 32 aortic tissue samples were embedded in paraffin blocks. For paraffin sections, aortic specimens were fixed in 10% formalin for one day, processed through graded alcohols and xylene, and embedded in paraffin. Tissue sections were then stained with haematoxylin and eosin (H&E) or immunostained (Figure 1). The specimens were painted with CD68 for macrophage, and THBS-2 (Thermo Fisher Scientific, TSP) and LECT-2 (Abcam) antibodies for immunohistochemical staining.

THBS-2 immunohistochemical staining showed myocyte and endothelial immunoreactivity in LiMA tissue (Figure 1). However, aortic punches showed positive staining in myoctes. LECT-2 immunohistochemical staining of aortic tissue showed positive staining in myocte but LiMA tissue showed myocyte and endothelial immunoreactivity. Aortic and LiMA THBS-2 and LECT-2 levels were measured by semiquantitative immunoreactivity method.

Statistical analyses

Continuous variables were expressed as the mean ± standard deviation (SD) or median (interquartile range), when appropriate. Categorical variables were expressed as percentages. The chi-square test was used to compare categorical variables. Spearman correlation coefficient was used to determine nonparametric measures of statistical dependence between two variables. To compare related variables (aorta and LiMA), Wilcoxon signed ranks test was used. Immunohistochemical and histopathological examinations were performed by two separate pathologist who were blinded to patient charactersitics. The intraclass correlation coefficient and the lower and upper limits the of the 95% CI were determined for interobserver variability. The Bland-Altman method for comparing paired measurements on aortic and LiMA THBS-2 and LECT-2 was used to determine interobserver agreement.

Two-tailed p values of less than 0.05 were considered to indicate statistical significance. All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

Results

Detailed characteristics of the study population are shown in Table 1 (mean age, 58±9 years; 77% of patients were male). The median AHA atherosclerosis grade was 2 (range, 0–5) in aortic tissue. Histological classifications of aortic specimens used in this study are shown in Table 2.
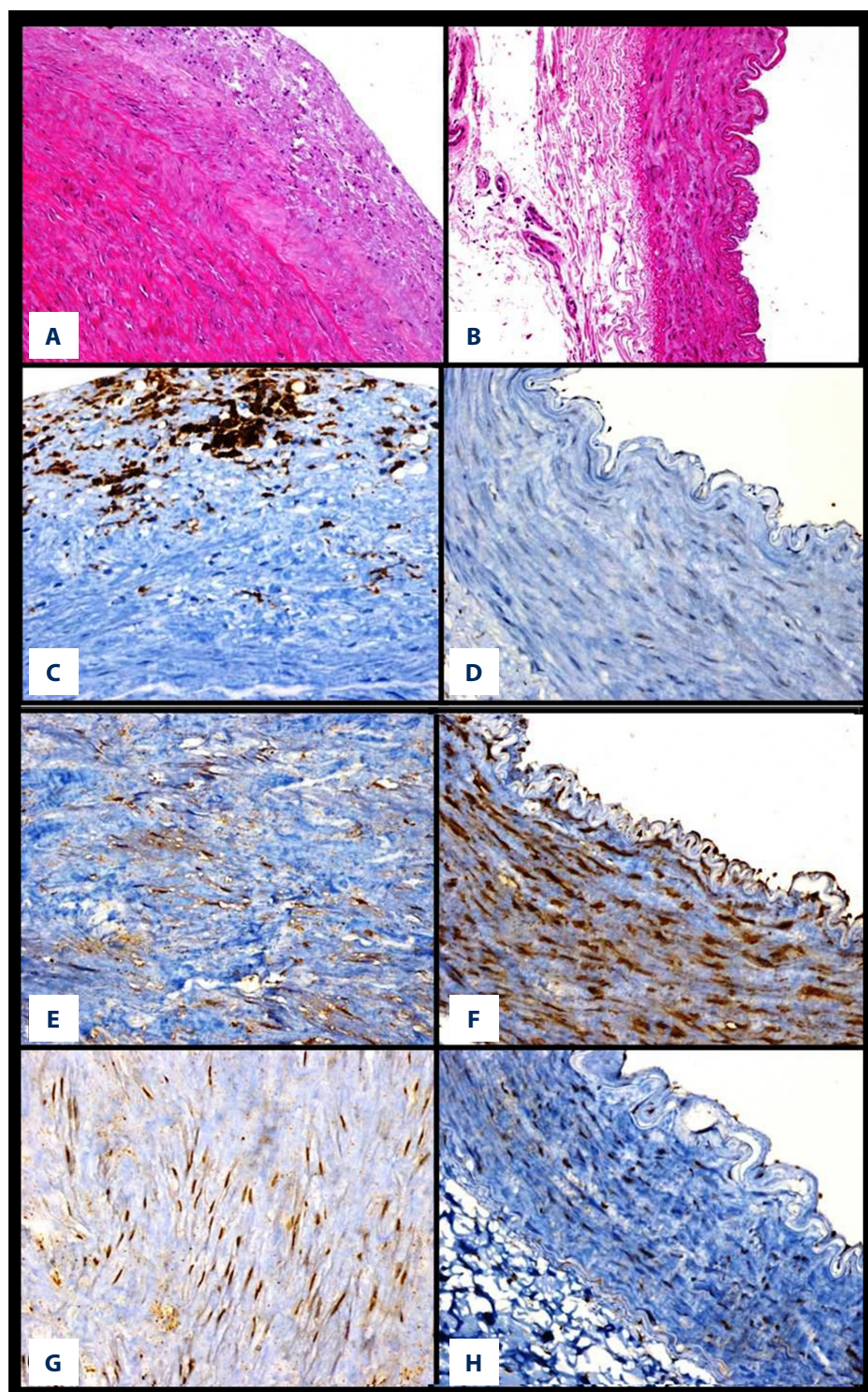
Figure 1. (**A**) Hematoxylin and Eosin stained aortic tissue ×200. (**B**) Hematoxylin and Eosin stained LiMA tissue ×200. (**C**) Immunohistochemical staining of aortic tissue with anti-CD68 antibodies ×200. (**D**) Immunohistochemical staining of LiMA tissue with anti-CD68 antibodies ×200. (**E**) Immunohistochemical staining of aortic tissue with anti-TSBN-2 antibodies ×200. (**F**) Immunohistochemical staining of LiMA tissue with anti-TSBN-2 antibodies ×200. (**G**) Immunohistochemical staining of aortic tissue with anti-LECT-2 antibodies ×200. (**H**) Immunohistochemical staining of LiMA tissue with anti-LECT-2 antibodies ×200.

Table 1. Baseline characteristics of group.

Variables	(n: 32)	Variables	(n: 32)
Patients characteristics		**Laboratory**	
Age	58±9 (40–74)	Creatinin mg/dl	0.8±0.2
Female/Male	6/26	e-GFR ml/min	100±22
Bmi kg/m^2	29.2±0.4	LDL mg/dl	136±40
Diabetes %	15/32	HDL mh/dl	44±8
Hypertension %	13/32	Hb gr/dl	14±1.7
Hyperlipidemia %	7/32	Wbc ×10^3	8.3±2.4
Smoking	18/32	Neutrophil ×10^3	5.2±1.8
Admission medication		Lymphocyte ×10^3	2.3±0.8
Aspirin %	14/32	Monocyte ×10^3	0.7±0.3
Beta blocker %	9/32	Platelet ×10^3	221±81
Digitals	0/32		
Ace/ARB %	9/32		
Statin %	6/32		
Diuretics %	0/32		
Clopidogrel %	0/32		
CaCB %	2/32		

ACE – angiotensin converting enzyme; ARB – angiotensin reseptor blocker; BMI – body mass index; e-GFR – estimated glomerular filtration rate; NLR – neutrophil to lymphocyte ratio; CaCB – Ca channel blocker; MLR – monocyte to lymphocyte ratio; Wbc – white blood cell. Results are expressed as mean ±SD or frequency (with in group percentage) and median (Interquartile range).

Table 2. Histological classification of aortic specimen used in the study.

Specimen containing	AHA classification	(n=32)
Adaptive intimal thickening	Type I	13
Intimal xanthoma	Type II	12
Pathological intimal thickening	Type III	2
Early fibroatheroma and Late fibroatheroma	Type IV/V	5

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CD68+ and monocyte level correlated significantly with AHA atherosclerosis grade (p=0.01, r=0.45 and p=0.001, r=0.56, Spearman's test) (Table 4). Table 4 shows Spearman correlation coefficients among all variables and AHA atherosclerosis grade.

CD68+ level correlated significantly with LECT-2 levels in atherosclerotic aortic tissue (p=0.026, r=0.392, Spearman's test) whereas aortic TSBN-2 levels were not.

Discussion

Human aortic atherosclerosis was evaluated in the present study. The results revealed a clear relationship between plaque morphology and CD68+ cell infiltration in proximal aortic atherosclerotic lesions. It also showed a clear relationship between THBS-2 and LECT-2 levels and atherosclerosis. This prospective observation indicated that THBS-2 and LECT-2 levels were strongly associated with aortic atherosclerosis when compare with LiMA representing non-atherosclerotic tissue.

It is well-known that atherosclerosis is a chronic inflammatory disease [1–9]. Histological classification of aortic tissue according

Table 3. CD68+,TBSN-2 and LECT-2 results between Aort and Lima.

(n=32)	Aort	Lima	p
CD68+	(0–3) (1–2)	(0–1) (0–0)	0.0001*
TSBN-2	14.4±9.9 (5–30)	36.9±13.0 (5–60)	0.0001#
Interobserver agrement (CI)	12.1 (10.6–13.6)	37.6 (32.7–44.7)	0.817–0.402
LECT-2	40.6±13.7 (30–50)	20.8±13.8 (10–30)	0.0001#
Interobserver agrement (CI)	44.5 (42.0–47.0)	17.2 (14.5–20.2)	0.255–0.305

Results are expressed as mean ±SD and min and maximal value (Interquartile range). CI – confidence interval. # Wilcoxon Signed Ranks and * Chi-square-test are used.

Table 4. Correlation analyses of variables.

N: 32		Aterosclerosis	CD68	Monocyte
Aterosclerosis	CC			
	p			
CD68	CC	.448 (**)		
	p	0.01		
Monocyte	CC	.562 (**)	.389 (*)	
	p	.001	.028	
LDL	CC	.281	–.074	.335
	p	.119	.688	.065
HDL	CC	–.505 (**)	–.434 (**)	–.225
	p	.004	.015	.224
TBSN-2	CC	–0.171	0.026	–.467 (**)
	p	0.350	0.888	0.007
LECT-2	CC	0.225	0.392	0.091
	p	0.216	0.026	0.622

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed). CC – correlation coefficient; TBSN-2 – thrombospondin-2; LECT-2 – leukocyte cell-derived chemotaxin-2 Spearman coleration test is used.

to the modified AHA classification system defines plaque morphology and complexity [8]. Monocytes play a pivotal role in the atherosclerotic process, and monocyte accumulation in the intima of the vessel is the first step in atherogenesis [1]. Macrophage colony-stimulating factor stimulates the monocytes and differentiates them into macrophages after entering plaque [10]. CD68+ infiltration reflects plaque activation; high levels of CD68+ infiltration are seen in high-grade atherosclerosis. In the present study, CD68+ infiltration was associated with aortic atherosclerosis grade, and in fresh aortic punches, CD68+ levels were significantly higher when compared with LiMA. The results from this study confirmed those from previous studies that evaluated aortic atherosclerosis in cadaveric tissues [11], in which HDL cholesterol was inversely correlated with AHA grade [12].

Thrombospondin is included a family of extracellular matrix glycoproteins. THBS-2 acts as a modulater for cell to matrix interactions and interactor for cell to surface receptors, cytokines, growth factors, proteases, and structural proteins [13]. TBSN-2 is adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions and can bind to fibrinogen, fibronectin, laminin, and type V collagen. Wang et al. suggested that THBS-2 influences atherosclerosis by regulating matrix metalloproteinase-2, which is associated with vulnerability of atherosclerotic plaque. This is because of THBS-2-null fibroblasts produced higher levels of this protein, which has been shown to be lower in atherosclerotic patients than the non-atherosclerotic group [4,14]. Additionally, in the THBS-2 absent mice, vascular and bleeding problems have been seen [15]. Finally,

Wang et al. have shown that THBS-2 genetic variants are associated with coronary atherosclerosis in Chinese population [2]. Presents study results for THBS-2 validate the findings from these prior studies.

Leukocyte cell-derived chemotaxin 2 (LECT-2), a hepatokine, was first identified as a neutrophil chemotactic factor especially expressed in liver and it can be found in the bloodstream. It has been related to skeletal muscle insulin resistance and obesity [6,16]. LECT-2 plays a role in macrophage activation via CD209 pathway [17]. Hwang et al. suggested that LECT-2 regulates all stages of atherosclerosis by inducing pro-inflammatory cytokines, as described by Stein et al. [7,18]. In our study, LECT-2 levels were strongly associated with aortic atherosclerosis and CD68+ levels correlated significantly with LECT-2 levels in atherosclerotic tissue when compare with LiMA that represents non-atherosclerotic tissue; these findings supports findings of previous studies.

Study limitations

There are two main limitations to this study. First, the study has relatively a small sample size. Second, multiple histopathological examinations of aortic tissue were not performed. Additional studies using different techniques and inflammatory markers can provide further valuable information.

Conclusions

The mechanism underlying atherosclerosis is still unclear. The present study has taken the first steps to highlight new markers in atherosclerosis by using immunohistochemical method. Second, results of this data suggest that the tissue levels of THBS2 and LECT-2 may correlate with the stage of atherosclerosis. However, further *in vivo* and clinical studies are needed to understand the exact role of THBS-2 and LECT-2 in pathogenesis of atherosclerosis

Declaration of interest

The authors report no conflicts of interest.

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