

Gene Expressions Associated with Ultrastructural Changes in Vascular Endothelium of Atherosclerotic Lesion

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Abstract—Attachment of the circulating monocytes to the endothelium is the earliest detectable events during formation of atherosclerosis. The adhesion molecules, chemokines and matrix proteases genes were identified to be expressed in atherogenesis. Expressions of these genes may influence structural integrity of the luminal endothelium. The aim of this study is to relate changes in the ultrastructural morphology of the aortic luminal surface and gene expressions of the endothelial surface, chemokine and MMP-12 in normal and hypercholesterolemic rabbits. Luminal endothelial surface from rabbit aortic tissue was examined by scanning electron microscopy (SEM) using low vacuum mode to ascertain ultrastructural changes in development of atherosclerotic lesion. Gene expression of adhesion molecules, MCP-1 and MMP-12 were studied by Real-time PCR. Ultrastructural observations of the aortic luminal surface exhibited changes from normal regular smooth intact endothelium to irregular luminal surface including marked globular appearance and ruptures of the membrane layer. Real-time PCR demonstrated differentially expressed of studied genes in atherosclerotic tissues. The appearance of ultrastructural changes in aortic tissue of hypercholesterolemic rabbits is suggested to have relation with underlying changes of endothelial surface molecules, chemokine and MMP-12 gene expressions.

Keywords—Ultrastructure of luminal endothelial surface, Macrophage metalloelastase (MMP-12), Real-time PCR.

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I. INTRODUCTION

SUSCEPTIBILITY of the vascular endothelium to pro-atherogenic low density lipoprotein (LDL) during development of atherosclerosis commonly affects the endothelial surface integrity [1], [2]. Expression of endothelial surface molecules and monocyte-chemoattractant protein (MCP) allows circulating monocytes to adhere and subsequently penetrate the endothelial surface and transform into macrophages in the vessel intima [3-5]. Development of atherosclerotic lesions has also been recognized to have relationship with extracellular matrix (ECM) turnover [6], [7]. In atherogenesis, the normal structure of the intimal extracellular matrix (ECM) is altered following production of matrix degrading enzymes produced by macrophages. ECM of aortic tissue which composed predominantly of collagen and elastin is susceptible to the reaction of matrix degrading enzyme known as matrix metalloproteases (MMPs) during formation of atherosclerotic plaque [8-10]. To date, several types of MMPs such as MMP-1, -2, -3, -9 and -12 have been identified in atherosclerotic tissues from humans and animal studies [11-13]. Excessive MMPs production in atherosclerosis will disrupt mechanical integrity of ECM resulting severe atherosclerotic lesions or plaque rupture. MMP-12 is recognized to be the predominant MMP produced by macrophages in atherosclerosis as demonstrated by its increased expression during macrophage accumulation [14-16]. Therefore, this study will show ultrastructural morphology of normal and hypercholesterolemic rabbits to ascertain changes of luminal surface and underlying genes during atherosclerosis.

II. MATERIALS AND METHODS

A. Cholesterol-fed rabbits

This study was conducted in accordance with Universiti Teknologi MARA guidelines for the care and use of laboratory animals (ACUC/CA/07(03)-UiTM). Young adult male New Zealand White Rabbits (2kg \pm 0.2kg) were individually caged for 8-week study. The animals were allowed for adaptation to the animal facility for 1-week period by feeding standard rabbit chow and water ad libitum. After stabilization, the rabbits were randomly assigned into two diet treatments. Control rabbits were fed with 100g per day standard rabbit pellet. The second group (high cholesterol-diet group) was fed with 100g per day 1% cholesterol-

supplemented rabbit pellet. Cholesterol was added to the rabbit pellet as diethyl-ether solution and was dried of the solvent at 40°C overnight before being used.

At the end of 8-week dietary treatment, rabbits were euthanized by an overdose injection of sodium pentobarbital (120mg/kg). The body cavity was opened and the aorta extending to the iliac bifurcation was excised. The aortic tissue was then cleansed from adhering fat and perfused in ice cold PBS buffer for scanning electron microscopy (SEM) study. Aortic tissue for matrix metalloproteinases gene expression study was snap frozen in liquid nitrogen and stored at -80°C until further use.

B. Ultra-Structural Morphology

Aorta segments were fixed overnight in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide for 2 hours. Then, the segments were washed twice with phosphate buffer at 15 minutes interval. Samples were mounted on stubs and examined by low vacuum mode (FEI Quanta FEG ESEM 200) without subjection to critical drying point and heavy metal coating.

C. Gene Expression Study of Endothelial Surface Molecules, Chemokine and MMP-12

Total RNA was extracted by homogenising frozen aortic tissue in Tri Reagent® (Ambion Inc) followed by purification using RNeasy column (Qiagen). First-strand cDNA was synthesized by OligodT primer using Sensiscript® Reverse Transcription (Qiagen). Real-time PCR was carried out for expressions of VCAM, ICAM, MCP-1 and MMP-12. Primers used were: VCAM forward: 5' CTG TCT GGG TCA GTC CCT CGT C 3', VCAM reverse: 5' TCT GCT CTT TCC AGC CAG GTT 3'; ICAM forward: 5' CAC GGA GCA GCA CTA CTG AG 3', ICAM reverse: 5' TTC TGC CAC CAT CAC TGT GT 3'; MCP-1 forward: 5' AGC ACC AAG TGT CCC AAA GA 3', MCP-1 reverse: 5' TGT GTT CTT GGG TTG TGG AA 3'; MMP-12 reverse : 5' AAA GCA TGG GCT ATG ACA CC 3'. Primers for housekeeping gene: GAPDH forward: 5' ATC ACT GCC ACC CAG AAG AC 3', GAPDH reverse: 5' TGA GTT TTC CGT TCA GCT CG 3'. Amplifications was performed using iCycler iQ with SYBR green fluorescence (Biorad). The reaction solution was assembled in a volume of 25µl, which comprised of iQ™ SYBR® Green Super Mix (Biorad), forward and reverse primers (final concentration 200nm each) and 50ng cDNA template. Real-time PCR cycling condition was as follows: 95°C for 3 minutes for activation of Taq DNA Polymerase followed by 40 cycles of denaturation, annealing and extension at 95°C for 10 seconds, 55°C – 60°C for 45 seconds, 72°C for 30 seconds respectively and final extension at 82°C for 1 second. All samples were normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) which gave constant Ct values in all studied samples. The normalized value for each target cDNA reflects the expression level of the corresponding gene in a test sample relative to the standard tissue.

D. Data Analysis

Data from Real-time PCR assay were generated using Genex software v1.10 (BioRad). Expression profiles were measured from mean Ct of triplicate samples of a specific gene of interest compared with normal control tissues. Expression level of the corresponding gene was calculated by normalization to GAPDH as the housekeeping gene.

III. RESULTS

A. Ultrastructural Morphology

The lumen endothelial surface of control and hypercholesterolemic rabbits examined by SEM showed some changes in the ultrastructural morphology. Aortic tissues of the control animal showed normal-appearance of convoluted endothelial surfaces (Fig. 1A). When exposed to 2 week cholesterol consumption, ultrastructure of the luminal surface showed discernible change with surface swellings (Fig. 1B). Following increase duration of 8 weeks cholesterol consumption, marked changes was observed exhibited by presence of 'craters' which indicated disintegration of the endothelial surface (Fig. 1C).

B. Expression Profiles of Endothelial Surface Molecules and Macrophage Metalloelastase (MMP-12)

Expression changes of genes studied from Real-time PCR assay were expressed as fold changes associated with relative quantification to the housekeeping gene (GAPDH). The endothelial surface molecules, VCAM and ICAM showed different expression profiles in week-2 and 8 of atherosclerosis (Table 1, Fig. 2A to 2D).

The endothelial surface molecule, ICAM showed upregulation from 0.274 ± 0.04 (expression level \pm SD) at week-2 and 2.896 ± 0.733 at the end of week-8. However, VCAM exhibited down regulation with 0.472 ± 0.103 and 0.581 ± 0.138 at week-2 and 8 respectively. The chemokine, MCP-1 displayed upregulation from 0.707 ± 0.118 at week-2 to 3.102 ± 0.263 at the end of week-8. Therefore, ICAM and MCP-1 respectively showed increased expression levels with 2.62 fold and 2.4 fold changes at the end of week-8.

Expression profiles of macrophage metalloelastase (MMP-12) exhibited slight increased with 1.81 ± 0.31 fold at week-2. Progression of atherosclerotic lesion demonstrated MMP-12 was highly expressed with 23.45 ± 5.78 fold at week-8 atherosclerosis (Table 1).

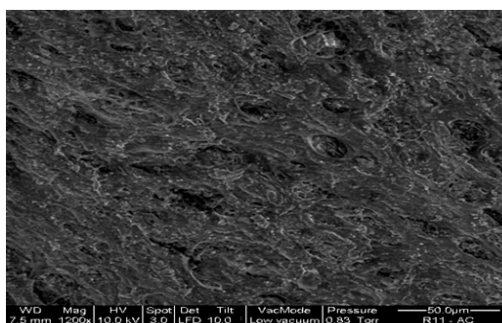
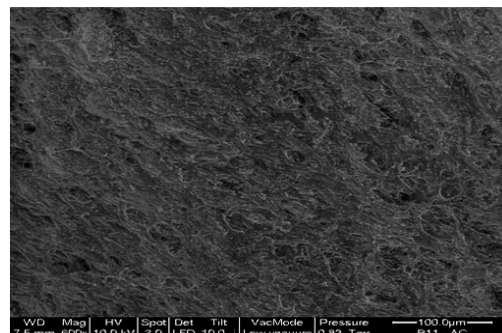
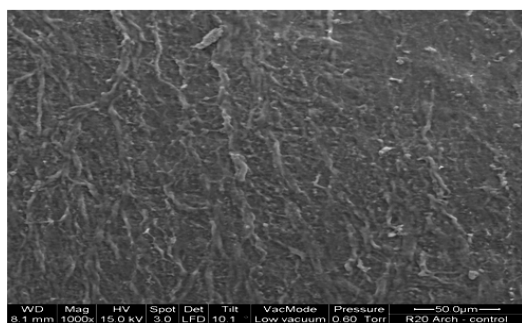
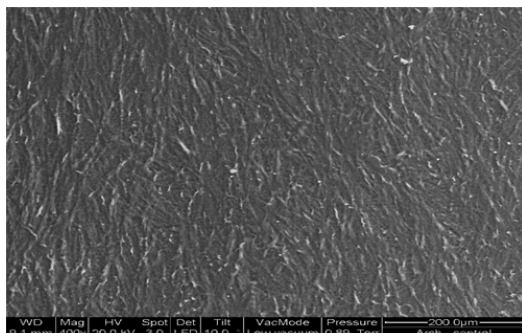


Fig. 1A Ultrastructural Morphology of Aorta from Normal Control Animal. Upper panel (200 μm) and lower panel (50 μm)

Fig. 1C Ultrastructural Morphology of Aorta from 8 Week Cholesterol Diet. Upper panel (100 μm) and lower panel (50 μm)

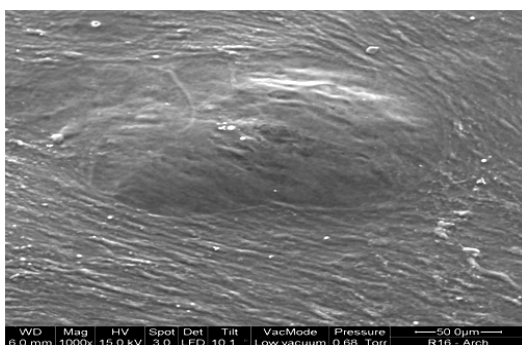
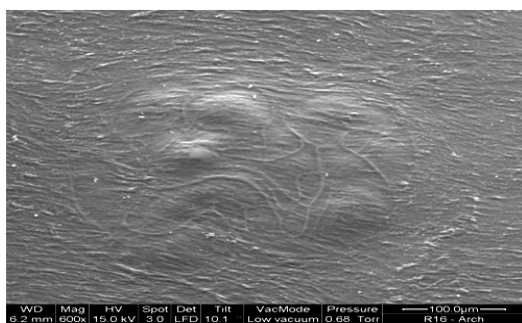


Fig. 1B Ultrastructural Morphology of Aorta from 2 Week Cholesterol Diet. Upper panel (100 μm) and lower panel (50 μm)

TABLE I
DIFFERENTIAL EXPRESSIONS OF ENDOTHELIAL SURFACE GENES, CHEMOKINE AND MMP-12 IN 2 AND 8-WEEK ATHEROGENESIS

Identifier	Mean *Ct	*Ct S.D	Expression	Expression S.D
VCAM (week-2)	29.7	0.283	0.472	0.103
VCAM (week-8)	29.43	0.115	0.581	0.138
ICAM (week-2)	31.77	0.153	0.274	0.040
ICAM (week-8)	28.40	0.173	2.896	0.733
MCP-1 (week-2)	30.63	0.115	0.707	0.118
MCP-1 (week-8)	28.10	0.100	3.102	0.263
MMP-12 (week-2)	27.87	0.12	1.81	0.31
MMP-12 (week-8)	24.22	0.08	23.45	5.78

* Ct = threshold cycle, a cycle at which the fluorescence exceeds the baseline threshold.

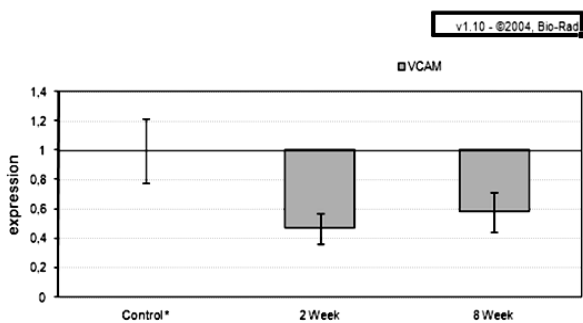


Fig. 2A Expressions of VCAM gene was down-regulated at both 2 and 8 week of atherogenesis

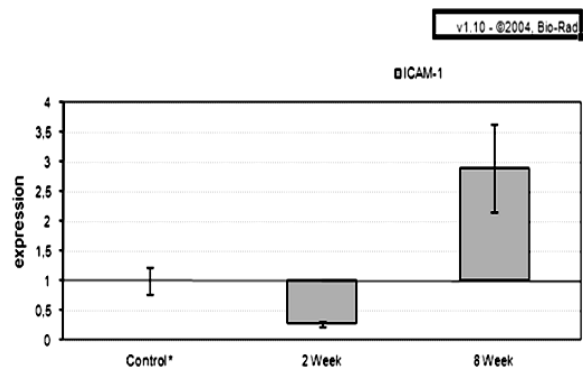


Fig. 2B ICAM-1 was differentially expressed of gene at 2 week and 8 week of atherogenesis

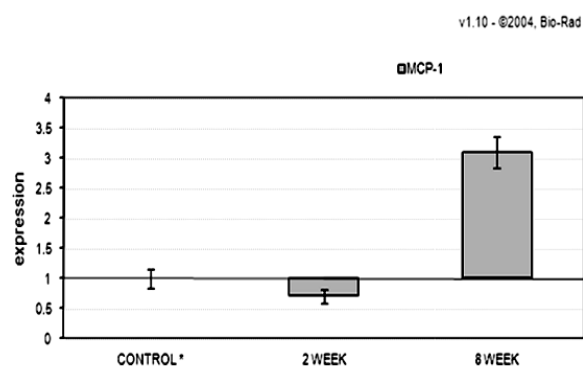


Fig. 2C MCP-1 gene was differentially expressed at 2 and 8 week of atherogenesis

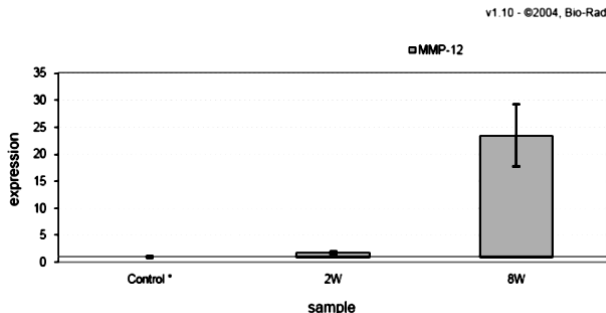


Fig. 2D MMP-12 gene was highly expressed at 8 week of atherogenesis

IV. DISCUSSION

Histo-morphology of endothelial tissues in humans and animal model has been extensively studied to reveal various stages of atherosclerotic plaque composition [14], [17-21]. Thickening of the intimal layer occupied by macrophage foam cells is a common histological feature observed in atherosclerotic tissues. Other histo-morphological features such as lipid core, fibrous cap and extracellular matrix are also recognized to be associated with atherosclerotic lesions [15].

The emergent interest in the usage of scanning electron microscopy (SEM) in the early 70's has supported the histo-morphological observations. SEM was employed as an analytical tool to examine ultrastructural changes of the endothelial cells associated with various stages of atherosclerotic lesions [22]. SEM of atherosclerotic endothelial cells displayed marked ultrastructural changes such as appearance of cuboidal cells, 'crater-like' defects and microvilli or cellular protrusion. Adherence of platelet and leukocytes to the endothelial cells was also frequently observed [1], [22].

The present study demonstrated disruptions of endothelial walls have occurred in various stages of atherogenesis as depicted by ultrastructural morphology of the endothelium (Fig. 1). Discernible changed of the ultrastructural morphology from surface swelling in 2-week atherogenesis to formation of 'craters' or 'cavity' at the end of 8-week atherogenesis suggesting occurrence of an endothelial injury which was denoted to have a relation with atherosclerosis [23].

To the best of our knowledge, this study is the first to describe ultrastructural changes of the endothelium by SEM using low vacuum mode. Many other studies on SEM for observation of endothelial surface of blood lumen used samples coated with gold as described by Kawamura *et al* (1974), Reidy *et al* (1978), Ragazzi *et al* (1993) and Walski *et al* (2002). In the present study, the aorta tissues were examined much closer to its natural conditions using a preparation without subjection to critical drying point and heavy metal coating. Although, preparations of samples were simplified, this technique effectively revealed the ultrastructural morphology by minimizing presence of artifacts which may mimic changes in the ultrastructural morphology. Findings from this study were comparable from other studies which using conventional sample preparation. The present report detected ultrastructural changes in the early atherogenesis (2-week cholesterol diet) presented as formation of surface 'swelling'. This finding is consistent with a study by de Bruijn *et al.* (1975) which showed 'bulging' in the endothelium at 2-3 weeks of cholesterol-diet animal. Similar SEM appearance of intimal swelling at 4 to 6-week cholesterol diet rabbits was also described by Reidy *et al.* (1978) which indicating occurrence of surface changes in atherosclerotic lesion development. Other appearance associated with loss of intimal surface integrity such as 'craters', 'cobble-stone', 'dome-shaped' and 'dentate' structure were also discerned by

SEM in 6 to 7-week cholesterol diet rabbits [22]. The present study also effectively showed presence of 'craters' at 8-week atherogenesis consistent with indication of endothelial surface disruption. SEM analysis of advanced human atherosclerotic plaque also showed wide spectrum of pathological alterations of the luminal surface such as appearance of cuboidal cells, 'microvilli' and 'craters' [1]. Evidence by ultrastructural morphology of vascular luminal surface in animal models and human samples was valuable to reveal important roles of endothelium in atherosclerotic lesion development [1], [22], [24-26]. Evaluation on endothelial function may give insights into the mechanisms underlying the initiation and progression of atherosclerosis [23]. In this study, endothelial integrity was effectively examined by SEM which revealed loss of endothelial integrity has a relationship with development of atherosclerotic lesion.

Disrupted endothelial surface renders its susceptibility to pro-atherogenic blood lipid components particularly the low density lipoprotein (LDL) that allows attraction of circulating monocytes to adhere to the endothelium. Interaction of endothelial cells with accumulated monocytes results with upregulation of cell adhesion molecules and chemokines. Recruitment of monocytes from the blood lumen into the vessel wall is designated as inflammatory process which is a key factor in initiation and progression of atherosclerosis. Expression of endothelial surface molecules plays important roles in mediating recruitment of the circulating monocytes [27]. VCAM, ICAM, L-selectin, P-selectin and E-selectin are the common adhesion molecules highly expressed in atherosclerotic tissues of experimental animals and human studies. The chemoattractant factor or chemokine such as MCP-1 was also found involved in regulating the attachment and migration of leukocytes into the arterial wall [28].

In the present study, the expression profiles of major adhesion molecules and chemokine including VCAM, ICAM, and MCP-1 were analyzed by Real-time PCR assay. Expression levels analyzed measured by fold changes of each studied gene compared to normal tissues after normalization with GAPDH as the housekeeping gene. Therefore, an expression level below 1.0 is considered low expression and vice-versa.

The genes of interest as stated above demonstrated differential gene expression as shown by the expression profiles at 2 and 8-week atherogenesis. The 2-week atherogenesis demonstrated 0.5 fold changes in the gene expression indicating low expression of VCAM by Real-time PCR. ICAM exhibited 0.4 fold change reflecting low expression as detected by Real-time PCR. The chemokine, MCP-1 started as low expression with 0.3 fold at 2-week atherogenesis and increased to 2 fold indicating high expression at 8-week atherogenesis. MCP-1, a chemokine that is encoded by the CCL2 gene, also involved in mediating monocytes recruitment and entry into vessel wall [29]. Different expression profiles of adhesion molecules and chemokine were detected from the present study suggesting their relationship with development of atherosclerosis. From this study, atherogenesis can be detected by deposition of lipid

in the intimal area from subsequent accumulation of blood lipids and the occurrence of endothelial disruption. The molecular event underlying endothelial disruption involving changed expression of endothelial molecules is then determined the extent of atherosclerotic lesion in the arterial wall.

The expression profile of macrophage metalloelastase (MMP-12) exhibited slightly increased in 2-week atherogenesis and continued to the highest expression level at 8-week atherogenesis. MMP-12 expression profile from the present study was comparable from other studies in humans and animals [14], [30]. This finding suggested the important role of macrophage metalloelastase (MMP-12) in determining the extent of atherosclerotic lesion. Macrophages were recognized as prominent in the production of MMP-12. Smooth muscle cells were also contributed to production of MMP-12 leading to remarkable degradation of ECM of the blood vessel manifested by serious clinical complications associated with thrombosis and plaque rupture.

V. CONCLUSIONS

The appearance of ultrastructural changes in aortic tissue of hypercholesterolemic rabbits seems to be associated with changes in endothelial surface molecules, chemokine and MMP-12 gene expressions.

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