

Intercellular Adhesion Molecule (ICAM)-1 and Vascular Cell Adhesion Molecule (VCAM)-1 at the Early Stages of Atherosclerosis in a Rat Model

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Abstract. *Background:* Members of the immunoglobulin superfamily of endothelial adhesion molecules, vascular cell adhesion molecule (VCAM-1) and intercellular cell adhesion molecule (ICAM-1), participate in leukocyte adhesion to the endothelium and play an important role in all stages of atherosclerosis. The aim of the study was to examine the expression of VCAM-1 and ICAM-1 in the aorta of rats at the early stages of atherosclerosis and the correlation with their plasma concentrations. *Materials and Methods:* Male rats (n=44), 10 weeks of age, were divided in 4 groups. Groups A and C (n=12) were fed with rich cholesterol diet for 12 and 16 weeks, respectively. Group B (regression group, n=12) was fed for the first 12 weeks with rich cholesterol diet and for another 4 weeks with normal diet. Group D (control group, n=8) was fed with normal diet for 12 weeks. We measured the serum lipid profile, the concentration of soluble ICAM-1 and the immunohistochemical expression of ICAM-1 and VCAM-1 in the endothelium, media and vasa vasorum of the aorta. *Results:* There were significant differences (p<0.05) in the expression of ICAM-1 between group C (maximum time of rich cholesterol diet) and all other groups in the 3 groups of the aorta studied. There was regression of the expression of ICAM-1 in group B

and significant differences (p<0.05) between group B and all the other groups, except group D in the expression of ICAM-1. There were no significant differences in the expression of VCAM-1 between any groups. The serum concentration of soluble ICAM-1 positively correlated with the expression of the molecule in the vasa vasorum (r=0.35, p<0.05) and fibroblasts/smooth muscular cells (r=0.34, p<0.05) of the aorta. *Conclusion:* A cholesterol diet plays a role in the expression of ICAM-1 but not in that of VCAM-1 in the rat aorta. The expression of ICAM-1 in the aorta regresses after the withdrawal of a cholesterol-rich diet. Soluble ICAM-1 is a reliable measure of ICAM-1 expression in the aorta, vasa vasorum and fibroblasts/smooth muscle cells.

Atherosclerosis is influenced by the interactions of cellular adhesion molecules. The cell-surface expression of these molecules in response to pathophysiological stimuli mediates the interaction between the endothelium and blood cells and is central to the development of atherosclerosis (1). Atherosclerosis affects discrete regions of the vasculature, such as vessel curvatures and bifurcations. These regions are characterized by disturbed oscillatory flow that induces up-regulation of proinflammatory adhesion molecules, such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (2). Chemokines then stimulate leukocytes and up-regulate $\alpha 4$ and $\beta 2$ integrin binding to VCAM-1 and ICAM-1 respectively, which themselves mediate later steps (2). VCAM-1 and ICAM-1 are closely related in structure and function. Both are cytokine-inducible Ig gene superfamily members that bind leukocyte integrins (3).

We investigated the expression of ICAM-1 and VCAM-1 in the aortic wall of Wistar rats in response to hypercholesterolemia. We also investigated the correlation between the expression of adhesion molecules, the lipid

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Key Words: Atherosclerosis, vascular cell adhesion molecule, intercellular adhesion molecule, high-density lipoprotein cholesterol, vasa vasorum.

Table I. Metabolic characteristics and soluble Intercellular Adhesion Molecule-1 (ICAM-1) values at the beginning of the study.

	Group A (n=12)	Group B (n=12)	Group C (n=12)	Group D (n=8)
Total cholesterol (mg/dl)	74.25±11.29	69.42±11.54	62.00±9.01	83.00±8.07
Triglycerides (mg/dl)	96.75±22.08	121.75±29.71	107.27±21.46	88.75±35.75
LDL cholesterol (mg/dl)	23.90±8.92	20.73±5.50	20.36±9.13	31.62±6.48
HDL cholesterol (mg/dl)	31.00±3.41	24.33±6.47	20.18±3.81	33.62±4.40
Soluble ICAM-1 (pg/ml)	27521±5020	21340±6835	17568±4711	21665±3825

LDL: Low-density lipoprotein-cholesterol; HDL: high-density lipoprotein-cholesterol.

profile and the plasma concentration of the soluble form of adhesion molecules.

Materials and Methods

Experimental model. The experiments were carried out on 44 male 10-week-old Wistar rats (220-300 g). The rats were housed in plastic cages according to European standards (Tecniplast, Buguggiate, Italy) in the Laboratory for Experimental Surgery and Surgical Research in a controlled environment at 20±2°C, with 55% relative humidity, central ventilation (15 air changes/h) and an artificial 12-h light-dark cycle. All animal experiments were carried out in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Academy of Sciences (4). The protocol was approved by the Institutional Animal Care and Use Committee of the University of Athens Medical School and Veterinary Directorate of the Athens Prefecture (permit number K/3512). After acclimatization for 1 week, the animals were randomly divided into 4 groups (A-D) of 8-12 rats each. Access to food and water was unrestricted for all groups.

Group A received a high fat diet for 12 weeks; group B received a high-fat diet for 12 weeks and then standard diet for 4 weeks; group C received a high fat rat diet for 16 weeks; and group D (control group) received standard diet for 12 weeks. The composition of the control diet was 20% protein, 40% carbohydrates, 5% fat, 3% fiber and other constituents composed 32%. The composition of the high-fat diet was enriched with 2% cholesterol.

Sampling. Blood samples were collected at the beginning of the study and after 4, 8, 10, 12 and 16 weeks for serum lipids and soluble ICAM-1 (sICAM-1). The blood was collected from the retro-orbital plexus of the rats, which were under anesthesia with i.m. ketamine at 150 mg/kg. For preserving the eyes of the animals topical 0,3 % tobramycin plus 0,1% dexamethasone eye solution for 2 days and tobramycin for another 2 days. The blood samples were placed into Eppendorf tubes containing heparin. The blood samples were collected at the same time of day for every measurement. Food was withdrawn 10 h before blood collection. All animals were killed by exsanguination at the end of the study for each group. Blood was then drawn from the inferior vena cava while the rats were under deep anesthesia with ketamine. All animals were then killed with xylazine at 30 mg/kg injected i.m.. Their aortas were dissected immediately for histopathological analysis. The plasma was separated by centrifugation (15 min at 3000 g) and was stored at -80°C until analyzed.

Lipid measurement. High-density lipoprotein cholesterol (HDL-C) was isolated by precipitating chylomicrons. Very low-density lipoprotein cholesterol (VLDL-C) and low-density lipoprotein cholesterol (LDL-C) were isolated by adding phosphotungstic acid and magnesium ions to the samples. Plasma cholesterol and HDL-C were determined enzymatically by the cholesterol oxidase peroxidase-amidopyrine method using a commercially available kit (Biosis®). Serum triglycerides (TGs) were measured by the enzymatic glycerol-3-phosphate-oxidase peroxidase-amidopyrine method using a commercially available kit (Biosis®, Athens, Greece). LDL-C was estimated by the Friedewald formula for values expressed in mg/dl: LDL-C=total cholesterol (TC) level – (HDL-C + TG level/5).

Histopathological staining. Part of the aorta was fixed in 10% formalin at room temperature. The tissues were then embedded in paraffin, sectioned and mounted on glass microscope slides. The sections were stained with hematoxylin-eosin. Immunohistochemistry was applied to 4-µm paraffin-embedded tissue sections. Antigen retrieval was performed by heating the slides in a microwave oven at 800 W for 20 min in 0.1 M citrate buffered (pH: 6.0) for ICAM-1 and VCAM-1. The following monoclonal antibodies were used: anti-ICAM-1 (MS-1094; Neomarkers, LabVision Corp. Fremont, Canada) at a dilution of 1:100 and anti-VCAM-1 (MCA-981; Serotec, UK) at a dilution of 1:50. The two molecules were detected by a two-step polymer-based technique (Envision K-5007, Dako Corp., Glostrup, Denmark). Diaminobenzidine was used as chromogen substrate and Harris hematoxylin as counterstain. For negative controls, tris-buffered saline substituted for the primary antibodies. Expression for ICAM-1 and VCAM-1 was seen in endothelial cells lining the intima and vasa vasorum. Partial expression of the molecules was also observed in the medial layer. ICAM-1 and VCAM-1 expression was assessed semiquantitatively on a scale of 0 to 3 as follows: 0, negative; 1, weak intensity staining; 2, medium intensity staining; 3, strong intensity staining. Evaluations were performed blindly and independently by two pathologists. The interobserver variability in all cases was <5%. In cases of disagreement, the slides were re-evaluated jointly, and a consensus reached.

sICAM-1 Measurement. An enzyme-linked immunosorbent assay (ELISA) kit (Rat sICAM-1 [CD54] Immunoassay catalog number RIC100; R&D Systems company, Minneapolis, USA) was used for sICAM-1 levels measurement. The final absorption was assessed in an ELISA photometer (Model 680; Bio-Rad).

Statistical analysis. Data are expressed as the mean±standard deviation for continuous variables and as percentages for categorical data. The Kolmogorov-Smirnov test and graphical methods were used

to assess whether the distribution of variables followed a Gaussian pattern. Analysis of variance (ANOVA) was used for multiple between-group comparisons. The comparison between the beginning and the end of the study was performed with paired *t*-test and Wilcoxon sign rank test for nonparametric data as appropriate. Linear relationships between quantitative variables were assessed with the Pearson's correlation coefficient for parametric data and Spearman's rho for nonparametric data. In cases of multiple comparisons, the Bonferroni-Holmes post hoc test was performed for locating individual differences. The family error rate was set to $p < 0.05$. All tests performed were two-sided. The null hypothesis was rejected with $>95\%$ confidence. SPSS 17.0 was used for the statistical analysis.

Results

Lipid profile. Total Cholesterol (TC) in group C at euthanasia was significantly higher compared with the other groups (group A: 76 ± 8 ; group B: 73 ± 14 ; group C: 85 ± 8 ; group D: 68 ± 12 mg/dl; $p < 0.05$ for all comparisons). No significant difference in TC levels was observed between the other three groups at euthanasia (Table II). HDL-C in group C at euthanasia was also significantly increased compared with the other groups (group A: 21 ± 6 ; group B: 21 ± 5 ; group C: 36 ± 7 ; group D: 26 ± 6 mg/dl; $p < 0.05$). No difference in HDL-C levels at euthanasia was observed among the other three groups (Tables I and II).

TG and LDL-C levels did not differ significantly among the groups at euthanasia (Tables I and II).

sICAM-1. sICAM-1 measurements of group C were lower compared with the other groups at baseline. However, after 16 weeks of hypercholesterolemic diet, group C animals had the highest sICAM levels at euthanasia (for all comparisons, $p < 0.05$). Group C was the only study group with a significant alteration of mean sICAM-1 concentrations between baseline and euthanasia. Group C sICAM-1 levels increased more than 50%, compared with baseline measurements ($p < 0.05$; Table II).

sICAM-1 at euthanasia was negatively correlated with baseline HDL-C levels ($r = -0.31$, $p < 0.05$). The difference in sICAM-1 concentration between the beginning of the study and euthanasia was positively correlated with the difference in TC ($r = 0.38$, $p < 0.05$), LDL-C ($r = 0.36$, $p < 0.05$) and HDL-C ($r = 0.46$, $p < 0.05$) for the same intervals.

Tissue ICAM-1. Group C strongly expressed ICAM-1 in all three layers of the aortic wall (endothelium, *vasa vasorum* and media, Figures 1 and 2) and there was a significant difference in the expression of tissue ICAM-1 from that of groups A, B and D (for all associations, $p < 0.05$) (Table II). There was no difference among groups A, B and D regarding ICAM-1 endothelial expression (Table II). There was a significant difference in ICAM-1 expression between groups A and B, as well as between A and D, regarding the *vasa vasorum* and *intima media* but no significant difference between groups B and D (Table II). There was a positive correlation between TC levels at euthanasia with ICAM-1

Table II. Metabolic characteristics, soluble Intercellular Adhesion Molecule-1 (sICAM-1) values and comparisons between groups at euthanasia.

	Group A (n=12)	Group B (n=12)	Group C (n=8)	Group D (n=12)	p-Value for comparison of Group		p-Value for comparison of Group	
					C vs. A	C vs. B	A vs. B	A vs. D
Total cholesterol	75.66±7.70 mg/dl	72.75±13.63 mg/dl	85.18±8.01 mg/dl	67.62±12.10 mg/dl	<0.05	<0.05	NS	NS
Triglycerides	108.00±26.67 mg/dl	92.08±29.92 mg/dl	76.00±31.00 mg/dl	71.75±22.51 mg/dl	<0.05	NS	NS	<0.05
LDL cholesterol	32.58±10.89 mg/dl	32.08±7.14 mg/dl	33.72±7.47 mg/dl	27.62±10.48 mg/dl	NS	NS	NS	NS
HDL cholesterol	21.5±6.33 mg/dl	21.41± 4.85 mg/dl	35.63±7.32 mg/dl	25.62±6.52 mg/dl	<0.05	<0.05	NS	NS
sICAM-1	22551±6707 pg/ml	20782±3431 pg/ml	26021±7971 pg/ml	18291±7206 pg/ml	NS	NS	NS	NS
T ₀ -T _e % Difference sICAM-1 concentration	-12.96±39.04	7.73±24.77	54.71±64.82	-13.87±35.77	<0.05	<0.05	NS	NS
Tissue ICAM-1 endothelium	0.417±0.66	0.333±0.65	1.333±0.77	0.250±0.70	<0.05	<0.05	NS	NS
Tissue ICAM-1 vasa vasorum	1.417±0.51	0.333±0.65	2.750±0.45	0.500±0.53	<0.05	<0.05	<0.05	NS
Tissue ICAM-1 Fibroblasts/smooth muscle cells	1.167±0.72	0.417±0.66	2.500±0.67	0.125±0.35	<0.05	<0.05	<0.05	NS

LDL: Low-density lipoprotein; HDL: high-density lipoprotein; ICAM-1: Intercellular Adhesion Molecule-1; sICAM-1: soluble Intercellular Adhesion molecule-1; Tissue ICAM-1 expression was assessed semiquantitatively on a scale of 0 to 3 as follows: 0, negative; 1, weak intensity staining; 2, medium intensity staining; 3, strong intensity staining; NS: not statistically significant; T₀-T_e: time between the start of the study and euthanasia.

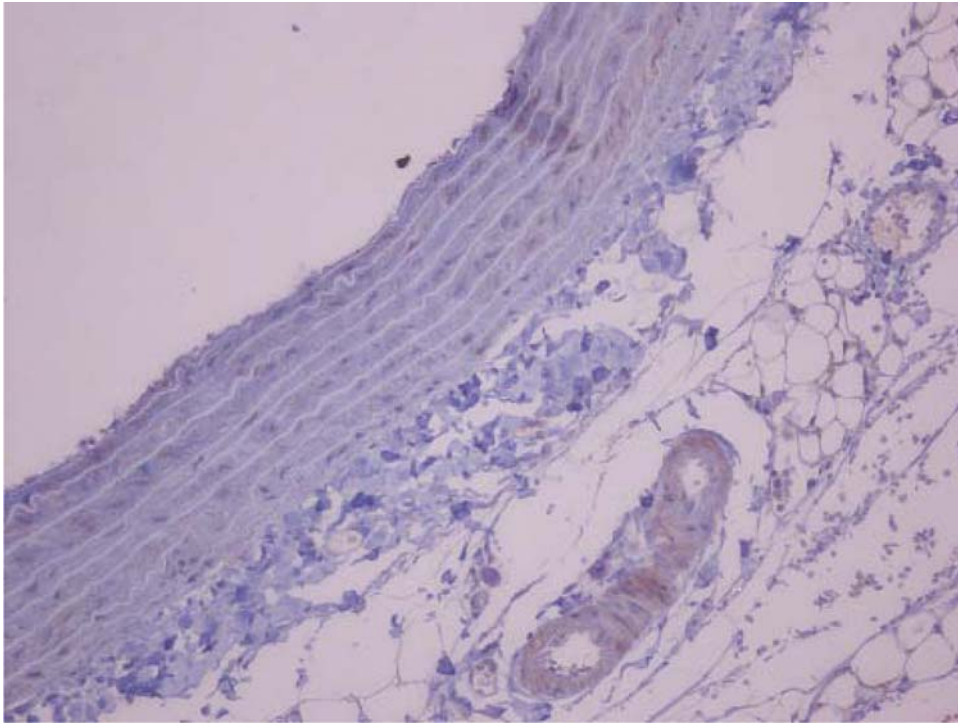


Figure 1. *ICAM-1* expression in the vasa vasorum and the intima media. Group C. Original magnification $\times 100$.

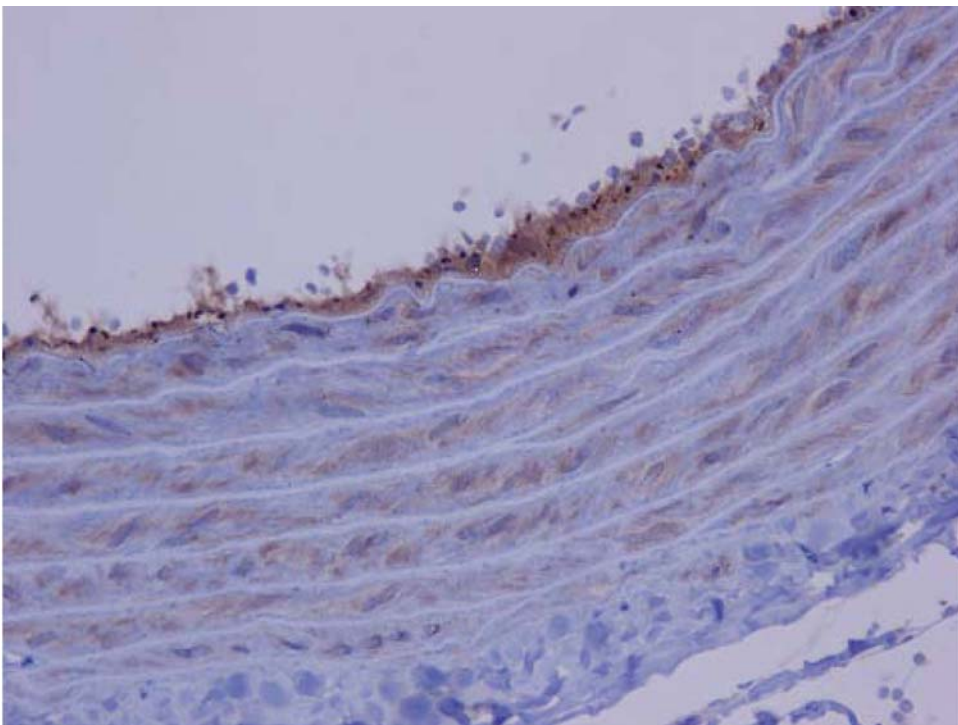


Figure 2. *ICAM-1* expression in the intima media and the endothelium. Group C. Original magnification $\times 200$.

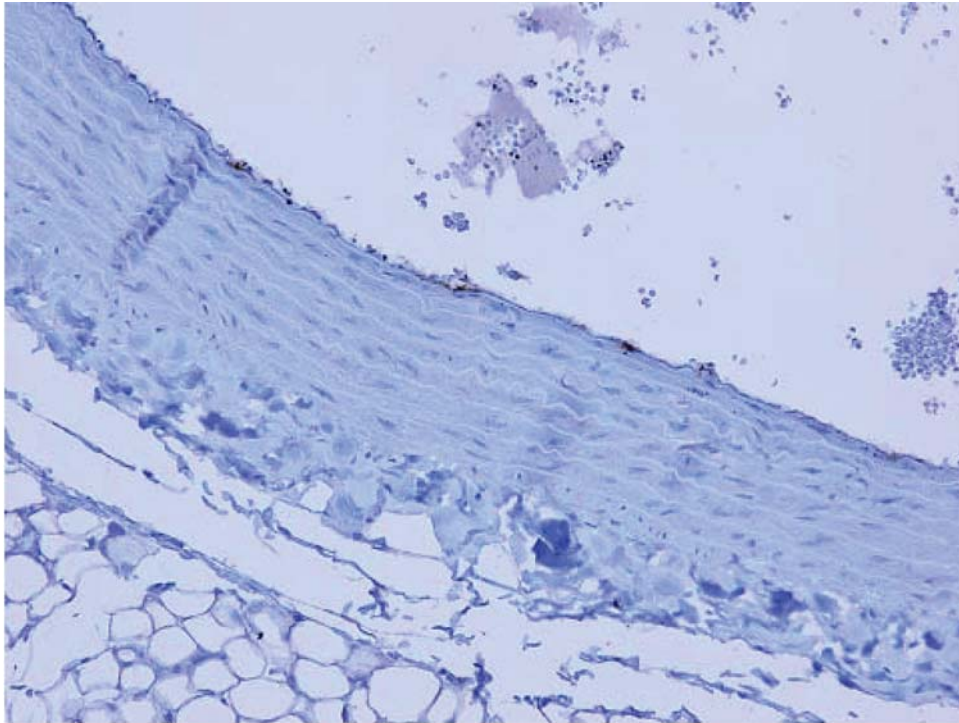


Figure 3. Weak VCAM-1 expression in the intima media and the endothelium. Group C. Original magnification $\times 100$.

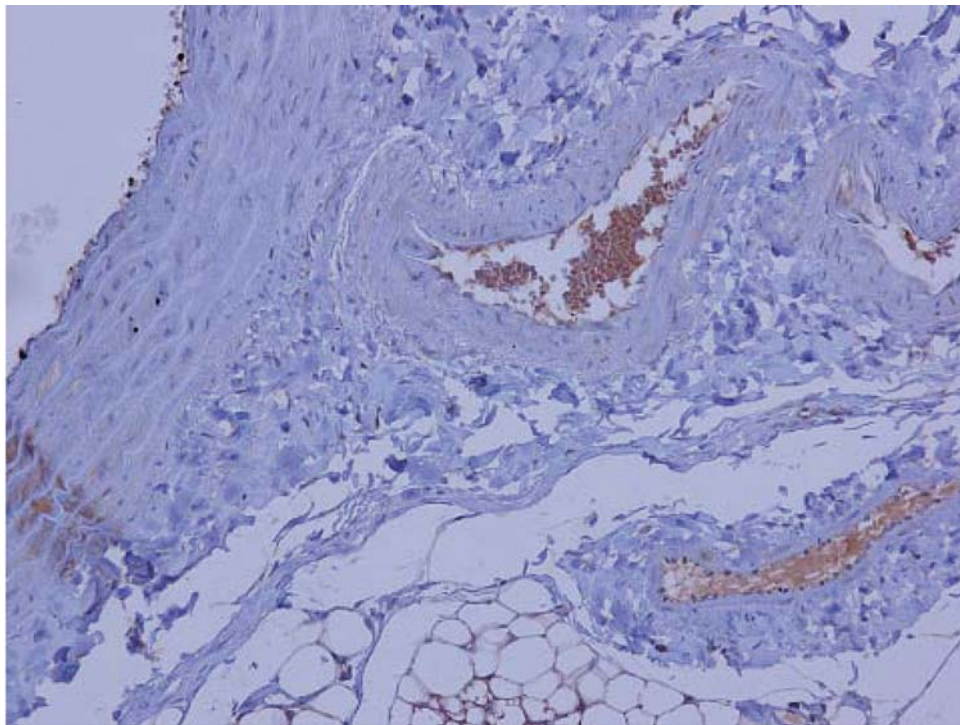


Figure 4. Weak ICAM-1 expression in the intima media and vasa vasorum. Group A. Original magnification $\times 100$.

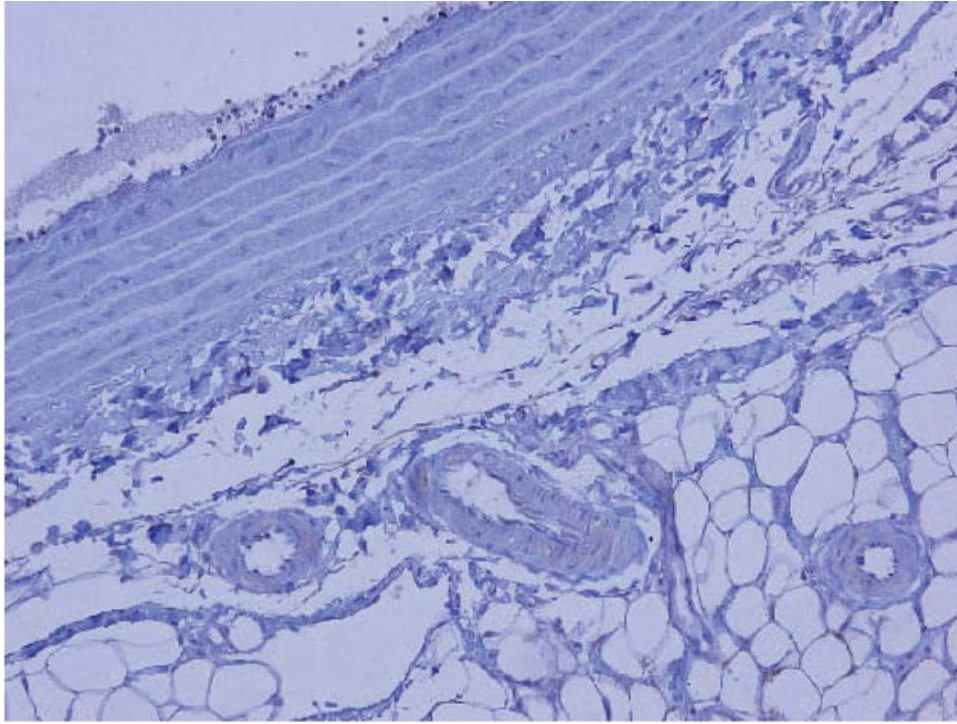


Figure 5. Weak ICAM-1 expression in the intima media and the vasa vasorum. Group B. Original magnification $\times 100$.

expression in the *vasa vasorum* ($r=0.41$, $p<0.01$) and *intima media* ($r=0.4$, $p<0.01$). Furthermore, the concentration of sICAM-1 at euthanasia was positively correlated with ICAM-1 expression in the *intima media* of the aortic wall ($r=0.34$, $p<0.05$) and *vasa vasorum* ($r=0.33$, $p<0.05$).

Tissue VCAM-1. There was a weak immunohistochemical expression of VCAM-1 in some of the subjects of the study (Figure 3). There was no significant difference among the groups nor was there any correlation with the lipid profile, ICAM-1 expression, or sICAM-1.

Discussion

The effect of a diet rich in cholesterol was an increase in TC and the induction of ICAM-1 expression in the endothelium, *vasa vasorum* and fibroblasts/smooth muscle cells of the aortic wall. The expression of this adhesion molecule was more pronounced in the *vasa vasorum* and the fibroblasts/smooth muscle cells. After 12 weeks of cholesterol-rich diet, the expression of ICAM-1 increased significantly compared with that in the control group. The effect of a cholesterol-rich diet for another 4 weeks (a total of 16 weeks; group C) led to a significant induction of ICAM-1 expression (Figure 1 and 2). In contrast, by withdrawing the harmful stimulus for 4 weeks (a total of 12 weeks plus 4 weeks of normal diet; group B),

the expression of ICAM-1 decreased to levels comparable with those of the control group (Figures 4 and 5). Due to a lack of cholesterol ester transfer protein (CETP) in Wistar rats, high cholesterol levels were combined with increased HDL-C levels. This makes HDL-C the major carrier of plasma cholesterol in this animal model (5). This experimental model could be used to study CETP inhibitors (*e.g.* anacetrapib and dalcetrapib), a novel group of drugs that may decelerate atherosclerotic lesion progression through HDL-C increase and HDL-C function improvement, as well as LDL-C and Lipoprotein (a) reduction (6, 7).

sICAM-1 was positively correlated with the expression of the molecule in the *vasa vasorum* and the media. The variation of the concentration of the soluble molecule was also correlated with the expression of ICAM-1 in the media and the *vasa vasorum*. This finding supports using the concentration of sICAM-1 as a reliable measure of the expression of the molecule in the tissue of the aortic wall.

Since ICAM-1 correlates with the development and expansion of atherosclerotic lesions, the soluble molecule could be used to reflect the extent of the lesions. Clinical studies using imaging carotid *intima media* thickness or the ankle-brachial index have also expressed this correlation (8, 9). The present study suggests the use of sICAM-1 as an index of expression of the molecule in tissues, especially in the *vasa vasorum* and the *intima media*.

sICAM-1 is a reliable predictor of cardiovascular events in those without any prior history of coronary artery or other vascular disease (10-16). Additionally, data from the Framingham Offspring study supports the correlation between sICAM-1 and risk factors such as smoking, high cholesterol levels, high glucose levels and obesity (17).

TC levels did not correlate with plasma ICAM-1 concentration. Only HDL-C levels correlated negatively with sICAM-1 concentration at the end of the study. This is in agreement with the results of studies supporting that soluble ICAM-1 does not correlate with TC and LDL-C levels, and negatively correlates with HDL-C (9, 12, 16). In contrast, the present study advocates that the absolute difference in sICAM-1 concentration between baseline measurements and the end of the study correlates with the absolute difference in TC, LDL-C and HDL-C levels, thus supporting the idea of using sICAM-1 to assess the role of lipids in triggering atherosclerotic lesions.

The expression of VCAM-1 in the aortic wall was very weak without any correlation being observed with either TC levels or the expression of ICAM-1 (Figure 3). These results confirm the histopathological findings in human tissues without pronounced atherosclerotic lesions where ICAM-1 is expressed. In contrast, VCAM-1 is not expressed as shown by the same studies (18, 19). Additionally, the results are in agreement with studies in large populations where sICAM-1 is increased in patients with progressive atherosclerotic lesions and has been used as a predictor of future events (20-24). In contrast, VCAM-1 expression is increased in patients with chronic already-established lesions such as in coronary artery disease or peripheral arterial disease, correlating positively with the extent of plaques (14, 22, 23, 25). There is no basal VCAM-1 expression but there is an increase in the proatherosclerotic state both in human and experimental models. VCAM-1 is a predictor of future cardiovascular events in patients with coronary artery disease (14, 25), diabetes mellitus (26) and unstable angina (27). This is not observed in healthy individuals.

The present study advocates that ICAM-1 expression is an early event in atherosclerotic lesions and subsides after withdrawing the risk factor, while VCAM-1 is probably expressed at a later stage. ICAM-1 expression in the endothelium, *vasa vasorum* and fibroblasts/smooth muscle cells correlated both with TC and HDL-C levels and their variation. Additionally, early intervention in TC levels can interrupt the development of atherosclerotic lesions through inhibiting the expression of ICAM-1 (28).

Several drugs target both ICAM-1 and VCAM-1 and their ligands. These drugs, however, have not yet been extensively studied regarding the prevention of atherosclerosis (29). Succinobucol is a stable metabolic analog of probucol with antioxidant properties that reduces the levels of TC (30). This drug also has the ability to reduce the expression of VCAM-1 and MCP-1 genes (31). The double blind Aggressive Reduction

of Inflammation Stops Events (ARISE) study included 6144 patient who had an acute coronary syndrome occurring within 14-365 days before recruitment; patients received succinobucol or placebo in addition to their standard treatment. Succinobucol was associated with a reduction in atherosclerosis-related clinical outcomes, such as cardiovascular death, resuscitated cardiac arrest, non-fatal myocardial infarction, non-fatal stroke and the occurrence of new-onset diabetes. These results were seen despite unfavourable changes in lipids, blood pressure, and C-reactive protein, suggesting that the antioxidant and anti-inflammatory effects of succinobucol might have favourably affected clinical outcomes (32). A novel gene therapeutic strategy was also developed that aims for a locally restricted effect at atherosclerotic areas of the vasculature targeting VCAM-1 binding in cell membrane and cytoskeleton (33). This method may provide directions to locally inhibit atherogenesis in the future.

In conclusion we have demonstrated in the present study that rich cholesterol diet acts as an inducing factor in ICAM-1 expression in the Wistar rat aorta. This action seems to be time dependent. This is mainly observed in the *vasa vasorum* and the fibroblasts/smooth muscle cells and to a lesser degree in the endothelium. In contrast VCAM-1 expression is not induced by hypercholesterolemia, at least at the early stages of atherosclerosis. This result supports the idea that ICAM-1 is one of the first events in atherosclerotic lesion formation. In addition the removal of this specific irritant factor results in ICAM-1 expression regression. It is also evident that soluble ICAM-1 concentration measurement in plasma is a reliable index of the molecule expression in the Wistar rat aorta, mainly in the *vasa vasorum* and the fibroblast/smooth muscle cells.

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