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Genomic rearrangements on VCAM1, SELE, APEG1 and AIF1 loci in atherosclerosis

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Abstract

The inflammatory nature of atherosclerosis has been well established. However, the initial steps that trigger this response in the arterial intima remain obscure. Previous studies reported a significant rate of genomic alterations in human atheromas. The accumulation of genomic rearrangements in vascular endothelium and smooth muscle cells may be important for disease development. To address this issue, 78 post-mortem obtained aortic atheromas were screened for microsatellite DNA alterations versus correspondent venous blood. To evaluate the significance of these observations, 33 additional histologically normal aortic specimens from age and sex-matched cases were examined. Loss of heterozygosity (LOH) was found in 47,4% of the cases and in 18,2% of controls in at least one locus. The LOH occurrence in aortic tissue is associated to atherosclerosis risk (OR 4,06, 95% CI 1,50 to 10,93). Significant genomic alterations were found on 1p32-p31, 1q22-q25, 2q35 and 6p21.3 where VCAM1, SELE, APEG1 and AIF1 genes have been mapped respectively. Our data implicate somatic DNA rearrangements, on loci associated to leukocyte adhesion, vascular smooth muscle cells growth, differentiation and migration, to atherosclerosis development as an inflammatory condition.

Keywords: inflammation - microsatellite DNA - loss of heterozygosity

Introduction

Over the past three decades many cellular and molecular steps of atherosclerosis development have been well recognized and understood. Atherosclerosis is a complex systemic condition where endothelium and vascular smooth muscle cells (VSMCs), macrophages, T-lymphocytes, and platelets interact with each other and produce in combination a vascular injury-like response [1,2]. Chemotactic factors that affect leukocyte chemotaxis, mitogens and growth factors that cause proliferation of connective tissue, macrophages, VSMCs and endothelial cells, promote atherosclerotic lesion formation [3]. Production and uptake of oxidized low-density lipoprotein (ox-LDL) by the endothelium and macrophages causes further injury to the vessel walls, while the formation of nitric oxide (NO) modulates vasomotor tone [4-6].

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The development and progression of atherosclerotic lesions is closely correlated to the intensity of this inflammatory response which may be modulated by both environmental stimuli and individual genetic determinants [7]. Infectious agents of both viral and bacterial nature as well as mechanical arterial stresses have been implicated in the initiation of vascular inflammation that could lead to atherosclerosis [8-11]. However, the triggering signals of this discontinuous anatomic distribution of inflammation remain to be elucidated.

Several previous studies identified genomic and epigenetic DNA alterations in atherosclerotic plaques from different arteries in man [12-21]. These observations seems to be the result of the focal monoclonal proliferation of genetically distinct VSMCs [14,15,22,23]. To elucidate the potential importance of DNA alterations in the pathology of atherosclerosis we screened DNA from aortic atheromas for microsatellite alterations in markers previously associated with the disease [12, 14, 17, 21]. Furthermore, we tested age and sex-matched histologically normal aortic specimens to clarify the specificity of the alterations observed.

Materials and methods

Specimens

Seventy-eight aortic atheroma specimens from autopsy cases (forty-two males and thirty-six females) ranging from 58 to 85 (mean ± SD, 72,1±8,3) years of age, and thirty-three histologically normal aortic specimens from autopsies as controls (eighteen males and fifteen females) aged between 60 to 88 (73,5±10,0) years were obtained along with correspondent venous blood from all cases and controls, at the Laboratory of Forensic Medicine, Medical School, University of Athens, Greece. All tissue samples were examined histologically and were selected not to contain calcified or significant fibrous components. The tissues were snap frozen after excision and stored at -80°C until DNA extraction. DNA was isolated from blood and tissues as previously described [14,24]. This study was approved by the Medical School, University of Crete Ethics Committee.

Microsatellite DNA markers and PCR amplification parameters

Sixty-three microsatellite DNA markers were amplified in panels of 3- and 4-plex reactions (Table 1). PCR reactions were performed by introducing 100ng of genomic DNA in a PCR reaction mixture containing 1X PCR buffer, 400 μM dNTPs, 2,66 mM $MgCl_2$ and 0,35U Taq DNA polymerase (Invitrogen Corp, Carlsbad, CA, USA). Amplification cycling parameters were: initial denaturation for 3 min; 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; final extension step at 72°C for 10 min. The PCR assays were done in a PTC-100 programmable thermal controller (MJ Research Inc., Waltham, MA, USA).

Digital imaging

The PCR products were analyzed by using 10% polyacrylamide gel electrophoresis (29:1 ratio of acrylamide to bisacrylamide) and silver staining. Gels were sealed in a plastic transparent bag and scanned on an Agfa SnapScan 1212u (Agfa-Gevaert N.V., Mortsel, Belgium). Integrated density (ID) was calculated using the ImageJ 1.32j software (Wayne Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). These measurements were used as quantitative parameter to score loss of heterozygosity (LOH) for a given DNA microsatellite marker in a heterozygous case when the ratio was calculated >1,49 or <0,58, with a 99,5% confidence interval, as determined from independent reproducibility experiments. The analysis in LOH positive cases was repeated three times and the results were reproducible. Representative examples of LOH are shown in Fig. 1. The fractional allele loss (FAL) value was calculated for each sample as (loci scored with LOH) / (total informative loci).

Statistical analysis

Data analysis was done with SigmaStat 3.00 statistical software (SPSS Inc., Chicago, IL, USA). Results are expressed as mean ± SD. Differences in the mean values of quantitative measurements

were tested with the Student's t or the Mann-Whitney U test. Kolmogorov-Smirnov goodness of fit test was performed for the examination of normal distribution of data sets. The statistical modeling using logistic regression was used to calculate the relative risk (odds ratio, OR) of microsatellite alterations for case-control study. ORs were expressed together with the 95% confidence interval (CI). The chi-square test was used for comparison of percentages. A p value of <0,05 was considered statistically significant.

Results

We assayed 78 aortic atheromas compare to correspondent venous blood DNA with a total of 63 microsatellite DNA markers. LOH was found in 37 out of 78 atherosclerotic cases (47,4%), most frequently on chromosomal region 6p21.3 (16,67%). Microsatellite DNA marker D6S2225 (28,1%) and D6S1002 (24,2%) were most frequently affected. The mean FAL value was 0,027±0,032 with a 99,5% confidence interval (CI) and the highest was 0,11 observed in two males aged 58 and 76 years respectively. Genomic alterations were observed in chromosomal regions 1p, 1q, 2q, 6p, 8p, 9p, 11p, 13q and 17q.

Thirty-three histologically normal aortic tissues in comparison to their correspondent blood samples were also examined as a control group. LOH was detected in 6 out of 33 specimens (18,2%). LOH was found in chromosomal regions 1p, 5q, 8p, 9p, 11p, 13q and 17q. The mean FAL value was 0,007±0,016 with a 99,5% confidence interval (CI) while the highest was 0,07 observed in a female aged 81 years. Microsatellite DNA marker FGR (4,5%) and D13S220 (4,5%) were most frequently affected.

Histograms of FAL and LOH frequencies per locus are presented in Fig. 2. The LOH incidence was found to be associated to atherosclerosis risk with an odds ratio value of 4,06, 95% CI 1,50 to 10,93. Comparison of the profiles of microsatellite DNA alterations between cases and controls revealed that 1p32-p31, 1q22-q25, 2q35 and 6p21.3 were significantly affected in atherosclerotic cases (p<0,05). In these genomic regions

Table 1. Microsatellite DNA markers tested

Marker	Genetic locus	PCR product size (bp)	Het
D1S186	1p31	82-106	0.84
D1S116	1p31.2	89-101	0.65
D1S159	1p32	147	0.67
CLN1	1p32	140-209	0.87
CRTM	1p35	102-110	0.67
D1S165	1p36	156-177	0.71
FGR	1p36.2-p36.1	135-143	0.69
D1S305	1q12	156-176	0.83
D1S104	1q21-q23	152-168	0.76
APOA2	1q21-q23	131-145	0.74
ATP1A2	1q21-q23	397-405	0.72
CRP	1q21-q23	127-145	0.60
D1S416	1q24	146-162	0.82
ACTN2	1q42-q43	91-107	0.50
D2S160	2p13-q15	204-218	0.78
D2S337	2p15-p13	233-255	0.88
D2S171	2p24-p21	253-281	0.86
D2S122	2q21	126-140	0.78
D2S141	2q21-q33	152-178	0.87
D2S111	2q21-q33	126-140	0.81
D2S105	2q23-q35	107-125	0.69
D2S164	2q33-q37	265-303	0.83
D2S311	2q35-qter	185-207	0.81
D3S1611	3p242-p22	252-268	0.66
D3S1270	3pter-p25	164-186	0.75
D5S207	5q31.3-q33.3	135-143	0.68
D5S376	5q32-q33.1	117-129	0.72
D6S2225	6p21.3	152	0.38
D6S1002	6p21.3-p22.1	240-244	0.32
D6S429	6p23-p25	222-238	0.74
D6S259	6p23-p25	267-285	0.73
D6S263	6p23-p25	90-114	0.82
D7S478	7p15-q22	118-130	0.69
D7S519	7p15-q22	256-268	0.81
PLAT	8p12-q11.2	105-149	0.77
ANK1	8p21.1-p11.2	107-113	0.55
D8S137	8p21.3-q11.1	150-162	0.67
D9S166	9p12-q21	233-261	0.83
D9S51	9p21-qter	135-159	0.84
D9S157	9p23-p22	133-149	0.84
D9S132	9pter-p22	156	0.75
D9S265	9q21.0	84-94	0.61
D9S287	9q22.3-q31	168-180	0.67
D9S109	9q31	219-229	0.70
D9S127	9q31	149-159	0.72
D9S103	9q33-qter	89-97	0.58
D9S157	9q34.2-2qter	133-149	0.84
D11S569	11p15.3	139-158	0.84
D11S576	11p15.5	137	0.89
D11S922	11p15.5	88-138	0.93
D13S289	13q12.1	260-276	0.74
D13S219	13q12.3-q13	117-127	0.64
D13S171	13q12.3-q13	227-241	0.73
D13S220	13q12.3-q13	191-203	0.66
D14S72	14q11.1-q11.2	257-271	0.83
D14S258	14q23-q24.3	170-182	0.8
D14S251	14q21-14q24.3	298-318	0.83
D14S292	14q32.1-q32.3	110-118	0.74
TP53	17p13.1	103-135	0.90
D17S379	17p13.3	342-362	0.74
D17S855	17q	145	0.82
D17S250	17q11.2-q12	151-169	0.91
D17S113	17q12-q24	146-160	0.64

Source: *http://mp.invitrogen.com/resources/apps/mappairs/*

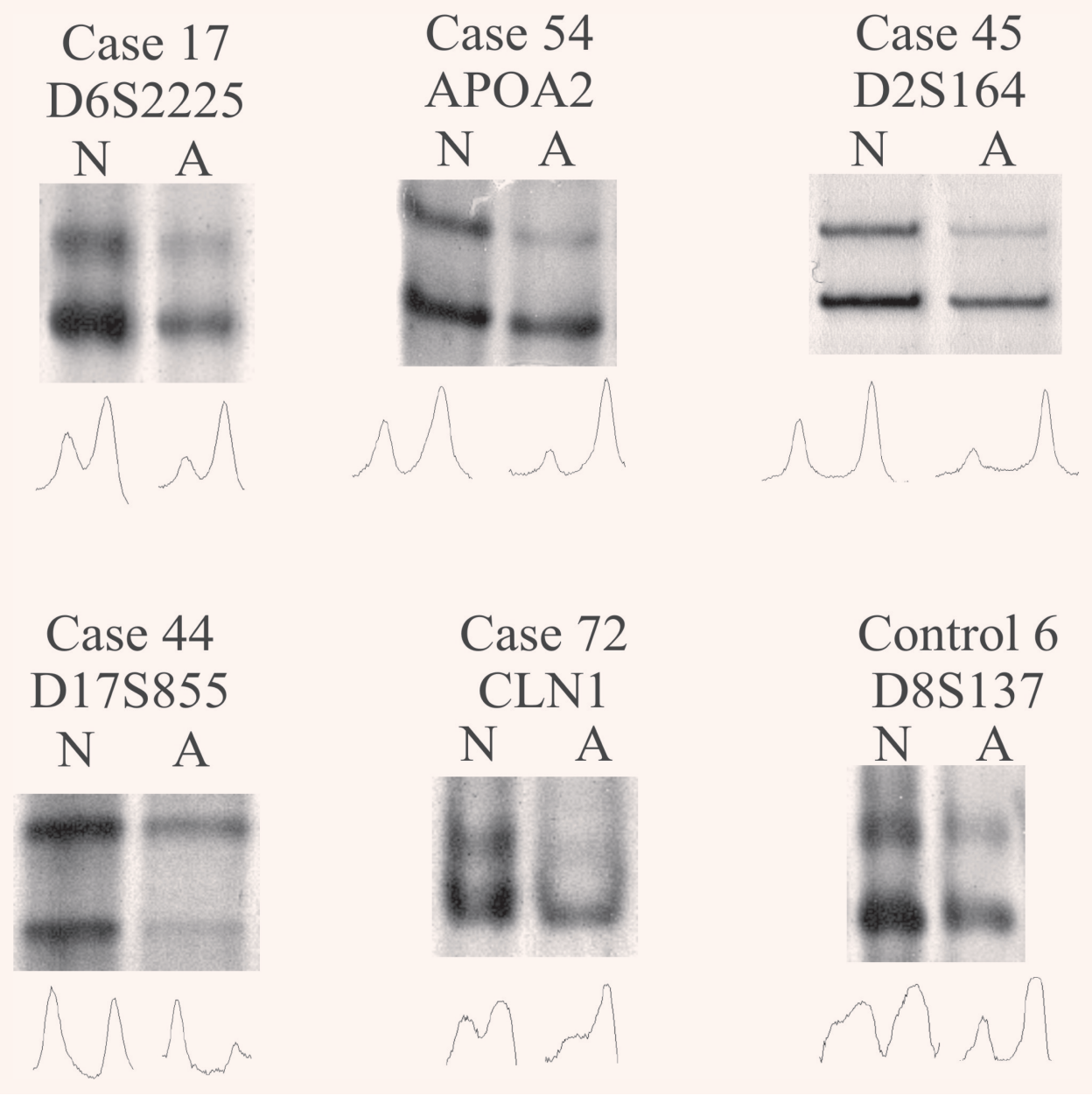

Fig. 1 Representative examples of LOH in aortic atheromas. A: aortic atheromas, N: correspondent venous blood, wave lines underneath each case represent the integrated density calculated by ImageJ 1.32j software.

VCAM1, SELE, APEG1 and AIF1 genes have been mapped respectively (http://www.ncbi.nlm.nih.gov/entrez/).

Discussion

Microsatellite alterations represent useful markers for clonally expanded cellular populations. It seems that the formation of both normal and atherosclerotic arterial wall occurs by clonal proliferation of VSMCs, so microsatellite DNA screening could provide important information [22]. The generation of allelic imbalance in close proximal short tandem repeats provides evidence of affected genes with extensive rearrangements.

LOH have been previously described in atherosclerotic tissue from different anatomic

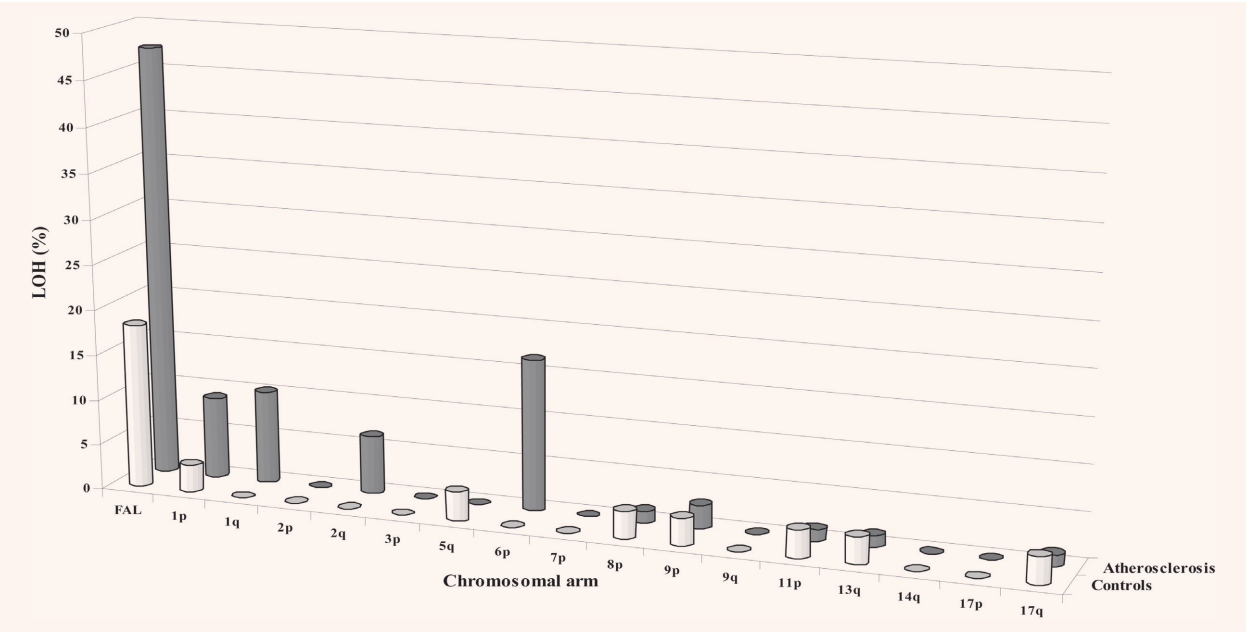

Fig. 2 Diagram presenting LOH incidence (%) per locus tested for aortic atheroma cases and normal aortic tissue controls.

locations and LOH on chromosomal arms 2p13-p22.3, 2q24.1-q32.1, 3p21.32-p21.1, 7q36, 8p12-q11.2, 9q31-34, 17p13 and 17q21 have been implicated with disease development [12,14,17,21, 25,26]. Here, we employed microsatellite DNA analysis, to identify genetic loci exhibited allelic imbalance in human aortic atheromas in a specific non-random manner.

Therefore, we examined 63 microsatellite DNA markers, located on 1p, 1q, 2p, 2q, 3p, 5q, 6p, 7p, 8p, 9p, 9q, 11p, 13q, 14q 17p and 17q chromosomal regions, in both atherosclerotic and normal aortic tissues compared to correspondent venous blood. This setting improves the strength of our study since microsatellite alterations have been reported in other non-malignant diseases as well as in phenotypically normal human tissues [27-33].

Genomic alterations were found in 47,4% of atherosclerotic cases versus 18,2% of controls in at least one locus. This finding suggests that allelic imbalance is associated to aortic atherosclerosis with an odds ratio value of 4,06 (95% CI 1,50 to 10,93). Comparison of mean FAL value (0,027± 0,032) observed in this study with previously reported for lung (0,243±0,021) and cervical (0,15±0,09) cancer from our group showed that LOH incidence per individual is substantially lower in atherosclerosis [33,34]. Comparison of LOH profiles between cases and controls revealed that genomic alterations on 1p32-p31, 1q22-q25, 2q35 and 6p21.3, which affect VCAM1, SELE, APEG1 and AIF1 genes, were significantly correlated with atherosclerosis.

Vascular cell adhesion molecule-1 (VCAM-1) is a cell surface protein expressed by cytokine activated vascular endothelium which mediates leukocyte adhesion to endothelial cells [35]. This type I membrane sialoglycoprotein, a member of the Ig superfamily, was found to be upregulated in atherosclerosis and has been implicated with early development of the disease [36,37].

The white blood cell adhesion molecule E-selectin (SELE) is also expressed by cytokine-stimulated endothelial cells. SELE has been proposed to be responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of cells to the vascular lining [38]. Also, its expression and polymorphisms has been suggested as important players of atherosclerosis development [39,40].

Aortic preferentially expressed protein 1 (APEG1) is expressed in differentiated and is markedly down-regulated in de-differentiated VSMCs. Expression of this gene is thought to serve as a marker for differentiated vascular smooth muscle cells which may have a role in regulating

growth and differentiation of this cell type [41,42]. Allograft inflammatory factor 1 (AIF1) is induced by cytokines and interferon and it has been suggested as an anti-inflammatory factor to vascular vessel wall trauma. It is a negative regulator of VSMCs growth but it promotes their migration through interaction and polymerization of F actin and regulation of Rac1 activity [43].

The genomic rearrangements described in atherosclerosis are less extensive than genomic instability in cancer. However the somatic genomic imbalances either in the form of amplification or loss of alleles may account for severe cellular phenotype alterations. The genomic rearrangements identified here predominantly in aortic atheromas may provide triggering signals of focal inflammatory response which could lead to atherosclerosis, through modifications of leukocyte adhesion, VSMCs growth, differentiation and migration. Our findings suggest an important link between somatic DNA mutations and inflammatory nature of atherosclerosis and provide a unified perspective of disease development. In conclusion, our data suggest that genomic rearrangements in atherosclerosis may be directly linked with disease development as an inflammatory vascular wound healing response. Genes involved in leukocyte adhesion, vascular smooth muscle cells growth, differentiation and migration, were found affected by somatic genomic alterations in atheromas and could provide the trigger to disease development.

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