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Protein and mRNA expression levels of VEGF-A and TGF-ß1 in different types of human coronary atherosclerotic lesions

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Abstract. It is well documented that inflammation plays a major role in the establishment and progression of atherosclerosis. Endothelial cells, vascular smooth muscle cells and monocytes/macrophages are involved in this process by expressing inflammatory factors. The aim of the present study was to evaluate potential association and risk of VEGF-A and TGF-ß1 in human coronary atherosclerotic lesions. Twenty-six fresh human coronary artery segments were collected at autopsy. Conventional histology was performed and samples were classified into: no lesion group (NL), fatty streak group (FS), plaque group (P) and complicated lesion group (CL) based on the atherosclerotic lesion type. RNA extraction-analysis with RT-PCR and immunohistochemistry was also performed. We observed that VEGF-A protein and mRNA expression increased during atherogenesis. The expression levels (protein and mRNA levels) of TGF-ß1 were decreased from NL to the FS group while, strong protein-staining and signal of mRNA expression in P and CL groups were observed. Our findings suggest a crucial role of VEGF-A in the development of coronary artery disease. The high protein and mRNA expression levels of TGF-ß1 in P and CL suggest that this factor may be implicated in the deposition of excessive extracellular matrix in the intima of the vessel wall, contributing to the expansion of the atheromatic plaque.

Introduction

Atherosclerosis has been characterised as a rather complex inflammatory disease. Several molecular mechanisms have been implicated in this process, the prolonged development of which leads to ischemia, plaque rupture and thrombosis. The accumulation of different molecules, such as oxidated LDL (oxLDL), into the intima, has been observed to play a crucial role in the formation and the kinetics of atherosclerotic lesion, leading to endothelial injury. The endothelial cells (ECs) provoke the recruitment of monocytes/macrophages from peripheral blood. In more advanced stages of atherosclerosis migration of vascular smooth muscle cells (VSMCs) and fibroblast is taking place, resulting in pathological and morphological changes of the intima of the artery (1). Vascular endothelial growth factor A (VEGF-A) and transforming growth factor beta 1 (TGF-ß1) are involved in the mechanism of atherogenesis and collateral vessel development (2).

VEGF-A is a glycoprotein of 45 kDa presented with 5 different isoforms deriving from a single gene following alternative exon splicing (3). Proliferation of endothelial cells has been shown to be linked with the expression of VEGF, which has also been implicated with enhanced vascular permeability through interaction with VEGF receptor 2 (VEGFR-2) in macrophages, ECs and VSMCs, as well as modulation of atheromatosis and thrombogenicity (4). Furthermore, VEGF receptor flt-1 is expressed in human monocytes provoking chemotactic response of these cells (5,6).

TGF-ß1 is a 25 kDa homodimeric, pleiotropic growth factor secreted by many cell types including macrophages, lymphocytes, smooth muscle cells and platelets (7). TGF-ß1 is a well recognised anti-inflammatory cytokine found to be expressed both in human and rat atherosclerotic plaques, suggesting a potential anti-atherogenic role (8-10). Its secreted inactive form is activated by plasmin (7), which is in turn produced from plasminogen by tissue plasminogen activator. Production of plasmin is blocked by competitive inhibition from lipoprotein (a) and plasminogen activator inhibitor 1. These molecules are therefore able to promote smooth muscle cell proliferation by relieving the inhibition caused by active TGF-ß (11,12).

The involvement of the above factors in atherogenesis has been previously established. Data on their involvement in the specific stages of plaque formation in human coronary arteries are very limited. In this work, the expression and synthesis patterns of the angiogenetic VEGF-A and the anti-

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inflammatory TGF-ß1 were examined in injured human coronary arteries (HCA). We evaluated the potential association and risk of these factors in atherogenesis and progress of the development of different stages of atherosclerosis in human coronary artery lesions.

Materials and methods

Sample collection took place from March 2002 until December 2003 in the General Mortuary (Department of the Forensic Medicine, Medical School, University of Athens). Twenty-six fresh coronary artery segments were collected at autopsy within 8 h after death, from 8 individuals (6 males and 2 females) aged from 41 to 81 years. Individual A (51/male): samples 1, 8, 13, and 20 in Table II. Individual B (41/female): samples 2, 10 and 25 in Table II. Individual C (55/male): samples 3, 5, 7, 14, 15, 21 and 23 in Table II. Individual D (51/male): samples 6, 9 and 26 in Table II. Individual E (74/male): samples 4 and 24 in Table II. Individual F (58/female): samples 11, 17 and 18 in Table II. Individual G (46/male): samples 12 and 16 in Table II. Individual H (81/male): samples 19 and 22 in Table II. Since we can not fully exclude the possibility of postmortem changes in autopsy samples we took every precaution to treat the samples in exactly the same way. A recent study demonstrated that there were no changes in the expression patterns of vascular autopsy materials (<12 h after death) compared to samples taken immediately after amputation (4). All samples were immediately separated in two parts. One part was collected and fixed immediately in 10% buffered paraformaldehyde solution, for conventional histology and immunohistochemistry. The second part from all samples was immediately stored at -70˚C for further RNA analysis.

The present study was approved by the institutional ethics committee of the General Mortuary (Department of the Forensic Medicine, Medical School, University of Athens). The investigation conforms to the principles outlined in the Declaration of Helsinki.

Immunohistochemistry. For immunohistochemical analysis the following antibodies were used: VEGF mouse monoclonal antibody (Pharmingen) in 1:100 dilution and TGF-ß1 rabbit polyclonal antibody (Santa-Cruz), in dilution 1:50. In serial sections we also determined the cellular phenotype of atherosclerotic plaques. To identify endothelial cells, macrophages/foam cells and smooth muscle cells the following mouse monoclonal antibodies were used respectively: CD34 mouse monoclonal antibody (clone:QBEnd/10. Biogenex) in 1:50 dilution, CD68 mouse monoclonal antibody (clone: KP1. Dako) in 1:50 dilution and α-smooth muscle actin monoclonal antibody (clone: 1A4. Dako) in 1:50 dilution. Non-specific immunoglobulin binding was assesed with a mouse monoclonal antibody for Chromogranin A (Dako, dilution1:200) and a rabbit polyclonal antibody for Prostate Specific Antigen (Dako, dilution 1:300). Immunohistochemistry was performed according to the indirect streptavidin-biotin-peroxidase method. In brief, 5 μm paraffin sections were maintained on poly-L-lysine-coated slides, dewaxed, rehydrated and incubated for 30 min with 0.3% hydrogen peroxide to quench the endogenous peroxidase activity. Unmasking of the related proteins was carried out. Sections were incubated with the appropriate primary antibody in the afore-mentioned dilution. Biotin-conjugated secondary antibody was added at a 1:200 dilution for 1 h at room temperature. The next stage comprised of 30-min incubation in Strept AB Complex (1:100 stock biotin solution, 1:100 stock streptavidin-peroxidase solution) (Dako). For color development 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) and haematoxylin as counterstain was used.

Histopathologic examination classified the lesions into four categories: a) no lesion (NL) no=7, b) fatty streak (FS) no=5 (lesions I-II in AHA classification), c) plaque (P) no=6 (lesions III-IV in AHA classification) and d) complicated lesion (CL) no=8 (lesions V-VI in AHA classification) (13). Semi-quantitative microscopical evaluation of the sections was done by one experienced pathologist in random order without knowledge of the origin of the samples. The specimens were graded using VEGF-A and TGF-ß1 immunostained sections by the following criteria: no detectable staining (-); weak staining (+), <10% of the cells in the area were positive for the studied signal; moderate staining (++), 10-50% of the cells in the area were positive for the studied signal; strong staining (+++), >50% of the cells in the area were positive for the studied signal (14).

Extraction and quantification of mRNA. Total RNA was isolated, from the frozen samples following homogenisation of the tissue, using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA). The RNA preparation was treated with DNase I to remove residual traces of DNA. One percent agarose gel electrophoresis and ethidium bromide staining were used to examine RNA integrity. RNA concentration and purity was determined on a UV spectrophotometer (Hitachi Instruments Inc., USA) by the 260 nm absorbance and 260-280 nm absorbance ratio respectively. cDNA synthesis was performed with 2 μg of total RNA using ThermoScript RT-PCR System (Invitrogen, USA) according to the manufacturer's instructions as previously described (2).

Semi-quantitative RT-PCR. PCR quantification included optimisation of all primer sets as shown in Table I. In brief, each quantification set included two PCR reactions [the target and the ß2-microglobulin (ß2-M) reference]. Each PCR reaction (target and ß2-M) was optimized individually for primer, Mg and Taq polymerase concentration using as a template a representative pool of all samples to be measured. Then the reactions were combined into a single tube in order to eliminate tube to tube variations. A new optimization was performed to ensure that there was no cross inhibition between the two PCR reactions. Subsequently another optimization was performed modulating the relative concentration of the two sets of primers to ensure that the two reactions reached the logarithmic phase of expansion in the same PCR cycles (reaction synchronization). Finally, we determined the cycle in which the reaction reached the middle of the logarithmic expansion phase. The set of conditions that were established regarding primer, Mg, Taq polymerase concentration and cycle number, was applied specifically to the set of samples that were used for the standardization (sample pool) and the corresponding target. The total standardization procedure

Table I. Oligonucleotide sequences and PCR conditions.

Primer set	Oligonucleotide sequence (5'-3')	Primer annealing temperature (°C)	Amplification cycles	Product size (bp)
VEGF-A	Fwd: gca gaa gga gga ggg cag aat c Rev: aca ctc cag gcc ctc gtc att	62	38	197
TGF-ß1	Fwd: acc aac tat tgc ttc agc tc Rev: tta tgc tgg ttg tac agg	56	38	198
ß2-Microglobulin	Fwd: agc gta ctc caa aga ttc agg tt Rev: tac atg tct cga tcc cac tta act at	55-65		297

Fwd, forward oligonucleotide primer. Rev, reverse oligonucleotide primer.

Table II. VEGF-A and TGF-ß1 protein expression levels in human coronary arteries.

Sample no.	Patient ID	Age/ sex	Lesion type	Cause of death	Anatomical site	VEGF-A				TGF-ß1			
						ECs	Intima	Media	Adventitia	ECs	Intima	Media	Adventitia
1	A	51/M	NL	Accidental	CA	-	+	+	++	+++	++	+++	+++
2	B	41/F	NL	Accidental	CA	-	-	++	-	++	++	+++	+++
3	C	55/M	NL	Suicide	CA	-	-	+	-	+++	-	+++	+++
4	E	74/M	NL	Infarct	CA	-	-	++	-	+++	++	+++	+++
5	C	55/M	NL	Suicide	CA	-	-	++	+	++	-	+++	+++
6	D	51/M	NL	Accidental	CA	-	-	-	-	++	-	+++	+++
7	C	55/M	NL	Suicide	CA	-	-	+	-	+++	++	+++	+++
8	A	51/M	FS	Accidental	CA	-	+++	-	-	++	-	+++	+++
9	D	51/M	FS	Accidental	CA	+	++	-	-	++	+++	+++	+++
10	B	41/F	FS	Accidental	CA	++	+++	+	+	++	-	+++	+++
11	F	58/F	FS	Accidental	CA	++	+++	+	-	++	-	+++	+++
12	G	46/M	FS	Accidental	CA	+	++	+	-	++	-	+++	+++
13	A	51/M	P	Accidental	CA	+	+++	+	+	+++	+++	+++	+++
14	C	55/M	P	Suicide	CA	+	+++	++	+	++	+++	+++	+++
15	C	55/M	P	Suicide	CA	+	+++	++	+	++	+++	+++	+++
16	G	46/M	P	Accidental	CA	+	+++	++	+	++	++	+++	+++
17	F	58/F	P	Accidental	CA	+	++	++	-	+++	+++	+++	+++
18	F	58/F	P	Accidental	CA	+	+++	++	+	++	+++	+++	+++
19	H	81/M	CL	Infarct	CA	+	+++	+++	++	++	+	+++	+++
20	A	51/M	CL	Accidental	CA	+	+++	+	+	++	+	++	+++
21	C	55/M	CL	Suicide	CA	+	++	+	+	++	+	++	++
22	H	81/M	CL	Infarct	CA	+	+++	+++	++	++	+++	+++	+++
23	C	55/M	CL	Suicide	CA	+	+++	++	++	++	+	+++	+++
24	E	74/M	CL	Infarct	CA	+	+++	+++	++	++	+	+++	+++
25	B	41/F	CL	Accidental	CA	++	+++	++	+	++	+++	+++	+++
26	D	51/M	CL	Accidental	CA	+	+++	+	+	++	+++	+++	+++

M, male. F, female. NL, no lesion. FS, fatty streak. P, plaque. CL, complicated lesion. CA, coronary artery. ECs, endothelial cells. -, no detectable staining. +, weak staining. ++, moderate staining. +++, strong staining.