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.

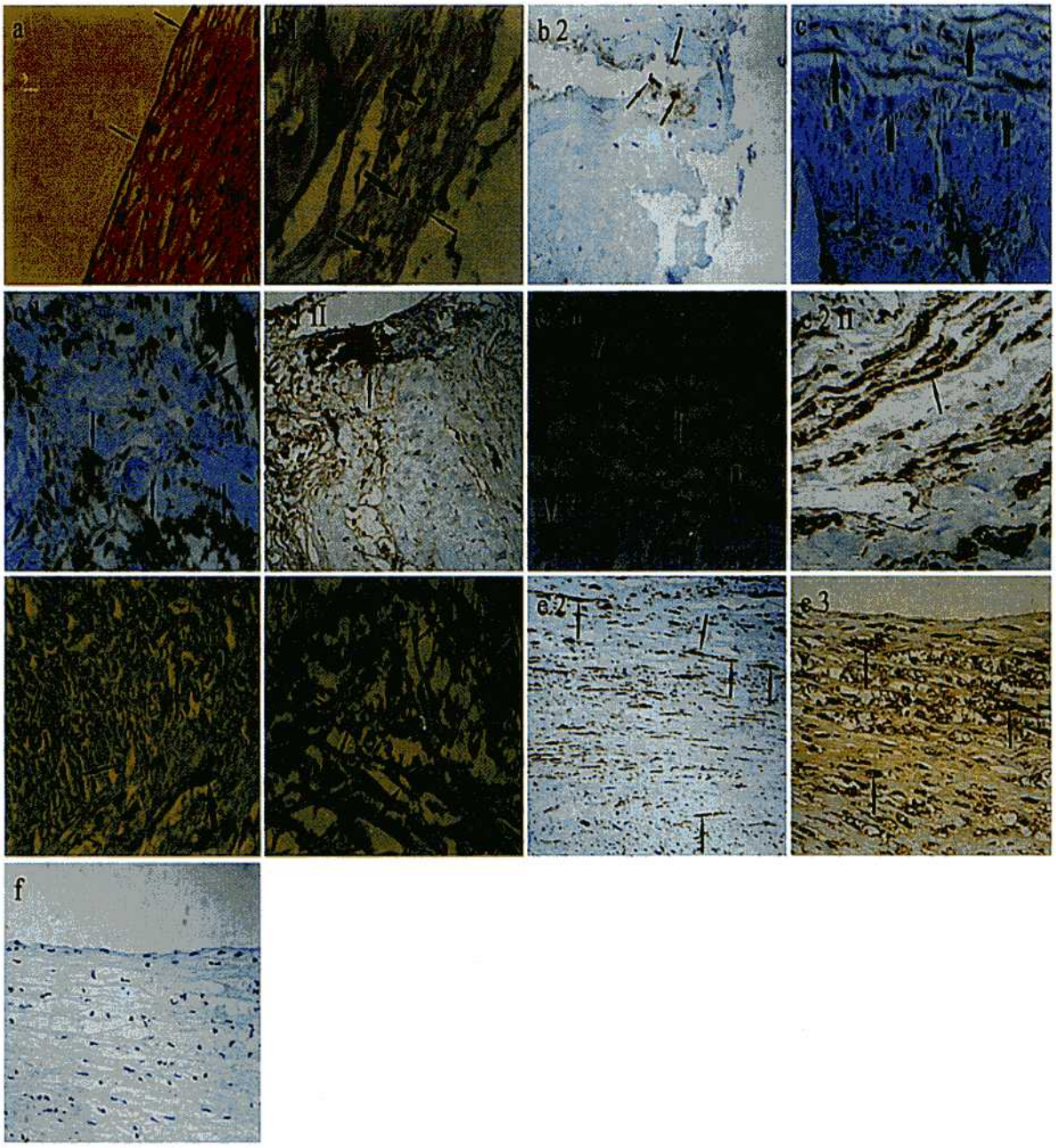

Figure 1. (a), Normal coronary artery stained for VEGF-A. Note that endothelial cells (thin arrows) and the underneath of intima are negative. Magnification, x400. (b1), Fatty streak stained for VEGF-A. Note weak staining of endothelial cells (thin arrows) and strong staining of cells of the lesion (thick arrow) in intima. Magnification, x400. (b2), Consecutive section of (b1). Note the stained endothelial cells (thin arrows) with anti-CD34. Magnification, x400. (c), Plaque stained for VEGF-A. Strong staining of cells of the plaque in the intima (thin arrow), moderate staining of adjacent media (thick arrow). Internal elastic lamina (double thin arrow). Magnification, x250. (c1I), Higher magnification of (c). Strong staining of the cells of the plaque in the intima (thin arrows). Magnification, x400. (c1II), Consecutive section of (c1I). Stain for macrophages/foam cells (arrow) with anti-CD68. The group of stained cells indicated by an arrow is the same group indicated by the two arrows in the upper right corner of (c1I). Magnification, x400. (c2I), Higher magnification of (c). Moderate staining of the smooth muscle cells of media (thin arrows). Internal elastic lamina (thick arrow). Magnification, x400. (c2II), Consecutive section of (c2I) stained with anti-α actin shows intense stain for smooth muscle cells (arrow). Magnification, x400. (d), Weakly stained media (thin arrows) adjacent to fatty streak. Internal elastic lamina (thick arrow). (e1), Plaque stained for VEGF-A. Both oval foam cells (thin arrows) and elongated smooth muscle cells (thick arrow) are stained. Magnification, x400. (e2), Consecutive section of (e1). Stained for smooth muscle cells (arrows) with anti-α actin. Note elongated cells are mainly stained. Magnification, x400. (e3), Consecutive section of (e1). Stained for macrophages/foam cells (arrows) with anti-CD68. Note oval cells are mainly stained. Magnification, x400. (f), Negative control. Stained with mouse monoclonal antibody for Chromogranin A.

was repeated for each quantification reaction (VEGF-A, TGF-ß1).

The general PCR protocol included 1X PCR reaction buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, and 0.6 U Platinum Taq DNA polymerase (Invitrogen, USA), with 10 pmol of each primer set. Cycling parameters of the reaction varied depending on primer sets used, as shown in Table I. ß2-M was used as an internal control in all PCR reactions.

PCR products were analysed by 10% polyacrylamide gel electrophoresis (29:1 ratio acrylamide/bis-acrylamide) and silver stained. Gels were scanned on an Agfa Snap-Scan 1212u (Agfa-Gevaert N.V., Belgium). The integrated density

of the bands was used as quantitative parameter and was calculated by digital image analysis (Scion image). The ratio of the integrated density of each gene divided by that of ß2-M was used to quantify the results.

Statistical analysis. Results are presented as mean values ± SEM. Non-parametric Mann-Whitney U test was used for comparisons between two groups and Kruskal-Wallis One-way ANOVA was used when more than two groups were compared. The statistical analysis was performed by SPSS statistical program (version 11).

Results

Immunohistochemical analysis of VEGF-A and TGF-ß1 in atherosclerotic lesions and normal coronary arteries. Protein expression levels of VEGF-A and TGF-ß1 were evaluated and compared using immunohistochemistry both in atherosclerotic lesions and normal coronary arteries. VEGF-A mouse monoclonal antibody and TGF-ß1 rabbit polyclonal antibody were used for the detection of VEGF-A and TGF-ß1 respectively. The results of the semi-quantitative analysis of the immunostanings in ECs, intima, media and adventitia in all 26 HCA are shown in Table II.

No VEGF-A expression was observed in the ECs of the 7 NL coronary arteries which were analysed by immunostaining (Fig. 1a), whereas from the total of 19 samples of FS, P and CL types, 18 showed low expression (Fig. 1b1 and b2). The FS sample 8 showed no expression of this factor in the ECs. In ECs the staining of VEGF-A was substantially higher in FS lesions (mean score 1.20, P=0.018), in plaques (mean score 1, P=0.001) and CL (mean score 1.13, P<0.001) when all three types were individually compared with NL. Comparison among the three different lesion types in ECs showed no statistically significant difference.

VEGF-A staining was more abundant in the intima of atherosclerotic lesions (Fig. 1. b1, c and c1I) versus the 6 NL, which showed no expression (Fig. 1a). Sample 1 was the only normal artery type which showed low staining in the intima for this growth factor. VEGF-A expression levels in the intima of the FS lesions (mean score 2.6) presented statistically significant differences (P=0.003) when compared with NL. Furthermore, statistical analysis was carried out comparing the increased expression of this growth factor in plaques (mean score 2.83, P=0.001) and CL (mean score 2.88, P<0.001) compared to NL. However, comparison among the three different lesion types in intima showed no statistically significant difference.

Staining of the media and adventitia of all arteries revealed fluctuation in the expression of VEGF-A. Increased levels of VEGF-A were detected in the media of P (Fig. 1c2I) (mean score 1.83, P=0.009) and CL (mean score 2.00, P=0.019) versus FS respectively (Fig. 1d) and also in adventitia of the CL (mean score 1.50, P=0.021 and P=0.006) when compared with NL and FS respectively.

It is noteworthy that staining of the intima of the arteries with atheromatosis demonstrated similar expression patterns in both cell types implicated with the development and progress of atheromatosis, VSMCs and foam cells (Fig. 1c1II, c2II, e1, e2 and e3).

The evaluation of the expression levels of TGF-ß1 among the different types of atheromatosis and the normal coronary arteries in ECs and the three vascular layers was carried out in the same manner as for VEGF-A. Elevated levels of TGF-ß1 were recorded in the intima of plaque samples (Fig. 2a1, a2 and a3) (mean score 2.83, P=0.005 and P=0.030) versus normal arteries and FS respectively (Fig. 2b). In addition, marginal statistical significance was observed in the expression levels of TGF-ß1 in the CL (mean score 1.75, P=0.045) versus FS.

Semi-quantitation of the mRNA for VEGF-A and TGF-ß1 in atherosclerotic lesions and normal arteries. A representative semi-quantitative RT-PCR assay demonstrating a range of differences in the production of VEGF-A and TGF-ß1 mRNA during the different stages of development of atherosclerotic lesions, ranging from normal arteries to CL (Fig. 3). The results of the quantitation of the mRNA for VEGF-A and TGF-ß1 are summarised in Table III.

Elevated levels of expression of VEGF-A mRNA was observed in FS (mean score 0.63, P=0.003), in P (mean score 1.1, P=0.001) and the CL (mean score 1.7, P<0.001), when compared with normal arteries. Further analysis revealed differences in the expression of this factor among the different types of atherosclerotic lesions. Thus, expression patterns of VEGF-A mRNA were increased in P (P=0.004), in CL (P=0.002) versus FS and increased in CL (P=0.029) versus P.

TGF-ß1 mRNA expression levels were increased in P (mean score 2.7, P=0.001, P=0.004 and P=0.001) when compared with NL, FS and CL respectively. Furthermore, higher levels of expression of mRNA in NL (mean score 0.9, P=0.030) and CL (mean score 1, P=0.045) versus FS were found.

Furthermore, we compared samples from the same donor, for the immunohistochemical detection and mRNA quantification of VEGF-A and TGF-ß1. The results were in agreement with the main findings of our study based on the analysis of the population as a total.

Discussion

We attempted to quantify both protein and mRNA expression levels of VEGF-A and TGF-ß1 growth factors in normal coronary arteries and also in coronary arteries presented at different stages of atheromatosis. Using these data we attempted to reveal the mechanism of action of these factors as regards to the development and progress of CAD.

Immunostaining results regarding VEGF-A revealed that there was no expression in protein level in ECs and in the intima of normal arteries, whereas it was mainly localized in the VSMCs of the media and the adventitia. On the other hand, the presence of this factor was detected in the ECs along with strong staining of the cells of the lesion in intima of all atherosclerotic types. Staining for VEGF-A in the intima of these arteries was also detected in the macrophage-foam cells and the VSMCs. Data gathered following immunostaining for VEGF-A are in complete agreement with the results of expression levels of mRNA for the same factor following semi-quantitative RT-PCR. Gradual increase in mRNA expression levels were also detected from the NL

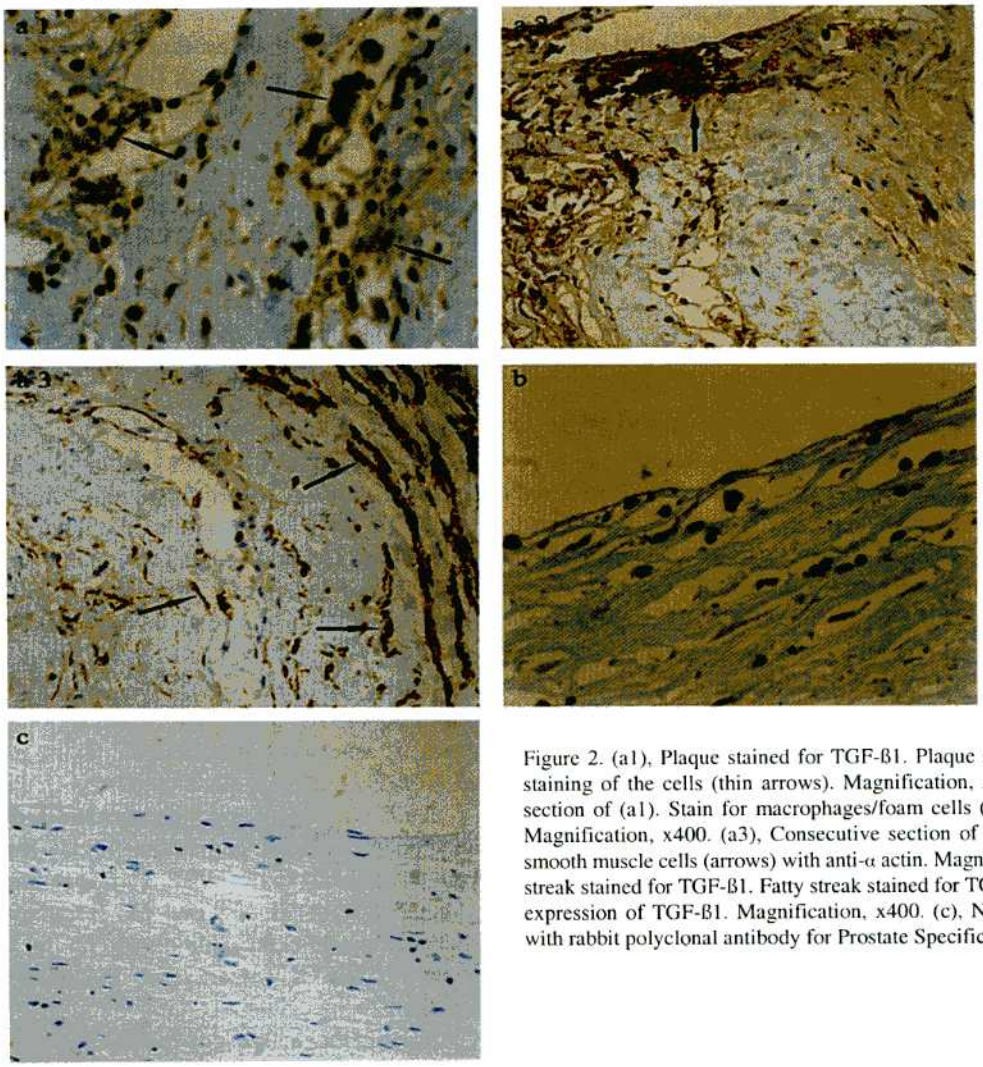

Figure 2. (a1), Plaque stained for TGF-ß1. Plaque showing strong TGF-ß1 staining of the cells (thin arrows). Magnification, x400. (a2), Consecutive section of (a1). Stain for macrophages/foam cells (arrow) with anti-CD68. Magnification, x400. (a3), Consecutive section of (a1) and (a2). Stain for smooth muscle cells (arrows) with anti-α actin. Magnification, x400. (b), Fatty streak stained for TGF-ß1. Fatty streak stained for TGF-ß1 showing moderate expression of TGF-ß1. Magnification, x400. (c), Negative control. Stained with rabbit polyclonal antibody for Prostate Specific Antigen.

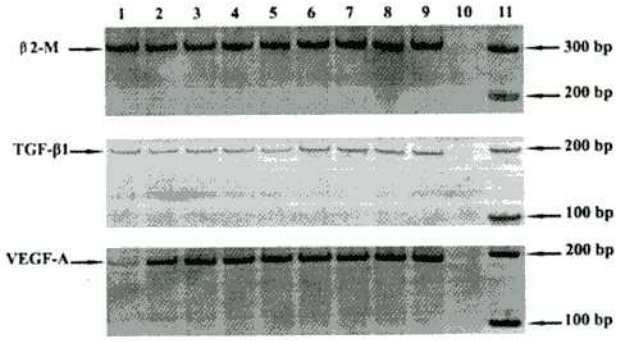

Figure 3. Acrylamide gel electrophoresis of RT-PCR products. RT-PCR products from 8 random human coronary arteries, 2 from normal human coronary arteries (lanes 1 and 2), 2 from fatty streak human coronary arteries (lanes 3 and 4), 2 from plaques of human coronary arteries (lanes 5 and 6) and 2 from complicated lesion of human coronary arteries (lanes 7 and 8) demonstrating the levels of mRNA expression of ß2-microglobulin (ß2-M), VEGF-A and TGF-ß1. Lane 9, positive control. Lane 10, negative control. Lane 11, ladder 100 bp.

Table III. Summary of the results on VEGF-A and TGF-ß1 expression in human coronary arteries.

Lesion type	VEGF-A/ß2-M	TGF-ß1/ß2-M
NL	0.12±0.15	0.89±0.17
FS	0.63±0.07	0.69±0.11
P	1.13±0.31	2.7±1.68
CL	1.73±0.53	1.7±0.28

Data are presented as mean ± SEM (standard error of the mean). The values represent the VEGF-A/ß2-microglobulin and TGF-ß1/ß2-microglobulin ratios which were calculated by the optical integrated density of each gene divided by that of ß2-microglobulin. NL, no lesion. FS, fatty streak. P, plaque. CL, complicated lesion.

types to the atherosclerotic lesions. It is noteworthy that a gradual increase of VEGF-A expression was also observed from FS type, to the plaque and that of CL.

Experiments on histological and molecular level identified the presence of VEGF-A in atheromatic plaque of coronary arteries. A growth factor with major role in angiogenesis and vasculogenesis in the embryo and promotion mobilization of endothelial precursor cells from bone marrow in adults (15), to adult vascular remodeling after repair and regeneration under normal circumstances (16). Overall, a growth factor with strong pro-inflammatory and chemiotactic role on macrophages and smooth muscle cells (inflammatory cells

present in the atherosclerotic lesion) (17). In recent reports Rutanen *et al* (4) and Inoue *et al* (18) showed using immunostaining and RT-PCR in atherosclerotic lesions of coronary and also in peripheral arteries, that protein expression levels similarly to mRNA expression levels of VEGF-A, increased with the progression of atherosclerosis in ECs and the intima of FS to CL. On the other hand, no protein or mRNA expression of this factor was detected in the ECs and the intima of normal, coronary or peripheral arteries.

VEGF-A expression was expected in ECs of all different types of atherosclerotic lesions due to the converted endothelial function of these cells from normal to inflammatory status. Given that VEGF-A not only stimulates proliferation of ECs and increases vascular permeability, it also induces migration of monocytes/macrophages and smooth muscle cells. Indeed, protein and mRNA expression of VEGF-A in ECs of atheromatic lesions was higher compared with normal samples, a rather expected result, as dysfunction of ECs is neither expected nor observed in normal arteries. Thus, no infiltration of circulating monocytes/lymphocytes was detected, which was the main concept introduced by Ross (19).

We previously showed statistically significant association of the increased VEGF-A levels in peripheral monocytes, in patients with stable angina and diabetes in coronary artery disease (2). Furthermore, Fleisch *et al* (20) showed that VEGF-A serum levels of patients suffering from 1- to 3-vessel coronary artery disease were increased by the degree of coronary atherosclerosis.

Statistically significant differences were observed regarding TGF-ß1 in the intima of different types of atherosclerotic lesions depending on the type of atheromatic injury involved. Protein expression levels of this growth factor showed an increase in P compared with NL and FS in the same manner as in CL when compared with FS. No statistically significant difference was observed either in protein levels of TGF-ß1 in NL compared with CL, or P compared with CL. The decrease of expression of TGF-ß1 from the NL to FS followed by its increase in P and CL. Protein expression data of TGF-ß1 correlate with those of mRNA, where again FS showed a decreased expression when compared with NL but also an increase in plaques and CL.

Mallat *et al* (21) showed that the inhibition of TGF-ß signaling following intraperitoneal injection with anti-hTGF-ß1, ß2 and ß3 2G7 monoclonal antibody in apoE knockout mice, accelerated the development of atherosclerotic lesions and thus the development of lesions with increased inflammatory component and decreased collagen content. Grainger *et al* (22) and Wang *et al* (23) showed that serum levels of TGF-ß1 were much lower in patients with advanced atherosclerosis, compared with individuals with normal coronary arteries. These results identify a major protective role for TGF-ß1 in atherosclerosis. These results suggest an anti-inflammatory role for this factor in the formation and progress of the disease.

However, histologically and histochemically the plaques actually are atherosclerotic lesions basically composed of a lipid core that precedes an increase in fibrous tissue (mainly collagen), that will subsequently change the nature of the intima above the lipid core. For the duration of this procedure atheromatous plaque may gradually develop to complicated fibrous plaque following the deposition of extracellular matrix proteins such as collagen. Furthermore, repeated disruptions of the lesion surface, hematomas, and thrombotic deposits result in the multilayered architecture of fibroatheroma (13). During this process, it has been shown that TGF-ß1 promotes the production of extracellular matrix by stimulating the synthesis of individual matrix components such as type I procollagen, by activated SMC in atherosclerotic human pulmonary arteries (24). The deposition of excessive extracellular matrix, which induces atherogenesis and the finding that TGF-ß1 induces thrombogenesis by down-regulating thrombomodulin (25), suggest that this factor could play a major role in the development and progress of atheromatic plaque both in coronary and peripheral arteries. Bobic *et al* (8) previously showed that protein expression levels of TGF-ß1 in both normal and atherosclerotic thoracic and abdominal aortas were higher in normal, FS and fibrous plaques. Our results for TGF-ß1 agree with previous studies on this factor. Consequently, TGF-ß1 could be characterized as a pro-inflammatory factor inducing the development of atherosclerotic lesions.

We would like to emphasize the importance of high expression levels for VEGF-A in the development and progress of the disease, factor with strong angiogenetic and pro-inflammatory profile. TGF-ß1 on the other hand could be characterized as a 'double-faced' factor, since anti-inflammatory but also pro-inflammatory properties have been attributed to this factor. However, our findings suggest that there is a complex balance of TGF-ß1-mediated responses influencing atherogenic outcomes. The debate is still open and hence future studies both *in vivo* and *in vitro* are considered necessary in order to determine the precise role of TGF-ß1 and the mechanism of action of VEGF-A.

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