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Vascular endothelial growth factor-D expression in human atherosclerotic lesions

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Abstract

Objective: Vascular endothelial growth factor-D (VEGF-D) is a recently characterized member of the VEGF family, but its expression in atherosclerotic lesions remains unknown. We studied the expression of VEGF-D and its receptors (VEGFR-2 and VEGFR-3) in normal and atherosclerotic human arteries, and compared that to the expression pattern of VEGF-A. **Methods:** Human arterial samples ($n=39$) obtained from amputation operations and fast autopsies were classified according to the stage of atherosclerosis and studied by immunohistochemistry. The results were confirmed by in situ hybridization and RT-PCR. **Results:** We found that while VEGF-A expression increased during atherogenesis, VEGF-D expression remained relatively stable only decreasing in complicated lesions. In normal arteries and in early lesions VEGF-D was mainly expressed in smooth muscle cells, whereas in complicated atherosclerotic lesions the expression was most prominent in macrophages and also colocalized with plaque neovascularization. By comparing the staining profiles of different antibodies, we found that proteolytic processing of VEGF-D was efficient in the vessel wall. VEGFR-2, but not VEGFR-3, was expressed in the vessel wall at every stage of atherosclerosis. **Conclusions:** Our results suggest that in large arteries VEGF-D is mainly expressed in smooth muscle cells and that it may have a role in the maintenance of vascular homeostasis. However, in complicated lesions it was also expressed in macrophages and may contribute to plaque neovascularization. The constitutive expression of VEGFR-2 in arteries suggests that it may be one of the principal mediators of the VEGF-D effects in large arteries.

Keywords: Arteries; Atherosclerosis; Gene expression; Growth

1. Introduction

Vascular endothelial growth factors (VEGFs) play fundamental roles in the growth, differentiation and maintenance of blood vessels [1]. VEGFs are also involved in many other physiological and pathological conditions, such as vessel remodelling, wound healing, bone growth, tumorigenesis, and tissue ischemia. The mammalian members of the gene family known to date are VEGF-A (six isoforms), placenta growth factor, VEGF-B, VEGF-C and VEGF-D. Their signaling is primarily mediated via three tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4) [1,2].

VEGF-A induces migration and proliferation of endothelial cells (ECs) and enhances vascular permeability mainly through VEGFR-2 [3,4]. VEGF-A is also a ligand

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for VEGFR-1 and induces monocyte/macrophage infiltration and activation in the vessel wall through VEGFR-1 [2]. VEGF-A expression in atherosclerotic arteries increases during atherogenesis [5,6]. VEGF-A is a cytoprotective and anti-apoptotic maintenance factor for endothelial cells (ECs) [7]. However, in atherosclerotic plaques VEGF-A is found in macrophages and in connection with plaque neovascularization [6,8]. Also, high plasma concentrations of VEGF-A induced atherosclerotic lesion formation in mice and rabbits [9]. These studies suggest that in addition to vascular protection VEGFs may play a role in atherogenesis.

VEGF-D is a novel member of the VEGF gene family initially described as a *c-fos*-induced growth factor [10]. It shares 48% homology at amino acid level with its closest relative, VEGF-C, that binds to the same receptors. Furthermore, VEGF-D is produced as a prepropeptide and further processed to a biologically fully active form that effectively binds to VEGFR-2 and -3 [11,12]. In adult human tissues VEGF-D is mostly produced in heart, lung, skeletal muscle, colon, and small intestine [11]. It induces angiogenesis [13] and is associated with tumorigenesis [14] and spread of metastasis via lymphatics [15]. VEGF-D is not upregulated by hypoxia but regulation by cell-to-cell interaction has been observed in fibroblasts [16]. In the vascular system, VEGF-D has been previously found in smooth muscle cells (SMCs) of small arterioles [14,17] but its expression in large atherosclerotic arteries remains unknown.

We studied the expression of VEGF-D and its receptors in human arteries at different stages of atherogenesis. It was found that VEGF-D is abundant in arteries regardless of the stage of atherosclerosis with only a reduction in the most advanced lesions. Also, VEGFR-2 was expressed in the artery wall at every stage of atherogenesis, whereas VEGFR-3 was only found in adventitial vessels.

2. Methods

2.1. Materials

Fresh human arterial samples were collected from amputation operations and fast autopsies within 12 h after death. We cannot exclude the possibility of some post mortem changes in autopsy samples, but there were no differences in expression patterns compared to the samples taken immediately after amputation. Also, no major changes were observed in previous studies conducted on similar tissue samples, compared with results obtained from organ donors or from perfusion fixed animals [18–20]. All samples were divided in two parts: one part was immersion-fixed in 4% paraformaldehyde–15% sucrose (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) and embedded in paraffin [18]. The other part was snap frozen in liquid nitrogen and stored at −70 °C for RNA analysis. The study protocol was approved by the Ethical Committee of the Kuopio University Hospital and the study conforms with the principles outlined in the Declaration of Helsinki.

2.2. Immunohistochemistry and in situ hybridization

Immunostainings were done in serially cut paraffin sections as described [18] using the following mouse monoclonal antibodies against human antigens: VEGF-D (VD1 [14], dilution 1:100 and R&D [cat no: MAB286], 1:25), α-actin (HHF-35, Dako, 1:50), macrophages (CD68, Dako, 1:150), VEGF-A (Santa Cruz Biotechnology [sc-7269], 1:500), VEGFR-2 (Santa Cruz Biotechnology [sc-6251], 1:500 and R&D [MFLK1Cabm], 1:200), and VEGFR-3 [21] (1:100). Also, a goat polyclonal antibody against human VEGF-D (Santa Cruz Biotechnology [sc-7602], 1:100) C-terminal end was used. For double immunostainings, avidin–biotin–HRP and alkaline-phosphatase systems with DAB and Vector Blue color substrates (Vector Laboratories) were used for signal detection, respectively. Control stainings with no primary antibodies and with class and species matched irrelevant antibodies were done for each specimen and antigen. Also, the signal for VEGF-D was blocked by preadsorption with recombinant VEGF-D protein before staining. The localization of VEGF-D, VEGFR-2, and VEGFR-3 mRNA was studied by in situ hybridization in paraffin sections. All antisense and control sense riboprobes (nucleotides 789–1113 of VEGF-D, 1723–2365 of VEGFR-2 and 1–595 of VEGFR-3) were synthesized from pBluescript (VEGF-D) or pGEM (VEGFR-2 and -3) plasmids using T7, T3 or SP6 polymerases in the presence of [^{33}P]UTP. In situ hybridizations were performed on pretreated (Proteinase K 0.5 mg/ml at 37 °C for 30 min) tissue sections (1×10^6 cpm per section) as described [18].

Lesions ($n=39$) were histopathologically classified into four categories: no lesion $n=5$, fatty streak $n=11$ (lesions I–II in AHA classification [22]), plaque $n=12$ (lesions III–IV [22]), and complicated lesion $n=11$ (lesions V–VI [22]). Semiquantitative microscopical evaluation of the sections was done by one experienced observer (JR) in random order without knowledge of the origin of the samples. The specimens were graded using VEGF-D and -A immunostained sections by following criteria: no detectable staining (−); weak staining (+), meaning that less than 10% of the lesion area was positive for the studied signal; moderate staining (++), meaning that 10–50% of the area was positive for the studied signal; strong staining (+++), meaning that more than 50% of the area was positive for the studied signal [23].

2.3. RT-PCR

Total RNA was extracted from the tissue samples using

Trizol reagent (Gibco-BRL). After DNase treatment cDNA synthesis was performed using random hexamer primers (Promega) with 3 μg of total RNA. RT-PCR for VEGF-A was performed as described earlier [6]. For VEGF-D RT-PCR primers were 5′-GTTGCAATGAAGAGAGCCTT-3′ and 5′-TCCCATAGCATGTCAATAGG-3′, and for β-actin RT-PCR primers were 5′-CCCTGAAGTACCCCAT-CGAG-3′ and 5′-GGGAGACCAAAAGCCTTCATA-3′. Each 50-μl reaction mixture contained 20 μl of cDNA primers (20 pmol), 200 μl of each deoxynucleotide triphosphate (200 μM, MBI Fermentas), 2.0 mM (VEGF-D) or 1.5 mM $MgCl_2$ (β-actin) and 1 U of Dynazyme polymerase (Finnzymes, Finland). PCR consisted of 39 cycles of 96 °C for 30 s, 53 °C for 40 s (VEGF-D) or 58 °C for 30 s (β-actin), and 72 °C for 90 s. Controls without reverse transcriptase were included in each run.

2.4. Statistical analysis

Statistical comparisons between the groups within vessel layers were done by Kruskal–Wallis test followed by multiple comparisons using Mann–Whitney *U*-test with Bonferroni's correction.

3. Results

3.1. Expression of VEGF-D and VEGF-A

The expression of VEGF-D and VEGF-A was studied using immunocytochemistry (Fig. 1). Two different monoclonal antibodies used for VEGF-D staining showed similar expression patterns (data not shown). VEGF-D and

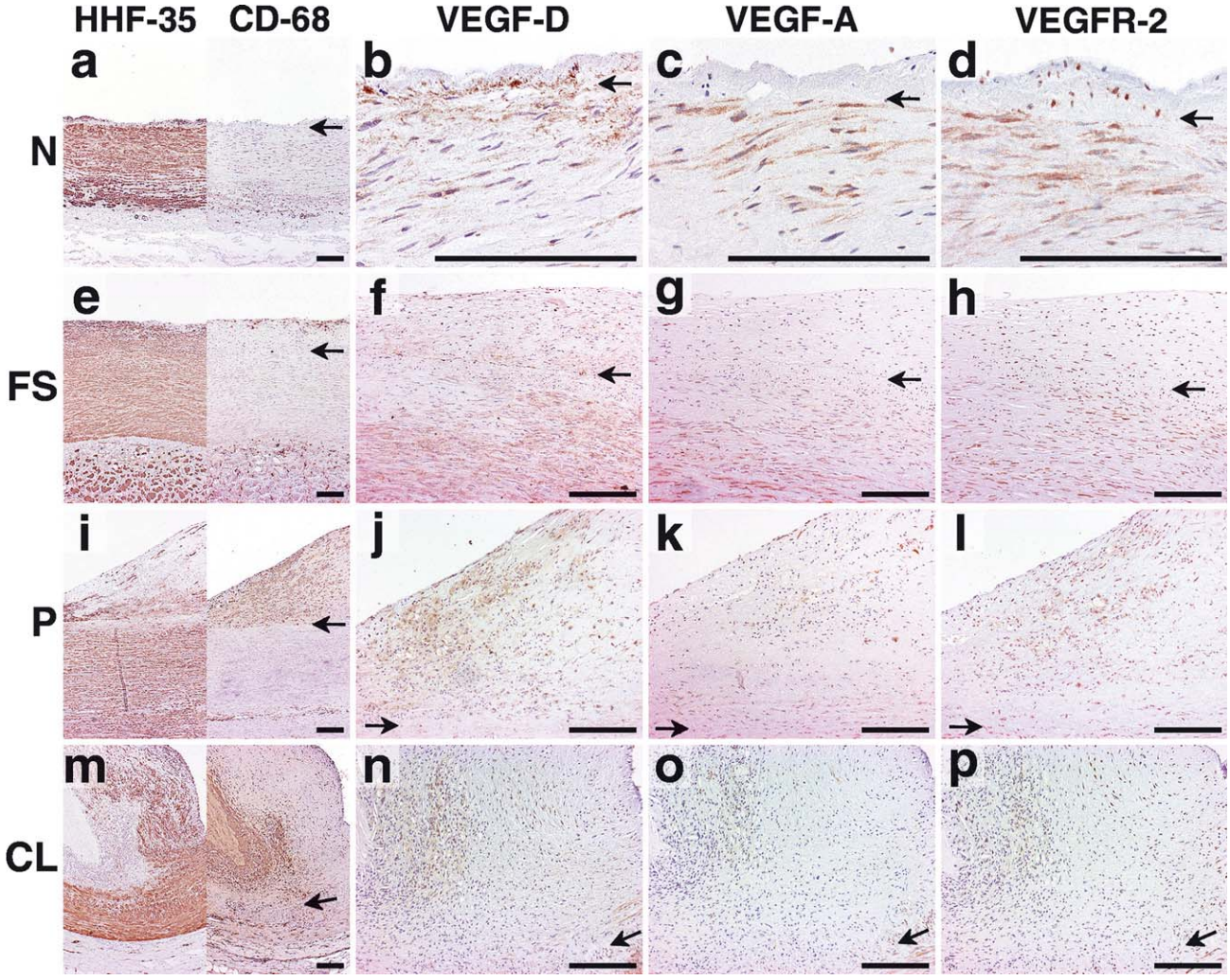

Fig. 1. Expression of VEGF-D, VEGF-A, and VEGFR-2 in human atherosclerotic lesions. Serial sections of normal artery (a–d), fatty streak (e–h), plaque (i–l), and complicated lesion (m–p). (a,e,i,m) HHF-35 (left) and CD-68 (right) immunostainings for SMCs and macrophages. (b,f,j,n) VEGF-D immunostainings. (c,g,k,o) VEGF-A immunostainings. (d,h,l,p) VEGFR-2 immunostainings. Brown color indicates positivity. N, normal; FS, fatty streak; P, plaque; and CL, complicated lesion. Arrows indicate the location of internal elastic lamina. Hematoxylin counterstain. Bars 200 μm.

VEGF-A analyses were done from sections stained with antibodies VD1 and sc-7269, respectively. For VEGF-D and VEGF-A semiquantitative analysis of the immunostainings in ECs, intima and media are presented in Table 1. For VEGF-D the majority of the samples showed at least weak staining in ECs. VEGF-D was abundant in ECs at all stages of atherosclerosis and there were no significant differences between the groups. The expression of VEGF-A in ECs changed during atherogenesis ($P<0.001$): VEGF-A was more abundant in plaques (mean score 1.50, $P=0.006$ versus normal arteries and $P<0.001$ versus fatty streaks ECs) and complicated lesions (mean score 1.45, $P=0.006$ versus normal arteries or fatty streaks) than in normal arteries (mean score 0) or fatty streaks (mean score 0.27). None of the normal arteries and only one fatty streak showed positive VEGF-A staining in ECs. Meanwhile, ECs in every sample graded as plaque or complicated lesion showed positive staining for VEGF-A.

VEGF-D was abundant in intima in every type of lesion studied. The majority of the samples showed strong expression. However, the staining for VEGF-D was significantly lower in complicated lesions (mean score 2.09) compared to the normal arteries (mean score 3.00, $P=0.039$) or plaques (mean score 2.92, $P=0.006$), and there was a trend towards decrease as compared to fatty streaks (mean score 2.73, $P=0.069$). Whereas the immunostaining of VEGF-A was mainly localized inside the cells (Fig. 1c,g,k,o), VEGF-D was also found in extracellular space

Table 1
VEGF-D and VEGF-A expression in human atherosclerotic arteries

Sample no.	Age/sex	Lesion type	Cause of death /amputation	Anatomical site	VEGF-D			VEGF-A		
					ECs	Intima	Media	ECs	Intima	Media
1	35/M	No lesion	Accidental	a. iliaca communis	+	+++	+++	−	+	+++
2	35/M	No lesion	Accidental	a. mesenterica	+	+++	+++	−	−	+++
3	35/M	No lesion	Accidental	a. iliaca	+	+++	+++	−	−	+++
4	35/M	No lesion	Accidental	a. iliaca	+	+++	+++	−	−	+++
5	35/M	No lesion	Accidental	a. iliaca	++	+++	+++	−	−	+++
6	38/M	FS	Accidental	Abdominal aorta	++	+++	+++	+	−	+
7	47/F	FS	Suicide	Abdominal aorta	++	+++	+++	−	++	++
8	30/M	FS	Homicide	Thoracic aorta	−	+++	++	−	+	++
9	30/M	FS	Homicide	Thoracic aorta	−	+++	++	−	−	++
10	36/F	FS	Suicide	Thoracic aorta	++	+++	++	−	−	++
11	35/M	FS	Accidental	a. iliaca communis	++	+++	+++	−	++	+++
12	35/M	FS	Accidental	a. iliaca	−	+	+++	−	−	+
13	35/M	FS	Accidental	a. iliaca	+	++	+++	−	+	++
14	35/M	FS	Accidental	a. iliaca	++	+++	+++	−	++	+++
15	25/F	FS	Suicide	Abdominal aorta	++	+++	++	++	+++	+
16	25/F	FS	Suicide	Abdominal aorta	++	+++	++	−	+	+
17	30/M	P	Homicide	RCA	+	+++	+++	+	+++	+++
18	35/M	P	Accidental	a. iliaca	+++	+++	+++	+	++	+++
19	69/M	P	Gangraena pedis	a. tibialis post.	++	+++	+++	++	+	+
20	30/M	P	Homicide	RCA	+	+++	+++	++	+	+++
21	35/M	P	Accidental	a. iliaca communis	+++	+++	+++	++	++	+++
22	40/M	P	Infarct	Thoracic aorta	++	+++	+++	+	++	++
23	84/M	P	Arrhythmia	LCX	++	+++	+++	+	+	+++
24	84/M	P	Arrhythmia	Abdominal aorta	++	+++	+++	+	++	+++
25	84/M	P	Arrhythmia	Abdominal aorta	++	+++	+++	+	++	+++
26	25/F	P	Suicide	RCA	++	+++	+++	++	+	+++
27	25/F	P	Suicide	RCA	+++	+++	+++	++	++	+++
28	25/F	P	Suicide	LAD	++	++	+++	++	+	+
29	30/M	CL	Homicide	RCA	+	+++	+++	++	+	+++
30	40/M	CL	Infarct	Thoracic aorta	+++	+++	+++	++	++	++
31	69/M	CL	Gangraena pedis	a. tibialis post.	++	++	++	++	+++	+++
32	82/F	CL	Gangraena pedis	a. tibialis post.	++	++	++	+	++	++
33	78/F	CL	Gangraena pedis	a. tibialis post.	++	+++	+++	+	+	++
34	30/M	CL	Homicide	RCA	+	+	+++	++	+	+++
35	84/M	CL	Arrhythmia	RCA	++	++	+++	+	+	+++
36	84/M	CL	Arrhythmia	RCA	+	++	+++	+	+	+++
37	84/M	CL	Arrhythmia	LCX	+++	++	+++	+	++	+++
38	84/M	CL	Arrhythmia	LAD	+	++	+++	+	+	+++
39	84/M	CL	Arrhythmia	LAD	+	+	+++	++	++	+++

M, male; F, female; FS, fatty streak; P, plaque; CL, complicated lesion; RCA, right coronary artery; LCX, left circumflex artery; LAD, left anterior descending artery; ECs, endothelial cells; −, not detectable staining; +, weak staining; ++, moderate staining; +++, strong staining.

(Figs. 1b,f,j,n and 2b). In normal arteries, fatty streaks and plaques rich in SMCs diffuse VEGF-D staining was found mostly around SMCs. In plaques and complicated lesions rich in connective tissue the diffuse VEGF-D staining was most prominent in macrophage-rich areas, whereas the staining in SMCs was mostly cell-associated (Fig. 2e,f). The expression of VEGF-A in intima was different between the study groups ($P=0.01$): the majority of the normal arteries did not show any expression (mean score 0.20) but there was at least weak expression in the majority of samples graded as fatty streak (mean score 1.09, $P=$NS compared to normal arteries), plaque (mean score 1.67, $P=0.006$), or complicated lesion (mean score 1.55, $P=0.009$). Whereas the positive cells in normal arteries and fatty streak lesions were mostly SMCs, in advanced lesions the positive cells were located almost exclusively in macrophage-rich areas. The VEGF-A expression in intima was mostly localized inside the cells and only weak positivity was found in the extracellular space. Both VEGF-A and VEGF-D where found in macrophages in association with plaque neovascularization (Fig. 2 f for VEGF-D; VEGF-A data not shown).

The proteolytic processing of VEGF-D was analyzed by comparing immunostainings with different antibodies: VD1 recognizes all forms of VEGF-D, whereas sc-7602 recognizes the C-terminal end of VEGF-D (i.e., unprocessed or partially processed VEGF-D). The staining with VD1 was abundant in all samples apart from complicated lesions rich in connective tissue (Fig. 2b,e). The staining with sc-7602 was notably less abundant than with VD1 and was localized almost exclusively inside the cells (Fig. 2c).

Both VEGF-A and VEGF-D were abundant in medial layers of all studied arteries and no differences in immunostaining patterns were found between the study groups (Fig. 1). The expression of VEGF-D in analyzed samples was verified using double immunostainings and in situ hybridization. The VEGF-D protein and mRNA was found in ECs, intimal SMCs, and macrophages (Fig. 3).

3.2. Expression of VEGFR-2 and VEGFR-3

In immunostainings VEGFR-2 was localized in SMCs in intima and media in every study group (Fig. 1d,h,l,p). Positive cells were also detected in endothelium of fatty streaks and more advanced lesions, and strong expression was localized on ECs of adventitial vessels. Both antibodies used gave similar expression patterns (data not shown). Thus, the expression of VEGFR-2 in SMCs was not related to the stage of atherosclerosis. With in situ hybridization VEGFR-2 mRNA was found in ECs and intimal SMCs in the same areas which were positive for immunostaining (Fig. 4c).

VEGFR-3 was undetectable in ECs, intima, or media either with immunostaining (Fig. 4d) or in situ hybridization (data not shown). Only some ECs of the adventitial

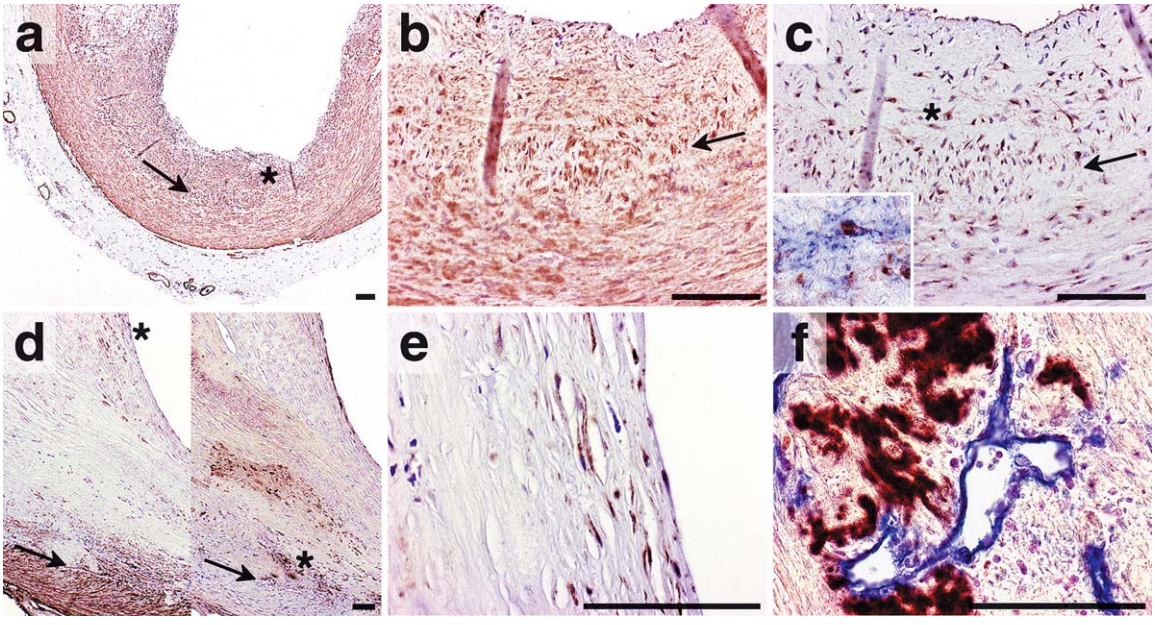

Fig. 2. VEGF-D expression, plaque composition and the presence of the processed form of VEGF-D in lesions. Serial sections of plaque (a–c) and complicated lesion (d–e). (a) HHF-35 staining for SMCs. (b) VEGF-D (VD1) immunostaining for all forms of VEGF-D. (c) VEGF-D (sc-7602) immunostaining for the unprocessed C-terminal end of VEGF-D. Insert: double staining with sc-7602 (brown) and VD1 (blue). (d) HHF-35 (left) and CD-68 (right) immunostainings for SMCs and macrophages. (e) VEGF-D (VD1) immunostaining. (f) Double staining for VEGF-D (VD1); brown and endothelial cells (CD-31); blue. Hematoxylin counterstaining in immunostainings and Mayer's carmalum in double stainings. An asterisk indicates the location of higher magnifications. Arrows indicate the location of internal elastic lamina. Bars 100 μm.

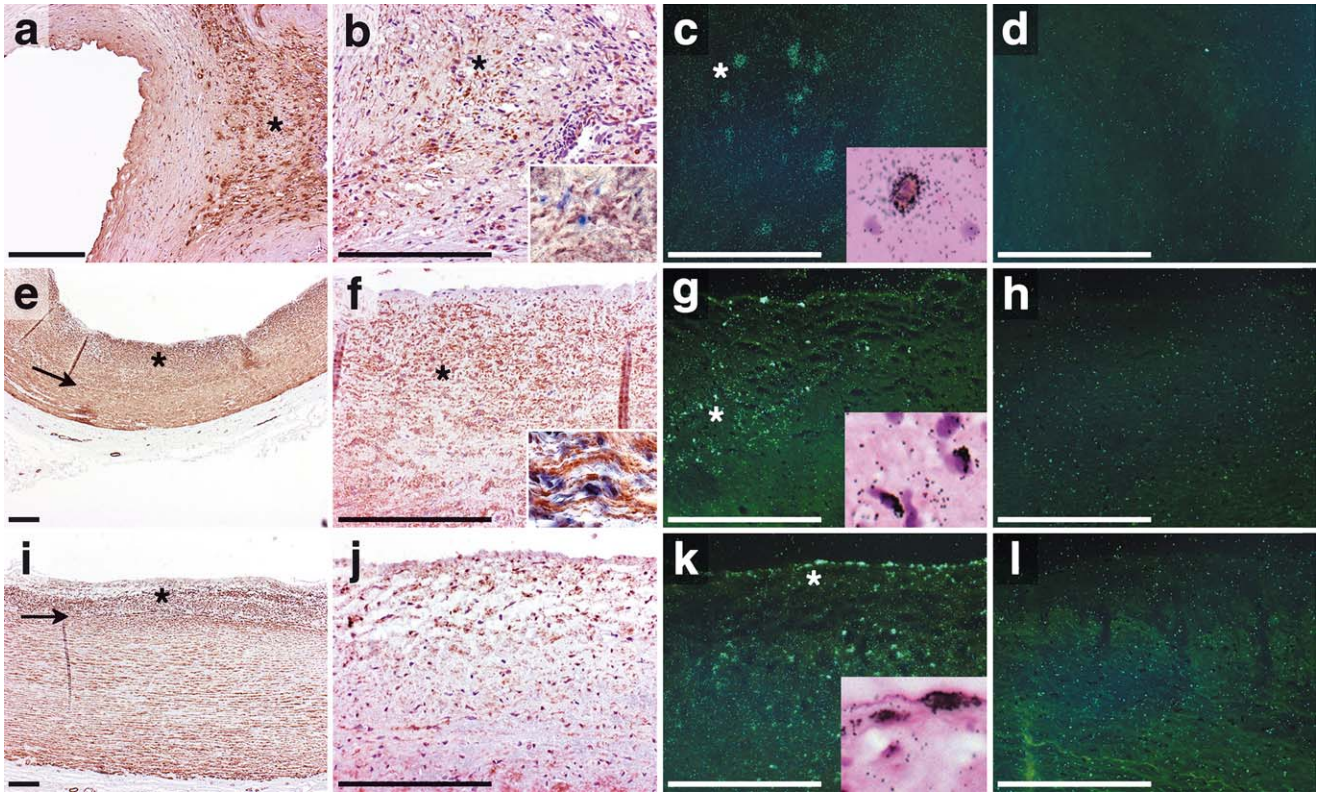

Fig. 3. VEGF-D protein and mRNA expression in macrophage and SMC rich areas. Serial sections of a complicated lesion (sample 25, Table 1) (a–d), a plaque (sample 18) (e–h), and a fatty streak (sample 8) (i–l). (a) CD-68 immunostaining for macrophages. (b,f,j) VEGF-D immunostaining. Inserts: (b) VEGF-D (brown) and CD-68 (blue) double staining. f VEGF-D (brown) and HHF-35 (blue) double staining. (c,g,k) Dark field images of sections hybridized with [^{33}P]UTP-labelled VEGF-D antisense riboprope. VEGF-D mRNA expression is shown as bright spots. Inserts in (c,g,k): higher magnifications with bright field illumination. (d,h,l) In situ hybridization controls hybridized with VEGF-D sense riboprobe. (e,i) HHF-35 immunostainings for SMC. An arrow indicates the location of internal elastic lamina. Hematoxylin counterstaining in immunostainings, Mayer's carmalum in double stainings and hematoxylin–eosin in in situ hybridizations. (c,d,g,h,k,l) are polarized light epiluminescence images. An asterisk indicates the location of higher magnifications. Bars 200 μm.

small vessels stained positive for VEGFR-3 immunostaining (Fig. 4d insert).

3.3. Analysis of expression of VEGF-D and VEGF-A by RT-PCR

The expression of VEGF-D and VEGF-A mRNA was verified by RT-PCR. As shown in Fig. 5, both VEGF-D and VEGF-A mRNAs were present in arteries regardless of the stage of atherosclerosis. In VEGF-A RT-PCR, two distinct bands of 356 and 428 bp were detected, corresponding to mRNAs for VEGF-A_{165} and VEGF-A_{189}. VEGF-D RT-PCR gave only one band of 213 bp in every studied sample. β-Actin RT-PCR showed equal results between the samples (data not shown).

4. Discussion

In this study we investigated the expression patterns of VEGF-D and VEGF-A in normal human arteries and atherosclerotic lesions. In ECs and intima the expression of VEGF-A increased with the progression of atherosclerosis. Meanwhile, VEGF-D was abundant in ECs and intima of each lesion group. The only change was a reduction in VEGF-D staining in intimas of complicated lesions. This suggests that the role of VEGF-D in adult vascular tissue differs from that of VEGF-A.

Immunostainings indicated that the expression of VEGF-D and VEGF-A in normal arteries and fatty streaks was mainly localized in SMCs. In plaques and complicated lesions these proteins were also detected in macrophages. The positive VEGF-D staining was diffuse in each studied sample, except in complicated lesions rich in connective tissue. In macrophage-rich lesions VEGF-D staining was found in macrophages near plaque neovascularization. In situ hybridization confirmed that VEGF-D mRNA was produced in macrophages, ECs, and intimal SMCs. Also, RT-PCRs showed that both VEGF-A and VEGF-D mRNAs were produced locally in arteries. So far, the only known mechanisms for VEGF-D regulation involve c-fos and cell-to-cell interactions in fibroblasts [10,16]. VEGF-D is initially produced as a prepropeptide and further processed to its functional form by extracellular proteases

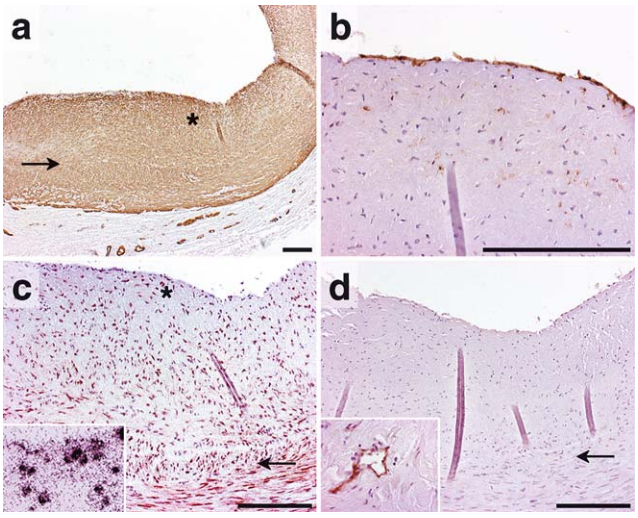

Fig. 4. VEGFR-2 and -3 protein and mRNA expression in atherosclerotic arteries. Serial sections of plaque (sample 18). (a) HHF-35 immunostaining for SMC. (b) CD-31 immunostaining for endothelial cells. (c) VEGFR-2 immunostaining. Insert: higher magnification of a section hybridized with [^{33}P]UTP-labelled VEGFR-2 antisense riboprope. mRNA expression is shown as black spots. (d) VEGFR-3 immunostaining. Insert: VEGFR-3 immunostaining from adventitial vessel as a positive control. An arrow indicates the location of internal elastic lamina. Hematoxylin counterstaining in immunostainings and hematoxylin–eosin in in situ hybridization. An asterisk indicates the location of higher magnifications. Bars 200 μm.

[12]. The staining with VD1 that recognizes all forms of VEGF-D was substantial both intracellularly and in extracellular space, whereas the staining with sc-7602 that binds to the C-terminal end of the unprocessed VEGF-D was strictly intracellular. Thus, this suggests that VEGF-D is effectively processed in the vessel wall.

The presence of VEGF-D in normal arteries suggests that it may have a constitutive or homeostatic role in the artery wall. In complicated lesions SMCs present in lacunae of connective tissue seem to be unable to secrete VEGF-D to extracellular space. This results in a reduction of VEGF-D in intima of complicated lesions with potential attention of the VEGF-D effects in the arteries. On the other hand, with the influx of macrophages into the lesions the high focal expression of VEGF-D in macrophages near plaque neovascularization suggests that VEGF-D may have a role in this process. Thus, constitutive expression of VEGF-D in normal arteries is most likely useful for the large arteries whereas in macrophage-rich inflammatory areas it may contribute to the pathological changes in advanced atherosclerotic lesions.

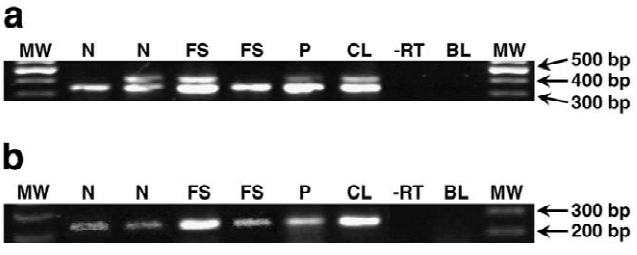

Fig. 5. RT-PCR analysis of human normal arteries, fatty streaks, plaques, and complicated lesions. (A) VEGF-A is expressed in normal arteries, fatty streaks and complicated lesions as two different isoforms, $VEGF_{189}$ corresponding to a 428-bp fragment and $VEGF_{165}$ corresponding to a 356-bp fragment. From the left: molecular weight marker (MW), two different normal arteries (N), two different fatty streaks (FS), plaque (P), complicated lesion (CL), control without reverse transcriptase (-RT), blank (BL), molecular weight marker (MW). (B) VEGF-D is expressed in normal arteries, fatty streaks, plaques, and complicated lesions. The size of the product is 213 bp. β-Actin RT-PCR showed similar loading of RNA on each lane (data not shown).

In this study we saw that the expression of VEGF-A in the artery wall increased during atherogenesis and that VEGF-A was found in macrophages in association with plaque neovascularization. VEGF-A can also have protective effects in arteries as it mediates anti-apoptotic effects in endothelium [7,24], protects against LDL toxicity [25], and induces nitric oxide production [26]. On the other hand, the capability of VEGF-A to stimulate monocyte/macrophage influx into the vessel wall [2], and to increase vascular permeability [4] suggests that VEGF-A may contribute to atherogenesis. Also, high plasma concentrations of VEGF-A induced atherosclerotic plaque formation and neovascularization in mouse and rabbit models of atherosclerosis [9]. However, it has remained unclear whether the net effect of local production of VEGF-A in the artery wall is protective or harmful.

In this study we demonstrated constitutive VEGFR-2 expression in arterial SMCs, macrophages, and ECs of atherosclerotic arteries. This is in agreement with previous findings that VEGFR-2 is expressed in cultured arterial SMCs [27] and in intimal SMCs of denuded rabbit aorta [28]. Also, VEGF-A stimulation leads to VEGFR-2 upregulation in ECs [29] which could explain why we found VEGFR-2 in ECs only in atherosclerotic arteries. The most pronounced biological responses to VEGFs, such as permeability changes and endothelial proliferation, are primarily mediated by VEGFR-2. Also, VEGFR-2 mediates SMC migration, but not proliferation, suggesting that VEGF-A is chemotactic for SMCs [27]. As VEGF-D and VEGF-A were strongly expressed in medial layers of arteries, the lack of angiogenic effects in media suggests that VEGFR-2 may have additional roles in SMCs apart from inducing angiogenesis via ECs.

VEGFR-3 plays a role in vascular development in embryos but in adult tissues it is almost exclusively present in lymphatic vessels and not found in vascular system apart from vasa vasorum [17,30]. Our results show that VEGFR-2, not VEGFR-3, is found in ECs, intima and media in large arteries. VEGFR-3 expression was only detected in ECs of adventitial, probably lymphatic vessels. This suggests that among the VEGF receptors characterized so far, VEGFR-2 is probably the most important receptor mediating the effects of VEGF-D in large human arteries.

In conclusion, our study demonstrates constitutive expression of VEGF-D in large atherosclerotic and non-atherosclerotic arteries. Also, our results suggest that the effects of VEGF-D in these arteries are mediated primarily through VEGFR-2. The widespread expression of VEGF-D in normal arteries and in early atherosclerotic lesions suggests that it may have a maintenance role in adult arteries. In advanced lesions, the expression in macrophages near neovessels may indicate that VEGF-D may be involved in plaque neovascularization.

5. Study limitations

The majority of the samples consisted of autopsy material and for practical reasons were collected within 12 h after death. Thus, we cannot completely exclude the possibility of some post-mortem changes in the analyzed samples. Also, we cannot fully exclude the possibility of some vascular bed-dependent differences. However, all samples were from large arteries and showed no major changes in the staining patterns between different vascular beds. It should also be kept in mind that immunostaining, in situ hybridization and RT-PCR are not quantitative analyses but, at best, can give semi-quantitative estimates of the analyzed parameters.

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