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HELENA VIITA

15-Lipoxygenase-1 in Atherosclerosis, Angiogenesis and Tumorigenesis

Studies with Retro- and Adenovirus Mediated Gene Transfer Techniques

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium, Mediteknia building, University of Kuopio, on Friday 4th December 2009, at 12 noon

Department of Biotechnology and Molecular Medicine
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University of Kuopio

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ABSTRACT

Atherosclerosis, a chronic vascular disease, leads to the formation of atherosclerotic plaques in the arteries. The key events in atherogenesis include lipoprotein oxidation, foam cell formation, induced expression of cell adhesion molecules, invasion of inflammatory cells, smooth muscle cell proliferation, accumulation of the extracellular matrix and calcification. Angiogenesis is a physiological event involved in many pathological conditions such as the development of atherosclerosis and neovascular ocular diseases, and tumor progression. Members of the vascular endothelial growth factor (VEGF) family are crucial molecules in the process of angiogenesis.

15-Lipoxygenase-1 (15-LO-1) is a lipid oxidizing enzyme the expression of which is induced in atherosclerotic lesions, but not in the normal vessel wall. 15-LO-1 has been suspected to play a role in tumor development, but the results are controversial. The aim of this thesis was to study the effects of 15-LO-1 in atherosclerosis, angiogenesis and tumorigenesis by virus mediated gene transfer techniques.

Retroviral transduction of 15-LO-1 *in vitro* led to induced expression of cellular adhesion molecules and the binding of inflammatory cells to the transduced cells. Intramuscular and intravitreal adenoviral gene transfers of VEGF family members significantly increased the size and number of the capillaries, and capillary perfusion and permeability were significantly increased in the rabbit skeletal muscles. 15-LO-1 blocked these angiogenic effects by preventing the expression of the growth factors, by down-regulating VEGF receptor 2 and by reducing the bioavailability of nitric oxide. Tail vein adenoviral 15-LO-1 gene transfers into mice resulted in increased 15-LO-1 expression mainly in liver, followed by induced lipid peroxidation and elevated caspase-3 activity. In a rat malignant glioma model, 15-LO-1 significantly prolonged the survival of the animals.

In conclusion, 15-LO-1 may be involved in the early phases of atherosclerosis by inducing adhesion molecule expression and the binding of inflammatory cells to the endothelium. These studies suggest that 15-LO-1 could have potential in the treatment of pathologic angiogenic conditions, for example in neovascular ocular diseases. In addition, the prolongation of survival by 15-LO-1 detected in rat malignant glioma model indicates that further studies should be conducted to evaluate the potential of 15-LO-1 in the treatment of malignant glioma.

National Library of Medicine Classification: QU 140, QU 475, QZ 52, QZ 266, WG 500, WG 550
Medical Subject Headings: Lipoxygenase; Atherosclerosis; Neovascularization, Pathologic; Neoplasms; Glioma; Gene Transfer Techniques; Transduction, Genetic; Retroviridae; Adenoviridae; NF-Kappa B; Vascular Endothelial Growth Factors; Cell Adhesion Molecules; Gene Expression; Inflammation; Endothelium; Capillaries; Capillary Permeability; Down-Regulation; Nitric Oxide; Growth Substances; Placenta; Lipid Peroxidation; Caspase 3; Apoptosis; Disease Models, Animal; Rats

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I owe my special gratitude to my former colleagues Pauliina Lehtolainen-Dalkilic, Hanna Kankkonen, Johanna Tietäväinen and Maija Päivärinta for their friendship. You were also part of the “old core”, which together with me, Marja Hedman, Annaleena Heikkilä, Anniina Laurema and Elisa Vähäkangas created memorable dance shows – as cats, mice, kidneys, placentas… - in SYH-group dissertation parties before the time of Apu Rah Alla theatre. I especially cherish my former roommate Anssi Mähönen for the discussions we shared about science, parenthood and life in general. The expertise of my former and present colleagues Tiina Tuomisto, Suvi Jauhiainen, Jonna Koponen and Ivana Kholova is acknowledged. My former colleague Jani Räty is thanked for his patient help with all the computer issues and questions.

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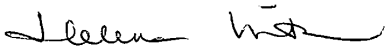
My deepest gratitude goes to my parents Ritva and Seppo, for their love and support. I want to thank my sister Johanna and her husband Antti and our sons’ furry “cousins” Nemo and Into for your support and for supplying my family with relaxing moments. I wish to express my gratitude for my mother-in-law Katja for being such a lovable and caring person. My father-in-law Matti and “avoppi” Pirkko are thanked for relaxing vacations. Finally, I want to thank my husband Markku for his support, for enduring the ups and downs of life with me, for his patience in waiting for this thesis finally to come to reality, and for giving me two great sons. Johannes and Julius, I want to thank you for giving so much meaning to life outside research and love beyond comprehension. Thanks for being such good sports and keeping my feet hardly on the ground of real everyday life. Julius deserves special thanks for checking the error bars in the histograms and for filing the thesis references in alphabetical order. And thanks for patiently waiting the “after dissertation” time, when the use of Mom’s computer is no longer restricted!

Lapinlahti, November 2009



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ABBREVIATIONS

12/15-LO	the mouse ortholog of human and rabbit reticulocyte 15-LO-1
15-LO-1	15-lipoxygenase-1
4-HNE	4-hydroxynonenal
AA	arachidonic acid
AAV	adeno-associated virus
apoE	apolipoprotein E
ATCC	The American Type Culture Collection
bp	base pair
cDNA	complementary deoxyribonucleic acid
CEU	contrast enhanced ultrasound
CMV	cytomegalovirus
DICE	differentiation control element
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EC	endothelial cell
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GATA	GATA-binding protein (transcription factor)
HDAC	histone deacetylase
HETE	hydroxyeicosatetraenoic acid
HIF-1	hypoxia inducible factor 1
hnRNP	heterogeneous nuclear ribonucleoprotein
HODE	hydroxyoctadecadienoic acid
HPETE	hydroperoxy-eicosatetraenoic acid
HPODE	hydroperoxy-octadecadienoic acid
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
kDa	kilo Dalton
LA	linoleic adic
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LO	lipoxygenase
LTR	long terminal repeat
MDA	malondialdehyde
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
˙NO	nitric oxide
NOS	nitric oxide synthase
NRP	neuropilin
NSAID	nonsteroidal anti-inflammatory drug
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PHGPX	phospholipid hydroperoxide glutathione peroxidase
PlGF	placental growth factor
PMA	phorbol 12-myristate 13-acetate
PPAR	peroxisome proliferator-activated receptor
RAASMC	rabbit aortic abdominal smooth muscle cell
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SMC	smooth muscle cell
STAT	signal transducer and activator of transcription
TNF	tumor necrosis factor
UTR	untranslated region
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vp	virus particle

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by their Roman numerals. In addition, some unpublished data are presented.

I Viita H, Sen CK, Roy S, Siljamäki T, Nikkari T, Ylä-Herttuala S.
High expression of human 15-lipoxygenase induces NF-κB-mediated expression of vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and T-cell adhesion on human endothelial cells.
Antioxid Redox Signal. 1999 Spring;1(1):83-96.

II Viita H, Markkanen J, Eriksson E, Nurminen M, Kinnunen K, Babu M, Heikura T, Turpeinen S, Laidinen S, Takalo T, Ylä-Herttuala S.
15-Lipoxygenase-1 prevents vascular endothelial growth factor A- and placental growth factor-induced angiogenic effects in rabbit skeletal muscles via reduction in growth factor mRNA levels, NO bioactivity and downregulation of VEGF receptor 2 expression.
Circ Res. 2008 Feb 1;102(2):177-84. Epub 2007 Nov 8.

III Viita H, Kinnunen K, Eriksson E, Lähteenvuo J, Babu M, Kalesnykas G, Heikura T, Laidinen S, Takalo T, Ylä-Herttuala S.
Intravitreal adenoviral 15-lipoxygenase-1 gene transfer prevents vascular endothelial growth factor A-induced neovascularization in rabbit eyes.
Hum Gene Ther. In press.

IV Viita H*, Pacholska A*, Ahmad F, Tietäväinen J, Naarala J, Ketola A, Wirth T, Ylä-Herttuala S.
15-Lipoxygenase-1 induces lipid peroxidation and apoptosis, and reduces tumor volume and extends survival in rat malignant glioma model.
Manuscript 2009

* Equal contribution.

TABLE OF CONTENTS

INTRODUCTION

Cardiovascular diseases are the leading cause of death worldwide, while cancers hold the third position after infections and parasitic diseases (World Health Organization, 2004). Atherosclerosis is the principal cause of many of the clinical cardiovascular complications such as coronary heart disease and peripheral artery disease. The main risk factors of atherosclerosis include high low density lipoprotein (LDL) cholesterol, high blood pressure, age, smoking, diabetes and male gender.

Angiogenesis, where new capillaries are formed from pre-existing vessels, is a physiological process occurring for example during wound healing and the female reproductive cycle (Risau, 1997). Pathological angiogenesis occurs for instance in tumor growth and metastasis and neovascular ocular diseases. Members of the vascular endothelial growth factor (VEGF) family are important angiogenic molecules in health and disease (Ferrara *et al.,* 2003).

15-Lipoxygenase-1 (15-LO-1) is an enzyme capable of oxidizing fatty acids and LDL cholesterol. It is expressed in the macrophages present in the atherosclerotic lesion, but not in the normal vessel wall, and its reaction products have been detected in atherosclerotic lesions. Both pro- and anti-atherogenic activities for 15-LO-1 have been presented (Cathcart *et al.,* 2000).

15-LO-1 may also be involved in tumor growth (Fürstenberger *et al.,* 2006). However, rather conflicting results have been reported about the role of 15-LO-1 in tumors, showing evidence of both anti- and pro-tumorigenic properties. Anti-tumorigenic effects of 15-LO-1 have been presented in mouse colorectal carcinoma xenograft models (Shureiqi *et al.,* 2003; Nixon *et al.,* 2004; Wu *et al.,* 2008), as well as in two different tumor models in transgenic mice over-expressing human 15-LO-1 (Harats *et al.,* 2005). In this latter study, 15-LO-1 also resulted in aberrant angiogenesis in lung metastases. However, in prostate cancer, 15-LO-1 displays quite an opposite effect, indicating that it plays a pro-tumorigenic role in this tissue (Nie *et al.,* 2001).

This thesis project was carried out to study the role of 15-LO-1 in atherogenesis, angiogenesis and tumorigenesis. Both retro- and adenovirus mediated gene transfer techniques were used *in vitro* in cell culture models, and recombinant adenoviruses were applied in animal experiments *in vivo* by various gene transfer routes.

REVIEW OF THE LITERATURE

PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis is a disease causing high morbidity and mortality in the industrial countries. It takes decades to develop clinically significant, advanced lesions, but the earliest signs can be detected already in the second decade of life. Atherosclerosis is a chronic, progressive disease initiated by lipid retention, oxidation and modification at susceptible sites of the vascular wall in major conduit arteries. The lesions predominantly develop into locations of adaptive intimal thickening (Insull, 2009). Atherosclerotic lesions can be classified into six classes, as depicted in **Table I** and **Figure 1** (Stary *et al.,* 1994; Stary *et al.,* 1995).

Table I. Classification and characteristics of atherosclerotic lesions (Stary *et al.,* 1994; Stary *et al.,* 1995).

Lesion type and description	Characteristics
I Macrophage foam cells	Increase in macrophages and lipid accumulation into macrophages, foam cell formation
II Fatty streaks	All type I changes Smooth muscle cell -derived foam cells Extracellular lipid
III Preatheroma, intermediate or transitional lesion	All type II changes Increased extracellular lipid as separate pools
IV Atheroma, fibrous plaque	All type III changes, except the extracellular lipid as a coalesced lipid core
V Fibroatheroma	All type IV changes Collagenous fibrous cap Microscopical hemorrhages
VI Complicated lesion	All type V changes Fissure, hematoma, plaque rupture, thrombus

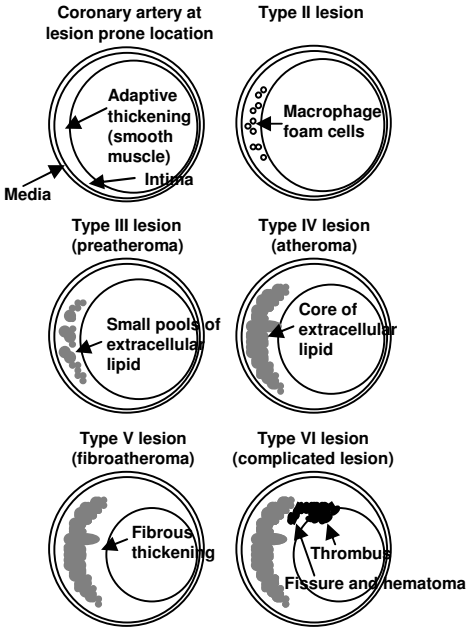

Figure 1. Lesion types of atherosclerosis. Modified from Stary *et al.,* 1995.

Foam cell and fatty streak formation

The sites of diffuse intimal thickening, where atherosclerotic lesions predominantly develop, contain extracellular matrix (ECM) proteins such as biglycan and decorin in their outer layer. The initial extracellular lipid deposits colocalize with these proteoglycans (Nakashima *et al.,* 2007). The neointimal thickening can be reduced by decorin, a small chondroitin/dermatan sulphate proteoglycan, which can reduce the ECM volume by inducing type I collagen synthesis and contraction (Järveläinen *et al.,* 2004).

The activation of endothelial cells (EC) induces the expression of several specific adhesion molecules, which together with inflammatory chemokines, produced by smooth muscle cells (SMC), mediate the adhesion of monocytes to the EC and their migration into the sub-endothelial space. The invading monocytes are transformed into intimal macrophages, which uptake modified low density lipoprotein (LDL) and become foam cells filled with lipid. These early lipid changes and the first grossly visible lesions, the fatty streaks, can be reversed and are not yet considered as established atherosclerosis (Insull, 2009).

Intermediate lesions and atheroma

Intermediate lesions are characterized by the accumulation of lipid-rich necrotic debris and SMCs. Plaques contain a central lipid core, which is mainly devoid of cells. In fibroatheroma, which is considered the hallmark of established atherosclerosis, the lipid core is separated from the arterial lumen by a fibrous cap consisting of ECM and SMCs (Insull, 2009).

More advanced lesions can be calcified and ulcerated at the luminal surface especially from the shoulder regions of the lesions. New fragile vessels growing into the lesion from the medial side can be vulnerable to produce haemorrhages within the blood vessel wall (Insull, 2009).

Advanced lesions, plaque rupture and thrombus formation

Advanced, complicated lesions can grow large enough to prevent blood flow causing, for example, stable ischaemic myocardial disease. The most important clinical complication is an acute rupture of the plaque, leading to thrombus formation occluding the vessel. This can evoke myocardial infarction or stroke (Insull, 2009).

ANGIOGENESIS

Angiogenesis is defined as the formation of new capillaries from pre-existing vessels (Risau, 1997). Physiological angiogenesis is an essential phenomenon in development, tissue regeneration and reproduction. Pathologic angiogenesis is involved in many diseases such as tumor angiogenesis, rheumatoid arthritis and neovascular ocular diseases, of which the most important are retinopathy of prematurity, proliferative diabetic retinopathy and the vascular form of age related macular degeneration (Ferrara, 2004).

TUMORIGENESIS

The development of tumors arises from abnormal function of genes. Tumor cells become self-sufficient in growth promoting signals and insensitive to growth inhibiting signals. They evade the normal pathway of programmed cell death and acquire unlimited potential for cell division. Angiogenesis is increased in order to supply the growing tumor tissue with oxygen and nutrients. Malignant cells also lose their interaction with ECM and can invade the surrounding tissue by degrading the basal membrane with proteolytic enzymes. Finally, cancer cells can metastasize to distant locations via blood and lymph vessels (Hanahan *et al.,* 2000).

VASCULAR ENDOTHELIAL GROWTH FACTORS AND THEIR RECEPTORS

Members of the VEGF family are key molecules in regulating both physiological and pathological angiogenesis and lymphangiogenesis (**Figure 2**) (Ferrara *et al.,* 2003). In addition, VEGF signalling is required for the assembly of embryonic blood vessels in vasculogenesis, but is not required for the maintenance of these vessels (Argraves *et al.,* 2002).

The mammalian VEGF family comprises five secreted glycoproteins, which share a common structure of eight characteristically spaced cysteine residues. In addition, VEGFs have been isolated also from viruses (VEGF-E) and snake venoms (VEGF-F). VEGF family members mediate their biologic effects by binding to their cognate receptors VEGF receptor (VEGFR)-1, VEGFR-2 and VEGFR-3. VEGF receptors are transmembrane receptor tyrosine kinases, which activate downstream signalling cascades upon ligand binding to their extracellular domain resulting in receptor dimerization and autophosphorylation of the intracellular receptor tyrosine kinases (Olsson *et al.,* 2006).

Neuropilins (NRP) 1 and 2 are semaphorin receptors, but they can also bind some members of the VEGF family and act as co-receptors in angiogenic signalling (Soker *et al.,* 1998; Gluzman-Poltorak *et al.,* 2000). In addition, heparan sulphate proteoglycans affect the bioactivity of VEGFs (Stringer, 2006)..

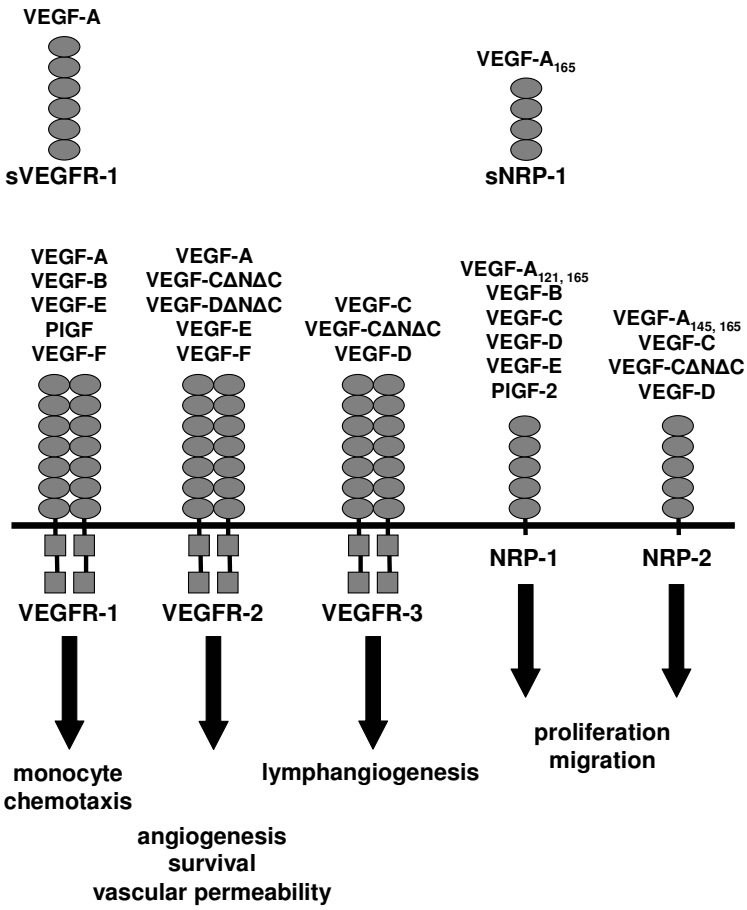

Figure 2. VEGFs and their receptors in angiogenic and lymphangiogenic signalling. sVEGFR-1, soluble VEGFR-1; sNRP-1, soluble NRP-1; VEGF-E, viral VEGFs; VEGF-F, snake venom VEGFs; VEGF-CΔNΔC, the mature short form of VEGF-C; VEGF-DΔNΔC, the mature short form of VEGF-D.

Vascular endothelial growth factor (VEGF) family

VEGF-A

Vascular endothelial growth factor (VEGF or VEGF-A) was first identified as a vascular permeability factor secreted by tumor cells (Senger *et al.,* 1983). VEGF-A was originally purified and cloned as a secreted EC mitogen (Leung *et al.,* 1989). The human VEGF-A gene is composed of eight exons and is differentially spliced to yield at least seven mature isoforms: $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ (Tischer *et al.,* 1991), $VEGF_{145}$ (Poltorak *et al.,* 1997), $VEGF_{162}$ (Lange *et al.,* 2003), $VEGF_{183}$ (Lei *et al.,* 1998), and $VEGF_{206}$ (Houck *et al.,* 1991). The numeric designation of the isoforms denotes the number of amino acids in each molecule. The different isoforms have distinct binding properties to heparan sulphate proteoglycan (Houck *et al.,* 1992). The predominant isoform $VEGF_{165}$ seems to have optimal bioavailability and biological potency due to its high mitogenic activity (Keyt *et al.,* 1996) and intermediate binding affinity to heparan sulphate proteoglycan (Houck *et al.,* 1992).

VEGF-A is a ligand for VEGFR-1 (de Vries *et al.,* 1992) and VEGFR-2 (Terman *et al.,* 1992; Quinn *et al.,* 1993), which are widely expressed on vascular ECs (Kaipainen *et al.,* 1993; Quinn *et al.,* 1993). $VEGF\text{-}A_{165}$ (Soker *et al.,* 1998; Mamluk *et al.,* 2002) and $VEGF\text{-}A_{121}$ (Pan *et al.,* 2007) also have affinity for the neuropilin receptor NRP-1, and $VEGF\text{-}A_{145}$ and $VEGF\text{-}A_{165}$ bind to NRP-2 (Gluzman-Poltorak *et al.,* 2000) (**Figure 2**).

VEGF-A transcription is induced in hypoxia (Shweiki *et al.,* 1992) and in ischemia (Banai *et al.,* 1994) by hypoxia-inducible factor 1 (HIF-1) (Forsythe *et al.,* 1996). HIF-1 also stabilizes the VEGF-A messenger ribonucleic acid (mRNA) (Liu *et al.,* 2002) as does also the ribonucleic acid (RNA) -binding protein HuR, which binds to the 3' untranslated region (UTR) of VEGF-A (Levy *et al.,* 1998). VEGF-A is expressed in multiple organs during development; in adults its expression is highest in lung and liver (Kaipainen *et al.,* 1993; Lagercrantz *et al.,* 1998; Maharaj *et al.,* 2006).

VEGF-A induces EC survival, proliferation, migration, sprouting and tube formation (Ferrara, 2004). It also induces vasodilatation of arteries (Ku *et al.,* 1993) via induction of the endothelial nitric oxide synthase (eNOS) (Hood *et al.,* 1998). The crucial role of VEGF-A in normal growth and survival as well as vascular development has been proven by VEGF inhibition (Gerber *et al.,* 1999) and by knockout mice, where lack of even one copy of VEGF-A causes embryonic lethality (Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996).

Transgenic mice overexpressing VEGF-A in the epidermis under keratin 14 promoter exhibit abundant cutaneous angiogenesis and a skin condition resembling psoriasis (Xia *et al.,* 2003). Over-expression of VEGF-A in skin under keratin K6 regulatory sequences induces angiogenesis, vascular permeability and accelerated tumor development (Larcher *et al.,* 1998). VEGF-A is expressed practically in all solid human tumors studied so far (Ferrara, 2004).

Results from phase III clinical trial revealed that bevacizumab (Avastin™), a monoclonal antibody against VEGF-A, significantly increased the survival of patients with metastatic colorectal cancer when used in combination with chemotherapy (Hurwitz *et al.,* 2004). Bevacizumab and several other drugs targeted against VEGF (VEGF Trap, a decoy VEGF receptor; pegaptanib, an aptamer binding to $VEGF\text{-}A_{165}$; ranibizumab, a monoclonal antibody fragment against VEGF-A) are currently being tested in phase III clinical trials for the treatment of different cancers and neovascular ocular diseases (U. S. National Institutes of Health,

http://www.clinicaltrials.gov, accessed October 2009).

Gene transfer studies have shown that VEGF-A can induce angiogenic effects in ischemic (Wright, 2002) and normoxic (Rissanen *et al.,* 2003b) rabbit hind limbs, in rabbit eyes after intravitreal gene transfer (Kinnunen *et al.,* 2006), and in normoxic mouse hind limb muscles (Kholová *et al.,* 2007).

VEGF-B

VEGF-B was cloned from human (Grimmond *et al.,* 1996) and murine (Townson *et al.,* 1996) brain complementary deoxyribonucleic acid (cDNA) libraries. The promoter region of VEGF-B contains binding sites for the early growth response 1 transcription factor, but on the contrary to VEGF-A, no HIF-1 binding sites (Silins *et al.,* 1997). Consequently, VEGF-B expression is not regulated by hypoxia (Enholm *et al.,* 1997; Ristimäki *et al.,* 1998). Two splice variants, VEGF-B_{167} and VEGF-B_{186}, are expressed in human and mouse (Olofsson *et al.,* 1996b) as a result of alternative splicing of exon 6 (Grimmond *et al.,* 1996; Olofsson *et al.,* 1996b; Townson *et al.,* 1996). Both isoforms have an identical N-terminal region, but differ in their C-terminal regions. VEGF-B_{167} has a C-terminal basic domain, which anchors it to the ECM (Olofsson *et al.,* 1996a), whereas VEGF-B_{186} has a hydrophobic tail and is secreted (Olofsson *et al.,* 1996b). VEGF-B binds to VEGFR-1 (Olofsson *et al.,* 1998) and NRP-1 (Makinen *et al.,* 1999) (**Figure 2**). It forms disulfide-linked homodimers as well as heterodimers with VEGF-A (Olofsson *et al.,* 1996a; Olofsson *et al.,* 1996b).

Expression of VEGF-B starts early in fetal development and is predominant in heart, central nervous system and brown fat tissue (Lagercrantz *et al.,* 1996; Lagercrantz *et al.,* 1998; Aase *et al.,* 1999). In the central nervous system, VEGF-B appears to be the only VEGF family member expressed at detectable levels (Lagercrantz *et al.,* 1998). In adults, VEGF-B is mainly expressed in heart, brain, skeletal muscle, kidney and pancreas (Olofsson *et al.,* 1996a; Olofsson *et al.,* 1996b; Townson *et al.,* 1996; Lagercrantz *et al.,* 1998).

The exact role of VEGF-B *in vivo* is not yet clear. VEGF-B knockout mice are healthy and fertile, but they have smaller hearts and impaired recovery from experimentally induced myocardial ischemia (Bellomo *et al.,* 2000).

Administration of VEGF-B protein or VEGF-B gene transfer to ischemic hind limb skeletal muscle (Wright, 2002; Silvestre *et al.,* 2003; Wafai *et al.,* 2009) or myocardium (Li *et al.,* 2008; Lähteenvuo *et al.,* 2009) results in increased vascularization, whereas VEGF-B does not show angiogenic effects in periadventitial tissue (Bhardwaj *et al.,* 2003) or in normoxic hind limb skeletal muscle (Rissanen *et al.,* 2003b). Transgenic overexpression of VEGF-B in ECs induces vessel growth without changes in vascular permeability (Mould *et al.,* 2005). In transgenic mice expressing VEGF-B only in cardiac myocytes, no increases in vessel numbers are detected, but VEGF-B induces concentric myocardial hypertrophy and alters lipid metabolism resulting in mitochondrial damage and elevated mortality (Karpanen *et al.,* 2008).

VEGF-C

VEGF-C was originally cloned from human PC-3 prostate carcinoma cells (Joukov *et al.,* 1996). VEGF-C expression is not regulated by hypoxia, but is induced by proinflammatory cytokines (Enholm *et al.,* 1997; Ristimäki *et al.,* 1998). It is synthesized as a precursor protein, but is proteolytically processed (Joukov *et al.,* 1996) from the N- and C-terminal ends, first

intracellularly by proprotein convertases (Siegfried *et al.,* 2003) and further extracellularly by plasmin (McColl *et al.,* 2003) to produce a fully active mature form. The precursor protein binds only to VEGR-3. The proteolytic processing not only increases this affinity but also introduces a new binding affinity, since the fully processed protein can also bind VEGFR-2 (Kukk *et al.,* 1996; Lee *et al.,* 1996; Joukov *et al.,* 1997) (**Figure 2**). VEGF-C also interacts with NRP-1 and NRP-2 (Kärpänen *et al.,* 2006).

VEGF-C is expressed during embryonic development especially in the regions, where the lymphatic vasculature has developed (Kukk *et al.,* 1996), whereas in adults, VEGF-C expression is detected in multiple tissues (Kukk *et al.,* 1996; Partanen *et al.,* 2000). VEGF-C induces mitogenesis, migration and survival of ECs and increases vascular permeability (Lee *et al.,* 1996; Joukov *et al.,* 1997; Zhao *et al.,* 2007). VEGF-C is indispensable in embryonic lymphangiogenesis, since embryos lacking VEGF-C are not viable and fail to form the initial lymph sacs required for the generation of lymphatic vasculature (Karkkainen *et al.,* 2004). Transgenic mice overexpressing VEGF-C either inducibly or constitutively in the epidermis or hair follicles have induced lymphatic vessel hyperplasia in the skin (Jeltsch *et al.,* 1997; Veikkola *et al.,* 2001; Lohela *et al.,* 2008). Inducible overexpression of VEGF-C in the vascular endothelium leads to lymphangiogenesis postnatally and in adult tissues, wheras in embryos can a strong induction in angiogenesis also be detected (Lohela *et al.,* 2008).

In experimental models, VEGF-C promotes tumor lymphangiogenesis, metastasis and growth (Karpanen *et al.,* 2001; Mandriota *et al.,* 2001). VEGF-C has shown potential in inducing therapeutic lymphangiogenesis in pathological conditions of lymphatic insufficiency (Karkkainen *et al.,* 2001; Szuba *et al.,* 2002; Tammela *et al.,* 2007; Liu *et al.,* 2008). It also induces therapeutic angiogenesis and lymphangiogenesis in diabetic wound healing (Saaristo *et al.,* 2006).

Gene transfer studies with VEGF-C have revealed that it induces angiogenesis in ischemic settings (Witzenbichler *et al.,* 1998; Pätilä *et al.,* 2006). Ectopic expression of recombinant VEGF-C induces angiogenesis in the mouse cornea and the chicken embryo (Cao *et al.,* 1998). In skin, VEGF-C induces lymphangiogenesis (Enholm *et al.,* 2001) and changes in blood vessel morphology, but no sprouting angiogenesis (Saaristo *et al.,* 2002). Prevention of VEGF-C expression was reported to inhibit lymphangiogenesis and tumor growth (He *et al.,* 2008). A correlation between VEGF-C expression and lymph node metastases has been detected in a number of different tumors (Alitalo *et al.,* 2002).

VEGF-D

VEGF-D (also called c-fos -induced growth factor) was originally cloned from mouse fibroblasts (Orlandini *et al.,* 1996) and mouse and human lung cDNA libraries (Yamada *et al.,* 1997; Achen *et al.,* 1998). VEGF-D is a secreted dimeric protein (Orlandini *et al.,* 1996). It is a ligand and activator of both VEGR-2 and VEGFR-3, but does not bind to VEGFR-1 (Achen *et al.,* 1998). VEGF-D also interacts with NRP-1 and NRP-2 (Kärpänen *et al.,* 2006).

VEGF-D is structurally most closely related to VEGF-C (Yamada *et al.,* 1997) and undergoes similar proteolytic processing of the N- and C-terminal ends by plasmin and proprotein convertases i.e. like VEGF-C (McColl *et al.,* 2003; McColl *et al.,* 2007). The proteolytic processing regulates the biological activity and receptor specificity of VEGF-D. The unprocessed, long form of VEGF-D binds predominantly to VEGFR-3. Proteolytic processing of the N- and C-terminal ends results in the short, mature

form of the protein (VEGF-DΔNΔC) containing only the VEGF homology region, which increases the binding affinity by 290-fold to VEGFR-2 and by 40-fold to VEGFR-3 (Stacker *et al.,* 1999; McColl *et al.,* 2003) (**Figure 2**). Mouse VEGF-D only binds VEGR-3 (Baldwin *et al.,* 2001a). VEGF-D is expressed in many tissues in adults (Yamada *et al.,* 1997; Achen *et al.,* 1998; Partanen *et al.,* 2000; Baldwin *et al.,* 2001b) and during embryonic development (Avantaggiato *et al.,* 1998; Partanen *et al.,* 2000), the expression being most prominent in lungs (Yamada *et al.,* 1997; Farnebo *et al.,* 1999; Stacker *et al.,* 1999).

VEGF-D has been proposed to have a role in tumor angiogenesis (Marconcini *et al.,* 1999) and lymphangiogenesis (Achen *et al.,* 2002). However, it does not seem to play a crucial role in embryonic lymphangiogenesis, since VEGF-D deficient mice do not display any profound defects in their lymphatic vessels (Baldwin *et al.,* 2005). Transgenic VEGF-D mice produced by lentiviral transgenesis have the highest expression levels of the mature form of human VEGF-D in skeletal muscle, skin, pancreas and heart. These animals have increased capillary densities in their skeletal and cardiac muscles and enhanced muscle regeneration after ischemia, but they also have increased susceptibility to tumor formation (Kärkkäinen *et al.,* 2009). Adenoviral gene transfer studies in various animal models show that VEGF-D is a potent stimulator of both angiogenesis and lymphangiogenesis. VEGF-DΔNΔC induces angiogenesis in rat cremaster muscle and in a mouse skin model (Byzova *et al.,* 2002), in rabbit and mouse hind limb skeletal muscle (Rissanen *et al.,* 2003b; Kholová *et al.,* 2007) and in pig myocardium (Rutanen *et al.,* 2004). Both VEGF-D and VEGF-DΔNΔC can induce angiogenesis in rabbit carotid arteries after periadventitial gene transfer (Bhardwaj *et al.,* 2003; Bhardwaj *et al.,* 2005). Lymphangiogenesis is induced by VEGF-DΔNΔC in mouse skin (Byzova *et al.,* 2002) and in lymph node-excised mice (Tammela *et al.,* 2007), and by VEGF-D in rabbit hind limb skeletal muscle (Rissanen *et al.,* 2003b).

Placental growth factor

Placental growth factor (PlGF) was originally isolated from human placenta cDNA library (Maglione *et al.,* 1991). It is also expressed in human vascular ECs (Barleon *et al.,* 1994) and has been detected in fetal liver, heart, lung and kidney (Kaipainen *et al.,* 1993). Four secreted PlGF isoforms have been described: PlGF-1 (Maglione *et al.,* 1991; Park *et al.,* 1994), PlGF-2 (Hauser *et al.,* 1993; Maglione *et al.,* 1993; Park *et al.,* 1994), PlGF-3 (Cao *et al.,* 1997) and PlGF-4 (Yang *et al.,* 2003). PlGFs form homodimers (Maglione *et al.,* 1991; Park *et al.,* 1994; Cao *et al.,* 1997), but also natural heterodimers with VEGF-A have been detected, with reduced EC mitogenic potential as compared with the VEGF homodimers (DiSalvo *et al.,* 1995; Cao *et al.,* 1996).

PlGFs mediate their effects via VEGFR-1 and do not bind to VEGFR-2 (Park *et al.,* 1994; Terman *et al.,* 1994; Sawano *et al.,* 1996). PlGF-2 binds also to NRP-1 (Migdal *et al.,* 1998; Mamluk *et al.,* 2002), and the PlGF/VEGF heterodimers to VEGFR-2 (Cao *et al.,* 1996) (**Figure 2**).

PlGF induces EC proliferation (Maglione *et al.,* 1991; Ziche *et al.,* 1997a), migration (Ziche *et al.,* 1997a; Migdal *et al.,* 1998) and angiogenesis (Ziche *et al.,* 1997a; Luttun *et al.,* 2002b; Roy *et al.,* 2005). PlGF modulates inter- and intramolecular crosstalk between VEGR-1 and VEGFR-2 by activating VEGFR-1, which evokes the transphosphorylation of VEGFR-2 (Autiero *et al.,* 2003), and it has also been shown to induce VEGF-A expression (Roy *et al.,* 2005). However, it

has also been postulated that the PlGF/VEGF heterodimers have an antagonistic effect on VEGF -mediated angiogenic signalling (Eriksson *et al.,* 2002; Xu *et al.,* 2006; Schomber *et al.,* 2007).

Overexpression of PlGF in the skin of transgenic mice leads to increased vascularization (Odorisio *et al.,* 2002; Marcellini *et al.,* 2006), enhanced vessel permeability, and expression of VEGFR-1 and VEGFR-2 (Odorisio *et al.,* 2002), as well as increased melanoma growth and metastasis spreading (Marcellini *et al.,* 2006). Mice deficient in PlGF suffer impaired angiogenesis, vascular permeability and arteriogenesis in pathological conditions (Carmeliet *et al.,* 2001; Luttun *et al.,* 2002a). PlGF has also been implicated in the induction of cutaneous inflammation in inflammatory angiogenesis (Oura *et al.,* 2003). Gene transfer studies with PlGF show that it has potential in inducing therapeutic angiogenesis in ischaemic hind limbs, resulting in increased exercise tolerance (Korpisalo *et al.,* 2008).

A role for PlGF in tumor vasculogenesis and angiogenesis has been proposed, but the current results are controversial (Matsumoto *et al.,* 2003; Li *et al.,* 2006a; Schomber *et al.,* 2007). PlGF may also play a role in arteriogenesis by activating monocytes (Pipp *et al.,* 2003; Scholz *et al.,* 2003), in epithelial differentiation (Zhang *et al.,* 2003), and in the development of atherosclerosis (Khurana *et al.,* 2005; Pilarczyk *et al.,* 2008), proliferative diabetic retinopathy (Khaliq *et al.,* 1998), and choroidal neovascularization (Rakic *et al.,* 2003).

Receptors for vascular endothelial growth factors

VEGF family members mediate their functions via a family of VEGFRs, which are high-affinity receptor protein-tyrosine kinases. They consist of an extracellular ligand-binding component containing seven immunoglobulin-like domains, a single transmembrane segment, an intracellular tyrosine kinase domain split by a kinase insert, and a downstream carboxy terminal domain (Olsson *et al.,* 2006) (**Figure 2**).

VEGF receptor 1

VEGFR-1 was isolated from normal human placenta RNA (Shibuya *et al.,* 1990). There is extensive expression of VEGFR-1 in human heart, lung, kidney, placenta and vascular ECs (Kaipainen *et al.,* 1993; Barleon *et al.,* 1994). The mouse homolog fms-like tyrosine kinase-1 is expressed in vascular ECs both during embryonic vascular development (Peters *et al.,* 1993; Breier *et al.,* 1995; Fong *et al.,* 1996) and in adult tissues (Peters *et al.,* 1993). The expression of VEGFR-1 is induced by hypoxia (Detmar *et al.,* 1997; Gerber *et al.,* 1997) via an HIF-1 dependent mechanism (Gerber *et al.,* 1997).

VEGFR-1 binds VEGF-A (de Vries *et al.,* 1992; Breier *et al.,* 1995; Clauss *et al.,* 1996), VEGF-B (Olofsson *et al.,* 1998) and PlGF (Park *et al.,* 1994; Terman *et al.,* 1994; Clauss *et al.,* 1996; Sawano *et al.,* 1996) with high affinities (**Figure 2**). However, VEGF-A binding to VEGFR-1 mediates only weak mitogenic signalling (Waltenberger *et al.,* 1994).

VEGFR-1 knockout mice have abnormal vascular channels and die during early embryogenesis (Fong *et al.,* 1995). Developmental studies have shown that VEGFR-1 plays a major role in vasculogenesis by establishing vascular endothelium (Fong *et al.,* 1996), by controlling EC division and sprout formation (Kearney *et al.,* 2002; Kearney *et al.,* 2004; Kappas *et al.,* 2008), and by modulating VEGFR-2 signalling (Roberts *et al.,* 2004; Kappas *et al.,* 2008). The tyrosine kinase domain of VEGFR-1 is

dispensable (Hiratsuka *et al.,* 1998), whereas the ligand-binding domain and the transmembrane domain are essential for normal development (Hiratsuka *et al.,* 2005). VEGFR-1 signalling promotes both angiogenesis and lymphangiogenesis by increasing bone marrow-derived macrophage recruitment (Murakami *et al.,* 2008). VEGFR-1 is also involved in monocyte/macrophage chemotaxis (Barleon *et al.,* 1996; Clauss *et al.,* 1996; Hiratsuka *et al.,* 1998; Sawano *et al.,* 2001; Tchaikovski *et al.,* 2008) and recruitment of endothelial (Li *et al.,* 2006a) and myeloid (Luttun *et al.,* 2002b) progenitor cells.

A soluble, truncated form of VEGFR-1 (sVEGFR-1) is produced through alternative splicing (Kendall *et al.,* 1993; He *et al.,* 1999b). The sVEGFR-1 inhibits VEGF-A mediated signalling by functioning as a VEGF-A sink, thus binding and depleting free circulating VEGF-A (Kendall *et al.,* 1993; Hornig *et al.,* 2000; Inoue *et al.,* 2000) as well as by heterodimerizing with VEGFR-2 (Kendall *et al.,* 1996). Adenoviral gene transfer of soluble VEGFR-1 into rat femoral muscle inhibits the formation of subretinal neovascularization, a serious complication in age related macular degeneration (Honda *et al.,* 2000). Adenovirus mediated gene transfer of soluble ligand-binding ectodomain of VEGFR-1 also prevents endogenous angiogenesis and inhibits perfusion in animals with experimental ischemia (Jacobi *et al.,* 2004).

VEGF receptor 2

VEGFR-2 was originally cloned from human EC cDNA library and named kinase insert domain-containing receptor (Terman *et al.,* 1991). It is expressed in multiple tissues during development (Kaipainen *et al.,* 1993), in cultured vascular ECs and in placenta (Barleon *et al.,* 1994). The murine homolog fetal liver kinase 1 isolated from hematopoietic tissue is also expressed in a wide variety of non-hematopoietic tissues (Matthews *et al.,* 1991), where the expression is restricted to the vascular endothelium during development (Millauer *et al.,* 1993; Quinn *et al.,* 1993). Fetal liver kinase 1 is highly expressed in the developing brain, but its expression is drastically reduced in adults when proliferation has ceased (Millauer *et al.,* 1993). VEGFR-2 expression is not transcriptionally regulated by hypoxia (Gerber *et al.,* 1997), but hypoxia decreases VEGFR-2 mRNA stability (Olszewska-Pazdrak *et al.,* 2009).

VEGFR-2 binds VEGF-A (Terman *et al.,* 1992; Quinn *et al.,* 1993), the mature forms of VEGF-C (Joukov *et al.,* 1997) and VEGF-D (Achen *et al.,* 1998), VEGF-E (Ogawa *et al.,* 1998) and VEGF-F (Yamazaki *et al.,* 2003) (**Figure 2**). VEGFR-2 transcription is induced by ligand binding, indicative of a positive feedback mechanism for VEGF action (Shen *et al.,* 1998).

VEGFR-2 is the major signal transducer of both physiological and pathological angiogenesis. VEGFR-2 deficiency (Shalaby *et al.,* 1995) and especially a mutation in the tyrosine residue 1173 (Sakurai *et al.,* 2005) are lethal early in embryogenesis since there is a lack of vasculogenesis. VEGFR-2 mediates EC migration and vascular permeability (Gille *et al.,* 2001), EC proliferation (Gille *et al.,* 2001; Li *et al.,* 2002), tube formation (Koolwijk *et al.,* 2001; Yang *et al.,* 2001) and hypotension (Li *et al.,* 2002). VEGFR-2 activates several signal transduction pathways in ECs (Kroll and Waltenberger, 1997; Wu *et al.,* 2000; Gille *et al.,* 2001), VEGF-A induces nitric oxide synthase (NOS) expression via VEGFR-2 (Kroll and Waltenberger, 1998) and VEGFR2 mediates nitric oxide (·NO) release from ECs (Kroll and Waltenberger, 1999). The importance of NOS for the VEGF-A induced angiogenesis has been presented by using NOS inhibitors (Papapetropoulos *et al.,* 1997; Ziche *et*

al., 1997b) and eNOS knockout mice (Fukumura *et al.,* 2001). In addition to mediating angiogenic signalling, VEGFR-2 may also be involved in lymphatic vessel enlargement, without affecting vessel sprouting (Wirzenius *et al.,* 2007).

Retrovirally mediated dominant-negative inhibition of VEGFR-2 suppresses the growth of many tumor types in an athymic mouse model, where cells producing truncated VEGFR-2 lacking the kinase domain are implanted subcutaneously together with tumor cells (Millauer *et al.,* 1994; Millauer *et al.,* 1996). Adenovirus mediated gene transfer of soluble ligand-binding ectodomain of VEGFR-2 prevents endogenous angiogenesis and inhibits perfusion in animals with experimental ischemia (Jacobi *et al.,* 2004). VEGFR-2 antibodies and tyrosine kinase inhibitors targeting either VEGFR-2 or multiple growth factor receptors are currently being tested for the treatment of different cancers in clinical trials (U. S. National Institutes of Health, http://www.clinicaltrials.gov, accessed October 2009).

VEGF receptor 3

VEGFR-3 (fms-like tyrosine kinase 4) was cloned from a human placenta cDNA library (Galland *et al.,* 1992). VEGFR-3 is expressed in multiple tissues during development (Pajusola *et al.,* 1992; Galland *et al.,* 1993; Kaipainen *et al.,* 1993; Borg *et al.,* 1995; Partanen *et al.,* 2000), especially in the ECs of developing vessels (Kaipainen *et al.,* 1993; Kukk *et al.,* 1996). In adulthood, VEGFR-3 expression becomes restricted to lymphatic ECs, some high endothelial venules and fenestrated capillaries (Kaipainen *et al.,* 1995; Partanen *et al.,* 2000), with the exception of tumor tissues, where it is expressed also in vascular ECs and is possibly involved in maintaining the integrity of the EC lining during angiogenesis (Kubo *et al.,* 2000; Witmer *et al.,* 2001; Petrova *et al.,* 2008). VEGFR-3 is also expressed in blood vessel endothelium in chronic inflammatory wounds (Paavonen *et al.,* 2000).

VEGFR-3 binds VEGF-C (Joukov *et al.,* 1996; Joukov *et al.,* 1997) and VEGF-D (Achen *et al.,* 1998), but not VEGF-A (Pajusola *et al.,* 1994) (**Figure 2**). Proteolytic processing of VEGF-C and –D increases their affinity to VEGFR-3 (Joukov *et al.,* 1997; Stacker *et al.,* 1999; McColl *et al.,* 2003). VEGFR-3 transduces signalling for lymphatic EC survival, growth and migration (Mäkinen *et al.,* 2001b).

Targeted inactivation of VEGFR-3 shows that VEGFR-3 signalling is essential in the development of functional blood vessels during embryogenesis (Dumont *et al.,* 1998). Recent studies with genetic targeting of VEGFR-3 and inhibition of VEGFR-3 signalling have revealed that VEGFR-3 is involved in angiogenic sprouting and regulation of the vascular network formation (Tammela *et al.,* 2008). The cooperative properties of VEGFR-2 and VEGFR-3 are required for lymphatic migration and proliferation, whereas either one of the receptors is sufficient to organize the lymphatic ECs into functional capillaries (Goldman *et al.,* 2007).

Studies with transgenic mice overexpressing VEGF-D or a VEGFR-3-specific mutant of VEGF-C under the keratin 14 promoter in the hair follicles have shown that activation of the VEGFR-3 signalling pathway is sufficient to induce lymphangiogenesis in the skin (Veikkola *et al.,* 2001). Transgenic mice expressing detectable levels of circulating extracellular ligand binding soluble form of VEGFR-3 under the keratin 14 promoter in keratinocytes have reduced lymphangiogenesis, resulting in lymphedema (Mäkinen *et al.,* 2001a). Inhibition of VEGFR-3 signalling in animal tumor models suppresses tumor lymphangiogenesis and lymph node metastasis (He *et al.,* 2002; Shimizu *et*

al., 2004; Lin *et al.,* 2005) as well as tumor angiogenesis and lymphangiogenesis with a consequent reduction in tumor growth in animal models (Laakkonen *et al.,* 2007; Tammela *et al.,* 2008).

LIPOXYGENASES

Classification

Lipoxygenases (LO) are a family of nonheme iron dioxygenases capable of inserting molecular oxygen regio- and stereospecifically into the 1,4-pentadiene structures of polyunsaturated fatty acids, thus producing their corresponding hydroperoxy derivatives (Yamamoto, 1992; Fürstenberger *et al.,* 2006). The primary lipid peroxidation products from arachidonic acid (AA) and linoleic acid (LA) are hydro-peroxyeicosatetraenoic acid (HPETE) and hydroperoxyoctadecadienoic acid (HPODE), respectively (Fürstenberger *et al.,* 2006). These are readily reduced to their respective hydroxy fatty acids, hydroxyeicosatetraenoic acid (HETE) and hydroxyoctadecadienoic acid (HODE). LOs have been isolated from plants, animals and micro-organisms (Kuhn *et al.,* 2002).

Mammalian LOs were first classified according to their positional specificity of oxygenating AA, resulting in nomenclature of 5-, 8-, 12- and 15-LOs (Kuhn and Thiele*,* 1999). However, this classification has proved to be over-simplified, since the positional specificity of the enzymes is not absolute, but depends on the structure of the substrate and the species in question. Thus, another classification based on the phylogenetic relatedness has been proposed, grouping the enzymes into four different phylogenetic groups, namely 12(S)/15(S)-LOs (the reticulocyte and leukocyte type LOs), platelet-type 12(S)-LOs, 5(S)-LOs and epidermis-type LOs (Fürstenberger *et al.,* 2002) (**Figure 3**). The phylogenetic group of the mouse epidermal 12(S)-LO has not yet been clearly identified (Fürstenberger *et al.,* 2007).

Substrate specificity, products and physiological roles

All LOs are intracellular enzymes involved in the regulated metabolism of AA (**Figure 4**), a common constituent of cell membrane phospholipids. In response to a diverse set of external stimuli, free AA is released from the membranes by the action of phospholipases. The released AA is consequently metabolized via the cyclo-oxygenase or the LO pathways (Sigal, 1991). In addition to AA, the LOs can also metabolize a number of other polyenoic fatty acids with various substrate specificities.

5-LO reacts predominantly with C_{20} fatty acids (Ochi *et al.,* 1983). 5-LO initiates leukotriene synthesis from AA together with 5-LO activating protein (Dixon *et al.,* 1990). The association of leukotrienes with asthma and allergic rhinitis have been established in clinical trials. 5-LO and leukotrienes have also been implicated as playing a role in other allergic diseases as well as in inflammatory diseases, pulmonary syndromes, cancer and atherosclerosis (Peters-Golden and Henderson*,* 2007).

The mouse 8(S)-LO can use AA and LA as a substrate (Jisaka *et al.,* 1997). There are three distinct isoforms of 12(S)-LOs, which differ in their substrate specificities: the platelet-type 12(S)-LO reacts mainly with AA, whereas the leukocyte-type 12(S)-LO displays a wide substrate specificity, and can react with C_{18} and C_{22} fatty acids as rapidly as with AA (Yamamoto, 1992). The mouse epidermal 12(S)-LO (Aloxe) produces mainly 12(S)-HETE and minor amounts of 15(S)-HETE from AA (Funk *et al.,*

1996). The physiological roles of the epidermal-type and platelet-type LOs are still largely unknown. Mouse 8-(S)-LO, its human ortholog 15(S)-LO-2, 12(R)-LO and e-LOX-3 have been suggested to be involved in the terminal differentiation of epidermal keratinocytes (Fürstenberger *et al.,* 2002; Fürstenberger *et al.,* 2007), and e-LOX-3 also in adipocyte differentiation (Fürstenberger *et al.,* 2007). 8-(S)-LO may also have a critical role in mouse skin tumor development (Fürstenberger *et al.,* 2002). Platelet-type 12(S)-LO has been suggested to be involved in skin tumor development (Krieg *et al.,* 1995).

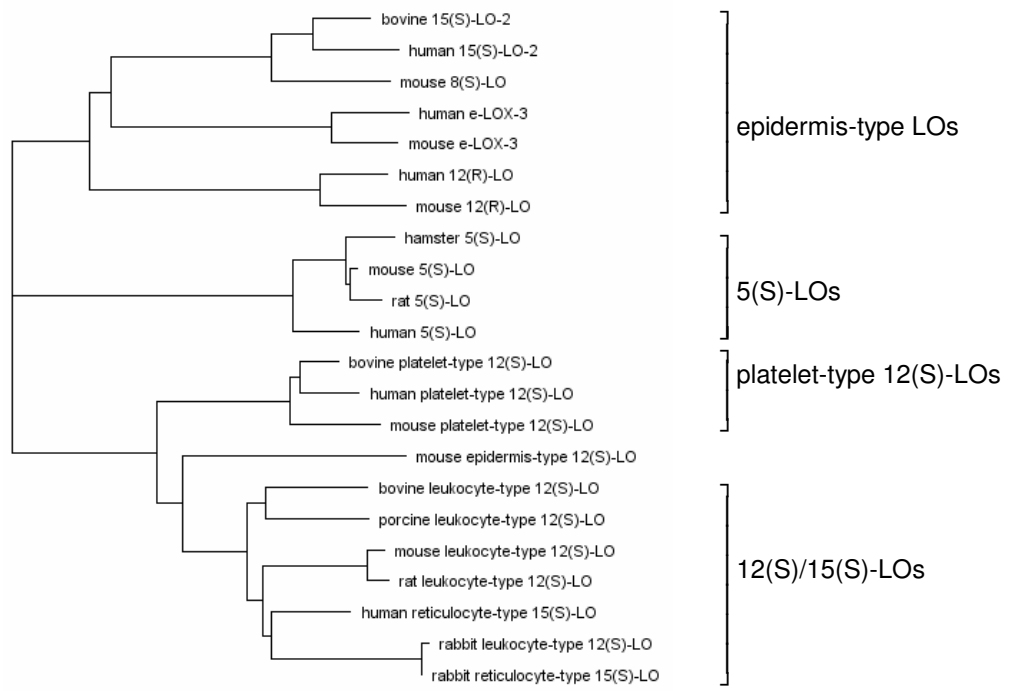

Figure 3. Phylogenetic tree of mammalian LOs. Genetic distance for all pairs was calculated with the Geneious Pro 4.5.4 software. Sequence accession numbers are according to Fürstenberger *et al.,* 2002.

Reticulocyte 15-LO (15-LO-1) shows wide substrate specificity in terms of carbon chain length: it can oxygenate unsaturated C_{18}, C_{20}, and C_{22} fatty acids (Yamamoto, 1992). The optimal substrate for 15-LO-1 is LA (Kühn *et al.,* 1993a), producing mainly 13(S)-HPODE (Kühn *et al.,* 1993b). The reaction with AA produces mostly 15(S)-HPETE, but also 12(S)-HPETE as a side product (Bryant *et al.,* 1982; Kühn *et al.,* 1993a; Kühn *et al.,* 1993b). In contrast, the 15-LO-2 isolated from human hair roots produces only 15(S)-HPETE from AA, and LA is a poor substrate (Brash *et al.,* 1997).

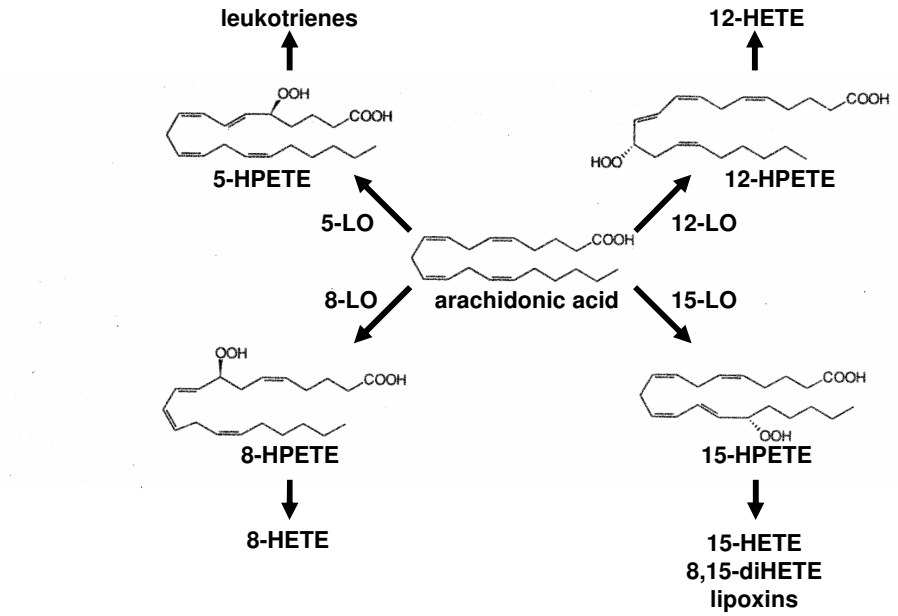

Figure 4. Arachidonic acid metabolism by mammalian lipoxygenases. Modified from Viita and Ylä-Herttuala, 2000.

15-LO-1 has been shown to be involved in the controlled degradation of reticulocyte mitochondria during red cell maturation (Schewe *et al.,* 1975; Rapoport *et al.,* 1979; Grüllich *et al.,* 2001). It preferentially acts on mitochondrial membranes compared with cell membranes (Rapoport *et al.,* 1979) and this leads to inactivation of respiratory enzymes (Kroschwald *et al.,* 1989). The disruption of the mitochondrial membrane is mediated by induction of collapse of the mitochondrial pH gradient (Vijayvergiya *et al.,* 2004) leading to the dissipation of mitochondrial membrane potential and release of cytochrome c (Maccarrone *et al.,* 2001).

15-LO-1 protein expression is temporally tightly regulated during red cell maturation: it is not expressed in bone marrow, but the expression begins in the transition phase from late erythroblast to early reticulocyte stages, peaks during reticulocyte maturation and then declines until no protein is expressed in the erythrocyte (Rapoport *et al.,* 1979; Thiele *et al.,* 1979; Thiele *et al.,* 1982; Nadel *et al.,* 1991). Production of characteristic 15-LO-1 hydroxy fatty acids can be induced in experimental reticulocytosis (Kühn *et al.,* 1990; Kühn and Brash, 1990).

A similar pattern of 15-LO-1 expression is detected in the central fibre cell of the eye lens, where degradation of the nuclei and organelles results in the formation of a transparent cell filled with crystallins. 15-LO-1 is localized to the region where organelle degradation occurs and is absent from the outer layers, which contain precursor and immature fibre cells as well as mature central fibres (van Leyen *et al.,* 1998). In rat liver, 15-LO-1 is involved in the disruption of peroxisomal membranes and programmed degradation of peroxisomes (Yokota *et al.,* 2001). A more generalized function for 15-LO-1

has been proposed in the programmed degradation of various intracellular organelles by membrane integration, induction of pore formation and permeabilization of the organelle membranes (van Leyen *et al.,* 1998; van Leyen, 1998; Vijayvergiya *et al.,* 2004).

There are two important differences between the reticulocyte 15-LO-1 and other mammalian LOs with respect to atherogenesis. The optimal substrate for reticulocyte 15-LO-1 is LA, which is abundant in LDL. 15-LO-2 is also capable of binding LA with a similar affinity, but the catalytic activity of 15-LO-1 is two to four fold higher (Kilty *et al.,* 1999). In addition, 15-LO-1 is able to oxidize not only free polyenoic fatty acids, but also esterified fatty acids in more complex substrates such as phospholipids, cholesterol esters, lipoproteins and biomembranes, without the preceding action of phospholipases (Kuhn *et al.,* 2002).

Reticulocyte 15-lipoxygenase (15-LO-1)

Structure and substrate binding

Reticulocyte 15-LO-1 consists of two domains, the C-terminal substrate binding domain containing a hydrophobic pocket and an N-terminal β-barrel domain (Gillmor *et al.,* 1997). The enzymatic activity is localized to the C-terminal catalytic domain, whereas both the N-terminal β-barrel domain and the C-terminal catalytic domain determine the membrane binding properties of the enzyme (Walther *et al.,* 2002). 15-LOs contain one non-heme iron molecule in their postulated catalytic region. They exist usually in the inactive ferrous (Fe^{2+}) state, but oxidation to the active ferric (Fe^{3+}) form is needed for catalysis (Boyington *et al.,* 1994).

The product specificity of human 15-LO-1 *in vitro* has been shown to depend on the substrate concentration (Schnurr *et al.,* 1996; Belkner *et al.,* 1998), incubation temperature (Murray *et al.,* 1988; Belkner *et al.,* 1998; Heydeck *et al.,* 2001) and incubation time (Lass *et al.,* 1996; Schnurr *et al.,* 1996; Belkner *et al.,* 1998; Heydeck *et al.,* 2001).

Cloning and expression

15-LO-1 was originally purifed from rabbit reticulocytes (Rapoport *et al.,* 1979). Cloning and sequencing of the rabbit and human reticulocyte 15-LO-1 cDNAs showed that they encode predicted polypeptides of 661 amino acids and approximately 75 kilo Dalton (kDa) (Sigal *et al.,* 1988; Fleming *et al.,* 1989). In addition to reticulocytes, 15-LO-1 is highly expressed in human eosinophils, interleukin (IL) -4 and -13 treated human monocytes and alveolar macrophages (Kühn, 1996). The protein is localized to the cytoplasm (Nadel *et al.,* 1991). Cloning of the murine leukocyte 12-LO revealed that it is the functional homolog of the human reticulocyte 15-LO-1. The murine 12/15-LO is expressed in peritoneal macrophages, lung, spleen, heart, liver, reticulocytes, pineal gland, pituitary gland and kidney (Chen *et al.,* 1994; Freire-Moar *et al.,* 1995).

Regulation of expression

Human reticulocyte 15-LO-1 and its species homologs rabbit reticulocyte 15-LO-1 and murine 12/15-LO are strictly regulated enzymes at the transcriptional, translational, and enzyme activity levels.

Transcriptional regulation

The reticulocyte 15-LO-1 gene promoter contains interleukin responsive elements (Kritzik *et al.,* 1997; Kelavkar *et al.,* 1998) and a GATA-binding protein

(GATA) binding region mediating repression of 15-LO-1 expression (O'Prey *et al.,* 1995; Kamitani *et al.,* 2000). A transcriptional silencer only functioning in non-erythroid cells is located in the 5' flanking region of the rabbit 15-LO-1 gene (O'Prey *et al.,* 1995), and one exact match to the consensus sequence of this element is also found in the human 15-LO-1 gene (Kritzik *et al.,* 1997).

Numerous studies have shown that IL-4 and -13 are able to induce 15-LO-1 mRNA and protein expression and enzyme activity both *in vitro* in various cell lines and *in vivo* (Kuhn *et al.,* 2002). IL-4/13 receptor activation is reported to be mandatory, but not sufficient, for the induction of 15-LO-1 expression in A549 lung carcinoma cell line (Brinckmann *et al.,* 1996).

The cytokine induced upregulation of 15-LO-1 gene requires expression of a signal transducer and activator of transcription (STAT) 6 (Heydeck *et al.,* 1998) and IL-4 dependent binding of STAT6 to the STAT6 response element in the 15-LO-1 promoter (Conrad *et al.,* 2000). A specific antibody against IL-13 receptor α1 can inhibit IL-13 induced 15-LO-1 expression and tyrosine phosphorylation of STAT6 (Krause *et al.,* 2006). Studies have shown that in addition to tyrosine phosphorylation of STAT6, also acetylation of both STAT6 and histones is required. It has been suggested that in the absence of IL-4, nuclear histones may be bound to regulatory elements of the 15-LO-1 gene, preventing its transcription. IL-4 stimulation causes rapid phosphorylation of STAT6, but its binding to the promoter appears to be prevented by nonacetylated histones. Once the histones have become acetylated, STAT6 binding sites may be unmasked so that the phosphorylated and acetylated transcription factor can bind and activate 15-LO-1 gene transcription (Shankaranarayanan *et al.,* 2001).

Translational regulation

15-LO-1 mRNA is abundant in young rabbit reticulocytes as a translationally inactive free cytoplasmic messenger ribonucleoprotein particle, but functional enzyme is not expressed (Thiele *et al.,* 1982). The 15-LO-1 mRNA 3' UTR contains a repeated sequence where regulatory proteins can bind, thus preventing translation (Fleming *et al.,* 1989; Ostareck-Lederer *et al.,* 1994). This differentiation control element (DICE) of rabbit 15-LO-1 is composed of ten subunits, whereas the human 15-LO-1 DICE has three repetitive structures (Reimann *et al.,* 2002). The regulatory proteins binding to the repeats have been isolated and identified as heterogeneous nuclear ribonucleoprotein (hnRNP) K and E_1, which cause inhibition of the 80S ribosome assembly on the 15-LO-1 mRNA. More specifically, the 3' UTR regulatory complex prevents joining of the 60S ribosomal subunit at the translation start codon, thus inhibiting the formation of the translation competent 80S ribosome (Ostareck *et al.,* 1997; Ostareck *et al.,* 2001).

Binding of the inhibitory proteins depends on the number of the repetitive structures. At least two repeat sequences of the DICE are required for translational inhibition by hnRNP E_1 suggesting that the binding of at least two hnRNP E_1 molecules activates or exposes a binding site to allow the complex to interact with the 5'-end of the mRNA and to prevent translation (Reimann *et al.,* 2002). Certain hnRNP K -homology domains of hnRNP E_1 and K have been identified as the DICE -binding domains (Messias *et al.,* 2006).

Translational silencing of DICE-bearing mRNAs can be overcome by a specific interaction with hnRNP K and c-Src, leading to c-Src activation and tyrosine phosphorylation of hnRNP K. This phosphorylation reversibly inhibits the binding of hnRNP K to DICE and specifically activates translation of the

silenced mRNA (Ostareck-Lederer *et al.,* 2002).

Posttranslational regulation

There are no major posttranslational modifications of 15-LO-1 protein e.g. no evidence of glycosylation has been detected (Kühn *et al.,* 1993a).

Regulation of enzymatic activity

Lipid hydroperoxides

The catalytic activity of 15-LO-1 is highly regulated by several factors. The dioxygenase reaction by 15-LO-1 typically has a kinetic lag phase (Ludwig *et al.,* 1987). This can be abolished by addition of hydroperoxy fatty acids (Ludwig *et al.,* 1987; Schnurr *et al.,* 1996), which oxidize the ferrous (Fe^{2+}) nonheme iron into the ferric (Fe^{3+}) form (de Groot *et al.,* 1975). Mechanistic studies with soybean 15-LO have revealed that lipid hydroperoxides activate 15-LO by binding to the enzyme, facilitating its activation, but remaining chemically unchanged in this process (Jones *et al.,* 1996).

Nitric oxide

Likewise, ˙NO is able to regulate the catalytic activity of 15-LO-1 by affecting the oxidation state of the nonheme iron. Short-term incubations of 15-LO-1 in the presence of ˙NO lead to a prolonged kinetic lag period, possibly due to the formation of a dissociable LO-˙NO complex, whereas long-term incubations shorten the lag phase by oxidizing the ferrous LO to a ferric form which is more susceptible to peroxide activation (Wiesner *et al.,* 1996; Holzhütter *et al.,* 1997). 15-LO-1 affects the bioavailability of ˙NO and ˙NO signalling by catalytically consuming ˙NO and inhibiting the activation of the soluble guanylate cyclase (O'Donnell *et al.,* 1999; Coffey *et al.,* 2001). In fact, elevated plasma ˙NO metabolites and induced eNOS expression are detected in 12/15-LO knockout mice suggesting that 15-LO-1 can affect the ˙NO bioavailability *in vivo* (Anning *et al.,* 2005).

Phospholipid hydroperoxide glutathione peroxidase

Phospholipid hydroperoxide glutathione peroxidase (PHGPX) is the only known selenoperoxidase capable of reducing 15-LO-1 peroxidation products, i.e. hydroperoxy lipids esterified to biomembranes and lipoproteins (Thomas *et al.,* 1990a; Thomas *et al.,* 1990b; Schnurr *et al.,* 1996). It also down-regulates 15-LO-1 activity, probably by reducing the hydroperoxy lipids necessary for the activation of the lipoxygenase reaction (Schnurr *et al.,* 1996; Huang *et al.,* 1999a), thus counteracting the effects of 15-LO-1.

Calcium

Calcium can modulate 15-LO-1 activity in polymorphonuclear leukocytes (Nichols *et al.,* 1991) and the calcium dependent association of 15-LO-1 to membranes stimulates 15-LO-1 activity (Watson *et al.,* 1994; Brinckmann *et al.,* 1998; Hsi *et al.,* 2001). Membrane association of 15-LO is primarily mediated via hydrophobic interactions between apolar amino acids and the hydrophobic core of membrane phospholipids, but calcium supports membrane binding probably by electrostatic forces (Walther *et al.,* 2004).

Self-inactivation

15-LO-1 is irreversibly inhibited during oxygenation of polyenoic fatty acids by its product 13-HPODE resulting in suicidal

inactivation (Rapoport *et al.,* 1979; Härtel *et al.,* 1982; Kühn *et al.,* 1993a). The self-inactivation was originally suggested to involve oxygenation of a single methionine to methionine sulfoxide (Rapoport *et al.,* 1984), but this kind of methionine oxidation was later shown not to be critical for enzyme inactivation (Gan *et al.,* 1995). Instead, 15-LO-1 catalyzed conversion of 15-HPETE to 14,15-epoxyleukotriene leads to the formation of reactive intermediates, which are capable of covalently binding to several active site amino acids and evoke the suicidal inactivation of the enzyme (Wiesner *et al.,* 2003).

15-LO-1 in atherosclerosis

Lipoprotein oxidation and foam cell formation

15-LO-1 can oxidatively modify LDL showing preferential oxygenation of LDL cholesterol esters (Belkner *et al.,* 1993; Belkner *et al.,* 1998) and phospholipids (Heydeck *et al.,* 2001). Retrovirus mediated gene transfer studies also support the role of 15-LO-1 in LDL oxidation. Overexpression of 15-LO-1 in murine fibroblasts leads to enhanced levels of lipoperoxides in LDL incubated with these cells, detected especially in the cholesteryl esters (Benz *et al.,* 1995; Ezaki *et al.,* 1995). In addition, 12/15-LO deficient mouse macrophages have a decreased ability to form foam cells, when incubated with LDL (Huo *et al.,* 2004). However, in an *in vitro* foam cell model based on the uptake of acetylated LDL by murine macrophages, 12/15-LO attenuates the intracellular lipid deposition and degrades internalized LDL lipids more rapidly, suggesting that 12/15-LO can impact on both lipid uptake and intracellular turnover (Belkner *et al.,* 2005).

Adhesion molecule expression and monocyte adhesion

Transient overexpression of 15-LO-1 in bovine aortic ECs increases tumor necrosis factor (TNF) induced vascular cell adhesion molecule 1 (VCAM-1) expression (Wölle *et al.,* 1996). Similarly, 15-LO-1 and its reaction product 15-HPETE induce adhesion molecule expression in ECs (Sultana *et al.,* 1996; Sordillo *et al.,* 2008) and 15-HPETE increases transendothelial migration of monocyte-like cells (Sultana *et al.,* 1996). Fibroblasts overexpressing 15-LO-1 generate bioactive, minimally oxidized LDL, which stimulates monocyte chemotaxis and adhesion to ECs (Sigari *et al.,* 1997; Lee *et al.,* 1999). In the presence of LDL, macrophage 12/15-LO induces monocyte adhesion to ECs by activating ECs and upregulating VCAM-1 expression (Huo *et al.,* 2004). Inhibition studies with short hairpin RNA to knock down the endogenous 12/15-LO expression in mouse macrophages and rat and mouse vascular SMCs have been shown to reduce oxidant stress, reduce chemokine and VCAM-1 expression, and reduce the monocyte adhesion to the SMCs (Li *et al.,* 2005).

Production of 12/15-LO metabolites is induced in diabetic *db/db* mice. Monocyte adhesion to ECs derived from these mice is increased. The increase in adhesion can be reduced by inhibiting 12/15-LO using either an adenovirus expressing a ribozyme to 12/15-LO or with the 12/15-LO inhibitor, cinnamyl-3,4-dihydroxy-α-cyanocinnamate. The adhesion is mediated by the interactions of monocyte integrins with endothelial VCAM-1, connecting segment 1 fibronectin and intercellular adhesion molecule 1 (ICAM-1) (Hatley *et al.,* 2003). Increased monocyte adhesion is mediated by these same factors also in ECs isolated from 12/15-LO transgenic mice, where ICAM-1 expression is induced severalfold (Reilly *et al.,* 2004) via activation of nuclear factor kappa B (NF-κB) by the 12/15-LO

reaction product 12(S)-HETE (Bolick *et al.,* 2005). On the contrary, in apolipoprotein E (apoE)/12/15-LO double knock-out mice NF-κB activation, ICAM-1 induction and monocyte adhesion to aortic endothelium are reduced as compared to the apoE knock-out control mice (Bolick *et al.,* 2006).

In vascular SMCs, 13-HPODE activates NF-κB as well as VCAM-1 transcription (Natarajan *et al.,* 2001). 12/15-LO overexpression in vascular SMCs induces VCAM-1 expression and the induction is prevented by inhibiting NF-κB (Dwarakanath *et al.,* 2008). However, in another study it has been shown that both PHGPX and 15-LO-1 overexpressions inhibit basal and IL-1 induced VCAM-1 expression. It has been suggested that constitutive overproduction of hydroperoxides can inhibit VCAM-1 expression by rendering the cells refractory to IL-1 stimulation, possibly by oxidizing protein thiols of the signalling system (Banning *et al.,* 2004). Overexpression of 12/15-LO can enhance growth factor induced binding of monocytes to vascular SMCs, whereas monocyte binding is reduced to vascular SMCs derived from 12/15-LO knockout mice (Cai *et al.,* 2004).

15-LO-1 expression in atherosclerotic lesions

15-LO reaction products are produced by atherosclerotic rabbit aortas when incubated with AA (Henriksson *et al.,* 1985; Simon *et al.,* 1989; Hugou *et al.,* 1995). mRNA and protein expression of 15-LO-1 are detected in rabbit and human atherosclerotic lesion macrophages colocalizing with oxidatively modified lipid products (Ylä-Herttuala *et al.,* 1990; Ylä-Herttuala *et al.,* 1991; Hiltunen *et al.,* 1995; Hugou *et al.,* 1995). 15-LO-1 mRNA expression is also detected in ECs and SMCs of rabbit atherosclerotic lesions (Ylä-Herttuala *et al.,* 1990; Hugou *et al.,* 1995). Specific 15-LO-1 oxidation products can be detected in lipids extracted from rabbit and human atherosclerotic lesions (Kühn *et al.,* 1994; Folcik *et al.,* 1995; Kühn *et al.,* 1997). The induction of 15-LO-1 mRNA and specific 15-LO-1 reaction products are most prominent in early atherosclerotic lesions (Kühn *et al.,* 1994; Hiltunen *et al.,* 1995; Kühn *et al.,* 1997). 15-LO-1 protein has also been detected in complex atheromatous lesions of transplant coronary artery disease (Ravalli *et al.,* 1995a; Ravalli *et al.,* 1995b).

In vivo gene transfer of 15-LO-1 into rabbit iliac arteries induces the appearance of lipid-protein adducts characteristic of oxidized LDL (Ylä-Herttuala *et al.,* 1995), whereas treatment with a specific 15-LO-1 inhibitor limits the progression of atherosclerosis in rabbits (Sendobry *et al.,* 1997; Bocan *et al.,* 1998).

Phenotypes of transgenic 15-LO-1 animals

LDL receptor (LDLR) knockout mice created by homologous recombination in embryonic stem cells (Ishibashi *et al.,* 1993) develop hypercholesterolemia and complex atherosclerotic lesions when fed with a high-fat diet (Ishibashi *et al.,* 1994). 15-LO-1 overexpression in LDLR-deficient mice under preproendothelin-1 promoter accelerates early atherosclerosis. These $LDLR^{-/-}$/15-LO transgenic mice express 15-LO-1 protein in aortic sinuses and the induced 15-LO-1 enzyme activity is detected in trachea, heart and aorta. LDL isolated from these mice is more susceptible to oxidation than the LDL from the control LDLR-/- mice (Harats *et al.,* 2000). 12/15-LO transgenic mice also develop atherosclerosis (Reilly *et al.,* 2004).

In contrast, 15-LO-1 transgenic rabbits expressing 15-LO-1 specifically in macrophages (Shen *et al.,* 1995) have smaller atherosclerotic lesions than their

nontransgenic littermates (Shen *et al.,* 1996). 15-LO-1 transgenic mice have also been created in conjunction with the apoE knockout background. ApoE knockout mice were created by homologous recombination in embryonic stem cells (Piedrahita *et al.,* 1992; Plump *et al.,* 1992). These animals develop marked hypercholesterolemia and extensive atherosclerosis on both low and high fat diet (Plump *et al.,* 1992; Zhang *et al.,* 1992). Similarly to the transgenic rabbits, macrophage-specific overexpression of 15-LO-1 under scavenger receptor promoter in apoE knockout mouse background protects against the development of atherosclerosis in aorta (Merched *et al.,* 2008).

Phenotypes of 12/15-LO knock-out mice

12/15-LO knockout mice were created by homologous recombination in embryonic stem cells (Sun *et al.,* 1996). Atherogenesis is decreased in double-knockout mice created by crossbreeding the 12/15-LO knockout mice with apoE deficient mice (Cyrus *et al.,* 1999; Cyrus *et al.,* 2001; Zhao *et al.,* 2005; Tang *et al.,* 2008) or LDLR knockout mice (George *et al.,* 2001). Atherosclerosis is also reduced in triple-knockout mice created by crossbreeding the 12/15-LO knockout mice with Apobec$^{-/-}$/LDLR$^{-/-}$ double-knockouts (Zhao *et al.,* 2002), which is a mouse model of human familial hypercholesterolemia (Powell-Braxton *et al.,* 1998). Decreased lipid peroxidation and autoantibodies against oxidized LDL have been detected in the 12/15-LO-apoE double knockout mice (Cyrus *et al.,* 2001; Tang *et al.,* 2008).

The involvement of 12/15-LO in the development of atherosclerosis was examined in a study, where bone marrow cells from 12/15-LO-apoE double-knockout (12/15-LO$^{-/-}$/apoE$^{-/-}$) mice were transplanted into apoE$^{-/-}$ mice coferring protection from atherosclerosis, whereas conversely transplantation of apoE$^{-/-}$ bone marrow cells into 12/15-LO$^{-/-}$/apoE$^{-/-}$ mice resulted in the more severe development of atherosclerosis typically detected in apoE$^{-/-}$ mice (Huo *et al.,* 2004).

As with the transgenic mouse models, contradictory results about the role of 12/15-LO in atherogenesis have also been presented with 12/15-LO knockout mice. Lack of 12/15-LO accerelates atherosclerosis in 12/15-LO$^{-/-}$/apoE$^{-/-}$ double knockouts compared to the control apoE$^{-/-}$ mice (Merched *et al.,* 2008). In addition, opposing atheroprotective results have also been obtained with bone marrow cell transplantations. Transplantation of bone marrow cells from 12/15-LO$^{+/+}$/apoE$^{-/-}$ donors to 12/15-LO$^{-/-}$/apoE$^{-/-}$ double knockouts leads to smaller lesion size than transplantation of the 12/15-LO$^{-/-}$/apoE$^{-/-}$ bone marrow back to the original 12/15-LO$^{-/-}$/apoE$^{-/-}$ donor. This is considered to reflect an atheroprotective effect of 12/15-LO, which is believed to be mediated by local biosynthesis of lipid mediators, including lipoxin A_4, resolvin D1 and protectin D1. These mediators can resolve the local inflammatory response on macrophages and vascular ECs (Merched *et al.,* 2008).

15-LO-1 and angiogenesis

Very little is known about the effects of 15-LO-1 on angiogenesis. Both anti- and pro-angiogenic roles for 15-LO-1 have been presented.

Transgenic mice overexpressing 15-LO-1 in the vascular wall under the preproendothelin-1 promoter (Harats *et al.,* 1995) have been used to test the effects of the vascular overexpression of 15-LO-1. In the Lewis lung carcinoma model, the lung metastases of the 15-LO-1 transgenic mice contain an extensive hemorrhagic necrotic core, a higher number of apoptotic cells and also multiple small blood vessels arranged in

a complicated network (Harats *et al.,* 2005). Recently, an anti-angiogenic 15-LO-1 metabolite, 15-oxo-eicosatetraenoic acid, has been identified. 15-Oxo-eicosatetraenoic acid inhibits EC proliferation by suppressing deoxyribonucleic acid (DNA) synthesis (Wei *et al.,* 2009).

Hypoxia studies with human retinal microvascular ECs have shown that hypoxia can induce 15-LO-1 expression and the production of 15(S)-HETE, which in turn stimulates EC migration and tube formation (Bajpai *et al.,* 2007). PC-3 prostate cancer cells stably overexpressing 15-LO-1 have been generated by plasmid transfection and selection for antibiotic resistance. These cells have elevated VEGF secretion and increased proliferation compared with the controls (Kelavkar *et al.,* 2001).

15-LO-1, apoptosis and tumorigenesis

The selenoenzyme thioredoxin reductase is an important regulator of the redox balance within cells (Arnér and Holmgren, 2000). 15-LO-1 metabolites inhibit thioredoxin reductase, cause cell cycle arrest at the G_1 phase and induce the pro-apoptotic BAX protein (Yu *et al.,* 2004). Depletion of PHGPX, the only known glutathione peroxidase capable of reducing phospholipid hydroperoxides (Thomas *et al.,* 1990a; Thomas *et al.,* 1990b) results in early embryonic lethality (Yant *et al.,* 2003). In order to study the molecular and cellular mechanisms of PHGPX, a conditional, inducible PHGPX knockout mouse has been created. Depletion of PHGPX in these mice causes massive lipid peroxidation and cell death. These events require functional 12/15-LO and are mediated by apoptosis-inducing factor (Seiler *et al.,* 2008).

13-HODE and enzymatic modification of LDL with 15-LO-1 induce apoptosis in monocytic cells (Jostarndt *et al.,* 2002), and enhanced 15-HPETE production or induced 15-LO-1 expression increases apoptosis (Sordillo *et al.,* 2005; Kim *et al.,* 2006) by activation of caspase-3 and -9 (Sordillo *et al.,* 2005). Overexpression of 15-LO-1 in the vascular endothelium of LDLR knock-out mice is associated with increased thymic apoptosis, which is mediated by caspase-3 (Afek *et al.,* 2004).

Rather conflicting results have been obtained from the cancer studies, with evidence for either pro- or anti-apoptotic, and pro- or anti-tumorigenic role for 15-LO-1, depending on the tumor and tissue type. Transgenic mice overexpressing 15-LO-1 in the vascular wall under the preproendothelin-1 promoter (Harats *et al.,* 1995) have been used in two different tumor models to test the effects of the vascular overexpression of 15-LO-1. 15-LO-1 significantly reduces the growth of lung metastases in the Lewis lung carcinoma model and significantly increases the survival of the animals. In a mouse mammary adenocarcinoma model, the development of primary tumors is prevented in the 15-LO-1 transgenic mice (Harats *et al.,* 2005).

The evidence points to strikingly different roles for 15-LO-1 in colorectal and prostate cancer. The studies implying an anti-tumorigenic role in colorectal cancer and a pro-tumorigenic role in prostate cancer will be reviewed in detail in the following chapters.

15-LO-1 in colorectal cancer

15-LO-1 expression is decreased in colorectal cancer cell lines (Shureiqi *et al.,* 1999). The 15-LO-1 products 13-HODE and 15-HETE can suppress cell proliferation and induce apoptosis in colorectal cancer cell lines *in vitro* (Shureiqi *et al.,* 1999; Chen *et al.,* 2003; Nixon *et al.,* 2004), as does also ectopic restoration of 15-LO-1 activity (Shureiqi *et al.,* 2005).

The earliest report about 15-LO-1 expression in human colorectal cancer tissues stated that 15-LO-1 is highly expressed in colorectal carcinoma epithelial cells (Ikawa *et al.,* 1999). However, further studies have indicated that 15-LO-1 expression and 13-HODE and 15-HETE production are decreased in human colon cancers (Shureiqi *et al.,* 1999; Chen *et al.,* 2003; Heslin *et al.,* 2005; Shureiqi *et al.,* 2005; Zuo *et al.,* 2009), and the reduced 15-LO-1 expression is associated with poorer overall survival in the patients with stage IV colorectal cancer (Heslin *et al.,* 2005). It has been suggested that reversal of expression of 15-LO-1 to cyclooxygenase-2 is associated with the development of colon cancer. 15-LO-1 mRNA expression is mostly detected in low-grade adenomas and not found in advanced carcinoma, whereas cyclooxygenase-2 protein production is detected mostly in invasive carcinomas. Significant concurrent down-regulation of 15-LO-1 and up-regulation of cyclooxygenase-2 are detected in invasive carcinomas (Yuri *et al.,* 2007).

In the athymic nude mouse xenograft model, subcutaneously transplanted tumors derived from 15-LO-1 overexpressing HCT-116 colorectal carcinoma cells are smaller than tumors derived from the control HCT-116 cells (Nixon *et al.,* 2004). Adenoviral 15-LO-1 gene transfer induces a therapeutic effect in colon cancer. 15-LO-1 expression downregulates the expression of anti-apoptotic proteins, activates caspase-3, induces apoptosis, and inhibits colon cancer cell survival *in vitro*. Local 15-LO-1 adenovirus injections into subcutaneously transplanted colon cancer xenografts *in vivo* in nude mice are able to inhibit the growth of the xenografts (Wu *et al.,* 2008).

Induction by non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAID), potential chemopreventive agents, have been shown to stimulate the 15-LO-1 pathway in human polymorphonuclear leukocytes (Vanderhoek *et al.,* 1984). NSAIDs also induce 15-LO-1 protein expression and enzymatic activity in colorectal cancer cell lines. This is associated with reduced cell growth and increased cell apoptosis (Shureiqi *et al.,* 2000a; Shureiqi *et al.,* 2000b; Heslin *et al.,* 2005). These effects are prevented by inhibiting 15-LO-1 and restored by adding exogenous 15-LO-1 reaction product 13-HODE (Shureiqi *et al.,* 2000a; Shureiqi *et al.,* 2000b). In addition, NSAID-induced apoptosis is increased in colorectal carcinoma cells overexpressing 15-LO-1 or treated with 13(S)-HODE (Yoshinaga *et al.,* 2007).

NSAIDs induce 15-LO-1 expression and concomitant apoptosis in colorectal carcinoma cell lines at the transcriptional level by down-regulating the expression of GATA-6, a transcription factor that suppresses 15-LO-1 expression (Shureiqi *et al.,* 2002). GATA-6 mRNA and protein expression are induced in colon cancer and inhibition of GATA-6 expression combined with NSAID restores 15-LO-1 expression and induces apoptosis in colon cancer cells (Shureiqi *et al.,* 2007). Participants are currently being recruited to a phase II clinical trial, where the primary objective is to determine whether the NSAID celecoxib can downregulate GATA-6 expression to upregulate 15-LO-1 expression and to induce apoptosis in human rectal tumors (U. S. National Institutes of Health, http://www.clinicaltrials.gov, accessed October 2009).

Induction by histone deacetylase inhibitors

Histone deacetylases (HDAC) are important epigenetic regulators of gene expression. They mediate changes in nucleosome conformation by deacetylating histones, which leads to chromatin compaction and transcriptional repression. HDAC inhibitors are involved in growth arrest, cell differentiation, cytotoxicity and induction of apoptosis (de Ruijter *et al.,* 2003), and have therefore emerged as potential therapeutic agents in the treatment of cancer (Marks *et al.,* 2001). Currently, there are almost a hundred clinical trials listed for the treatment of different cancers with HDAC inhibitors (U. S. National Institutes of Health, http://www.clinicaltrials.gov, accessed October 2009).

Interestingly, HDAC inhibitors induce 15-LO-1 expression in colorectal carcinoma cell lines by stimulating 15-LO-1 promoter activity (Kamitani *et al.,* 1998; Kamitani *et al.,* 2001; Hsi *et al.,* 2004; Shureiqi *et al.,* 2005; Zuo *et al.,* 2008a; Zuo *et al.,* 2009). The induced 15-LO-1 expression correlates with 13(S)-HODE production, growth inhibition, differentiation and apoptosis in these cell lines (Kamitani *et al.,* 1998; Hsi *et al.,* 2004; Shureiqi *et al.,* 2005; Zuo *et al.,* 2009).

As with NSAIDs, down-regulation of GATA-6 expression combined with an HDAC inhibitor restores 15-LO-1 expression and induces apoptosis in colon cancer cell lines (Shureiqi *et al.,* 2007). Histone acetylation correlates with 15-LO-1 expression both in colorectal carcinoma cells and in colorectal tissue suggesting a role for histone acetylation in the regulation of 15-LO-1 expression in colorectal carcinoma (Kamitani *et al.,* 2001). The acetylation of histones H3 and H4 by histone acetyltransferase KAT3B is critical for 15-LO-1 transcriptional activation, independently from STAT6 (Zuo *et al.,* 2008a). A recent report has shown that recruitment of the nucleosome remodelling and histone deacetylation repression complex to 15-LO-1 promoter can repress 15-LO-1 transcription, whereas a specific HDAC1 and HDAC2 inhibitor restores transcription. Transcriptional downregulation of the essential repression complex components induces 15-LO-1 transcription (Zuo *et al.,* 2009).

Induction by methyltransferase inhibitors

In addition to histone deacetylation, DNA methylation is another important epigenetic mechanism involved in regulating gene expression (Jones *et al.,* 2001). Hypermethylation of tumor suppressor genes by DNA methyltransferases is often encountered in many human cancers (Mund *et al.,* 2006). Similarly to NSAIDs and HDAC inhibitors, also methyltransferase inhibitors induce 15-LO-1 expression in colorectal cancer cell lines and this leads to increased 13(S)-HODE production, growth inhibition and apoptosis (Hsi *et al.,* 2005). A specific 15-LO-1 promoter demethylation is necessary for HDAC inhibitor mediated activation of 15-LO-1 transcription (Zuo *et al.,* 2008a). Transcriptional activation of 15-LO-1 by HDAC inhibitor can be suppressed by DNA methyltransferase 1 binding to the 15-LO-1 promoter without affecting promoter methylation (Zuo *et al.,* 2008b).

Role of peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors that have been linked to adipocyte differentiation (Chawla *et al.,* 1994) and the control of cellular lipid uptake (Tontonoz *et al.,* 1998). The 15-LO-1 products 13-HODE and 15-HETE are endogenous activators and ligands of PPAR-γ (Nagy *et al.,* 1998; Huang *et al.,* 1999b). Treatment of colon cancer cells

with 15(S)-HETE inhibits cell proliferation and viability and induces apoptosis via a PPAR-γ dependent pathway (Chen *et al.,* 2003).

PPAR-δ deficiency decreases colon tumorigenesis (Park *et al.,* 2001) and NSAIDs suppress the activity of PPAR-δ in colon cancer cells (He *et al.,* 1999a). 13-HODE binds to PPAR-δ leading to decreased expression and activation of PPAR-δ in colorectal cancer cells. Induction of 15-LO-1 is a critical step in the NSAID mediated suppression of PPAR-δ activity and the resultant induction of apoptosis. The *in vivo* relevance of this mechanism has been demonstrated in nude mouse xenograft model, where NSAID reduced the tumor growth and down-regulated PPAR-δ protein expression, but these effects were prevented, when 15-LO-1 expression was blocked (Shureiqi *et al.,* 2003).

The interplay between 15-LO-1 and PPARs β, δ and γ has been further evaluated in colon cancer cells. The 15-LO-1 metabolite 13(S)-HODE mediates the activation of PPAR-γ by downregulating PPARs β and δ, resulting in the induction of apoptosis (Zuo *et al.,* 2006).

15-LO-1 in prostate cancer

Human prostate tumors and prostate cancer cell lines express 15-LO-1 and produce the 15-LO-1 metabolite 13-HODE (Spindler *et al.,* 1997; Kelavkar *et al.,* 2000). 15-LO-1 mRNA expression is significantly higher in human prostate cancer tissue when compared to normal prostate tissue (Kelavkar *et al.,* 2006a). The intensity of 15-LO-1 immunostaining correlates with the degree of malignancy (Kelavkar *et al.,* 2000). Similarly, expression and activity of 12/15-LO are induced in transgenic mouse models of prostate carcinoma, and the expression increases with the progression of the disease (Shappell *et al.,* 2003; Kelavkar *et al.,* 2004). In the FLiMP mouse model, where 15-LO-1 is expressed in the luminal epithelial cells of adult mice, the conditional expression of 15-LO-1 is sufficient to promote epithelial proliferation and prostatic intraepithelial neoplasia (Kelavkar *et al.,* 2006b). The same effect is also seen with adenovirus mediated 15-LO-1 gene transfer into mouse prostate tissue (Sen *et al.,* 2006). Stable overexpression of 15-LO-1 in human prostate cancer cells increases cell proliferation and subcutaneous inoculation of the 15-LO-1 overexpressing cells into nude mice evokes an increase in the frequency of tumor formation, tumor size and tumor angiogenesis suggesting a growth promoting role for 15-LO-1 in the prostate (Kelavkar *et al.,* 2001).

Epigenetic regulation for 15-LO-1 expression has also been shown in prostate cancer, where hypermethylation of a specific CpG dinucleotide in the 15-LO-1 promoter leads to upregulation of 15-LO-1 expression and enzyme activity and contributes to prostate cancer initiation and progression. In contrast to the situation in colon cancer cells, combination treatment with DNA methyltransferase inhibitor and HDAC inhibitor reduces 15-LO-1 expression in prostate cancer cells (Kelavkar *et al.,* 2007).

STAT6, a transcriptional activator of 15-LO-1, is highly expressed in prostate cancer tissues. Down-regulation of STAT6 in prostate cancer cell lines *in vitro* leads to decreased 15-LO-1 expression, induction of apoptosis, inhibition of cell migration, reduced cell viability and transcriptional down-regulation of anti-apoptotic Bcl-X_L protein (Das *et al.,* 2007).

VIRAL GENE TRANSFER VECTORS

Viruses have been exploited in gene expression studies and applied to gene therapy due to their natural capabilities for transferring genetic material into eukaryotic cells. The viral vectors used in gene therapy are generally modified in such a way that they lose their ability to multiply in all other cells except genetically modified packaging cells, which are used for the production of the replication-incompetent recombinant viruses.

The first viral vectors used for gene therapy were retroviral vectors based on murine leukemia virus (oncoretrovirus). Currently, the adenoviral vectors have become the most widely used virus derived vectors (**Figure 5**). In addition to retro- and adenoviral vectors, several other virus-derived gene transfer vectors have been developed. They are reviewed in the following chapters and summarized in **Table II**.

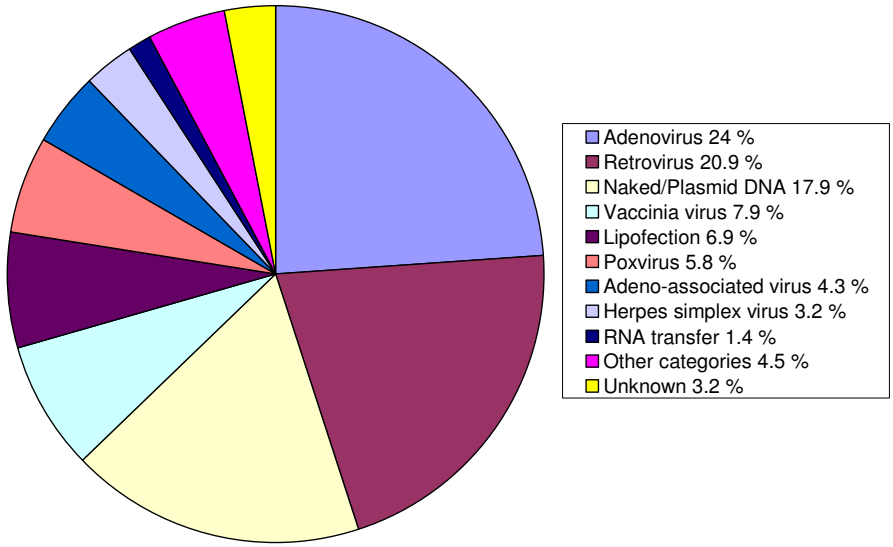

Figure 5. **Vectors used in gene therapy trials.** According to Journal of Gene Medicine, http://www.wiley.co.uk/genmed/clinical, accessed October 2009.

Table II. Summary of viral gene transfer vectors (Varmus, 1988; Buchschacher, 2001; Goff, 2001; McConnell *et al.,* 2004; Sinn *et al.,* 2005; Büning *et al.,* 2008; Daya *et al.,* 2008; Airenne *et al.,*2009). kb = kilobase

Viral vector	Particle size	Insert size	Localization	Expression	Transduction of non-dividing cells	Benefits	Drawbacks
Oncoretrovirus	100 nm	8 kb	Random integration	Stable	No	Stable expression	Risk of replication competent virus production and insertional mutagenesis, low transduction efficiency
Adenovirus	70-90 nm	5-8 kb for first-generation vectors Up to 37 kb with gutless vectors	Mainly episomal	Transient	Yes	Easy to produce in high titers	Immune response, inflammation, risk of replication competent virus production
Adeno-asociated virus	25 nm	< 5 kb, up to 9 kb by *trans*-splicing	Mainly episomal	Stable, mainly long term episomal, integration rarely	Yes	Non-pathogenic, low immunogenicity	Small insert size, difficulties in large-scale and helper virus free production, risk of insertional mutagenesis
Lentivirus	100 nm	9 kb	Integration	Stable	Yes	Stable expression	Risk of insertional mutagenesis
Baculovirus	250-300 nm	>100 kb	Non-integrating	Transient	Yes	Non-pathogenic, easy to produce in high titers	Low distribution

Retrovirus vectors

Oncoretroviruses

Retrovirus particles consist of a viral protein core, which is surrounded by an envelope made up of cellular membrane-derived lipid bilayer and viral-encoded glycoproteins. Oncoretroviruses have two copies of a single stranded RNA genome, which contains genes *gag*, *pol* and *env* encoding the viral structural and catalytic proteins. *Gag* encodes the viral core proteins (matrix, capsid and nucleocapsid), *pol* encodes the viral replication enzymes (protease, reverse transcriptase and integrase) and *env* encodes the envelope glycoprotein. During virus replication, the RNA is reverse transcribed into an intermediate double stranded DNA, which integrates randomly into the target cell DNA as a provirus having long terminal repeats (LTR) at each end of the proviral genome. The 5' LTR contains the viral promoter and enhancers, whereas the 3' LTR contains the transcription termination signal. During the virus replication cycle, full-length transcripts of the viral genome, which contain the packaging signal, are packaged into virus particles assembled from the newly synthesized structural proteins. The virus particles bud off from the surface of the target cell and their tropism depends on which glycoproteins they carry in their envelope (Varmus, 1988; Sinn *et al.,* 2005).

Oncoretroviral vectors encode the gene of interest between the viral LTR's, whereas the viral structural proteins are supplied by a packaging cell line stably expressing these genes. The resulting recombinant oncoretroviruses carry and integrate the gene of interest into the target cell genome leading to stable expression of the transgene, but without further virus propagation (Buchschacher, 2001).

Retroviral vectors derived from oncoretroviruses are able to transduce only dividing cells, since they lack a nuclear transport function (Lewis *et al.,* 1994). The most important limitations of oncoretroviral vectors in gene therapy are their limited transduction efficiency and the potential production of replication competent viruses, as well as the possibility of integration into important genome sites, which could lead to deleterious effects, e.g. inactivation of a tumor suppressor or activation of an oncogene. (Sinn *et al.,* 2005).

Lentiviruses

Lentiviruses belong to the class of retroviruses, but they have a more complex genome than the simple oncoretroviruses. Due to their genome complexity, lentiviruses have some benefits over the oncoretroviruses with regard to gene therapy. Unlike oncoretroviruses, lentiviruses can transduce also non-dividing cells. The most widely used lentivirus vectors are based on human immunodeficiency virus 1, due to the fact that the structure and biology of this virus have been extensively studied ever since acquired immunodeficiency syndrome was found to be caused by this virus (Varmus, 1988; Sinn *et al.,* 2005).

In order to prevent the formation of replication competent retroviruses, the lentiviral vectors have been extensively modified. The lentiviral vector plasmids contain the transgene expression cassette and a minimal amount of viral sequences, i.e sequences required for the transfer and expression of the transgene into target cells. Virus sequences required for the packaging of viral particles are supplied by additional plasmids (Sinn *et al.,* 2005).

Adenovirus vectors

Adenoviruses cause mild respiratory, gastrointestinal, and ocular diseases. There are more than 50 human adenovirus serotypes, of which the most widely used in gene therapy studies are serotypes 2 and 5.

Adenoviruses are non-enveloped icosahedral viruses about 70-90 nm in diameter. They have a protein coat, which shields the inner nucleoprotein core containing a linear double-stranded DNA genome of approximately 36 kb. The major structural protein of the capsid is a trimeric hexon protein. The vertices of the capsid consist of penton bases, which anchor the fiber proteins, whose knobs are the primary protein moieties responsible for the attachment of the virus to the coxsackievirus B and adenovirus receptor on the host cell. Cell surface integrins binding to the penton base mediate the internalization of the virus by receptor-mediated endocytosis.

The inverted terminal repeats (ITR) at each end of the viral genome serve as the origins of DNA replication. The adenovirus genome is divided into early and late genes, referring to genes that are transcribed early, i.e. before viral DNA replication, and genes that are transcribed late, i.e. after the onset of viral DNA replication.

Adenoviruses are able to transduce a wide variety of eukaryotic cells, both quiescent and actively dividing. However, the expression of the therapeutic gene is transient, since adenoviral genome does not integrate into the host cell genome, but remains extrachromosomal. The most important limitations of adenoviruses in gene therapy are the immune response and inflammatory reactions they cause in their host cells (McConnell *et al.,* 2004; Goncalves *et al.,* 2006).

Adeno-associated virus vectors

Native adeno-associated viruses (AAV) are small (25 nm) single-stranded DNA viruses of the parvovirus family. Their genome of approximately 5 kb is packaged into an icosahedral, non-enveloped capsid and it contains two open reading frames between ITRs. The *rep* open reading frame codes for a family of multifunctional non-structural proteins (Rep) involved in viral genome replication, transcriptional control, integration and encapsidation of AAV genomes into preformed capsids. The *cap* open reading frame codes for the three capsid proteins VP1, VP2 and VP3, which differ in their N-terminus and thus define the specificity towards cellular receptors and the tissue tropism of the different AAV serotypes. The ITRs serve as origin of replication and play a key role in viral integration into the host cell genome (Büning *et al.,* 2008; Daya *et al.,* 2008). Most recombinant AAV vectors are based on AAV-2, which enters the host cell by using the receptor heparan sulphate proteoglycan. The extent of internalization is enhanced by several coreceptors (Daya *et al.,* 2008).

The most important benefits of AAVs as gene therapy vectors are their non-pathogenicity to humans and fairly low immunogenicity. AAV vectors are able to transduce both dividing and quiescent cells and even though they remain extrachromosomal, long-term gene expression is achieved. The major drawback of the AAV vectors for gene therapy purposes is their cumbersome production procedure, which is extremely difficult to scale up. In addition, since their productive infection requires the presence of a helper virus, production of helper virus free recombinant AAVs is difficult (Büning *et al.,* 2008; Daya *et al.,* 2008).

Baculovirus vectors

Baculoviruses are large, rod-shaped insect viruses with a double-stranded circular DNA genome. Due to their large packaging capacity, they have been widely used in biotechnology for protein production in insect cells. Baculoviruses are non-pathogenic to humans and efficiently transduce both dividing and quiescent cells. They can achieve transient gene expression, since they do not integrate into the host genome. Their construction and production in large quantities are relatively easy, and due to their large genome size, they can accommodate large transgenes (Airenne *et al.,* 2009).

AIMS OF THE STUDY

The aims of the study were to explore the role of 15-LO-1 in the development of atherosclerosis, and in angiogenesis and tumorigenesis, by using retro- and adenovirus mediated gene transfer techniques both *in vitro* and *in vivo*.

In more detail, the specific aims of the study were:

I Create eukaryotic cells over-expressing 15-LO-1 by retro- and adenovirus mediated gene transfer methods *in vitro*, study the mRNA and protein expression as well as biological activity of 15-LO-1 in the transduced cells, and examine what kinds of gene expression changes are induced by 15-LO-1 over-expression.

II Study the effects of 15-LO-1 on VEGF family mediated induction of angiogenesis by adenovirus mediated intramuscular gene transfer into rabbit hindlimb skeletal muscle.

III Investigate the effects of 15-LO-1 in VEGF-A_{165} mediated induction of angiogenesis in a rabbit eye model by intravitreous adenovirus mediated gene transfer.

IV Examine the effects of 15-LO-1 overexpression in mouse by adenovirus mediated gene transfer via the tail vein and the effects of 15-LO-1 in a rat malignant glioma model.

MATERIALS AND METHODS

CELL CULTURE (I-IV)

The cell lines and culture conditions used in this study are summarized in **Table III**.

PRODUCTION OF RETROVIRUSES AND RETROVIRAL TRANSDUCTIONS (I)

Transfections and transductions for retrovirus production were performed as described (Ausubel *et al.,* 1992). Retroviral vectors pLZRNL (Sharkey *et al.,* 1990; Ylä-Herttuala *et al.,* 1995) and pLLORNL (Benz *et al.,* 1995; Ylä-Herttuala *et al.,* 1995) code for the *Escherichia coli* β-galactosidase (Kalnins *et al.,* 1983) and human 15-LO-1 (Sigal *et al.,* 1988), respectively, under the LTR of Moloney murine leukemia virus. The vectors also contain Tn5 neomycin phosphotransferase gene expressed from an internal Rous sarcoma virus promoter for selection of clones resistant to the neomycin analog geneticin. Ecotropic retroviruses were produced in the Ψ2 packaging cell line by calcium phosphate transfection, concentrated by polyethylene glycol precipitation or by centrifugation, and used to transduce the amphotropic PA317 packaging cells. Stably transduced geneticin-resistant PA317 clones were isolated, expanded and analyzed for 15-LO-1 mRNA production, protein expression and enzymatic activity. Concentrated amphotropic retroviruses from chosen clones were used to transduce ECV304 cells.

PRODUCTION OF ADENOVIRUSES (II-IV)

Human 15-LO-1 cDNA (Sigal *et al.,* 1988) was digested at 2245 base pairs (bp) with BglII to dissect out part of the 3' UTR and the polyA signal. This shortened cDNA fragment was subcloned into an expression vector containing human cytomegalovirus (CMV) immediate early I promoter and intron A with splicing signals, and bovine growth hormone transcription termination signals and polyA region. The expression cassette was subsequently subcloned into the BglII-site in the pAdBglII-vector (Barr *et al.,* 1994) to produce pAd15-LO-1 adenovirus vector. All recombinant adenoviruses used in this study and summarized in **Table IV** were produced in 293 cells by homologous recombination (Kozarsky *et al.,* 1993).

ADENOVIRAL TRANSDUCTIONS *IN VITRO* (II-IV)

In vitro adenoviral transductions were performed for rabbit abdominal aortic smooth muscle cells (RAASMC), primary human umbilical vein endothelial cells (HUVEC), ECV304 cells and RAW264.7 mouse macrophages. Transductions were carried out under serum free conditions for 1 h.

ADENOVIRAL GENE TRANSFERS *IN VIVO* (II-IV)

All animal experiments described in the following sections were approved by the Experimental Animal Committee, University of Kuopio.

Table III. Cell lines and culture conditions.

Cell line	Description	Source	Culture conditions	Publication
Ψ2	Ecotropic mouse fibroblast packaging cell line for recombinant retroviruses	Mann *et al.,* 1983	Dulbecco's Modified Eagle's Medium (DMEM), 5 % newborn calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 400 µg/ml geneticin for selection	I
PA317	Amphotropic mouse fibroblast packaging cell line for recombinant retroviruses	The American Type Culture Collection (ATCC) CRL-9078	DMEM, 5 % newborn calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 400 µg/ml geneticin for selection	I
ECV304	Originally documented as a spontaneously transformed human endothelial cell line, but later reported to be a variant of the human bladder cancer line T-24 derived by cross-contamination	ATCC CRL-1998	Medium M199, 10 % fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 600 µg/ml geneticin for selection	I, III
Jurkat T clone E6-1	T lymphocytes	ATCC TIB-152	RPMI1640, 10 % FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 110 mg/l sodium puryvate, 2 mM L-glutamine	I
293	Human embryonic kidney cell line containing the left end of human adenovirus 5; packaging cell line for recombinant adenoviruses	ATCC CRL-1573	DMEM, 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin	II-IV
RAASMC	Rabbit abdominal aortic SMCs	Isolated from New Zealand White rabbit aortas according to Pietilä *et al.,* 1980	DMEM, 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin	II
HUVEC	Human umbilical vein ECs	Primary cells isolated from human umbilical cords obtained from Kuopio University Hospital maternity ward	EBM Endothelial Cell Basal Medium supplied with EGM SingleQuots	II
RAW264.7	Mouse macrophage cell line	ATCC TIB-71	RPMI1640, 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine	IV
BT4C	Rat glioma cell line	a gift from Dr. Rolf Bjerkvig (Bergen, Norway)	DMEM, 10 % FBS, 50 µg/mL gentamicin, 2 mM sodium pyruvate, 2 mM glutamine	IV

Table IV. Recombinant adenoviruses used in the study.

Vector	Description	Application	Publication
AdlacZ	Recombinant adenovirus containing *E. coli* β-galactosidase under CMV enhancer and chicken β-actin promoter	*in vitro*, RAASMC and HUVEC; *in vivo*, intramuscular, rabbit hind limb skeletal muscle and the ischemia model	II
		in vitro, ECV304	III
		in vitro, RAW264.7; *in vivo*, mouse tail vein	IV
Adh15-LO-1	Recombinant adenovirus containing human 15-LO-1 under CMV promoter/enhancer with splice signals	*in vitro*, RAASMC and HUVEC; *in vivo*, intramuscular, rabbit hind limb skeletal muscle and the ischemia model	II
		in vitro, ECV304; *in vivo*, rabbit intravitreous	III
		in vitro, RAW264.7; *in vivo*, mouse tail vein and rat glioma model	IV
AdhVEGF-A_{165}	Recombinant adenovirus containing human VEGF-A_{165} under CMV promoter	*in vitro*, RAASMC and HUVEC; *in vivo*, intramuscular, rabbit hind limb skeletal muscle and the ischemia model	II
		in vivo, rabbit intravitreous	III
AdmPlGF-2	Recombinant adenovirus containing mouse PlGF-2 under CMV promoter	*in vivo*, intramuscular, rabbit hind limb skeletal muscle	II
AdhVEGF-DΔNΔC	Recombinant adenovirus containing mouse interleukin 3 signal sequence, flag-tag and the short mature form of human VEGF-D (VEGF-DΔNΔC) under CMV promoter	*in vivo*, intramuscular, rabbit hind limb skeletal muscle	unpublished
		in vivo, rabbit intravitreous	unpublished
AdCMV	Recombinant adenovirus containing CMV promoter without any insert	*in vivo*, rabbit intravitreous	III

Rabbit hind limb skeletal muscle (II)

Intramuscular injections of 1.0 x 10^{11} virus particles (vp) of each recombinant adenovirus (AdlacZ, Adh15-LO-1, AdhVEGF-A_{165}, AdmPlGF-2, AdhVEGF-DΔNΔC, and combinations of Adh15-LO-1 with either AdhVEGF-A_{165}, AdmPlGF-2 or AdhVEGF-DΔNΔC [combination

groups]) in a total volume of 1 ml divided into ten separate 100 µl injections were performed into the semimembranosus thigh muscles. The animals were sacrificed six days after gene transfer, when the maximal gene transfer effects are known to be present (Rissanen *et al.,* 2003a).

Rabbit hind limb ischemia model (II)

Intramuscular injections of Adh15-LO-1 and AdlacZ (1.0 x 10^{11} vp/ml) were performed into rectus femoris (5 x 100 µl), gastrocnemius (5 x 100 µl) and tibialis anterior (5 x 100 µl) muscles. Three days after the gene transfers unilateral ischemia was induced by surgically removing the superficial femoral artery and ligating the deep femoral and lateral femoral circumflex arteries (Rissanen *et al.,* 2002). The animals were sacrificed three days after the operations and samples were taken for immunohistochemistry.

Rabbit intravitreal gene transfers (III)

Intravitreal gene transfers were performed by injecting a total of 1.0 x 10^{11} vp (AdCMV, Adh15-LO-1, AdhVEGF-A_{165} or AdhVEGF-DΔNΔC, and a combination of Adh15-LO-1 with AdhVEGF-A_{165} or AdhVEGF-DΔNΔC [treatment group]) in a total volume of 100 µl (Kinnunen *et al.,* 2006).

Rabbit eyes were photographed before the gene transfers and one day before sacrifice with a digital fundus camera (Zeiss, FF450 PLUS IR, Jena, Germany). Photographs were taken from the anterior parts. Funduses were evaluated by a slit lamp and ophthalmoscopy and photographed after dilation of the pupils with one drop of tropicamid (5 mg/ml) and phenylephrine hydrochloride (100 mg/ml).

The animals were sacrificed six days after the gene transfers, when the concentration of the transduced human VEGF-A protein is maximal in the vitreous humour (Kinnunen *et al.,* 2006).

Rat glioma model (IV)

Rat glioma BT4C cells (a gift from Dr. Rolf Bjerkvig, Bergen, Norway) were obtained from fetal BDIX rats exposed to N-ethylnitrosourea (Sandmair *et al.,* 1999). Syngeneic BDIX male rats (Charles River Laboratories International, Inc., Wilmington, MA, USA) were injected with 10^4 BT4C cells resuspended in 10 µl of Optimem (GIBCO-BRL, Gaithersburg, MD, USA) to a depth of 2.5 mm into the right corpus callosum (Sandmair *et al.,* 1999). The rats were divided into two cohorts: group I, which received Adh15-LO-1 gene therapy (n=12) and group II, which was a control group (n=5) and did not receive any treatment. Tumor growth was verified 14 days after cell injection by magnetic resonance imaging (MRI). On the next day, the animals received Adh15-LO-1 (2.7 x 10^{10} plaque forming units/ml) injection to a depth of 1.5, 2.0 and 2.5 mm (2.5 µl per site), followed by two additional injections applied on the following day (at 2 mm depth at an angle, anteriorly and posteriorly, made by flexing the C-arm of the microinjection unit). Tumor growth was assessed by MRI again two and four weeks after the treatment.

Those animals that did not exhibit any signs of a tumor on the first MRI, as well as those that had shown an extra-cranial tumor growth, were excluded from the study. The rest of the animals were used in the survival studies. Animals were sacrificed upon human endpoints. Survival was calculated in days from the day of BT4C cell inoculation.

TISSUE PROCESSING (II-IV)

Rabbit muscle samples (II)

Rabbits were perfusion fixed with 1 % paraformaldehyde (PFA) in 0.05 M citrate buffer (pH 3.5). Muscle tissue samples were immersion fixed in 4 % PFA/7.5 % sucrose (pH 7.4) for 4 h, rinsed in 15 % sucrose (pH 7.4), embedded in paraffin and cut into paraffin sections (Rissanen *et al.,* 2003a). Another set of animals was used to obtain snap frozen, fresh muscle tissue samples for RNA, protein, and enzyme activity analyses. In addition, muscle samples were snap frozen in isopenthane and processed into frozen sections.

Rabbit eye samples (II)

Vitreous samples collected from each animal were snap frozen in liquid nitrogen and stored at -70 °C until analyzed. One set of animals was perfusion fixed with 1 % PFA in 0.05 M citrate buffer (pH 3.5) (Rissanen *et al.,* 2003a). The eyes were enucleated and cut in half. The half containing the optic nerve was immersion fixed in 4 % PFA/7.5 % sucrose (pH 7.4) for 4 h, rinsed in 15 % sucrose (pH 7.4) overnight and embedded in paraffin (Kinnunen *et al.,* 2006).

Another set of animals was sacrificed without perfusion fixation. The eyes were enucleated and cut in half from the side of the optic nerve. The half containing the optic nerve was embedded in 33 % optimal cutting temperature (OCT) compound and processed for frozen sections. The other half was cut into longitudinal segments, which contained parts from the back of the eye all the way to the cornea, i.e. parts of the outer layer (sclera and cornea), the middle layer (choroid, ciliary body and the iris) and the innermost layer (the retina). The segments were snap frozen in liquid nitrogen and stored at -70 °C for RNA analysis.

A third set of animals was sacrificed without perfusion fixation, the superior part of the eyes was identified for orientation, the eyes were enucleated, and the retinal whole-mounts were detached from the sclera and were post-fixed for 24 h in the same fixative solution.

Mouse tissue samples (II)

Mice were perfused with phosphate buffered saline (PBS). Tissue samples were fixed for 1 h in 4 % PFA/PBS and immersed in PBS until being processed into paraffin sections. Fresh tissue samples were snap frozen in liquid nitrogen and stored at -70 °C until processed for nucleic acid isolations, lipid peroxidation analysis and caspase-3 activity measurements.

NUCLEIC ACID ISOLATIONS (I-IV)

mRNA (I, IV) was isolated from transduced cells using oligo(dT)-cellulose (Ausubel *et al.,* 1992). Total RNA (II-IV) and genomic DNA (IV) were isolated from adenovirally transduced cells and tissues using TRIZOL reagent according to the manufacturer's instructions.

NORTHERN BLOTTING (I, IV)

mRNA samples were electrophoresed on 1 % agarose/formaldehyde gels and transferred to a nylon membrane. Random-primed ^{32}P-cDNA probes were synthesized using [^{32}P]dCTP and Prime-a-Gene labelling system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Northern blot hybrdizations were performed as described (Sambrook *et al.,* 1989).

POLYMERASE CHAIN REACTIONS (PCR) (II-IV)

Distribution of the transduced 15-LO-1 was detected with polymerase chain reaction (PCR) from genomic DNA samples (IV). mRNA expression of the adenovirally transduced genes (II-IV) and endogenous rabbit α-actin (II-III) was detected with reverse transcription PCR (RT-PCR) from the total RNA samples after DNAse treatment and cDNA synthesis (Jalkanen *et al.,* 2003). Each PCR mixture contained DNA or cDNA template, 1 U of DyNAzyme^{TM} EXT DNA Polymerase (Finnzymes, Espoo, Finland), 20 pmol of each primer, 200 µM of each nucleotide (Fermentas Life Sciences, Burlington, Ontario, Canada), and 1.5 mM $MgCl_2$ (3.5 mM for rabbit α-actin) in 1 x PCR buffer (Fermentas Life Sciences, Burlington, Ontario, Canada) in a total volume of 50 µl.

The primers for human 15-LO-1 were specific for the transduced gene, since the 5' primer binds to the CMV promoter area of the adenovirus vector 5' to the splice signals and the 3' primer binds to the cloning junction of 15-LO-1. These primers result in the amplification of a 956 bp fragment from the unspliced Adh15-LO-1 vector and a 129 bp fragment from the spliced messenger RNA produced from the recombinant Adh15-LO-1 virus. All the primers and PCR reaction conditions of the end-point PCRs are summarized in **Table V**.

Quantitative RT-PCR was used to quantify the mRNA expression of endogenous rabbit PPAR-γ and VEGFR-2 (II-III). The sequence-specific oligonucleotide primers for rabbit PPAR-γ (GenBank accession number AY166781) and VEGFR-2 (GenBank accession number AB017155) were 5'-TGAGCCTTGACTTGAACGAC-3' and 5'-TGAGGACCCCGTCTTTATTC-3', and 5'- ATGTACCAGACCATGCTTGA-3' and 5'-AGTCTCTGACATCGGAAGAA-3', respectively. Rabbit 18S rRNA (GenBank accession number X06778) was used as a reference gene (Hofstaetter *et al.,* 2004). The assays and data calculations were performed as described (Roy *et al.,* 2005). In addition, quantitative RT-PCR was used to study the endogenous expression of eNOS and VEGFR-2 mRNA in HUVECs by using TaqMan® Gene Expression Assays for human eNOS (Hs00167166_m1, Applied Biosystems, Foster City, CA, USA) and human VEGFR-2 (Hs00176676_m1, Applied Biosystems, Foster City, CA, USA), and TaqMan® ribosomal RNA control reagents for human 18s rRNA (Part Number 4308329, Applied Biosystems, Foster City, CA, USA), which served as the control gene.

PROTEIN ISOLATION, SDS-PAGE AND WESTERN BLOTTING (I)

Retrovirally transduced cells (I) were suspended in 10 % sucrose/PBS, homogenized on ice with a tissue homogenizer and the supernatant was isolated by ultracentrifugation. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10 % gels in 0.025 M Tris/0.19 M glycine buffer pH 8.3 containing 0.1 % sodium dodecyl sulfate. Transfer to nitrocellulose membranes for Western blotting was performed at 4 °C for 2 h at 200 mA. 15-LO-1 protein was detected using polyclonal rabbit anti-human recombinant 15-LO-1 antibody (1:500 dilution) (Sigal *et al.,* 1990), horseradish peroxidase-conjugated secondary antibody (1:3000 dilution) and 4-chloro-1-naphthol substrate.

Table V. PCR primers and PCR reaction conditions used in the study. All primers are written 5' → 3'.

Amplified product	Forward primer	Reverse primer	PCR fragment size in base pairs (bp)	Annealing temperature °C	Number of cycles
human 15-LO-1	CACGCTGTTTTGACCTCCATAG	CCATCTTGCTCCCGAATTTC	956 bp from vector; 129 bp from spliced mRNA	58	35
human VEGF-A_{165}	TCAGATCCATGAACTTTCTGC	TCTCTCCTATGTGCTGGCCT	366 bp	64	30
mouse PlGF-2	GGTGCCTTTCAACGAAGTGT	GCCTTTGTCGTCTCCAGAAT	300 bp	55	35
rabbit α-actin	ACCAACTGGGACGACATGGAAAA	GTCAGGATCTTCATGAGGTAGTC	353 bp	60	25
human VEGF-DΔNΔC	TTGCCAGCTCTACCACCA	TTCATTGCAACAGCCACCAC	292 bp	56	30

HYDROXY FATTY ACID ANALYSIS (I-IV)

Transduced cells were broken either by mechanical shearing or by three freeze-thaw cycles. The broken cell suspensions were incubated at 37 °C for 15 min with 50 µM LA. Specific 15-LO inhibitor PD146176 (Sendobry *et al.,* 1997; Bocan *et al.,* 1998) (a generous gift from Dr. Joseph Cornicelli, Pfizer, Ann Arbor, MI, USA) was tested in some experiments at a concentration of 10 µM. The inhibitor was added 15 min before the LA incubation. After the LA incubation, 25 µg of butylated hydroxytoluene was added as an antioxidant and 25 µg of triheptadecanoin as an internal standard. Lipids were extracted, hydrogenated, saponified, esterified, and acetylated, and the functionality of the transduced 15-LO-1 protein was confirmed by measuring 15-LO-1 enzyme activity with gas chromatographic hydroxy fatty acid analysis (Nikkari *et al.,* 1995). Protein concentrations were measured according to (Lowry *et al.,* 1951) using bovine serum albumin as a standard.

NUCLEAR EXTRACTION AND ELECTROPHORETIC MOBILITY SHITF ASSAY (I)

Retrovirally transduced ECV304 cells (I) were activated with either TNF-α, phorbol 12-myristate 13-acetate (PMA), thapsigargin, or H_2O_2 for 2 h. Nuclear extracts were prepared using 10 % Nonidet P-40 solution (Sen *et al.,* 1996b). Electrophoretic mobility shift assays were performed by incubating the nuclear extracts with poly(dI-dC) and 32-P labeled NF-κB consensus oligonucleotide probe for 30 min at room temperature (Roy *et al.,* 1998). Proteins were separated by electophoresis in a native 6 % polyacrylamide gel and detected by autoradiography. The specificity of the NF-κB band was demonstrated by treating the nuclear protein extract with an excess of cold competitor probe or a cold mutant probe for 15 min before incubation with a labeled consensus NF-κB probe (Sen *et al.,* 1996a).

ADHESION MOLECULE EXPRESSION (I)

Retrovirally transduced ECV304 cells (I) were activated with either PMA or TNF-α for 24 h and with or without pre-treatment with α-lipoate for 48 h. The expression of VCAM-1 and ICAM-1 was analyzed by flow cytometry using monoclonal VCAM-1 antibody (Immunotech, Cedex, France) followed by fluorescein isothiocyanate (FITC) - labeled goat anti-mouse IgG monoclonal antibody (Coulter, Miami, FL, USA), and FITC-labeled monoclonal ICAM-1 antibody (Immunotech, Cedex, France), respectively (Roy *et al.,* 1998).

CELL-CELL ADHESION ASSAY (I)

Jurkat T cells (ATCC TIB-152) were fluorescently labeled with 5 µM calcein acetoxymethyl ester (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C (Roy *et al.,* 1998). Retrovirally transduced ECV304 cells were cultured in 96-well tissue culture plates and activated with 100 nM PMA for 24 h. Labeled Jurkat T cells were co-cultured with the ECV304 clones for 1 h at 37 °C. The adhesion of the labeled Jurkat T cells to the ECV304 clones was analyzed by measuring the fluorescence intensity of each well (Roy *et al.,* 1998).

IMMUNOHISTOCHEMISTRY (II-IV)

The primary antibodies used in immunohistochemistry are summarized in **Table VI**. The avidin-biotin-horseradish peroxidase system (Vector Laboratories, Burlingame, CA, USA) and 3'-5'-diaminobenzidine (DAB) color substrate (Zymed Laboratories, South San Francisco, CA, USA) were the detection agents used in all immunostainings.

Table VI. Primary antibodies used in the immunostainings.

Antibody	Source	Publication
Mouse monoclonal anti-human CD31, clone JC/70A (endothelial cell marker)	DAKO, Glostrup, Denmark	II,III
Goat polyclonal anti-human 15-LO-1	Santa Cruz Biotechnology, Santa Cruz, CA, USA	II
Mouse monoclonal anti-human VEGF	Santa Cruz Biotechnology, Santa Cruz, CA, USA	II, III
Mouse monoclonal anti-human eNOS	BD Biosciences, San Jose, CA, USA	II
Mouse monoclonal anti-nitrotyrosine, clone 1A6	Upstate, Lake Placid, NY, USA	II
Mouse monoclonal anti-human PPAR-γ	R&DSystems, Minneapolis, MN, USA	II
Mouse monoclonal anti-fetal liver kinase 1 (VEGFR2)	Santa Cruz Biotechnology, Santa Cruz, CA, USA	II
FITC-conjugated anti-lectin (*Lycopersicon esculentum*, tomato)	Sigma-Aldrich, St. Louis, MO, USA	III
Mouse monoclonal anti-glial fibrillary acidic	Sigma-Aldrich, St. Louis, MO, USA	III
Rabbit polyclonal anti-human 15-LO antibody CheY	Sigal *et al.,* 1990	IV
Rabbit polyclonal anti-human active caspase-3	Promega, Madison, WI, USA	IV

ASSESSMENT OF ANGIOGENIC EFFECTS (II-III)

Capillary perfusion was measured from rabbit hind limbs with contrast enhanced ultrasound (CEU) after a bolus injection of a second generation contrast agent (2 x 10^8 bubbles/ml, mean diameter 2.5 µm, SonoVue, Bracco, Italy) via the ear vein (Rissanen *et al.,* 2005). Vascular permeability was analyzed by a modified Miles assay, where Evans Blue dye (Sigma-Aldrich, St. Louis, MO, USA) (30 mg/kg) was injected intravenously 30 min before sacrifice. Animals were perfusion fixed with 1 % PFA in 0.05 M citrate buffer (pH 3.5). Evans Blue dye bound to the plasma proteins in the muscle tissue was extracted by incubation in formamide for 24 h at 60 °C. The amount of bound Evans Blue dye was quantified with a spectrophotometer at 610 nm and the permeability ratio was calculated as the ratio between transduced tissue and intact control tissue after normalization for the tissue weights (Rissanen *et al.,* 2003a).

Capillary mean area (μm^2) and capillary density (capillaries/myocytes) were measured from CD31-immunostained sections by AnalySIS software (Soft Imaging System) (Rissanen *et al.,* 2003a).

Four peripheral areas of medullary rays from each whole-mounted retina stained with FITC-conjugated lectin were digitally imaged with Olympus DP50 (Olympus, Tokyo, Japan) camera system operating on an Olympus BX40 transmitted microscope. Retinal blood vessel diameters were measured using Adobe Photoshop CS3 software (Adobe Systems Incorporated, San Jose, CA, USA).

NOS ACTIVITY (II)

NOS activity was measured as relative nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity from rabbit muscle tissues homogenized in solubilization buffer (0.1 M Tris-HCl, pH 7.4 containing 0.5 % (v/v) Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Complete, Roche, Basel, Switzerland)). The homogenate supernatants were incubated with NADPH diaphorase medium (20 mg/ml β-NADPH, 0.5 mg/ml nitroblue tetrazolium and 0.5 % (v/v) Triton X-100 in 0.1 M Tris-HCl buffer, pH 7.4) at 37 °C for 30 min. Incubations were also performed in the absence of β-NADPH (blank) (Baum *et al.,* 2002). Absorbance of the produced formazan was measured at 560 nm. The protein concentrations were measured by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The results were calculated as relative NADPH activity (ΔE/mg protein/30 min) by subtracting the absorbance values obtained from the blank reactions from the absorbances obtained from the reactions containing β-NADPH (Baum *et al.,* 2002).

ELISA (II-III and unpublished)

Muscle tissues were homogenized in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) and the total protein concentrations of the tissue homogenates were measured with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The protein expression of the transduced VEGF-A_{165} (II-III), PlGF-2 (II) and VEGF-DΔNΔC (unpublished) was quantified from transduced muscle tissues by enzyme linked immunosorbent assays (ELISA) (Quantikine human VEGF, Quantikine mouse PlGF-2 and Quantikine VEGF-D, R&D Systems, Minneapolis, MN, USA). ELISAs were also performed from

HUVEC cell culture media and vitreous samples.

LIPID PEROXIDATION ASSAY (IV)

Lipid peroxidation, an indicator of oxidative stress, was measured from snap frozen liver samples according to the colorimetric BIOXYTECH® LPO-586™ lipid peroxidation assay (Oxis International, Inc., Portland, OR, USA). The assay is based on the reaction of a chromogenic agent, N-methyl-2-phenylindole, with malondialdehyde (MDA) and 4-hydroxyalkenals, which are decomposition products of the unstable lipid peroxides. For the assay, the snap frozen tissue samples were homogenized in ice-cold 20 mM Tris buffer, pH 7.4, containing 5 mM butylated hydroxytoluene to prevent sample oxidation. The homogenates were centrifuged at 4 °C 3000 g for 10 min to remove large particles. The supernatants were used for the lipid peroxidation assay and for protein determination by Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

DETECTION OF APOPTOSIS (IV)

Apoptosis was detected from mouse liver paraffin sections using polyclonal antibody against the active form of caspase-3 (**Table VI**). Enzymatic caspase-3 activity in mouse liver samples was detected fluorometrically from tissue homogenates by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC (Calbiochem, La Jolla, CA, USA) at excitation 380 nm and emission 465 nm (Pulkkanen *et al.,* 2000). Protein concentrations of the tissue homogenates were determined with the Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

MAGNETIC RESONANCE IMAGING (IV)

MRI was used for tumor visualization. The rats were anesthetized with isoflurane (induction 5 % and maintenance 1.5 % with carrier gas of O_2 30 % and N_2O 70 %) and fixed to a stereotactic holder. MRI data were acquired using a horizontal 4.7 T magnet (Magnex Scientific Ltd, Abington, UK) interfaced to a Varian (Palo Alto, CA, USA) UnityINOVA console and using an actively decoupled volume transmission coil and a quadrature surface receiver coil (Rapid Biomedical, Rimpar, Germany). T2-weighted images were measured using a spin echo sequence (echo time (TE) = 40 ms, repetition time (TR) = 2 s). A total of 17 slices, each of 1 mm thickness, were imaged. The matrix size was 256x128 zero-filled to 256x256, with field of view (FOV) 40 mm^2 yielding an in-plane resolution of 156 µm.

Total tumor volume was processed and analysed using Matlab version 7.04 (The MathWorks, Inc., Natick, MA, USA). Tumor volumes were calculated by delineating the tumor area in all the image slices, using premade macros. To minimize human errors, tumor volumes were measured independently by two individuals with the mean values of the two measurements being used. A multiplication factor to convert to tumor volumes, thus obtained in pixels into mm^3, was calculated using the following formula; FOV in mm divided by the pixel size and squared. Multiplication factor was 0.0244.

STATISTICS (II-IV)

Results are presented as mean ± SD. All data were analysed by GraphPad Prism Software package. Statistical significance was evaluated using One-Way ANOVA, followed by Newman-Keuls Multiple Comparison test, or Kruskal-

Wallis test or Mann-Whitney test. Survival analysis was done using Kaplan-Meier log rank test and tumor volume comparison was done with unpaired T-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

CHARACTERIZATION OF THE RECOMBINANT RETRO- AND ADENOVIRUSES CONTAINING HUMAN 15-LO-1 cDNA (Original publications I-IV)

Functionality of the recombinant retro- and adenoviruses containing human 15-LO-1 cDNA was confirmed at the mRNA, protein and enzyme activity levels. Northern blot analysis of the retrovirus-derived clones from both the packaging cell line PA317 and from ECV304 cells showed that the clones produced a full-length transcript from the proviral DNA between the LTRs (I). Adenovirus mediated gene transfer of 15-LO-1 led to detection of a specific PCR fragment from 15-LO-1 mRNA with all three *in vivo* gene transfer techniques that were applied: in rabbit hind limb skeletal muscle after local intramuscular gene transfer (II), in rabbit eye tissue after intravitreal gene transfer (III), and in several organs after tail vein gene transfer in mice (IV).

15-LO-1 protein expression was detected as an approximately 75 kDa protein from the retroviral 15-LO-1 clones by Western blotting, whereas no expression was detected from the control cells (I). Expression of 15-LO-1 after adenovirus mediated gene transfer was confirmed by immunohistochemistry from rabbit skeletal muscles, where 15-LO-1 protein expression was localized in the capillaries (II). After tail vein gene transfer in mice, the transgene expression was predominant in liver where there was uniform expression. In spleen, 15-LO-1 expression was localized to the interface between the white pulp and the red pulp (IV).

Enzymatic activity of 15-LO-1 was confirmed from retroviral PA317 and ECV304 cell clones (I) and from adenovirally transduced RAASMC (II), ECV304 cells (III) and RAW264.7 mouse macrophages (IV) by a gas chromatographic method measuring hydroxy fatty acids (Nikkari *et al.,* 1995). Incubation of the cells with LA led to specific induction in the production of the 15-LO-1 metabolite 13-HODE. The proportion of 13-HODE of all the HODEs detected was 77 % on average. Induction of 13-HODE production was prevented by the specific 15-LO inhibitor PD146176 (**Figure 6**).

INDUCTION OF ADHESION MOLECULE EXPRESSION AND T CELL ADHESION (Original publication I)

The activation of NF-κB, a transcription factor sensing various inflammatory stimuli and oxidative stress, was potentiated in the 15-LO-1 expressing ECV304 cell clone in response to several stimuli, including PMA and TNF-α. The constitutive expressions of the adhesion molecules ICAM-1 and VCAM-1 were similar in retroviral clones expressing either *E. coli* β-galactosidase or human 15-LO-1. However, the expression in 15-LO-1 clone was inducible by PMA and TNF-α via an NF-κB mediated mechanism, and the induction of ICAM-1 was shown to be inhibited by the antioxidant α-lipoate. Stimulation of the 15-LO-1 expressing ECV304 cells with PMA also led to significantly increased adhesion of T cells (**Figure 7**).

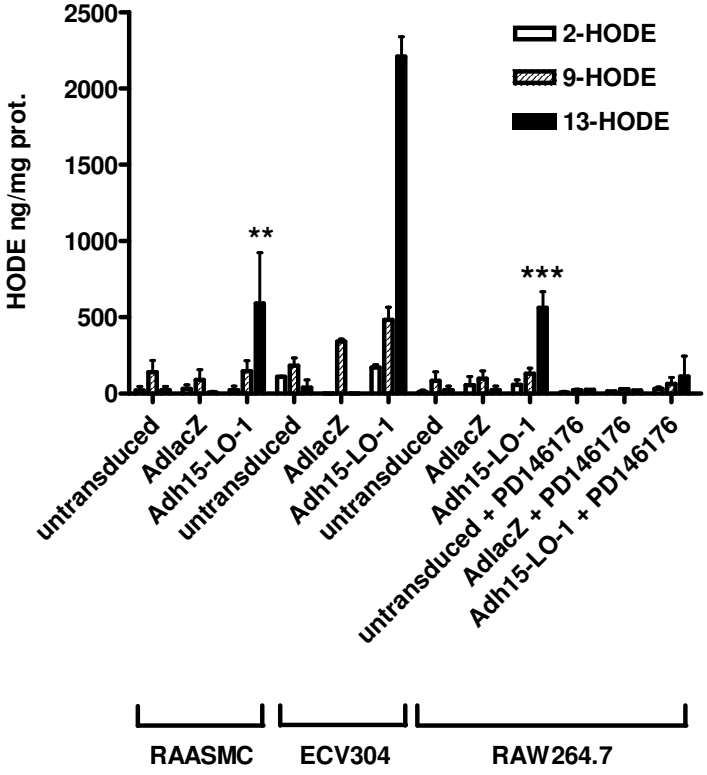

Figure 6. **Gas chromatographic HODE analysis from the adenovirally transduced cells after incubation with LA.** Results are presented as mean ng HODE/mg protein ±SD. **, $p<0.01$ Adh15-LO-1 vs. untransduced and vs. AdlacZ; ***, $p<0.001$ Adh15-LO-1 vs. untransduced and vs. AdlacZ; One-Way ANOVA followed by Newman-Keuls Multiple Comparison test. Combined from original publications II-IV.

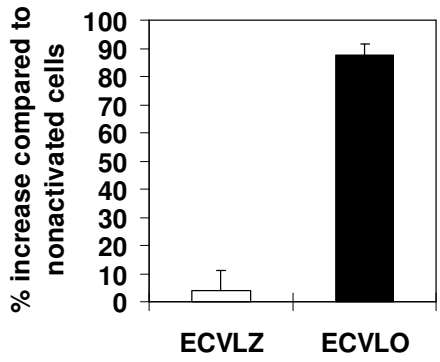

Figure 7. Increased adherence of Jurkat T cells to ECVLO cells in response to PMA stimulation. Retrovirally transduced ECV304 cells expressing either *E. coli lacZ* (ECVLZ) or human 15-LO-1 (ECVLO) were stimulated with 100 nM PMA for 24 h and then co-cultured with fluorescently labelled Jurkat T cells for 1 h. The results are presented as percent increase in the number of adhered Jurkat T cells adhering to ECVLO and ECVLZ cells following PMA stimulation. Three separate experiments were done in triplicate. $P<0.0001$, unpaired t test. From original publication I.

PREVENTION OF ANGIOGENESIS INDUCED BY VEGF FAMILY MEMBERS (Original publications II and III)

In articles II and III, the angiogenic findings previously reported for intramuscular VEGF-A_{165} gene transfer in rabbit hind limb skeletal muscles (Rissanen *et al.,* 2003b), and in rabbit eyes after intravitreal gene transfer (Kinnunen *et al.,* 2006) were confirmed. In addition, there were new findings that 15-LO-1 almost completely abolished all of the angiogenic effects induced by VEGF family members in the skeletal muscles and by VEGF-A_{165} in rabbit eyes. Capillary perfusion was significantly induced by VEGF-A_{165} in the skeletal muscles and PlGF-2 was even more potent than VEGF-A_{165} in this regard. 15-LO-1 almost completely prevented the growth factor induced increases in perfusion (**Figure 8**).

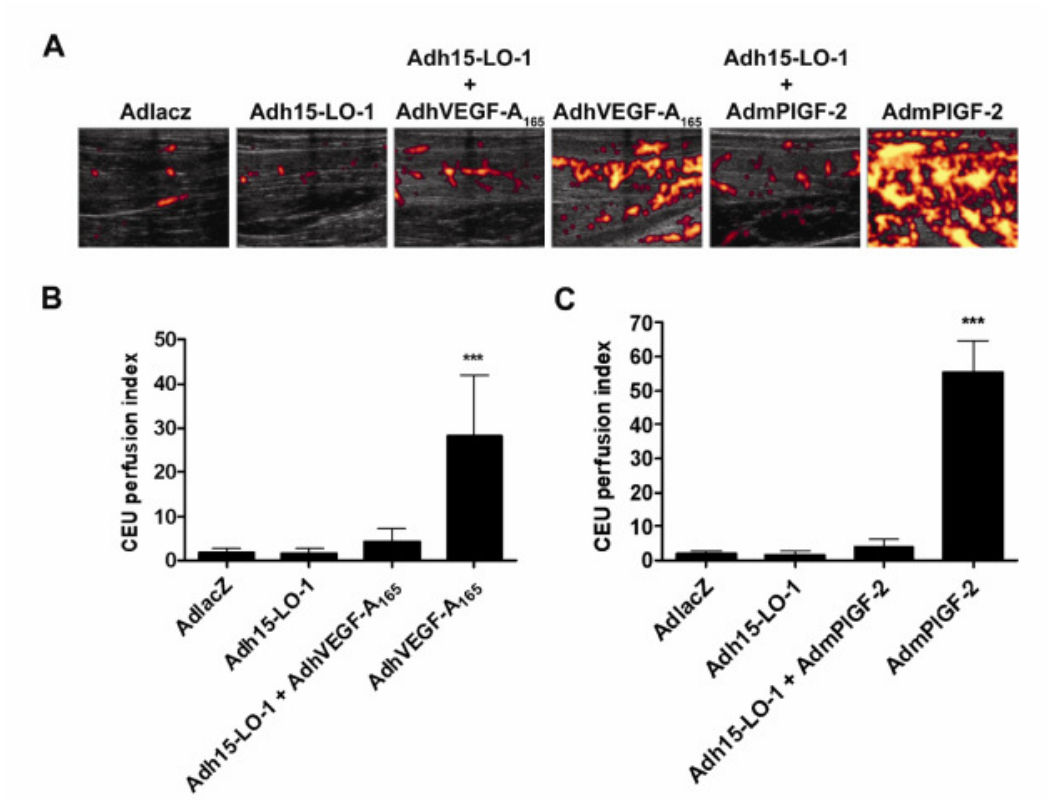

Figure 8. **Adh15-LO-1 prevents AdhVEGF-A_{165} and AdmPlGF-2 induced increases in capillary perfusion**. (A) CEU perfusion images were taken six days after gene transfer from semimembranosus muscles transduced with AdlacZ, Adh15-LO-1, Adh15-LO-1 + AdhVEGF-A_{165}, AdhVEGF-A_{165}, Adh15-LO-1 + AdmPlGF-2, and AdmPlGF-2 (see Table IV). Quantitative analysis of perfusion by CEU perfusion index between the transduced and intact semimembranosus muscles shows that AdhVEGF-A_{165} (B) and AdmPlGF-2 (C) significantly induce the CEU perfusion index and Adh15-LO-1 prevents this induction. n = 4 for Adh15-LO-1 + AdmPlGF-2 and AdmPlGF-2, and n = 8 for all other groups. The results are presented as mean ±SD. ***, $p<0.001$ AdhVEGF-A_{165} and AdmPlGF-2 vs. all other groups, One-Way ANOVA followed by Newman-Keuls Multiple Comparison test. From original publication II.

Capillary size and number were significantly increased by VEGF-A_{165} both in the rabbit skeletal muscle and in the papilla of the rabbit eyes and 15-LO-1 very efficiently prevented these increases (**Figure 9**). In the retina, a significant increase in the capillary size was detected after VEGF-A_{165} gene transfer, whereas in the anterior parts of the eyes VEGF-A_{165} increased the number of the capillaries. 15-LO-1 prevented all these effects (original publications II and III). Capillary enlargement was also detected in muscles transduced with AdhVEGF-DΔNΔC, and the increase in capillary sixe was prevented by 15-LO-1 (unpublished results).

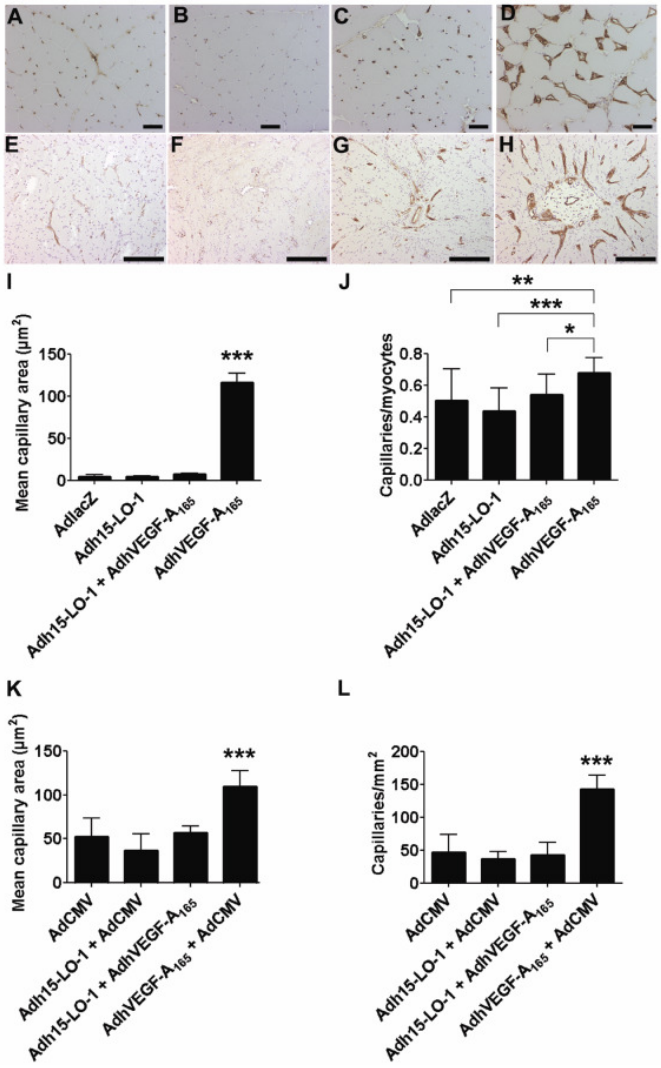

Figure 9. 15-LO-1 prevents VEGF-A_{165} induced increases in the size and number of the capillaries in rabbit skeletal muscle and papilla. (A-D) CD31 immunohistochemistry from semimembranosus muscles transduced with AdlacZ (A), Adh15-LO-1 (B), Adh15-LO-1 + AdhVEGF-A_{165} (C), and AdhVEGF-A_{165} (D). Magnification 200x, scale bar 50 µm. (E-H) CD31 immunohistochemistry from papilla after adenoviral intravitreal transductions with AdCMV (E), Adh15-LO-1 + AdCMV (F), Adh15-LO-1 + AdhVEGF-A_{165} (G), and AdhVEGF-A_{165} + AdCMV (H). Magnification 100x, scale bar 100 µm. Quantitative analysis of the capillaries shows that Adh15-LO-1 blocked the AdhVEGF-A_{165} induced increase in the mean capillary area (µm^2) in skeletal muscle (I) and in papilla (K) (***, $p<0.001$ AdhVEGF-A_{165} vs. all other groups). Adh15-LO-1 also prevented the induction in the capillary number by AdhVEGF-A_{165} in skeletal muscle (J) (**, $p<0.01$ AdhVEGF-A_{165} vs. AdlacZ, ***, $p<0.001$ AdhVEGF-A_{165} vs. Adh15-LO-1, *, $p<0.05$ AdhVEGF-A_{165} vs. Adh15-LO-1 + AdhVEGF-A_{165}) and in papilla (***, $p<0.001$ AdhVEGF-A_{165} vs. all other groups). n = 12-15/group for the muscles and n = 4-6/group for the eyes. Results are presented as mean ±SD. One-Way ANOVA followed by Newman-Keuls Multiple Comparison test. From original publications II and III.

VEGF-A $_{165}$ resulted in a significant 52-fold increase in capillary permeability in the rabbit skeletal muscles and 15-LO-1 prevented this induction (II). VEGF-A_{165} could mediate the angiogenic effects via ˙NO, since it induced a significant 1.5-fold induction in NOS activity in the skeletal muscles. The amount of nitrotyrosine positive cells was significantly increased, as was also eNOS protein expression, whereas 15-LO-1 prevented all these effects (II). In addition, both VEGF-A_{165} and PlGF-2 gene transfers induced the mRNA and protein expression of PPAR-γ and VEGFR-2 (II) (**Figure 10**), which may be evidence of a potentiation of the angiogenic signalling mediated by VEGFR-2. Induction of PPAR-γ and VEGFR-2 mRNA expression were also discovered in the eye tissue after intravitreal VEGF-A_{165} gene transfer (III), but these increases were not statistically significant.

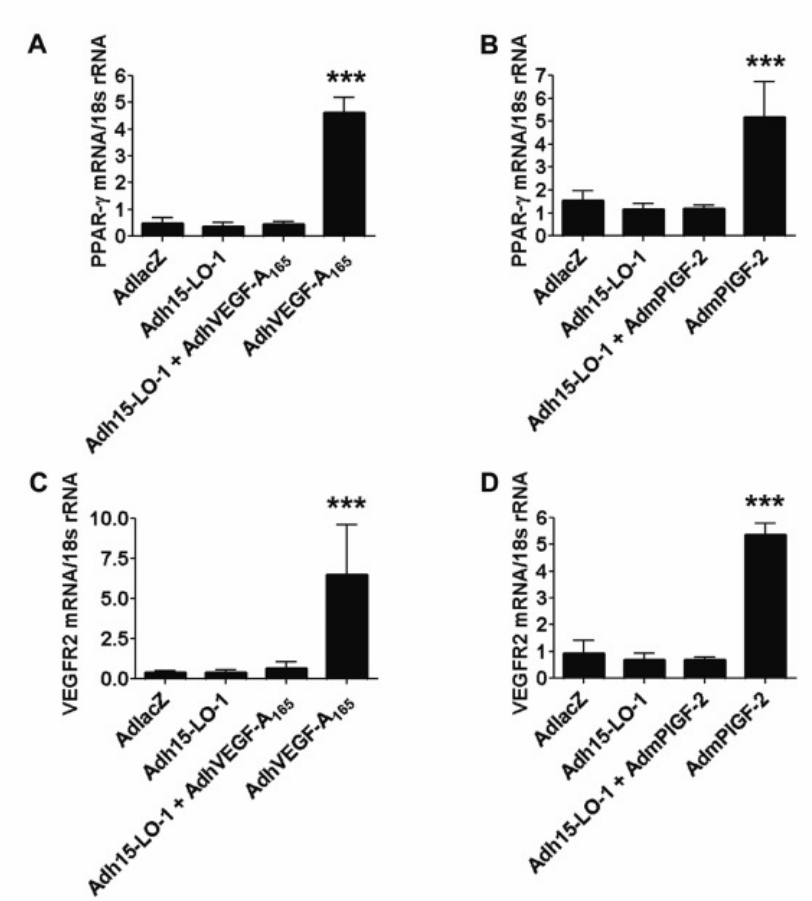

Figure 10. Adh15-LO-1 prevents the AdhVEGF-A_{165} and AdmPlGF-2 induced expression of PPAR-γ and VEGFR2. (A and B) Quantitative RT-PCR analysis of endogenous rabbit PPAR-γ mRNA expression from the transduced semimembranosus muscles. (A) ***, p<0.001 AdhVEGF-A_{165} vs. all other groups, (B) ***, p<0.001 AdmPlGF-2 vs. all other groups. (C and D) Quantitative RT-PCR analysis of endogenous rabbit VEGFR2 mRNA expression from the transduced semimembranosus muscles. (C) ***, p<0.001 AdhVEGF-A_{165} vs. all other groups, (D) ***, p<0.001 AdmPlGF-2 vs. all other groups. n = 3-5/group. Results are presented as mean ±SD. From original publication II.

15-LO-1 reduced the expression of VEGF-A_{165}, PlGF-2 and the short, mature form of VEGF-D (VEGF-DΔNΔC, unpublished results) already at the mRNA level (II, III), resulting in reduction in protein expression of these growth factors in rabbit skeletal muscles (II), in transduced HUVECs (II) and in vitreous humor (III) (**Figure 11**). 15-LO-1 also reduced endogenous angiogenesis induced by ischemia in rabbit skeletal muscles (II).

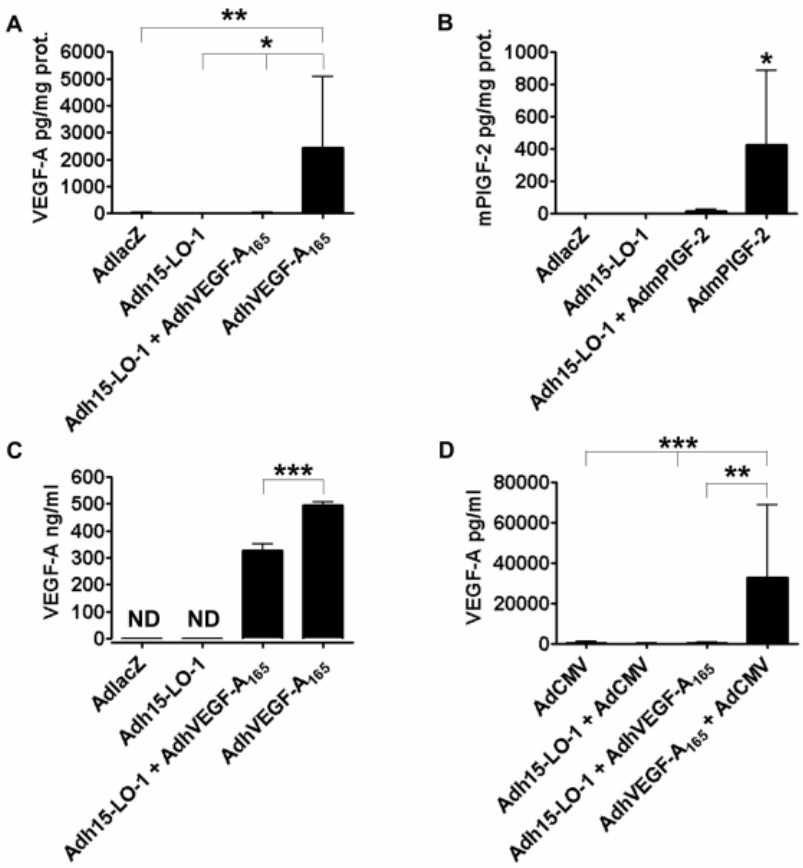

Figure 11. Adh15-LO-1 reduces the production of the transduced human VEGF-A_{165} and mouse PlGF-2 protein. Human VEGF-A (A) and mouse PlGF-2 (B) protein production in the transduced semimembranosus muscles. Results are presented as mean ±SD, n = 4-5/group. (A) **, $p<0.01$ AdhVEGF-A_{165} vs. AdlacZ, *, $p<0.05$ AdhVEGF-A_{165} vs. Adh15-LO-1 and Adh15-LO-1 + AdhVEGF-A_{165}; (B) *, $p<0.05$ AdmPlGF-2 vs. all other groups, One-Way ANOVA followed by Newman-Keuls Multiple Comparison test. From original publication II. (C) VEGF-A production in HUVEC cell culture media 72 h after transduction. Results are presented as average VEGF-A ng/ml ± SEM, n = 6/group. ***, $p<0.001$ AdhVEGF-A_{165} vs. Adh15-LO-1 + AdhVEGF-A_{165}, One-Way ANOVA followed by Newman-Keuls Multiple Comparison test. ND = not detectable. From original publication II online data supplement. (D) Concentration of human VEGF-A in the vitreous. Results are presented as mean ±SD, n = 16/group. $p<0.001$ AdhVEGF-A_{165} + AdCMV vs. AdCMV, $p<0.001$ AdhVEGF-A_{165} + AdCMV vs. Adh15-LO-1 + AdCMV, $p<0.01$ AdhVEGF-A_{165} + AdCMV vs. Adh15-LO-1 + AdhVEGF-A_{165}. Kruskal-Wallis test followed by Dunn's multiple comparison test. From original publication III.

INDUCTION OF LIPID PEROXIDATION AND APOPTOSIS (Original publication IV)

Tail vein gene transfer of Adh15-LO-1 led to highest expression of the transgene in mouse liver and spleen. 15-LO-1 induced lipid peroxidation in mouse liver, detected as an increased production of lipid peroxidation end products MDA and 4-hydroxynonenal (4-HNE) (**Figure 12**).

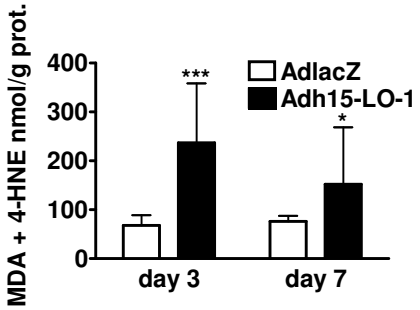

Figure 12. Lipid peroxidation analysis from the mouse livers. The results are presented as mean nmol MDA and 4-HNE/g protein ±SD. n = 8 for AdlacZ and n = 12-13 for Adh15-LO-1. ***, p<0.001; *, p<0.05, Mann-Whitney test. From original publication IV.

Induced lipid peroxidation was followed by induction of caspase-3 mediated apoptosis (**Figure 13**).

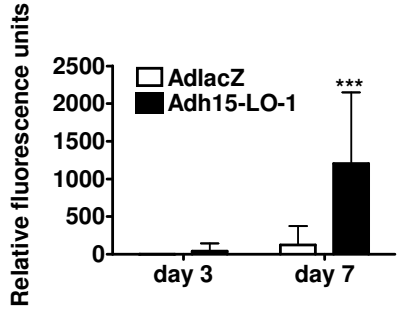

Figure 13. Caspase-3 enzymatic activity from mouse livers. The results are presented as mean relative fluorescence units ±SD. n = 3-4 for AdlacZ and n = 7-8 for Adh15-LO-1. ***, p<0.001, Mann-Whitney test. From original publication IV.

PROLONGED SURVIVAL IN RAT MALIGNANT GLIOMA MODEL (Original publication IV)

15-LO-1 and its reaction product 13(S)-HODE have been shown to be pro-apoptotic and anti-tumorigenic in various cell lines *in vitro*, in several animal models and also *in vivo* in some cancer types (Kuhn *et al.,* 2002). However, since conflicting effects of 15-LO-1 in tumorigenesis have been reported in various models depending on the tissue and cancer type, the effect of 15-LO-1 on tumorigenesis in a rat malignant glioma model was tested in this study. A tendency towards reduced tumor volume was detected in the 15-LO-1 treated animals and in this model 15-LO-1 significantly prolonged the survival of the animals (**Figure 14**).

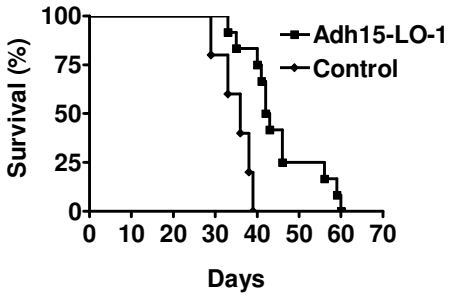

Figure 14. Kaplan-Meier survival analysis of Adh15-LO-1 gene therapy. Rats after implantation of BT4C cells were treated with Adh15-LO-1 (n=12) or followed up as control animals (n=5). The median survival of the treated and control animals were 42.5 days and 36.0 days, respectively (p=0.001). From original publication IV.

DISCUSSION

CHARACTERIZATION OF THE RECOMBINANT RETRO- AND ADENOVIRUSES CONTAINING HUMAN 15-LO-1 cDNA (Original publications I-IV)

Western blotting analysis of the PA317 clones confirmed that the recombinant 15-LO-1 retrovirus produced correct size of 15-LO-1 protein, approximately 75 kDa (I), in good agreement with previous reports (Sigal *et al.,* 1990; Kühn *et al.,* 1993b).

After tail vein gene transfer in mice, the transgene expression was predominant in liver (IV), in accordance with previous experiments using soluble macrophage scavenger receptor A (Jalkanen *et al.,* 2003) and with the general hepatotropism of adenovirus (Wood *et al.,* 1999). In spleen, 15-LO-1 expression localized to the interface between the white pulp and the red pulp (IV), as previously seen after intravascular gene delivery in rabbits and was possibly related with loose fenestrated endothelium in this region (Hiltunen *et al.,* 2000).

INDUCTION OF ADHESION MOLECULE EXPRESSION AND T CELL ADHESION (Original publication I)

15-LO-1 induced NF-κB mediated expression of the adhesion molecules VCAM-1 and ICAM-1, as well as T cell adhesion on the 15-LO-1 expressing ECV304 cells. These results suggest that 15-LO-1 may have pro-atherogenic properties in the early phases of atherosclerosis by inducing adhesion molecule expression and by mediating the adhesion of inflammatory cells to the endothelium.

Several reports support our findings in revealing similar effects of 15-LO-1 on the expression of adhesion molecules and on the adhesion of inflammatory cells. Transient overexpression of 15-LO-1 in aortic ECs increases TNF induced VCAM-1 expression (Wölle *et al.,* 1996). ICAM-1 expression is also induced in aortic ECs by 15-LO-1 or 15-HPETE (Sordillo *et al.,* 2008). Fibroblasts overexpressing 15-LO-1 generate bioactive, minimally oxidized LDL, which stimulates monocyte chemotaxis and adhesion to ECs (Sigari *et al.,* 1997). Inhibition studies with short hairpin RNA to knock down the endogenous 12/15-LO expression in mouse macrophages and vascular SMCs leads to reduced oxidant stress, chemokine and adhesion molecule expression and cellular adhesion (Li *et al.,* 2005).

Production of 12/15-LO metabolites 12(S)-HETE and 13(S)-HODE is induced in diabetic mice. Monocyte adhesion to ECs derived from these mice is induced, and the induction can be prevented by inhibiting 12/15-LO. The adhesion is mediated by the interactions of monocyte integrins with endothelial VCAM-1, connecting segment 1, fibronectin and ICAM-1 (Hatley *et al.,* 2003). Increased monocyte adhesion is mediated by these same factors also in 12/15-LO transgenic mice, where ICAM-1 expression is induced several-fold (Reilly *et al.,* 2004) via activation of NF-κB (Bolick *et al.,* 2005).

PREVENTION OF ANGIOGENESIS INDUCED BY VEGF FAMILY MEMBERS (Original publications II and III)

Previous studies from our research group have explored the angiogenic effects of different VEGF family members in rabbit hind limb skeletal muscles by adenovirus mediated gene transfers. The VEGF family members induce capillary perfusion and vascular permeability, and also increase both number and size of the capillaries (Rissanen *et al.,* 2003b). Our group has also created a rabbit model, where intravitreal VEGF-A_{165} gene transfer induces neovascularization and increases vascular leakage in rabbit eye, resembling the pathological changes detected in diabetic retinopathy (Kinnunen *et al.,* 2006). In articles II and III, the angiogenic findings previously reported with intramuscular VEGF-A_{165} gene transfer in rabbit hind limb skeletal muscles, and in rabbit eyes after intravitreal gene transfer were confirmed.

The results also showed that VEGF-A_{165} could mediate the angiogenic effects in the skeletal muscles partly by inducing NOS activity and eNOS protein expression. Endothelial ˙NO is required in arteriolar vasodilatation (Ignarro *et al.,* 1987), and stimulation of ˙NO release both endogenously and exogenously can increase capillary diameters (Bloch *et al.,* 1995). Previous studies by our group have shown that AdhVEGF-A_{165} gene transfer induces arteriogenesis, as nearly all enlarged capillaries in the AdhVEGF-A_{165} transduced muscles have a pericyte coverage positive for α-smooth muscle actin (Rissanen *et al.,* 2003b; Rissanen *et al.,* 2005). Therefore, it is likely that the capillaries, which have been shifted towards an arteriolar phenotype, can undergo ˙NO mediated vasodilatation. In the combination gene transfer groups, the induction of vasodilatation was prevented by 15-LO-1. This can be partly mediated by the prevention of growth factor expression and the signalling cascade mediated by the growth factors, but also partially by catalytic consumption of ˙NO by 15-LO-1 (O'Donnell *et al.,* 1999; Coffey *et al.,* 2001) (**Figure 15**).

In addition to inducing eNOS expression and NOS activity, VEGF-A_{165} and PlGF-2 could also mediate the angiogenic effects by inducing the expression of PPAR-γ and VEGFR-2 (II-III). 15-LO-1 product 13-HODE is an endogenous activator and ligand of PPAR-γ (Nagy *et al.,* 1998). Interestingly, it has been shown in a rabbit eye model that PPAR-γ binding to the VEGFR-2 promoter induces VEGFR-2 expression, but ligand binding to PPAR-γ actually results in an inhibition of VEGFR-2 expression (Sassa *et al.,* 2004).

Based on our findings, we hypothesize that 15-LO-1 affects VEGF-A_{165} induced neovascularization both in transduced cells and via its secreted products. Thus, 15-LO-1 expression in the cells next to the vascular ECs can directly inhibit VEGF-A_{165} expression and prevent angiogenic signal transduction events mediated by secreted VEGF-A_{165}, whereas in 15-LO-1 transduced ECs 15-LO-1 products binding to PPAR-γ prevent the VEGF-A_{165} induced expression of VEGFR-2 (**Figure 15**). PPAR-γ ligands have previously been shown to reduce VEGFR-2 expression and inhibit angiogenesis *in vitro* (Xin *et al.,* 1999), as well as to inhibit choroidal (Murata *et al.,* 2000) and corneal neovascularization (Xin *et al.,* 1999; Panigrahy *et al.,* 2002; Sarayba *et al.,* 2005) *in vivo*.

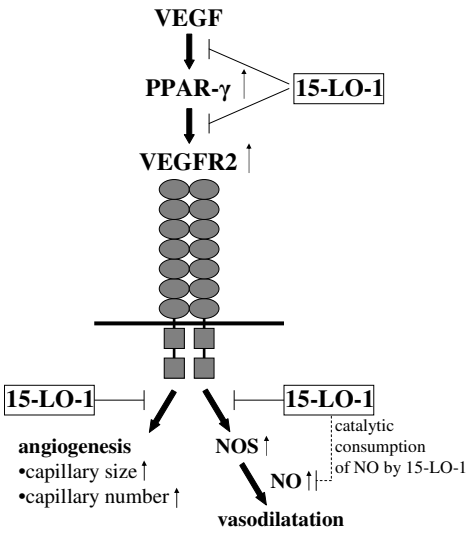

Figure 15. Proposed mechanism for the inhibitory effect on angiogenesis by 15-LO-1. VEGF mediated angiogenic signalling involves VEGF binding to VEGFR-2 and increased ·NO production resulting in angiogenesis and vasodilatation of the capillaries. We hypothesize that the 15-LO-1 mediated prevention of growth factor expression blocks these signalling cascades by preventing VEGF induced increases in PPAR-γ and VEGFR-2 expression as well as increases in NOS activity. From original publication II.

Very little is known about the effects of 15-LO-1 on angiogenesis. Transgenic mice overexpressing 15-LO-1 in the vascular wall under the preproendothelin-1 promoter (Harats *et al.,* 1995) have been used to test the effects of the vascular overexpression of 15-LO-1. In the Lewis lung carcinoma model, the lung metastases occurring in the 15-LO-1 transgenic mice have an extensive hemorrhagic necrotic core, a higher number of apoptotic cells and also multiple small blood vessels arranged in a complicated network (Harats *et al.,* 2005).

These studies showed that 15-LO-1 could reduce the expression of the VEGF family growth factors already at the mRNA level, resulting in very low production of the growth factor proteins. 15-LO-1 might affect the production of the transduced human VEGF-A_{165}, mouse PlGF-2 and human VEGF-DΔNΔC either by preventing their transcription or by destabilizing the transcripts, or both. VEGF-A_{165} mRNA production and stability are both highly regulated. VEGF-A_{165} transcription is induced in hypoxia by HIF-1α (Forsythe *et al.,* 1996) and the mRNA is stabilized by several factors binding to the 3' UTR (Levy *et al.,* 1998; Shih *et al.,* 1999; Liu *et al.,* 2002). PlGF transcription is induced by BF-2 (Zhang *et al.,* 2003) and also in hypoxia, by metal response element-binding transcription factor 1 (Green *et al.,* 2001).

However, the constructs used in these studies lack all the regulatory elements of the endogenous human VEGF-A_{165}, mouse PlGF-2 and human VEGF-DΔNΔC, containing only the protein coding regions under CMV promoter. It is unlikely that the reduction in the mRNA expression of the transduced growth factors would be attributable to blocking of the CMV promoter, since the human 15-LO-1 cDNA in our adenovirus construct is also under a CMV promoter, and there was no reduction in the expression of the transduced human 15-LO-1 in the combination gene transfer groups. Thus, the most likely explanation is that 15-LO-1 expression leads to destabilization of the growth factor transcripts by an as yet unidentified mechanism that would affect the coding sequences of these growth factors. The independence of the 15-LO-1 effect on the constructs is further supported by our finding that 15-LO-1 also reduced endogenous angiogenesis induced by ischemia in rabbit skeletal muscles (II).

INDUCTION OF LIPID PEROXIDATION AND APOPTOSIS (Original publication IV)

15-LO-1 induced lipid peroxidation in mouse liver, detected as MDA and 4-HNE. The induced synthesis of the lipid peroxidation end products was followed by increased activity of caspase-3. These lipid peroxidation end products, especially 4-HNE, have been shown to induce apoptosis in several different cell lines via caspase activation (Liu *et al.,* 2000; Ji *et al.,* 2001; Zhang *et al.,* 2001; Choudhary *et al.,* 2002; West *et al.,* 2004; Ferrington *et al.,* 2006; Li *et al.,* 2006b). Our findings support the pro-apoptotic role of 15-LO-1 and its reaction products, and indicate that the lipid peroxidation induces apoptotic process mediated by increased caspase-3 activity. Thus, 15-LO-1 could be a potential therapeutic molecular target for the modulation of apoptosis in human disease.

PROLONGED SURVIVAL IN RAT MALIGNANT GLIOMA MODEL (Original publication IV)

15-LO-1 showed a tendency in reducing the tumor volume and significantly prolonged the survival of the animals in the rat malignant glioma model. In a previous study, PPAR-γ ligand has been shown to inhibit the growth of primary glioblastoma by suppressing angiogenesis via reduction in vessel density and EC proliferation, as well as by reduction of VEGF production by the tumor cells (Panigrahy *et al.,* 2002).

There are no previous reports about the outcomes of 15-LO-1 gene therapy in animal tumor models of malignant glioma. This study reveals that adenoviral 15-LO-1 gene transfer significantly prolonged the survival of the animals in BT4C rat malignant glioma model. These results highlight the importance of performing further studies to evaluate the potential of 15-LO-1 in the treatment of malignant glioma.

SUMMARY AND CONCLUSIONS

Studies included in this thesis provide new information about the role of 15-LO-1 in the development of atherosclerosis, and in angiogenesis and tumorigenesis. Both retro- and adenovirus mediated gene transfer techniques were successfully applied to produce functional 15-LO-1 protein in *in vitro* and *in vivo* gene transfer studies.

These studies support the pro-atherosclerotic role of 15-LO-1 in the early development of atherosclerosis by showing that 15-LO-1 potentiates the activation of the inflammation and redox sensitive transcription factor NF-κB, leading to induction in the expression of NF-κB regulated adhesion molecules and consequently, increased adhesion of inflammatory cells.

Our findings show that 15-LO-1 very efficiently prevents the angiogenic effects mediated by VEGF family members in two different animal models, i.e. in rabbit hind limbs and eyes. In addition, reduced endogenous angiogenesis by 15-LO-1 was shown in an ischemic rabbit hind limb model. Based on these results, 15-LO-1 appears to have potential in the treatment of pathologic angiogenic conditions, for example in neovascular ocular diseases.

In addition, the detected prolongation of survival by 15-LO-1 in rat malignant glioma model indicates that further studies are needed to evaluate the potential of 15-LO-1 in the treatment of malignant glioma.

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