# The effect of sunitinib and erlotinib on smooth muscle cell proliferation in vitro

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Tiivistelmä – Referat – Abstract

Chronic allograft nephropathy is still the major cause for loss of transplanted kidneys. A prominent feature is luminal narrowing of blood vessels due to proliferating and migrating smooth muscle cells (SMCs). The mechanism is much like that of atherosclerosis.

We hypothesized that platelet derived growth factor (PDGF), vascular endothelial growth factor and epidermal growth factor play an important role in the process. This was based on the observation that drugs inhibiting these growth factors decreased luminal narrowing in a rat model. To test the hypothesis SMC were cultured in vitro. They were stimulated to proliferate with PDGF. After this, two growth factor inhibitors, sunitinib and erlotinib, were administered to the culture in three different doses.

The results are clear: both sunitinib and erlotinib inhibit SMC proliferation in a dose dependent matter. If SMC proliferation and migration could be prevented, it could potentially result in a decrease of late allograft loss.

(150)

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# 1 Introduction

The first kidney transplantation in Finland was done in 1964 when a mother donated one of her kidneys to her son. More than 5300 kidney transplants have been done in Finland since then. 350 of these kidneys are from living donors. Approximately 175 kidney transplantations are performed annually. In the year 1980 1-year graft survival was only slightly more than 60%. As we learned how to prevent and manage hyperacute and acute rejection 1-year graft survival had risen to 94% by the year 2007 (1). The indication for kidney transplantation is end-stage renal disease. Diseases leading to this include diabetic nephropathy, glomerulonephritis, chronic pyelonephritis, obstructive nephropathy and haemolytic uraemic syndrome.

Rejection is an immune response inducing injury and destruction in transplanted organs. Rejection is defined as hyperacute, acute and chronic, which is also called chronic allograft nephropathy (CAN) when kidney transplants are concerned. Hyperacute rejection occurs minutes to hours after transplantation. It is characterized by the development of cyanosis within the graft. Evidence of progressive cortical necrosis can be seen necessitating removal of the graft. Hyperacute rejection is caused by sensitization to alloantigen prior to the transplant. Acute rejection is seen days to weeks after transplantation and develops in a previously well functioning graft. Acute rejection occurs when transplantation has been performed despite of a positive T cell cross-match. The recipient can also developed antibodies against donor HLA class 1 antigens after transplantation. Endothelial injury is the central feature of acute rejection (2). CAN is the primary reason for late allograft loss and returning to dialysis after a transplantation (3, 4). On of the most important features of CAN is the proliferation of smooth muscle cells (SMC) and their migration into the intima of blood vessels of the graft (5). This leads to luminal narrowing and decreased blood flow.

Growth factors play an important role in the proliferation and migration of SMCs (6, 7, 8). Through these actions they also have significant impact on the development of

atherosclerosis. The growth factors considered in this study are platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). They all belong to the receptor tyrosine kinases and inhibitors to them are used in clinical oncology. Inhibitors of receptor tyrosine kinases are potential new drugs in oncology. The first small molecular growth factor inhibitor approved for clinical use was Imatinib (GLIVEC®; Novartis) in 2001. The first indication for Imatinib was treatment of chronic myelogenous leukemia in blast crisis, in accelerated phase, or in progression of chronic phase after one interferon treatment. The indications for Imatinib have since then been widened to include gastrointestinal stromal tumors, among others (9).

Sunitinib (SUTENT®; Pfizer) is a small molecule receptor tyrosine kinase inhibitor. Its inhibition concerns the class III / IV split kinase domain subgroup of receptor tyrosine kinases. This group contains platelet derived growth factor receptors (PDGFRs), vascular endothelial growth factor receptors (VEGFRs), the stem cell factor receptor (c-KIT) and fibroblast growth factor receptor 1 (FGFR-1). Erlotinib (TARCEVA®; Roche) is an orally bioavailable receptor tyrosine kinase selectively inhibiting the epidermal growth factor receptor (EGFR). Both sunitinib and erlotinib are used in clinical oncology.

Aorta denudation experiments are used as a model for accelerated atherosclerosis. In previous experiments our group has denudated the aorta of male Wistar rats (Scanbur, Sollentuna, Sweden) with balloon cathetry. Postoperatively the rats received either sunitinib or erlotinib treatment in different doses. After 14 days the area of neoitima of midaortic sections was analysed. In that in vivo study sunitinib and erlotinib decreased neotimal formation in a dose dependent manner. The immunohistochemistry revealed the expression of PDGF-A and PDGF-B in the neointimal areas of the control rats treated with vehicle only. PDGF-B was expressed mainly on the luminal surface of the neointima. Sunitinib decreased the expression of these two growth factors (10).

The aim of this study is to verify the results of our previous rat aorta denudation experiments. This time SMC will be studied in vitro. They are stimulated to proliferate with PDGF-B and then the effect of sunitinib and erlotinib on SMC proliferation will be

studied in three different doses. The hypothesis is that the effect of the medication on cell growth will show a similar dose dependent result as the one found in the denudation experiments. An important purpose of this study is also to develop a new protocol for SMC culturing in our laboratory. This will provide us with a valuable tool for further studies.

# 2 Literature Review

## 2.1 Chronic allograft nephropathy

CAN is still the major problem in kidney transplantation. As 1-year graft survival is up to 94% due to excellent immunosuppressive therapy, CAN is an even increasing reason for late allograft loss (1). In a study with over 850 kidney transplant patients done by Ponticelli 11,2% of the patients lost the allograft due to CAN. In the same study, CAN was responsible for 49,2% of all allograft losses. The second most common cause was death 35,5%. This is to say that CAN is an even greater cause of allograft loss among the patient who survive (11). Histologically a mild form of CAN can be seen in 94,2% of adult patients one year after the transplantation (3).

CAN can be seen from months to years after transplantation. Its clinical manifestations are gradual decrease in renal function accompanied by hypertension and hyperuricemia. (12, 13). CAN is characterized by tubular atrophy, interstitial fibrosis, glomerular changes and fibrous intimal thickening (4). The histological changes are seen early post-transplant before alterations in renal function are evident (12). Vasculopathy is a prominent histological feature of CAN leading to vascular remodeling and concentric luminal narrowing due to intimal thickening caused by SMC proliferation and migration. This accelerated atherosclerosis of the blood vessels of the graft is believed to be one major factor in late graft loss in response to chronic rejection (5). It is hypothesized that acute rejection could cause the primary injury leading to CAN. Acute rejection causes induction of reparative mechanisms, resulting in fibrosis and

mesenchymal cell proliferation (14). Acute rejection episodes have proven to have a negative influence on graft survival (15). A part of this effect is probably due to subacute episodes, which cause no symptoms and therefore cannot be treated (16). Certain characteristics of the donor and recipient influence the risk of CAN (12). These include obesity, hypertension, hyperlipidemia and diabetes.

## 2.2 Smooth muscle cells in atherosclerosis

Many different cell types, including macrophages, lymphocytes, endothelial cells, and SMCs, are involved in the formation of atherosclerotic lesions. Damage to the endothelium or removal of it results in an increased SMC proliferation and migration (17). This is because the damaged endothelium produces less nitric oxide and attracts leucocytes. Nitric oxide increase SMC apoptosis and leucocytes secrete growth factors.

Regions subjected to atherosclerotic plaque formation contain abundant SMCs whereas regions that are more resistant contain fewer. The medial layer contain the majority of the SMCs in the vessel walls but areas of the intima called intimal thickenings also contain large number of SMCs (18). Two types of thickenings have been identified: eccentric and diffuse. Eccentric intimal thickenings often involve up to half of the circumference of the arterial wall. They are found mainly in arterial branch points and areas of turbulent blood flow. Regions of eccentric thickening correlate with the later formation of atherosclerotic lesions. Diffuse thickenings occur throughout the vascular system and increase with age (18).

The primary initiating event in atherosclerosis is the accumulation of low density lipoprotein (LDL) in the subendothelial matrix (19). The trapped LDL particles undergo modification, including oxidation, lipolysis, proteolysis and aggregation, and the modification leads to their contribution in inflammation and foam-cell formation (21). The majority of foam cells are thought to derive from macrophages. However also SMCs express LDL receptors and variety of other cholesterol uptake receptors and give rise to a significant number of lipid laden cells (20).

Atherosclerosis is a form of chronic inflammation and SMCs contribute to the production of cytokines involved in this process. The extra cellular matrix accumulates during disease progression (20) and although endothelial cells and macrophages both contribute to extra cellular matrix production, SMCs are known to be the major producers of connective tissue both in the healthy and atherosclerotic vessels (22).

## 2.3 Growth factors

### 2.3.1 Platelet derived growth factor

Ligands and receptors

PDGF belongs to the receptor tyrosine kinases which exert their effect by catalyzing the transfer of the of the γ phosphate of ATP to hydroxyl groups of tyrosines on target proteins (23). Four different PDGF chains designated A through D have been identified, the PDGF-C and –D chains as late as in 2000 and 2001, respectively (24, 25). The A and B chains are ~100 amino acid residues long and show approximately 60% homology in amino acid sequence identity (6) while the C and D are 345 and 370 residues long and share a 43% homology (26). Activated platelets secret PDGF -A, -B and -C but not PDGF-D (27). PDGF-A and -B form both homo- and heterodimerized functional polypeptide chains but PDGF-C and –D form only homodimers (26). All chains have to be proteolytically cleaved to be activated (28, 29). Human platelets whose α-granules are the major storage site for PDGF contain AA, BB and AB dimers suggesting that the dimers are randomly assembled (30). PDGF dimerizes two receptors upon binding. α-receptors bind all but PDGF-D chains and β-receptors bind -B and -D chains (29).

PDGF C and D are expressed in many organs. High levels are found in heart, pancreas, kidney and ovary. PDGF-D expression is generally lower than PDGF-C expression. In all tissues expressing PDGF -C and -D the classical PDGF-A and PDGF-B are co-expressed (26). In another recent study PDGF-C and PDGF-D were found to be expressed in macrophages, SMCs and endothelial cells in human atherosclerotic plaques

(31). This suggests that PDGF-C and PDGF-D, like PDGF-A and PDGF-B, play important roles in atherosclerosis.

The receptor dimerization allows the cytoplasmic portions of the receptors to autophosphorylate each other on tyrosine recidues (32). It has also been shown that cell adhesion via integrin receptors leads to PDGFR activation (33). Receptor activation leads to the initiation of a number of different signaling pathways. Phosphatidylinositol$_3$-kinase (PI$_3$-kinase), phospholipase C (PLC)-γ, the Src family of tyrosine kinases, the tyrosine phosphatase SHP-2 and GTPase activating protein (GAP) activating Ras are themselves enzymes phosphorylating and activating transcriptional factors (6). The strength of the signals is modulated by the simultaneous activation of stimulatory and inhibitory signals. The inhibition is conveyed by activation of tyrosine phosphatases, also stimulated by PDGF (34).

Normal physiology

PDGF is synthesized by many different cell types, as previously mentioned. The rate of synthesis is often increased in response to external stimuli, such as exposure to low oxygen saturation (35), thrombin (36) or stimulation by growth factors and cytokines (6). The expression of the different PDGF chains is independently regulated (37).

Also the amount of receptors is dynamic and modulated by external stimuli. The cell's response to the stimuli depends on the receptor type or types expressed on the cell (32). The classical target cells for PDGF, fibroblasts and SMCs, express both α- and β-receptors, but generally higher levels of β-receptors (6). The expression of the β-receptors on connective tissue cells is low but increases during inflammation (37). The stimulation of vascular SMCs with basic fibroblast growth factor selectively increases the expression of α-receptors but not β-receptors (38).

PDGF is an important growth factor during development by means of paracrine function (6). It also bears an important role in maintaining the interstitial fluid pressure (39), probably through its ability to stimulate interactions between connective tissue cells and molecules of the extracellular matrix (6).

PDGF is important in wound healing and this also explains why it is an important factor in the development of atherosclerosis. As noted earlier, the quantity of β-receptors is up-regulated during inflammation. PDGF stimulates mitogenicity and chemotaxis of fibroblasts and SMCs (6), secretion of collagenase by fibroblasts (40), chemotaxis of neutrophils and macrophages, growth factor production by macrophages and the production of several matrix molecules like fibronectin, collagen, proteoglycans, and hyaluronic acid (6). This leads to an increase in the formation of granulation tissue, rich in fibroblasts and glycosaminoglycans (41). PDGF is released by platelets, activated macrophages, thrombin-stimulated endothelial cells, SMCs of damaged arteries, activated fibroblasts, as well as by epidermal keratinocytes (6). Reepitehelization and neovascularization also proceed with an increased rate, PDGF thus makes wound healing faster (41) while over activity of PDGF may be involved in the overhealing response of keloids (42).

Pathophysiology

PDGF is a major mitogen for SMCs and fibroblasts (6). SMCs make a big contribution to the formation of lipid laden cells, production of extra cellular matrix and cytokines (21, 22). This way PDGF has a central role in atherosclerosis. PDGF-A and smaller amounts of PDGF-B are found in cells in atherosclerotic lesions. The expression of PDGFR is also increased (43). SMCs in atherosclerosis often have altered gene expression patterns. PDGF-D stimulation can result in these kinds of alterations resulting in SMC accumulation. PDGF-D levels are up-regulated in endothelial cells exposed to hemodynamic forces (44).

PDGF also not only stimulates cells to divide, but actively prevent them from dying (7). This is supported by the over expression of PDGF or PDGFR in various forms of cancer, such as glioblastoma and sarcoma (6). PDGF has been shown to have an angiogenic effect (45) and exert a feedback control effect on platelet aggregation. An increase in PDGF stimulation leads to decreased platelet aggregation (46).

Clinical applications

PDGF inhibitors are approved in the treatment of a number of malignancies. Imatinib is for example used in Philadelphia chromosome positive chronic myelogenous leukemia,

gastrointestinal stromal tumors and metastatic dermatofibrosarcoma protuberans (9). Sunitinib on the other hand is used in gastrointestinal stromal tumors and metastatic renal cell carcinoma (47). Nilotinib (TASIGNA®; Novartis) a newly approved PDGF inhibitor is indicated for chronic myelogenous leukemia when treatment with Imatinib has failed (48).

### 2.3.2 Vascular endothelial growth factor

Ligands and receptors

There is a whole family of VEGFs but when using only VEGF it usually designates VEGF-A. Nine major splice forms of VEGF-A (from now on called only VEGF) have been identified in humans and are produced by alternative exon splicing. VEGF-165 is the major splice form due to its biological characteristics. The longest splice forms bind to the extra cellular matrix via the heparin-binding domain while the shorter splice forms lacking this domain are diffusible (49). The extra cellular matrix bound forms can be released by heparin and heparinase or by plasmin cleavage (50). A loss of the heparin binding domain results in reduced mitogenic activity (51). VEGF-121 and VEGF-165 induce mitogenic and permeability-enhancing activity on endothelial cells, whereas the longer splice forms trigger only permeability- enhancing activity (50). Most cell types produce several VEGF splice forms simultaneously (52).

VEGF binds to VEGFR-1, VEGFR-2 and to neuropilin1 (NP1), a co-receptor (53). All these three belong to the receptor tyrosine kinases. The binding sites on the VEGF are located at opposite ends of the molecule and can link two receptors to form homo- or heterodimers (52). VEGFR-1 expression is up-regulated by hypoxia and the function it mediates depends on the developmental stage and cell type. VEGFR-1 has been proposed to bind to VEGF and sequester it. This makes VEGF less available for binding to VEGFR-2. This way VEGFR-1 serves as a negative regulator to VEGFR-2 signaling (54). Another role of VEGFR-1 might be to regulate the paracrine release of tissue specific growth factors; VEGFR-1 stimulation has been found to augment the paracrine release of for example hepatocyte growth factor and IL-6 in liver sinusoidal cells (53, 55). An alternative splice form of VEGFR-1, called sFlt-1, has been shown to be an

inhibitor of VEGF activity (54). VEGFR-2 has been accepted to be the major mediator of the angiogenic, mitogenic, growth inducing and permeability-enhancing effects of VEGF (53).

VEGFR-1 and VEGFR-2 are expressed mainly on endothelial cells but additional cell types express one or both of the receptors (52). VEGFR-1 is for example found on hematopoetic stem cells, monocytes, vascular SMCs and renal mesangial cells whereas VEGFR-2 is found on circulating endothelial progenitor cells (56). In addition to the three receptors described above an additional VEGF-165 specific receptor has been found. Its expression is unusually large in several breast and prostate cancer cell lines (52).

Normal physiology

VEGF is a survival factor for endothelial cells both in vitro and in vivo and it promotes the growth of endothelial cells derived from arteries, veins and lymphatics. Furthermore mitogenic effects have been reported on retinal pigment cells, Schwann cells and pancreatic duct cells among others (53). VEGF also modifies the spectrum of released growth factors from endothelial cells. The VEGF mediated increase of fibroblast growth factor 2 (FGF-2) and a decrease of transforming growth factor β1 (TGF-β1) leads to SMC migration and proliferation (8). VEGFR activation also leads to activation of proteases required for the breakdown of the basement membrane and integrin expression, both required in angiogenesis, and initiation of cell migration (52). VEGF also promotes vasculogenesis (56).

VEGF makes inflammatory reactions more severe by increasing the vascularity at the site of inflammation (57). VEGF induces an increase in hydraulic conductivity of isolated microvessels through increased calcium influx (58) as well as induces endothelial fenestrations in some vascular beds (59) hence VEGF is also called vascular permeability factor (VPF). VEGF may also be important for glomerular endothelial cell repair after injury (60).

Inhibition of VEGF during development results in apoptotic changes in the vasculature, something not found during adulthood. It has been proposed that coverage by pericytes would be one of the factors making the vasculature independent of VEGF (53).

VEGF expression is stimulated by hypoxia and hypoglycemia (52), hormones such as estrogen (61), UV-B radiation and $H_2O_2$ (56) and growth factors such as TGF-α, TGF-β, EGF, keratinocyte growth factor, insulin like growth factor 1, FGF and PDGF (53). Hypoxia not only stimulates the induction of transcription but also stabilize the mRNA by protein binding (52). Antigen-, IL-2-, or hypoxia-activated T cells can produce VEGF and express VEGFR2. VEGF can in turn push the T-cells toward Th1 phenotype, which is associated with more cytotoxic T-cell activity (62).

Pathophysiology

Vascular changes due to up-regulation of VEGF are associated with cardiovascular disease, rheumatoid arthritis, diabetic retinopathy, delayed type hypersensitivity and asthma (57). Some of the previous studies point to that VEGF also promotes the progression of atherosclerosis (63). VEGF is expressed in aggregating macrophages in atherosclerotic lesions (64). Angiogenesis and growth of neointimal size in atherosclerotic plaques is thought to be mediated by VEGFR-2. VEGFR-1 mediates the migration of circulating monocytes into the vessel wall. This results in macrophage accumulation in the developing plaques (65).

VEGF mRNA has been found to be expressed in the majority of human tumors. As tumors expand and the distance from the cells to the nearest blood vessel increases the tumorigenic cells respond to the hypoxia by stimulating VEGF production (53).

Clinical applications

Bevacizumab (AVASTIN®; Genentech/Roche) and ranibizumab (LUCENTIS®; Novartis) are humanized anti-VEGF monoclonal antibodies. Bevacizumab is used in combination with other antineoplastic drugs in treatment for metastatic colorectal carcinoma, metastatic breast cancer, advanced non-small cell lung cancer (NSCLC) and advanced renal cell carcinoma (66). The inhibition of VEGF function may also attenuate the tumour's means to metastasize, possibly because the tumour cells come into contact with a lesser concentration of blood vessels (52). Ranibizumab is indicated for neovascular age-related macular degeneration (67).

### 2.3.3 Epidermal growth factor

Ligands and receptors

EGF is a single-chain acidic polypeptide of 53 aminoacid residues containing three intramolecular disulfide bonds, which are required for the proper tertiary structure. EGF is found in most bodily fluids, especially in milk (68). Early studies with EGF in the intact animal demonstrated its stimulatory effect on epidermal proliferation and inhibitory effect on gastric acid secretion (69).

Epidermal growth factor receptor (EGFR, also known as HER- 1 or ErbB1) belongs to the class of membrane bound tyrosine kinases. Its basolateral location in the epithelium makes it a mediator of signals between mesenchyme and epithelium. EGFR is expressed in cells of mesodermal and ectodermal origin (70). The epidermal growth factor receptor family (ErbB family) consists of the receptors ErbB 1 through to 4 and are also called type 1 receptor tyrosine kinases. Ligand binding with the EGFR results in dimerization of the receptor at the cell surface (71), followed by internalization of the dimerized receptor after which the cytoplasmic tyrosine kinase domain is subjected to autophosphorylation (72).

The EGFR is directly activated by EGF, TGFα, heparin-binding EGF, amphiregulin, betacellulin and epiregulin. The receptor is also activated by other factors, including membrane depolarizing agents, agonists for G-protein-coupled receptors, cytokine receptors such as prolactin and growth hormone and adhesion receptors, for example integrins. Also environmental stress factors such as ultraviolet irradiation, γ-irradiation, oxidizing agents, heat shock and hyperosmotic shock activate EGFR (73).

Normal physiology

The autophosphorylated EGF tyrosine kinase residues serve as binding sites for the recruitment of signal transducers and activators of intracellular substrates. These include $PI_3$ -kinase, Ras mitogen-activated protein kinase cascades (74) and phospholipase Cγ (PLCγ). These pathways regulate multiple biologic processes such as gene expression, cellular proliferation, division, differentiation, migration, angiogenesis and inhibition of apoptosis (72, 70). $PI_3$ –kinase promote cell survival and Ras is associated with cell

proliferation (74). Signaling through EGFR has been implicated in promoting proliferation and differentiation of the epithelial component of skin, lung, pancreas and the gastrointestinal tract (70). EGF is known to cause membrane ruffling formation due to extensive actin polymerization (75). Steroid hormones can augment the effect of EGFR signaling by activating the transcription of genes encoding for EGF ligands (70).

Pathophysiology

In normal skin EGFR expression is restricted to the basal layers whereas both the receptor and its ligand amphiregulin are expressed through the entire epidermal layer in psoriatic skin. EGFR is over expressed in the majority of solid tumors, including breast cancer, head-and-neck cancer, non-small-cell lung cancer (NSCLC), renal cancer, ovarian cancer, colon cancer and brain tumors. This promotes tumor growth, tumor cell motility, adhesion and metastasis (70, 72). EGFR activation also stimulates VEGF production, which is the primary inducer in angiogenesis (72). The hepatitis B virus (HBV), Epstein-Barr virus (EBV) and human papilloma virus (HPV) alter the expression of the EGFR (70).

Clinical applications

At the moment there are two predominant classes of anti-EGFR agents used in clinical cancer treatment: small-molecule tyrosine kinase inhibitors competing with ATP for the binding to the intracellular catalytic domain of the receptor and monoclonal antibodies blocking the ligand binding extracellular domain preventing activation (76). Cetuximab (ERBITUX®; Merck) is a monoclonal antibody. It can be used in some forms of EGFR-expressing metastatic colorectal cancers (77). Erlotinib is a receptor inhibitor and is used in treatment of metastatic pancreatic cancer and NSCLC (78).

## 2.4 Sunitinib

Sunitinib (SUTENT®; Pfizer) is a small molecule receptor tyrosine kinase inhibitor. It competitively inhibits VEGF dependent tyrosine phosphorylation of VEGFR and PDGF dependent phosphorylation of PDGFRβ and signaling through c-KIT. Even signaling

mediated by the tyrosine kinase activity of FGFR-1 is inhibited but in a lower portion. (79) Sunitinib also inhibits fms-related tyrosine kinase (FLT3/FLK-2/Stk-2) signaling (80). All of these molecules belong to the class III / IV split kinase domain subgroup of receptor tyrosine kinases. The inhibition is highly selective for its substrates and no receptor tyrosine kinases outside this subgroup are inhibited (79, 81). Enzymatic activity of nonkinase enzymes and receptors are not affected by sunitinib (81).

As hyperproliferation, migration, survival, differentiation, neoangiogenesis and invasion of tumors is enhanced by receptor tyrosine kinase signaling, sunitinib is used in cancer therapy. Clinical activity has been found in treatment for breast carcinomas, metastatic renal cell carcinomas and gastrointestinal stromal tumors (81).

## 2.5 Erlotinib

Erlotinib (TARCEVA®; Roche) is an orally bioavailable small molecule quinazoline derivative that selectively inhibits the EGFR tyrosine kinase (74). It prevents autophosphorylation by competing with adenosine triphosphate (ATP) for its binding site on the intracellular domain of EGFR (72). It is selective for EGFR and leads to the induction of apoptosis (82). It induces the disruption of mitochondrial function with loss of mitochondrial membrane potential and release of cytocrome c. The mechanism might be by facilitation of the formation of a megachannel in the outer mitochondrial membrane (83). Erlotinib treatment causes cell cycle arrest at G1/S phase because of accumulation of $p27^{KIP1}$ protein in the nucleus of the cells. The accumulation is due to promotion of gene expression and decrease in degradation (74).

The anticancer effects of erlotinib have been demonstrated in head-and-neck cancer, NSCLC, ovarian cancer and breast cancer (72). Erlotinib has been approved for treatment of locally advanced or metastatic NSCLC after failure of at least one chemotherapy regimen. It has also been approved for use in combination with gemcitabine as first-line treatment of patients with pancreatic cancer based on the improvement of overall and progression-free survival in a phase III trial (84). Erlotinib has also been shown to significantly reverse certain forms of multi drug resistance in

cancer cells by inhibiting the drug efflux and thus increasing the intracellular accumulation of the anticancer drugs (76)

# 3 Material and methods

## 3.1 Cell culture

The rat coronary artery SMCs (provided by Dariusz Lesczynski; Finnish Centre for Radiation and Nuclear Safety, Helsinki, Finland) were cultured in a culturing chamber in 37°C and a $CO_2$ concentration of approximately 5%. The cells were fed once every 24 hour with a media containing DMEM (GibCo) with 10% fetal bovine serum (FBS), 1% glutamine (GibCo), 2,5% penicillin and streptomycin (GibCo). The cells were split approximately once a week or when microscopic viewing showed a lack of space for the cells on the ground of the bottle.

## 3.2 Study design

At the beginning of the studies the cells were detached from the culturing bottle with trypsin/EDTA after which they were counted using a Burker counting chamber and appropriate equation. 550 cells were pipetted onto a 96-well breeding ground. The cells were allowed to attach to the ground in a FBS containing media for 24h after which the media was changed to a media lacking FBS and the cells were left in serum starvation for 72h. N = 6 for sunitinib experiments and n = 5 for erlotinib experiments.

### 3.3 Drug administration

After 72h of starvation the cells were stimulated to proliferate with 2ng/ml of PDGF and treated with either sunitinib or erlotinib raising the final concentrations in the wells to 0,05 μM, 0,1 μM and 0,2 μM of sunitinb or 1 nM, 2 nM and 5 nM of erlotinib. The concentrations were based on information about IC50 values available on the EMEA website (78, 85). The sunitinib capsules and the erlotinib powder were dissolved in Dimethyl sulfoxide (DMSO, Sigma).

### 3.4 Fixation and calculation

24h after drug administration the cells were fixed to the breeding ground using a 2% paraformaldehyde (PFA) solution and dyed with Mayer. This made it possible to count the cells without haste and to later return to the wells and recount them. Four microscopic fields using a 10x magnification were counted from each chamber.

### 3.5 Statistical analysis

The results are expressed as mean ± standard error of mean. Probability (p) $<0.05$ was accepted as significant. The significance between groups was determined by parametric analysis of variance, SPSS® (Statistical Package for the Social Sciences).

## 4 Results

In order to analyze the results a value called proliferation index was created. The non-stimulated control wells were given the proliferation index 1,0. When the other wells had been counted they were all compared to the wells serving as control. These wells received proliferation indexes in their ratio to the control wells.

Sunitinib

The data from sunitinib experiments (mean ± SD) is summarised in Table 1.

| Wells | Proliferation index | SD |
|---|---|---|
| Control | 1 | 0 |
| PDGF | 2,7 | 0,9 |
| Sunitinib 0,05uM | 1,3 | 0,6 |
| Sunitinib 0,1 uM | 1,0 | 0,3 |
| Sunitinib 0,2 uM | 0,9 | 0,3 |

Table 1.

We found that PDGF stimulated the SMCs to proliferate. This is evident since PDGF stimulated cells 2,7 times compared to control cells. We also found that administration of sunitinib ameliorated cell proliferation. Already the lowest sunitinib concentration (0,05μM) inhibited the PDGF induced proliferation significantly. No significant difference was detected in proliferation between the lowest sunitinib dose treated cells and the non-stimulated control cells. Increasing the sunitinib dose decreased the proliferation even further although no significant difference was seen between the sunitinib doses. This is graphically illustrated in Figure 1.

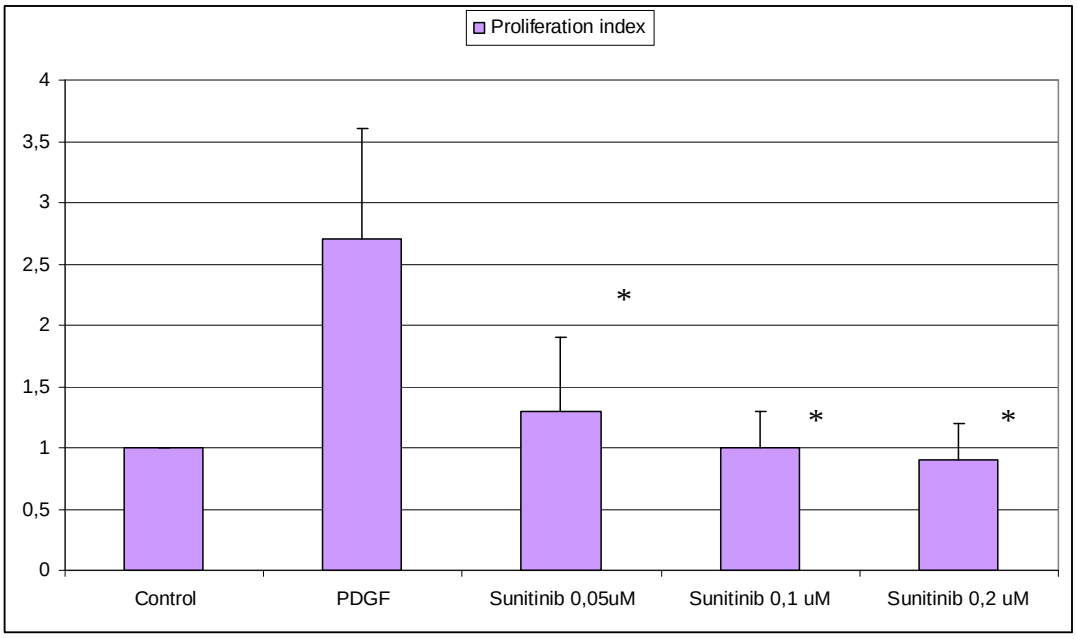


Figure 1. The results of sunitinib administration on PDGF induced SMC proliferation.

* are significant ($p<0,0001$) compared to PDGF induced control cells.

Erlotinib

The data from the erlotinib experiments (mean ± SD) is summarised in Table 2.

| Wells | Proliferation index | SD |
|---|---|---|
| Control | 1 | 0 |
| PDGF | 2,1 | 0,6 |
| Erlotinib 1nM | 0,8 | 0,3 |
| Erlotinib 2nM | 0,5 | 0,2 |
| Erlotinib 5nM | 0,8 | 0,5 |

Table 2.

Very similar results were detected with erlotinib as with sunitinib. The administration of erlotinib also inhibited PDGF induced proliferation of SMCs. Erlotinib decreased the proliferation ratio to values under the non-stimulated control cells already with the lowest concentration (1nM). Increasing the dose led to a slight increase in inhibition but the difference was not significant. The difference between the cells treated with the lowest erlotinib concentration and the control was not significant. The results are illustrated in Figure 2.

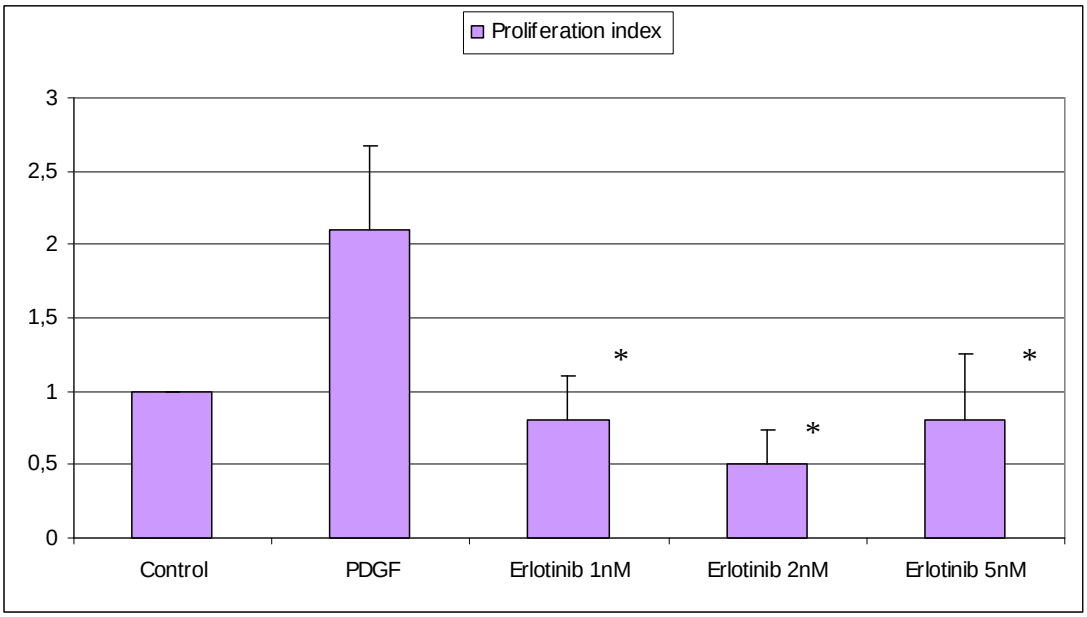


Figure 2. The results of erlotinib administration on PDGF induced SMC proliferation. * are significant ($p<0,0001$) compared to PDGF induced control cells.

# 5 Discussion

CAN is still the major problem in kidney transplantation. One of the central features of CAN is luminal narrowing in the vasculature of the graft. The formed neointima contains abundant SMCs (5). If the SMC proliferation and migration could be prevented, it would hopefully result in a better long-term graft outcome.

In this study the aim was to investigate if the tyrosine kinase receptor inhibitors sunitinib and erlotinib would decrease the proliferation of SMCs in vitro. The results are unequivocal; both sunitinib and erlotinib prevent SMC proliferation. Sunitinib inhibits proliferation in a dose dependent matter. Already the lowest concentration decreases the proliferation to the level of controls. When increasing the sunitinib dose even further inhibition is seen although no statistical difference is seen between the different doses. Similar results are seen with erlotinib. Also erlotinib inhibited the proliferation to the level of controls with the lowest does used. No further inhibition is achieved with higher concentration.

Our results concerning sunitinib are consistent with our previous experiments. Sunitinib ameliorates the neointima formation after arterial endothelial injury. This is through a combined inhibition of PDGF and VEGF signaling (10). The role of PDGF in initiating atherosclerosis is clear but the role of VEGF is still uncertain. VEGF seems to both cause plaque progression (63) and inhibit restenosis (86). In that study we demonstrate that the combined PDGF and VEGF-inhibition with sunitinib monotherapy prevented neointima formation almost completely. This indicates that PDGF has a bigger role in restenosis formation than VEGF has in preventing restenosis. Based on that data it seems that the inhibition of VEGF signaling with sunitinib still decreased neointima formation (87).

Sunitinib selectively inhibits VEGF-dependent endothelial cell proliferation, vascular sprouting and tube formation at IC50 values between 4-55 nM (85). However, much higher IC50 values ranging from 0,1μM to 0,25μM have been needed to inhibit signaling through other growth factor receptors. We used the following sunitinib concentrations: 0,05 μM, 0,1 μM and 0,2 μM, suggesting that in our study the inhibitory effect of sunitinib was also due to inhibition of other growth factors than VEGF.

The results from these sunitinib proliferation studies are also consistent with other results concerning PDGF/VEGF tyrosine kinase inhibition. Imatinib, another receptor tyrosine kinase inhibitor, inhibits signaling through PDGF, c-KIT and bcr-Abl receptors. It has been shown to prevent SMC proliferation in vitro (90, 91) and in vivo (92). Inhibition of SMC proliferation is probably due to its PDGFR inhibition. PTK787,

also receptor tyrosine kinase inhibitor, inhibits both PDGF and VEGF receptors and has been shown to partially inhibit PDGF induced SMC proliferation (91).

In recent studies done in our laboratory erlotinib has had a dose dependent influence on the formation of neointima in denuded rat aortas (88). Results from the present study suggest that this neointima formation, at least to some extent, is due to SMC proliferation and migration. Our findings are also consistent with the results found in an experimental pulmonary hypertension model. In that study EGFR blockers led to apoptosis of abnormally proliferated SMCs. This led to reversal of progressive vascular remodelling (89). If erlotinib could prevent the vascular remodelling in the graft, we would have a very potent drug against CAN.

The IC50 value for erlotinib's inhibition of EGFR signaling has been 2nM in previous in vitro studies (78). We used concentrations equal to the IC50 values (1 nM, 2 nM and 5 nM of erlotinib) indicating that the inhibition was due to selective EGF inhibition.

There are less studies concerning EGF in vascular biology. Genistein conjugated to EGF inhibits SMC migration in vitro and decreases the formation of neointima after vascular injury (93). Genistein inhibits the activities of tyrosine-specific protein kinases. In this case inhibition of EGF activity was due to competiton with ATP and the formation of nonproductive enzyme-substrate complexes (94). EGF binding sites are up-regulated in the neointima after balloon injury to the vasculature. They are concentrated to the cell layers closest to the lumen. This is also the site where the most sustained proliferative activity occurs (95). This confirms the role of EGF in SMC proliferation.

In conclusion we state that sunitinib and erlotinib inhibit SMC proliferation in vitro. Inhibition of SMC proliferation could be a potential intervention site to inhibit the accelerated atherosclerosis seen in dysfunctioning kidney transplants.

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