Chapter

SYNOPSIS
THIS thesis focuses on the diagnostic and therapeutic potential of VEGF. Since the recognition of its pivotal role in physiological and pathological angiogenesis research has evolved from cell culture and animal experiments to application in humans. The studies described in this thesis aim to contribute to the evaluation of circulating VEGF as a surrogate marker in the quantification of angiogenesis and of the therapeutic role of VEGF especially in therapeutic angiogenesis using gene therapy.

A review of the literature, presented in chapter 2, describes several potential clinical applications emerging in animal studies on therapeutic angiogenesis. These studies demonstrated that intramuscular (i.m.) gene therapy with the naked plasmid DNA encoding for VEGF165 could potentially address the limitations observed with the administration of the recombinant VEGF protein. VEGF exerts a site specific effect, due to the ischemia-induced upregulation of VEGF receptors, minimizing the risk of systemic side effects which occurs after systemic administration of the recombinant VEGF protein. Early clinical data (phase I and II studies) in peripheral limb and ischemic heart disease provide evidence of the proof of concept of these principles. Definite efficacy can only be proven by the results of placebo controlled phase III studies as described in chapter 9 of this thesis.

Another important direction in angiogenesis research is the quantification of angiogenesis by means of circulating VEGF measurements as a biomarker. In chapter 3 we conclude that the largest pool of the total circulating VEGF is contained in peripheral blood cells. This in support of the findings of other authors who also found that circulating VEGF is derived from various blood cells, in particular the platelets. From this line of evidence it was speculated that VEGF in the blood circulation may, at least in part, be stored and/or produced by peripheral blood cells, whereas originally it was thought to be only tumor derived. A relative new insight from our study, however, is that the predominant part of circulating VEGF resides in the neutrophilic granulocytes as confirmed by fluorescence-activated cell sorting (FACS). Fifty eight percent of the total circulating VEGF in healthy volunteers (n=15) and 69% in cancer patients (n=4) was found in the granulocytes whereas approximately 34% of the circulating VEGF in healthy volunteers and 11% in patients with cancer reside in platelets. The finding that the VEGF level per granulocyte in cancer patients (median 164 µg VEGF/L) was significantly elevated compared to that of the healthy volunteers (median 77 µg VEGF/L) may point to a biological significance of these cells in tumor angiogenesis. Future measurements of circulating VEGF aimed at prognostic or intervention targets in cancer patients, should take the various compartments of cell bound VEGF into account. More research into the mechanisms that influence circulating VEGF levels seems relevant, we attributed studies in chapters 5 and 6. Chapter 4 is a general introduction to the broad field of angiogenesis.

Circulating VEGF as surrogate marker

From studies on the optimal timing of resection during the menstrual cycle in pre-menopausal women with axillary node positive breast cancer without metastasis it was hypothesized that survival disadvantage in patients operated on during the proliferative phase of the menstrual cycle is dictated by cycle related variations in circulating VEGF. However, studies into
this relation are controversial.13,14 In chapter 5 we evaluated menstrual cycle-related variations in circulating VEGF levels and found that circulating VEGF, was inversely related with progesterone levels (r = -0.6, P=0.012). Circulating VEGF decreased throughout the menstrual cycle indicating that the lowest VEGF level occurred during the luteal phase. However, we found no differences in VEGF levels between the two menstrual phases. Therefore, according to the present data, it is unlikely that selection of timing for surgery in relation to VEGF levels on an individual basis would influence the individual prognosis in breast cancer patients.

In chapter 6 the effects of coronary artery bypass graft (CABG) surgery on systemic VEGF levels. Circulating VEGF levels were increased up to 6 days post CABG. VEGF levels correlated closely with leukocyte and platelet count but not with ischemic markers (troponin and creatin phosphokinase). The transient increase of VEGF levels indicates that circulating VEGF may be related to the inflammatory response following surgery, irrespective of global myocardial ischemia. As future anti- as well as therapeutic angiogenic therapy might be part of a multi modality treatment regimen in which surgery is a corner stone this finding is of clinical importance. Following surgery and in case of activated leukocyte and platelet counts care must be taken into account in the interpretation of the clinical significance of systemic circulating VEGF as a marker for disease activity or as a marker for treatment strategies.

Chapter 7 describes circulating VEGF levels in cancer patients (n=42) compared to the levels in healthy volunteers (n=28) and diabetic (n=37) patients with extensive vascular disease. A profile of elevated VEGF levels in cancer and diabetic patients may support the notion of VEGF as a potent predictive marker for treatment response. The number of patients with elevated VEGF levels among cancer patients was not different from that in the healthy subjects (3/42 versus 1/28). In contrast, relative to these groups, the diabetic patient group comprised significantly more patients (10/37) with elevated VEGF levels (P=0.015), and the mean level was also significantly elevated compared to that of the cancer patients and the healthy subjects (P=0.0001). Our data indicate that the use of circulating VEGF levels as a selection criterion for anti-angiogenic therapy in cancer patients is limited, whereas circulating VEGF may be a potent target in interventional studies or as a surrogate marker in diabetic patients.

**Diagnosis of critical limb ischemia**

Sodium fluoresceïne leakage studies in the patients with diabetes related critical ischemia showed no increased leakage as compared to controls in chapter 8. This probably indicates that the very low perfusion pressures mitigate any increase in capillary filtration. Following VEGF plasmid injection, but not after placebo, capillary leakage tended to increase.

**Therapeutic angiogenesis with VEGF**

The development of new methods of treatment for critical limb ischemia (CLI) would fulfil an eagerly awaited unmet medical success. This is especially relevant for diabetic patients who
have limited options beyond amputation due to distal and diffuse micro-vascular occlusive disease leaving no alternatives for invasive therapy. Therapeutic angiogenesis by means of intramuscular delivery of the VEGF gene may be an alternative treatment strategy for these patients. However, early attempts have not equivocally confirmed the positive results observed in the pioneering study performed by Baumgartner et al.\textsuperscript{6,15,16,17}

In chapter 9 results are presented of a double blind placebo controlled study performed in 54 adult diabetic patients with CLI. The primary aim of the study was to assess the effects of intramuscular (IM) phVEGF\textsubscript{165} (total of 4000 ug) on amputation rate at 100 days. Secondary aims were a 15\% increase in pressure indices (ABI/TBI), clinical improvement (skin, pain and quality of life) and safety. A major amputation was performed in 3 of the phVEGF\textsubscript{165} treated patients and in 6 of the control patients (not significant). A significant overall clinical (i.e. hemodynamic and clinical) improvement was seen in 14 versus 3 of the phVEGF\textsubscript{165} treated patients and the placebo treated patients respectively ($P=0.003$). No grade 3 and 4 adverse effects were seen. This relatively small randomized study is an important contribution to gene therapy in CLI with VEGF and should serve to restart the return of therapeutic angiogenesis to human trials on CLI.

**Physical methods in gene delivery**

The inconsistency in the early reports on gene therapy in CLI may be related to gene transfection issues. Therefore we considered to investigate physical methods, which facilitate DNA uptake into cells by the formation of transient pores in the cell membrane in order to improve the transfection efficiency of plasmid based gene delivery to skeletal muscle.\textsuperscript{18,19} We have compared (chapter 10) two of these physical methods, namely the electroporation technique or the use of ultrasound. Tibialis anterior muscles of mice (C57Bl/6) were injected using plasmid DNA encoding an intracellular protein (P53) followed by electroporation or ultrasound. Histochemical analysis of the P53 treated muscles showed a 36 fold increase in transfection efficiency with the electroporation technique relative to the sonoporation technique ($P=0.015$). No in-vivo short term toxicity was observed in the mice after i.m. injection and no damage was detected on stained tissue. The electroporation technique, compared to the sonoporation technique, may be a potential advance in the development of i.m. plasmid based gene transfer to the clinic.

**FUTURE PERSPECTIVES**

*Circulating VEGF as surrogate marker*

In the past few years, research on the quantitation of angiogenesis has resulted in a multitude of studies on circulating angiogenic factors which focus mainly on the prognostic implications in cancer patients. Although these studies indicate generally that high levels of for example
Diagnostic and Therapeutic Aspects

VEGF correspond with more malignant disease, no clear-cut clinically applicable marker function has emerged for any angiogenic growth factor.\textsuperscript{20,21}

Rather then further studies on circulating VEGF as a prognostic factor in cancer, the biological role of circulating VEGF in various pathological conditions deserves further attention. In particular studies in conditions which lead to activated platelets and leukocytes such as in physiologic inflammatory responses (chapter 6), or in a variety of autoimmune and infectious diseases\textsuperscript{22} or other non-cancer diseases\textsuperscript{23,24} (chapter 7) could yield worthwhile information.

The elevated VEGF levels found in diabetic patients (chapter 7) are interesting in that respect as they indicate that circulating VEGF may be a useful biomarker for end stage vessel disease in diabetic patients. The high levels of circulating VEGF suggest a pathophysiologic role in the development of diabetic vascular complications, but the therapeutic implications of this profile of elevated VEGF levels are not yet fully clear. Anti-VEGF therapy was already found to be successful in the treatment of diabetic retinopathy with ranibizumab, a smaller derivative of bevacizumab.\textsuperscript{25,26}

**Therapeutic angiogenesis using VEGF**

Early clinical trials on therapeutic angiogenesis progressed from the introduction of the recombinant protein or gene therapy with a single growth factor agent to the introduction in experiments of a combination of growth factors to achieve synergism, such as between bFGF and VEGF\textsuperscript{27} or ANG1 and VEGF.\textsuperscript{28,29} Out of concern that angiogenesis may be too complex a process to be stimulated effectively a separate line of research has explored ways to develop complementary strategies that would provide 'substrate' together with 'ligand': i.e. the so called cell-based "supply side angiogenesis" which has gained increasing popularity. In this concept the delivery of circulating monocytes or platelets, to increase local release of a cocktail of growth factors,\textsuperscript{30,31} or the delivery of endothelial progenitor\textsuperscript{32} or bone marrow cells\textsuperscript{33} is envisioned in order to overcome translational problems of the complexity of the angiogenic process. However, regardless of all the new avenues to introduce new angiogenic ligands or substrates, at least as important for clinical application is the recent observation that a critical period of growth factor-expression, i.e. a prolonged duration of growth factor exposure, is required to prevent regression of the newly acquired vessels.\textsuperscript{34,35} Thus, stable and prolonged - expression of transgenes encoding for angiogenic factors may be a prerequisite in order to achieve a clinically relevant angiogenic response. The electroporation technique seems to be the most promising physical method (chapter 10) to improve transfection efficiency\textsuperscript{36} and to prolong transgene–expression.\textsuperscript{37} The combination of plasmid based gene therapy with physical aid such as with electroporation should be considered in future studies on therapeutic angiogenesis. Optimization of electroporation parameters and conditions may be feasible for application in human diseases.\textsuperscript{38}

An important question is how to apply therapeutic angiogenesis to the best use in patients. Patients selected in the current trials on therapeutic angiogenesis mainly are patients with end stage vessel disease and critically threatened tissue viability. These patients may represent the group least likely to respond to angiogenic stimulation. Ideal candidates are
those with ischemic but viable tissue. Representing these groups are patients with intermittent claudication, who were already found to improve significantly compared to placebo treated with intra-arterial bFGF. In addition, this would also allow for the use of endpoints in studies which are clinically more relevant, such as exercise tolerance, which can no longer be used in end stage vessel disease.

The selection of patients who are likely to respond to angiogenic therapy requires, however, much further attention. Identification of factors that attenuate angiogenic responses, such as hypercholesterolemia and diabetes may have implications for developing new treatment strategies. Stratification should also account for these factors in future studies.
REFERENCES


