Summary

Cancer is one of the leading causes of death in the world. Current therapies are mainly focusing on eliminating the rapidly growing tumor cells by surgery, radiotherapy and/or chemotherapy. The major disadvantages of these approaches are the poor accessibility of tumor cells, development of drug resistance, and toxicity of drugs towards non-tumor cells. Therefore, new therapeutic strategies need to be developed.

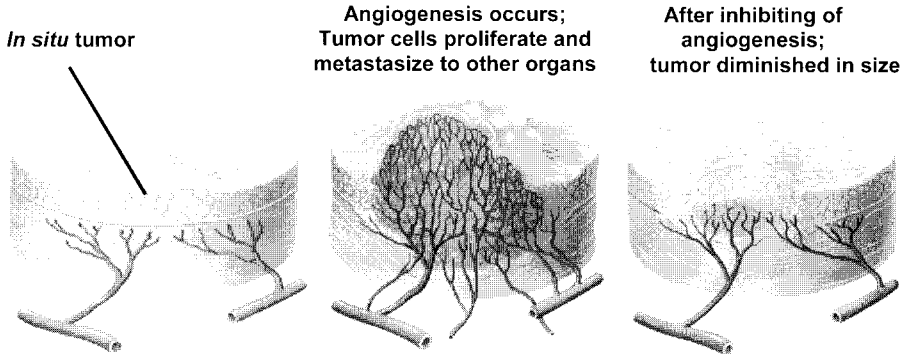

Figure 1. *Schematic presentation of the importance of angiogenesis for tumor growth. Angiogenesis transforms a small, relatively harmless, cluster of tumor cells (known as an in situ tumor) into a large tumor mass that can negatively influence organ function and can metastasize to other organs. Drugs that interfere with angiogenesis or otherwise block the tumor blood supply can reduce the size of a tumor. (Modified from Folkman, 1996).*

In the early 1970's, it was recognized that the formation of new blood vessels from pre-existing ones, also known as angiogenesis, is an important process in tumor growth (Fig. 1). Growth and survival of tumor cells depend on oxygen and nutrients supplied by the blood. As a consequence, the size of a tumor is restricted to a few cubic millimeters without the recruitment of new blood vessels. For a long time, it was thought that angiogenesis only occurs by sprouting of pre-existing vessels. Recently, it has become apparent that circulating endothelial progenitors, mobilized from the bone marrow, can contribute to neovessel formation as well. Since, in general, a large number of tumor cells depends on a small number of blood vessels for their growth and survival, inhibition of the blood supply is an effective approach to destroy solid tumors. It should be mentioned here that angiogenesis is also critically involved in chronic inflammation, and would, therefore, be an attractive target in the treatment of

chronic inflammatory diseases as well. However, in this thesis we focus on tumor angiogenesis as target for our strategies.

Endothelial cells are prominent players in angiogenesis. This, together with the fact that endothelial cells are easily accessible and genetically stable, makes them attractive targets for therapeutic intervention. Furthermore, inhibition of angiogenesis is, theoretically, independent of the type and location of a tumor, making anti-angiogenic therapies suitable for many tumors. Drug targeting approaches are a valuable tool for selective delivery of drugs that either do not reach the target cells sufficiently or are too toxic to non-target cells when applied systemically.

For successful drug targeting to angiogenesis-associated blood vessels, angiogenic endothelial cells need to be discriminated from the normal quiescent endothelium. In the past decade, using cellular and molecular biological approaches, many receptors, e.g., $\alpha_v\beta_3$, VEGFR, MMP-2/-9, Endoglin (CD105), and Aminopeptidase N (CD13), have been identified to be upregulated on tumor endothelial cells. In theory, these receptors can be explored as target epitopes for drug targeting purposes. Besides epitopes on endothelial cells, the ED-B domain of fibronectin (B-FN), a component of the extracellular matrix, has been described as a target epitope for tumor vasculature targeting. One of the most-studied target epitopes on angiogenic neovasculature is the $\alpha_v\beta_3$ integrin. This integrin mediates cell adhesion to extracellular matrix proteins by recognizing an Arg-Gly-Asp (RGD) sequence. RGD peptides with a constrained configuration have a high affinity for $\alpha_v\beta_3$ integrin, and can be used to target drugs to angiogenic endothelial cells.

Several approaches exploiting different target epitopes were investigated for their potential to interfere with the neovascular endothelium. For instance, toxic drugs (Arap *et al.*, 1998; Arora *et al.*, 1999), apoptosis-inducing agents (Ellerby *et al.*, 1999), cytokines (Carnemolla *et al.*, 2002; Curnis *et al.*, 2000), and therapeutic genes (Hood *et al.*, 2002) were delivered (in)to tumor endothelial cells, leading to reduced tumor growth. Furthermore, angiogenesis and tumor growth were inhibited upon occlusion of blood vessels by targeting coagulation factors to tumor vasculature (Huang *et al.*, 1997; Nilsson *et al.*, 2001; Ran *et al.*, 1998). Recently, immune effector cells were targeted to angiogenic blood vessels, leading to lysis of tumor endothelial cells and suppression of tumor growth (Niederman *et al.*, 2002).

The research described in this thesis aimed at the development of RGD-containing protein conjugates for the delivery of pharmacologically active agents or immune effector cells to $\alpha_v\beta_3$ on tumor vasculature. **Chapter 1** summarizes the current status and development of endothelial cell-specific drug targeting strategies for the treatment of cancer and chronic inflammation. The role of

endothelium in normal physiology, cancer and chronic inflammatory diseases, the development of macromolecular drug carriers, drugs of interest for interfering with endothelial cell (dys)function, and *in vitro* and *in vivo* experimental approaches to study (intracellular) drug delivery (in)to endothelial cells are reviewed.

In **Chapter 2** the aim of the research described in this thesis is presented. **Chapter 3** and **Chapter 4** describe the synthesis and characterization of multivalent RGD-modified proteins under development for drug targeting to $\alpha_v\beta_3$ on angiogenic endothelium. We chemically conjugated RGD (cRGDfK) and mismatched control RAD peptides (cRADfK) to a human antibody backbone (HuMab) as a model protein. The peptide:protein ratio was varied in the syntheses to obtain conjugates with different peptide load. We demonstrated that upon conjugation, the resulting RGDpep-HuMab conjugates specifically bound to both murine (H5V) and human endothelial cells (HUVEC), whereas the control RADpep-HuMab conjugate did not. Moreover, RGDpep-HuMab conjugates completely inhibited the adhesion of HUVEC to $\alpha_v\beta_3$ ligand vitronectin, indicating that the binding was $\alpha_v\beta_3$ specific. The binding of RGDpep-HuMab conjugates was increased in the presence of divalent cations, which is in agreement with the fact that interaction of $\alpha_v\beta_3$ with a ligand is dependent on divalent cations (Legler *et al.*, 2001). Furthermore, we demonstrated that multivalent RGDpep-HuMab conjugates bind to endothelial cells with dramatically increased avidity. The affinity of the conjugate with the highest peptide load was increased over 1000-fold as compared to the uncoupled peptide. Most likely, clustering of integrins by multivalent interaction of RGDpep-HuMab conjugates leads to an increase in avidity (van Kooyk and Figdor, 2000). From these data, we concluded that RGD-modified proteins can be further exploited for selective targeting of drugs or effector cells to $\alpha_v\beta_3$ on tumor vasculature.

In addition, internalization of radiolabeled RGD-modified proteins into primary human endothelial cells was investigated (**Chapter 4**). RGDpep-HuMab(IV), the conjugate with 23 peptides coupled per protein backbone, was internalized by HUVEC and degraded via the lysosomal pathway. This implies that RGD-modified proteins can be exploited for intracellular delivery of drugs into $\alpha_v\beta_3$-expressing angiogenic endothelial cells. Although we hereby demonstrated internalization of RGD-modified proteins into primary endothelial cells *in vitro*, the endocytotic capacity of angiogenic endothelial cells *in vivo* remains to be established.

In **Chapter 5**, the biodistribution and pharmacokinetics of RGD-modified proteins in mice bearing subcutaneous B16.F10 melanoma tumors are presented. The RGDpep-HuMab(IV) conjugate localized in the tumor specifically at the endothelium after intravenous administration in tumor-bearing mice. Additionally,

RGDpep-HuMab(IV) was taken up by liver and spleen, probably by $\alpha_v\beta_3$-expressing macrophages. The half-life of RGDpep-HuMab(IV) was prolonged as compared to the half-life of RGD peptides reported in literature ($t_{1/2}$ = 90 min and < 10 min, respectively). This is an advantage in terms of targeting potential, since the target cells are exposed to the conjugate for a longer time period.

Retargeting of cytotoxic T lymphocytes (CTLs) in order to specifically lyse tumor cells has been extensively studied as an approach to treat cancer. However, at present, this approach has not yet been successful, due to, among others, lack of infiltration of CTLs in the tumor tissue and the large number of target cells that needs to be killed. Retargeting of CTLs to tumor endothelial cells could be an alternative approach. Not only will accessibility of the target cells be much better compared to tumor cell retargeting strategies, but also endothelial cell-directed immunotherapy will have an amplification effect, i.e., less target cells have to be destroyed to induce a strong anti-tumor response.

By conjugating RGD peptides to a monoclonal anti-CD3 antibody, we prepared bifunctional conjugates that bound to both CD3 on CTLs and $\alpha_v\beta_3$ on tumor endothelial cells (**Chapter 6**). Furthermore, both RADpep- and RGDpep-anti-CD3 conjugates were able to activate CTLs via CD3 binding. We demonstrated that RGDpep-anti-CD3 conjugates were able to selectively redirect the cytolytic capacity of CTLs towards HUVEC, whereas RADpep-anti-CD3 conjugates had no effect.

Taken together, coupling of monocyclic RGD peptides to a protein backbone results in a multivalent macromolecular conjugate with an increased avidity for $\alpha_v\beta_3$, improved pharmacokinetic behaviour, and the ability to specifically localize at the tumor vasculature *in vivo*. In addition, RGD-modified anti-CD3 antibodies were able to cross-link immune effector cells with angiogenic endothelium and induce specific lysis of $\alpha_v\beta_3$-expressing endothelial cells *in vitro*. Based on this concept, drugs can be attached to the protein backbone or proteins with functional activity on their own can be conjugated with RGD peptides to be targeted to the tumor endothelium. Future studies using animal tumor models will have to reveal the potential of RGD-modified macromolecular conjugates to induce anti-tumor effects mediated via the vascular component.