Molecular Mechanisms of Action of Histidine-rich Glycoprotein in Angiogenesis Inhibition

CHUNSIK LEE
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Abstract

Angiogenesis, de novo synthesis of blood vessels from the pre-existing vasculature, is required both during embryonic development and in pathophysiological conditions. In particular, tumor growth needs new capillary vessels in order to both deliver oxygen and nutrients and to remove toxic metabolites. Growth of most solid tumors would be restricted to a microscopic size in the absence of neovascularization. Angiogenesis ensues as a result of a shift in the balance between pro- and anti-angiogenic molecules.

Histidine-rich glycoprotein (HRGP) is a heparin-binding plasma protein. We showed that HRGP inhibits endothelial cell migration and adhesion to vitronectin. As a consequence, HRGP attenuates growth and vascularization of mouse model tumors. The anti-angiogenic effect of HRGP is mediated by the central histidine/proline (His/Pro)-rich domain, which must be released from the parent molecule to exert its effect. A 35-amino acid residue peptide denoted HRGP330, derived from the His/Pro-rich domain, was identified as a minimal active anti-angiogenic domain of HRGP. HRGP330 induces disruption of molecular interactions required for cell motility, such as the integrin-linked kinase/paxillin complex. Moreover, HRGP330 inhibits VEGF-induced tyrosine phosphorylation of α-actinin, a focal adhesion kinase (FAK) substrate. Consequently, the motility of endothelial cells is arrested. By use of a signal transduction antibody array, we identified FAK, paxillin and growth factor receptor-bound 2 (Grb2) as tyrosine phosphorylated in HRGP330-treated cells. We confirmed that HRGP targets focal adhesions in endothelial cells, thereby disrupting the cytoskeletal organization and the ability of endothelial cells to assemble into vessel structures. A critical role of FAK in HRGP-inhibition of angiogenesis was validated using a FAK inhibitor, geldanamycin, which allowed rescue of endothelial cell actin rearrangement.

We identified another potential mechanism in the HRGP/HRGP330 anti-angiogenic effects, exerted through regulation of tumor-associated macrophages (TAMs). HRGP/HRGP330 treatment led to reduced TAM infiltration, which in turn caused a marked decrease in VEGF and MMP-9 levels in the tumor.

Taken together, our present studies show that HRGP/HRGP330 target endothelial cell adhesion, migration, focal adhesions, and furthermore, that HRGP is involved in regulation of macrophage infiltration.

Keywords: anti-angiogenesis, angiogenesis inhibitor, endothelial cell, histidine-rich glycoprotein, focal adhesion, tumor-associated macrophages, VEGF, MMP

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To my parents and family
List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.


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### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK</td>
<td>Activin-like receptor kinase</td>
</tr>
<tr>
<td>BCE</td>
<td>Primary bovine adrenal cortex capillary endothelial</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>Endostatin</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>HRGP</td>
<td>Histidine-rich glycoprotein</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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</table>
Introduction

Vasculogenesis and angiogenesis

Blood vessels supply oxygen and nutrients to the tissues of the body. During embryogenesis, the development of the vascular system occurs via two processes, vasculogenesis and angiogenesis \(^1,2\). Vasculogenesis involves the differentiation of endothelial cells from mesoderm-derived precursor cells, hemangioblasts \(^3\). The hemangioblasts aggregate to form primary blood islands. The cells in the interior part become hematopoietic cells, whereas the cells in the periphery layer differentiate into angioblasts, which then cluster and reorganize to form capillary-like structures (Figure 1) \(^4,5\).

Figure 1. Schematic illustration of vasculogenic and angiogenic processes in developing embryos. Figure modified from Saaristo et al., 2000 \(^5\).

After the primary vascular plexus is established, new capillaries form by sprouting or by splitting (intussusception) from pre-existing vessels in the process called angiogenesis \(^1,5\). During sprouting angiogenesis, endothelial
cells degrade the underlying basement membrane (BM), migrate, proliferate, and reassemble into tubes. Further remodeling steps occur when new vessels mature and form vessels of different sizes. Some vessels fuse to form large caliber vessels while others are regressed or pruned. During maturation, endothelial cells attract supporting pericytes and smooth muscle cells and synthesize a specialized basement membrane.

The angiogenic response is regulated by a range of growth factors/growth regulators, which may be directly or indirectly controlled through a number of mechanisms. One important regulator of angiogenesis is hypoxia, i.e. reduced oxygen tension. The response of cells to oxygen deprivation is mediated by hypoxia-inducible factor (HIF)-1, a heterodimeric transcription factor consisting of α and β polypeptides. HIF-1 binds to the hypoxia response element (HRE) in the promoter region of a large number of genes, to induce transcriptional activity. HIF-mediated angiogenesis results from the transcriptional activation of several angiogenic factors including vascular endothelial growth factor (VEGF).

Vascular endothelial growth factor and receptor (VEGF/VEGFR) family

VEGF, also referred to a vascular permeability factor (VPF), has been identified as one of the most important growth factors of endothelial cell physiology mediating vascular development, patho-physiological angiogenesis, as well as vascular permeability. VEGFs promote angiogenesis by stimulating endothelial cell proliferation, migration, and survival in newly formed blood vessels.

There are five mammalian VEGF family members (VEGF-A, B, C, D, and placenta growth factor; PlGF). VEGFs are secreted glycoproteins that form disulfide-linked bound dimers, whose subunits are arranged in an antiparallel manner. The VEGFs differ in their expression patterns, receptor specificities, and biological activities, although they are structurally highly related. Further diversity is attained by alternative splicing of the VEGFs, which in the case of VEGF-A regulates the ability to bind heparin and extracellular matrix. Furthermore, the biological function of VEGF/VEGFR is influenced by proteolytic processing of the ligands and by heterodimerization both of ligands and receptors. Genetic ablation studies show that deletion of a single VEGF allele results in embryonic lethality due to the fact that blood islands and vessels are abnormally formed. This phenotype is even more pronounced in embryos lacking both alleles of VEGF. Additionally, tumor studies in nude mice using Vgfl-deficient embryonic stem (ES) cells have shown a remarkably reduced tumor growth,
indicating that VEGFs also play an important role in pathological angiogenesis 19.

Figure 2. Schematic illustration of VEGF/VEGFR expression patterns and ligand specificity. Figure modified from Cross et al., 2003 13.

The VEGFs mediate their effects via several related but distinct receptor molecules denoted, VEGFR-1, -2, and -3. The VEGFRs are receptor tyrosine kinases organized in an extracellular part composed of seven immunoglobulin-like domains, a single transmembrane domain, and a kinase domain interrupted by a non-catalytic insert sequence. VEGFR-1, also referred to Flt-1 (Fms-like tyrosine kinase-1), is expressed in endothelial cells, pericytes, monocytes/macrophages, dendritic cells, hematopoietic stem cells, trophoblasts, and osteoclasts 20. It also exists as a soluble form (sVEGFR-1, i.e. the extracellular portion of VEGFR-1) 21. VEGFR-1 binds VEGF-A, B, and PlGF. The tyrosine kinase activity of VEGFR-1 is weak and it can be easily detected only in overexpression models such as the Baculovirus system 22. VEGFR-1 signaling has been shown to be involved in monocyte/macrophage migration and it also has a role in the recruitment and survival of bone marrow-derived progenitor cells 23-26. Genetic studies of Vegfr-1 deficient mice and mice expressing a kinase domain deletion of Vegfr-1, have suggested that VEGFR-1 acts as a negative regulator during embryonic development and functions as a decoy receptor to regulate the bioavailability of VEGF 27, 28. Notably, sVEGFR-1 has been correlated to the development of gestational toxemia and pre-eclampsia 29, 30.

VEGFR-2, also known as Flk-1 (fetal liver kinase-1) in mice and as KDR (kinase insert domain containing receptor) in humans, is the earliest marker...
for hemangioblasts. In addition to expression in EC, VEGFR-2 is expressed in neuronal cells, osteoblasts, pancreatic duct cells, retinal progenitor cells, megakaryocytes and hematopoietic stem cells. Vegfr-2 knockout mice are embryonic lethal at E8.5-9.5 due to defective blood island formation and vasculogenesis. Thus, VEGFR-2 is regarded as a major regulator of vasculogenesis as well as angiogenesis. The current understanding of VEGFR-2-induced signal transduction pathways are outlined in Figure 3. Interestingly, Ebos et al. reported that a soluble form of VEGFR-2 can be detected in plasma but it remains to be elucidated whether it is a result of proteolytic cleavage or alternative splicing.

**Figure 3. Schematic illustration of intracellular signaling pathways of VEGFRs.** Binding of signaling molecules to certain phosphorylation sites initiates signaling cascades, which lead to specific biological responses. Abbreviations: JM, juxta-membrane domain; TK, tyrosine kinase domain; KI, kinase insert; CT, carboxy-terminus; HPC, hematopoietic progenitor cell; EC, endothelial cell; eNos, endothelial nitric oxide synthase; TSAd, T cell specific adaptor; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; DAG, diacylglycerol; PLCg, phospholipase C gamma; FAK, focal adhesion kinase; MEK, MAPK and Erk kinase; MAPK, mitogen-activated protein kinase. Figure modified from Olsson et al., 2006.

VEGFR-3 has a distinct feature in which the 5th immunoglobulin-homology domain is proteolytically cleaved and the resulting polypeptide chains re-
main linked via a disulfide bond. The VEGF-C/VEGFR-3 signaling pathways are crucial for the development of the lymphatic vessels. VEGFR-3 gene targeted mice die at E9.5 due to cardiovascular defects before the lymphatic system is established. These data indicate that VEGFR-3 is critical also for blood vascular development. The sprouting and migration of differentiated lymphatic vessels from the embryonic cardinal vein is mediated by VEGFR-3 signaling and this signaling is essential for the survival and maintenance of the lymphatic system during embryogenesis and in the adult.

Other factors in vascular biology

Angiopoietins and Tie-receptors

Angiopoietins (Ang) are involved in the formation and maintenance of the vascular system. Their receptors, Tie-1 and Tie-2 (alternatively referred to as Tek), are expressed in endothelial cells and are critical for vessel remodeling, maturation, and stabilization. Mice lacking Ang-1 or Tie-2 or overexpressing Ang-2 in vascular endothelial cells undergo embryonic death with similar vascular defects. Moreover, transgenic mice overexpressing Ang-1 in the skin display increased vascularization and reduced vascular leakage even in the presence of excess VEGF. The effects of angiopoietins on tumor angiogenesis are still unclear and future studies are required to determine the definitive effects of angiopoietins in tumor angiogenesis.

Neuropilins

Neuropilins (NP) are transmembrane non-tyrosine kinase receptors for the collapsin/semaphorin family which regulate neuronal cell guidance. NPs are expressed in the vascular system, where NP-1 is mainly detected in arterial endothelium, whereas NP-2 is found in lymphatic vessels and at low levels in veins. Additionally, NPs bind VEGF in an isoform-specific manner. As accessory receptors, NPs seem to function to regulate the signaling of VEGF/VEGFRs by complex formation.

Heparan sulfate proteoglycans (HSPG)

HSPG are ubiquitously expressed on the cell surface and in the extracellular matrix. There are five human VEGF-A mRNAs encoding VEGF isoforms of 121, 145, 165, 189, and 206 amino acids produced by alternative splicing. VEGF121 and VEGF165 are the predominantly expressed isoforms but VEGF121 lacks exon 6 and 7 which correspond to the HSPG binding domains. Several tyrosine kinase receptors are known to require HSPG as a coreceptor. VEGFR-1 and VEGFR-2 contain a heparin-binding site in their extracellular domain. It has recently shown that heparin enhances signaling by VEGF165, but not by VEGF121, through VEGFR-2. Furthermore, Ja-
kobsson et al. have reported that HSPG in trans fully support the functional VEGFR signaling complexes in endothelial cells, which lead to sustained and prolonged signaling properties in response to VEGF$_{165}$ \(^5\). Thus, HSPG have important effects on the activities of the key angiogenic factor, VEGF and its receptors.

**Fibroblast growth factors (FGF)**

Several of the 22 different FGF family members have been implicated in regulation of angiogenesis \(^5\). Thus, FGF-2 was first identified as a tumor angiogenesis factor \(^5\). However, FGFs display pleiotropic effects, acting on a variety of cell types including endothelial cells, interacting with HSPG and tyrosine kinase receptors (FGFRs). FGFs play a significant role in wound healing, inflammation, angiogenesis, vasculogenesis, and tumor growth. Interestingly, several experimental data demonstrate the possibility of FGF/VEGF cross-talk such that tumor angiogenesis is induced by FGF-2 indirectly via activation of the VEGF/VEGFR complex. Seghezzi et al. \(^4\) demonstrated that systemic administration of neutralizing VEGF antibody dramatically reduces FGF-2-induced angiogenesis and both endogenous and exogenous FGF-2 modulates VEGF expression in endothelial cells \(^5\). On the other hand, Giavazzi et al. \(^6\) demonstrated that FGF-2 and VEGF stimulate vascularization synergistically but with distinctive effects on vessel functionality and tumor survival in tumor xenograft animal models \(^6\). Thus, under certain conditions, FGFs may act independently of VEGF to regulate angiogenesis.

**Platelet-derived growth factors (PDGF)**

The PDGFs consist of homo- and heterodimers formed by four different monomers; PDGF-A, -B, -C, and -D \(^5\). These factors exert their effects through two tyrosine kinase receptors; PDGF receptor-α and -β. Genetic studies in mice have revealed a crucial role for the PDGF-B/PDGFR-β signaling pathway in recruiting pericytes (PC) to newly formed blood vessels. The mice lacking either Pdgfb or Pdgfrb suffer perinatal death caused by vascular and renal dysfunction \(^5\). The primary cause of abnormal vasculature is pronounced reduction in PC recruitment and coverage of microvessels \(^5\). Loss of PCs leads to endothelial hyperplasia and abnormal junctions as well as compensatory upregulation of VEGF resulting in vascular leakage and hemorrhage \(^6\).

**Transforming growth factor-β (TGF-β)**

TGF-β is a multifunctional growth factor involved in a variety of cellular processes including proliferation, differentiation, migration, and survival. TGF-β is secreted in a latent form which needs to be cleaved by proteases before it binds to type I and type II serine/threonine kinase receptors \(^5\). TGF-β binds to both ALK (activin-like receptor kinase)-1 and -5 in endothe-
TGF-β signaling in endothelial cells is complex. It has been suggested that the balance between ALK-1 and ALK-5 guides the endothelium to enter one of two different phases: maturation or proliferation \(^ {65}\). The activation of the ALK-1-Smad1/5 pathway induces the expression of pro-angiogenic genes (e.g. Id1, endoglin) thereby affecting endothelial cell proliferation. On the other hand, the ALK-5-Smad2/3 signaling pathway induces the expression of maturation specific genes (e.g. connexin 37, plasminogen activator inhibitor-1) leading to endothelial maturation \(^ {66, 67}\). Interestingly, imbalance in TGF-β signaling via ALK-1 and -5 has been implicated in vascular defects such as impaired recruitment and differentiation of smooth muscle cells in HHT (hereditary hemorrhage telangiectasia; an autosomal dominant disorder resulting in vascular dysplasia) patients \(^ {68}\).

Ephrins and Ephs

Ephrins are membrane-bound ligands which bind to tyroine kinase receptors, Ephs on opposing cells thereby transmitting bi-directional signals between cells. The Ephrins/Ephs signaling is important in embryonic patterning and tissue morphogenesis, especially in axonal guidance during neuronal development \(^ {69}\). EphrinB2 is expressed primarily on arterial endothelium whereas its EphB4 receptor is expressed on venous endothelium \(^ {70}\). Mice lacking ephrinB2 or EphB4 die at E10-E11 due to defective remodeling of the primary vascular plexus and defective arterial-venous remodeling \(^ {71}\).

Tumor angiogenesis

The growth of a tumor mass is arrested at a size where oxygen diffusion becomes limiting (1-2 mm\(^3\)) unless it is supplied with vasculature to provide oxygen and nutrients \(^ {72}\). Since this hypothesis was introduced, it has been widely tested and essentially proven valid.

Many studies indicate that tumors secrete factors that elicit the entire series of events required in the formation of new capillaries \(^ {8, 73}\). These factors may act directly on blood vessels, or indirectly, e.g. on inflammatory cells that infiltrate the tumor where they in turn produce and release a battery of growth factors and proteases that affect blood vessels \(^ {74}\). Two important tumor associated factors are FGF-2 and VEGF.

The induction of angiogenesis is counteracted by inhibitors that commonly are fragments or conformational variants of endogenous proteins present in plasma or the basement membrane, such as angiostatin, endostatin, tumstatin, and interferons \(^ {75, 76}\). Physiological angiogenesis is considered to be tightly regulated by the balance between angiogenesis stimulators (pro-
angiogenic factors) and inhibitors (anti-angiogenic factors) \(^{77, 78}\). In pathological angiogenesis, this balance is lost (denoted the “angiogenic switch”) (Figure 4) \(^{78}\).

VEGF has been intensively investigated as a target for therapeutic angiogenesis. A number of studies have shown that the expression of VEGF is correlated with the onset of neovascularization. Elevated VEGF levels in the serum have been detected in the cancer patients \(^{79, 80}\). Moreover, a critical role of VEGF during tumor angiogenesis has been tested in animal models by use of neutralizing VEGF antibodies. In 2004, the US Food and Drug Administration finally approved bevacizumab, a humanized anti-VEGF antibody, for the treatment of colorectal cancer \(^{81}\).

![Figure 4. The angiogenic balance.](image)

Vascular homeostasis is regulated by the angiogenic balance between pro-angiogenic factors and anti-angiogenic factors. Figure modified from Hanahan et al., 1996 \(^{78}\).

Characteristics of tumor blood vessels

Tumor blood vessels are described as immature. They display several abnormal features that have implications for the development of novel therapies targeting tumor angiogenesis. Thus, tumor vessels have a chaotic structure and lack the regular structural architecture seen in normal tissue \(^8\). They are leaky due to unusually large gaps between adjacent endothelial cells, an abnormal basement membrane and extensive fenestration of the endothelial cells \(^{82, 83}\). The leakiness of tumor vessels might be ascribed to VEGF produced by tumor cells, as one of the prominent features of VEGF is potent induction of vessel permeability \(^{11, 84}\). Consequently, the blood flow in tumors is torpid and the interstitial fluid pressure is elevated \(^{85, 86}\). The high
interstitial fluid pressure restrains delivery of therapeutic agents and as a consequence, the efficacy of chemotherapy is decreased \(^{87,88}\). Furthermore, tumor vessels commonly display a loose association with pericytes and defects in their basement membrane coating, resulting in abnormal shapes and irregular thickness of tumor vessels (Figure 5) \(^{82}\).

How can tumor neovascularization be controlled? This is a vital question in the development of novel tumor therapeutics. For this purpose, we need to better understand the molecular mechanisms of tumor angiogenesis. It has been established that tumors induce vessel ingrowths from pre-existing vessels through a process denoted sprouting angiogenesis. However, several different mechanisms may operate to enhance tumor vascularization. These include i) recruitment of circulating endothelial precursors, ii) cooption, in which tumors grow along an already established vessel and iii) vasculogenic mimicry, the formation of vascular conduits by tumor cells \(^{83,89,90}\). It is likely that for different types of tumors, the mechanism of vascularization differ. Therefore, blood vessel-targeted therapy probably has to be tailored to suit the particular tumor type.
Endogenous angiogenesis inhibitors

Dr. Judah Folkman hypothesized that tumor growth is dependent on vascularization. Based on this idea, many studies have identified angiogenesis inhibitors that suppress tumor growth in mouse models, by blocking the vascular supply. Targeting endothelial cells rather than tumor cells is promising, since endothelial cells are regarded as genetically stable and it is unlikely that treatment of endothelial cells will lead to drug resistance.

The maintenance of vascular integrity is in part exerted via interaction of endothelial cells with the underlying BM. The components of the vascular BM are known to modulate endothelial cell behavior and support their functional and structural roles at various stages of the angiogenic process both during physiological and pathological condition. A number of endogenous angiogenesis inhibitors have been identified from proteolytic fragments of BM components (Table 1).

<table>
<thead>
<tr>
<th>Angiogenesis inhibitor</th>
<th>Parent protein</th>
<th>Inhibits proliferation</th>
<th>Inhibits migration</th>
<th>Induces apoptosis</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse endostatin</td>
<td>α1 chain of type XVIII collagen</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>α5β1 integrin glypican</td>
</tr>
<tr>
<td>Human endostatin</td>
<td>α1 chain of type XVIII collagen</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>α5β1 integrin</td>
</tr>
<tr>
<td>Tumstatin</td>
<td>α3 chain of type IV collagen</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>αvβ3 integrin</td>
</tr>
<tr>
<td>Endorepellin</td>
<td>C-terminal domain V of perlecan</td>
<td>NA</td>
<td>Yes</td>
<td>NA</td>
<td>α2β1 integrin</td>
</tr>
</tbody>
</table>

Table 1. Basement membrane-derived inhibitors of angiogenesis
Abbreviations: NA, not analyzed. Table modified from Kalluri, 2003.

Angiogenesis inhibitors exert their anti-angiogenic effects via multiple mechanisms. A plethora of studies have pointed out the collapse of the actin cytoskeletal organization and decreased endothelial cell migration as main endpoints of anti-angiogenic signals. Among many important regulators of cell migration, focal adhesion kinase (FAK) is a key player in the orchestration of signals from extracellular stimuli and growth factors, via integrins and growth factor receptors. The disturbance in molecular communication between FAK and the actin cytoskeleton, resulting in changes in actin dynamics and subversion of adhesion formation, disassem-
bly and turnover appears to be a common mechanism in anti-angiogenesis\textsuperscript{101,105,106}. The molecular mechanisms of action of a number of endogenous inhibitors are reviewed below.

**Endostatin (ES)**

Folkman and colleagues identified endostatin (ES) by screening fractions generated by chromatographic separation of medium conditioned by a hemangioendothelioma (EOMA) cell line; the conditioned medium inhibited endothelial cell responses \textit{in vitro}\textsuperscript{107}. ES is a 20 kDa fragment derived from the C-terminal NC1 domain of collagen XVIII\textsuperscript{108,109}. It is cleaved and released from the mother protein by several proteolytic enzymes including matrix metalloproteinases (MMP)-7, cathepsins, and pancreatic elastase-like enzyme\textsuperscript{110,111}.

A wide spectrum of molecular mechanisms have been implicated in the inhibition of tumor growth by ES, including inhibition of endothelial cell motility, induction of cell cycle arrest, and induction of apoptosis\textsuperscript{112,113}. ES has also been reported to inhibit the activity of some MMPs, including MMP-2, -9, -13, and membrane type-1-matrix metalloproteinases (MT1-MMP)\textsuperscript{114}. However, many investigations have shown that inhibition of endothelial cell migration is a consistent consequence of exposure of endothelial cells to ES\textsuperscript{101,115-117}. Thus, treatment with ES leads to a dose-dependent inhibition of VEGF-induced migration of human umbilical vein endothelial cells (HUVEC).

ES also affects FGF-2 induced reorganization of actin filaments, and tyrosine phosphorylation, alternatively, complex-formation, involving FAK, paxillin, and β-catenin, suggesting that ES interferes with cell-matrix interactions\textsuperscript{101}. The interference with cell-matrix interactions is probably a consequence of binding of ES to α5β1 integrins and cell surface heparan sulfate (HS) exposed on endothelial cells, which in turn results in Src-dependent activation of p190RhoGAP\textsuperscript{117,118}. As a consequence, RhoA activity is down-regulated, eventually resulting in disassembly of actin filaments and inhibition of cell migration\textsuperscript{117}. The exact site in ES mediating binding to α5β1 has not been identified. In contrast, a double mutant in which the ES arginine residues 193 and 194 were replaced by alanine residues have allowed exact mapping of the HS binding site on ES\textsuperscript{119}. Moreover, the composition of HS for binding of ES has been identified and shown to contain N-sulfated and N-acetylated disaccharide units (SAS-domain)\textsuperscript{120}. In accordance, glypican-4, a small shedded HS proteoglycan expressed on endothelial cells, has been shown to bind ES with an intermediate affinity\textsuperscript{121}.

In a transgenic mouse model of pancreatic islet cell carcinogenesis (Rip-Tag), systemic treatment with ES led to tumor shrinkage during early stages of tumorigenesis while late-stage tumors were relatively unaffected by the treatment\textsuperscript{122}. Interestingly, Schaffhauser \textit{et al.} recently reported that trans-
genic RipTag/ES mice moderately repress tumor progression. Moreover, the angiogenic switch was inhibited in RipTag;RipES mice. The data indicate that ES secreted by β-cells is not freely diffusible and may be sequestered in the islets of Langerhans.

Tumor studies in collagen XVIII/ES knockout mice showed similar tumor growth rates in wild type and collagen XVIII/ES null mice. However, subsequent studies where tumors were documented not to express collagen XVIII, indicated a clear reduction in tumor growth in the knockout animals. These data suggest that physiological levels of endogenous inhibitors of angiogenesis regulate tumor expansion.

Tumstatin
Tumstatin is a MMP-9-generated fragment (28 kDa) derived from the NC1 domain of the α3 chain of type IV collagen (Figure 6). Tumstatin inhibits blood vessel formation and tumor growth in several animal models. It has been proposed that the anti-angiogenic activity of tumstatin is exerted via binding to αvβ3 integrin, resulting in the inhibition of Cap-dependent protein synthesis. This inhibition is acting through FAK, PI3K, Akt, target of rapamycin (mTOR), and translation initiator factor 4E-binding protein (4E-BP1) signaling pathways.

Tumor studies on the α3 chain of collagen IV/tumstatin null mouse have provided genetic evidence for an in vivo role of tumstatin as an endogenous angiogenesis inhibitor. Whereas tumors grow to a larger size in the knockout animals than in the wild type control, treatment of the knockout animals with exogeneously administered tumstatin reduced the growth rate of the tumors back to the wild type level. These data suggest that physiological levels of circulating tumstatin (300-360 ng/ml in mice) allow efficient suppression of tumor growth in mice.

Endorepellin (ER)
ER was identified as the 85 kDa C-terminal domain V of the perlecan protein core. It contains three laminin-like globular (LG) domains separated by two sets of epidermal growth factor (EGF)-like repeats. This fragment is liberated by bone morphogenetic protein-1 (BMP-1)/Tolloid family of metalloproteases. Recombinant endorepellin inhibits VEGF-mediated endothelial cell migration; it blocks blood vessel growth and prevents endothelial tube formation. Endorepellin interferes with adhesion of endothelial, fibrosarcoma and colon carcinoma cells. The effects of endorepellin are mediated by α2β1 integrin and transmitted through increased cAMP levels, activation of protein kinase A (PKA), induction of phosphorylated FAK (pFAK) and transient increases in phosphorylated-p38 MAPK and pHSP27. Combined, these endorepellin-dependent events result in disassembly...
of the actin cytoskeleton and focal adhesions. Tumor studies in endorepelin/perlecan knockout mice have not yet been reported.

Tumor-associated macrophages (TAMs) in tumor progression

Macrophages have multiple functions which are essential for tissue remodeling, inflammation, and immune responses against cancer. Tumor-associated macrophages (TAM) constitute a major inflammatory component of leukocyte infiltration in many tumors. Activated macrophages can be cytotoxic for tumor cells mediated by anti-tumor mechanisms as an immune surveillance system. On the other hand, there is accumulating evidence that TAMs may promote tumor growth for example, by delivering pro-angiogenic factors, such as VEGF, to the tumor site. The detailed mechanisms underlying this apparently dual and opposing functions of TAMs are not fully understood, but may depend on different activation states of macrophages possibly regulated by different intrinsic properties of tumor cells.

Notably, accumulating data have identified that macrophages constitute two different populations classified by means of the expression level of chemokines, cytokines, membrane receptors, and effector molecules. TAMs are ‘alternatively activated’ (polarized) M2 macrophages (type II macrophages) which promote angiogenesis but on the other hand ‘classically activated’ M1 macrophages (type I macrophages) are effector cells that are cytotoxic for microorganisms and tumor cells. It appears that factors derived from tumor cells play a critical role to regulate different activation states of macrophages.

Regulation of TAMs

Chemotactic factors produced by tumor cells mediate migration of TAMs and inflammatory cells to the lesions. Tumor cells induce monocytes/macrophages by production of chemokines. A number of chemokines have been found in neoplastic tissues produced by tumor or stromal cells. For instance, CCL-2 (also, referred to as monocyte chemotactic protein-1; MCP-1) mediated macrophage attraction seems to be essential for tumor growth. CCL-2 is produced by various human tumors and also by fibroblasts, endothelial cells, and mononuclear phagocytes. Low CCL-2 concentration in the tumor leads to only modest monocyte infiltration, while higher CCL-2 secretion correlates with massive infiltration of monocytes/macrophages and destruction of the tumor mass. Moreover, CCL-2 induces production of MMP and urokinase-type plasminogen activator
Regulation of tumor growth by TAMs

TAMs have been known to be a source of potent soluble mediators of tumor growth and dissemination. They release a number of pro-angiogenic factors such as VEGF, FGF, PDGF, MMP, and uPA in order to promote angiogenesis and ECM remodeling. By production of these positive factors, TAMs regulate tumor growth and progression. In human breast cancer, CCL-2 expression is associated with TAM accumulation and with accumulation of various angiogenic factors such as VEGF, tumor necrosis factor-α (TNF-α), and IL-8. These findings indicate that TAMs are important for tumor progression in terms of regulation of angiogenic factors. Another study demonstrated that the number of infiltrating macrophages is proportional to the number of tumor cells in adenocarcinoma tissues. In addition, interaction of tumor cells and macrophages enhances VEGF release from macrophages in vitro. This study supports the notion that VEGF secreted by infiltrating TAM represents an essential support for tumor growth and angiogenesis. However, it should be indicated that macrophages secrete thrombospondin-1 (TSP-1), interferon-α (IFN-α), and interferon-β (IFN-β) which have anti-angiogenic properties. Some cytokines (e.g. TGF-β1, IL-1β) are also known to have pleiotropic effects, promoting or inhibiting angiogenesis under certain conditions. The detail mechanisms have not yet been elucidated as to how these signals and activities of macrophages and other inflammatory cells are concerted.

MMPs are a family of proteolytic enzymes that degrade ECM molecules and basement membrane. MMPs are involved in wound healing, angiogenesis, and in pathological conditions. During tumor angiogenesis, degradation of ECM components is necessary for blood vessel formation to promote migration, proliferation, and remodeling of endothelial cells. MMPs can liberate angiogenic factors sequestered by HSPG and ECM/basement membrane to enhance their availability. MMPs are not only produced by tumor cells but also by surrounding stromal cells including fibroblasts and infiltrating inflammatory cells. Particularly, macrophages, mast cells, neutrophils, and lymphocytes produce MMP-9 which is an important player in tumor angiogenesis.

Analysis of murine pancreatic islet model (Rip-Tag) has identified MMP-9 as critical in the onset of the angiogenic switch via release of VEGF. MMP-9 activates proteolytically cleaved TGF-β to promote tumor growth, invasion, and angiogenesis. Additionally, in MMP-9 deficient mice, tumor growth was decreased compared with wild type mice. However, contrary to these pro-angiogenic roles of MMP-9, recent studies demonstrate that MMPs can negatively regulate tumor angiogenesis. A number of MMPs can...
clease precursor forms of endostatin and angiostatin to generate active forms of these endogenous angiogenesis inhibitors \(^{111, 167}\). Interestingly, Pozzi. et al. showed that low levels of MMPs are necessary to enhance angiogenesis by facilitating endothelial cell migration and release of angiogenic factors, whereas high levels of MMPs might confer opposite effects \(^{168}\). Furthermore, a recent study has correlated the generation of tumstatin by MMP-9 mediated proteolysis of type IV collagen with the suppression of tumor growth \(^{127}\). In conclusion, Figure 6 briefly illustrates the contribution of TAM to tumor progression and angiogenesis \(^{169}\).

**Figure 6. Contribution of macrophages to tumor progression.** Macrophages play a critical role as a source of mitogenic factors, facilitating tumor angiogenesis, and metastasis. Figure modified from Weinberg, 2006 \(^{169}\).

**Signal transduction in cell migration**

Cell migration is a central process for every type of cell. The orchestrated movement of cells to a specific location is essential during embryonic development, wound repair, and immune response. Errors during this process is a feature of many pathological process. Of interest here is the contribution to vascular disease and tumor metastasis. A better understanding of the molecular details of cell migration may allow the development of new strategies of drug development. This section will discuss key players in cell migration.
Integrins

Integrins are composed of heterodimers of α and β subunits. The integrin receptor family consists of 18 α and 8 β subunits in the mammalian system, which together generate at least 24 combinatory receptors 170, 171. Major ECM components such as fibronectin, laminin, collagen, and vitronectin ligate specifically to different integrins and this engagement is crucial for a variety of biological processes including cell adhesion, migration, spreading, proliferation, differentiation, apoptosis, wound healing, and metastasis of tumor cells 172. In addition, cell-cell adhesion is mediated by certain integrins to transduce bi-directional signals with proper binding partners, generally immunoglobulin superfamily members such as vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) 170, 173.

Upon ligand binding, integrins undergo a conformational change and their cytoplasmic domains recruit signaling molecules and cytoskeletal proteins 174. The ligation of integrins can activate diverse intracellular signaling molecules including FAK, Erk, c-Jun N-terminal kinase (JNK), MAPK, integrin-linked kinase (ILK), and Rho family GTPases (Figure 7) 171.

Figure 7. Integrin signaling pathway. Integrin and growth factor receptor signaling pathways regulate cell migration, proliferation, and survival. Abbreviations: C3G, guanine nucleotide exchange factor; DOCK180, downstream of Crk-binding protein 180 kDa; NF-κB, nuclear factor κB; RAP, Ras-like small GTPase; JNK, c-Jun N-terminal kinase; PAK, p21-activated kinase; PtdIns, phosphatidylinositol; Shc, SH2 domain protein C1; MEK, MAPK/Erk; SFKs, Src family kinases. Figure modified from Guo et al., 2004 171.
Vascular endothelial cells express a subset of integrins including \( \alpha_1 \beta_1 \), \( \alpha_2 \beta_1 \), \( \alpha_3 \beta_1 \), \( \alpha_5 \beta_1 \), \( \alpha_6 \beta_1 \), \( \alpha_6 \beta_4 \), \( \alpha_\nu \beta_3 \), and \( \alpha_\nu \beta_5 \) \textsuperscript{175}. The expression of integrins in quiescent endothelium is different with only barely detectable levels of \( \alpha_2 \beta_1 \), \( \alpha_\nu \beta_3 \), and \( \alpha_\nu \beta_5 \) integrins \textsuperscript{176-179}. However, their expression is induced in angiogenic endothelial cells. In addition to these integrins, the de novo expression of the \( \alpha_4 \) subunit has been reported in angiogenic endothelial cells \textsuperscript{180}. \( \alpha_4 \) is generally expressed on hematopoietic cells or tumor cells and it has been described that \( \alpha_4 \beta_1 \) plays an important role in leukocyte infiltration in inflammation processes and therefore, in shaping of the tumor microenvironment \textsuperscript{181, 182}. Particularly, \( \alpha_\nu \beta_3 \) appears to be a critical regulator of tumor angiogenesis since a number of animal models demonstrate that small inhibitors or a neutralizing anti-\( \alpha_\nu \beta_3 \) monoclonal antibody (LM609) block tumor vascularization in response to growth factors \textsuperscript{183}. In gene targeting studies, \( \alpha_\nu \) deficient mice show extensive angiogenesis and brain bleeding but mice lacking \( \beta_3 \) or \( \beta_5 \) are viable and fertile \textsuperscript{184-186}. Moreover, \( \beta_3/\beta_5 \)-double knockout mice are also viable and fertile and do not display any vascular defects \textsuperscript{187}. Combined, these studies suggest that \( \alpha_\nu \beta_3 \) and/or \( \alpha_\nu \beta_5 \) are not required for angiogenesis. Interestingly, mice lacking \( \beta_3 \) or both \( \beta_3 \) and \( \beta_5 \) subunits show enhanced tumor angiogenesis \textsuperscript{187}. Why is there enhanced tumor angiogenesis in mice lacking one or both of these integrins? Reynolds \textit{et al.} have suggested that VEGFR-2 signaling in response to VEGF is enhanced in mice lacking \( \beta_3 \), leading to increased tumor angiogenesis \textsuperscript{188}. Endothelial cells lacking \( \beta_3 \) show 2-fold elevation of cellular levels of VEGFR-2 and 2-fold higher activation and phosphorylation of VEGFR-2 in response to VEGF. \( \alpha_\nu \beta_3 \) also mediates anti-angiogenic effects of several endogenous angiogenesis inhibitors including tumstatin and TSP. Interestingly, tumor angiogenesis in \( \beta_3 \)-deficient mice is not inhibited by tumstatin, suggesting that \( \alpha_\nu \beta_3 \) is required for the action of tumstatin \textsuperscript{127}.

**Focal adhesion kinase (FAK)**

FAK is a non-receptor tyrosine kinase which is composed of a FERM domain (protein 4.1, ezrin, radixin, and moesin homology) in the N-terminus, a kinase domain in the central, proline-rich region; the C-terminus has a focal adhesion targeting (FAT) domain \textsuperscript{103, 189}. FAK is phosphorylated on many tyrosine residues (Y397, Y407, Y576/577, Y861, and Y925) and activated upon integrin-mediated adhesion (Figure 8) \textsuperscript{103, 190}. FAK is auto-phosphorylated on Tyr397 and phosphorylated Tyr397 creates a binding site recognized by SH2-domain containing signaling proteins such as Src, phospholipase C\( \gamma \) (PLC\( \gamma \)), suppressor of cytokine signaling (SOCS), and the p85 subunit of PI3K \textsuperscript{189, 191, 192}. Tyr576/577 is phosphorylated by Src leading to enhancement of the catalytic activity of FAK \textsuperscript{193}. Src activates and phosphorylates FAK at Tyr925 which then allows the recruitment of Grb2 to this site \textsuperscript{194}. Grb2 binding to pTyr925 of FAK appears to dislocate FAK from
focal contacts \(^{195}\). FAK also contains four potential sites of serine phosphorylation (S722, S732, S843, and S910) but the role of these sites is not well defined \(^{196}\).

**Figure 8. Domain structure of FAK and phosphorylation sites.** FAK contains a FERM (protein 4.1, ezrin, radixin, moesin) domain, a kinase domain, and a FAT (focal adhesion targeting) domain. PR denotes proline-rich regions. Figure modified from Mitra SK. et al., 2005. Abbreviations: ASAP1, Arf-GTPase-activating protein; GSK3, glycogen synthase kinase3; Cdk5, cyclin-dependent kinase 5.

FAK activation has been shown to play a critical role in cell spreading, adhesion, apoptosis, proliferation, and survival. Cell migration requires the turnover of focal contacts, and FAK is known to regulate this process. The continuous remodeling of focal complexes to focal adhesions, and vice versa must be maintained to allow cell movement. In agreement, fibroblasts lacking FAK exhibit larger and more stable focal adhesions and have lost the ability to migrate both randomly and in a directed fashion \(^{196}\). How is FAK activated in response to VEGF? Le Boef et al. recently demonstrated that VEGF-mediated activation of the RhoA-dependent kinase ROCK results in ROCK-dependent phosphorylation of FAK on Ser732, which in turn allows autophosphorylation of FAK on Tyr407 \(^{197}\). FAK may also regulate other cellular processes in endothelial cells, such as survival and cell cycle. Thus, activation of PI3-kinase in endothelial cells is dependent on VEGF-induced FAK phosphorylation on Tyr397, which is a known PI3K-binding site \(^{198}\). Moreover, endothelial FAK signaling is implicated in the ECM-specific activation of Rac \(^{199}\). Rac, in turn, stimulates cyclin D1, thereby inducing phosphorylation of cyclin-dependent kinases (cdk)-1 and -6 and transition to the S phase.

That FAK plays a critical role in endothelial cell function has been clearly demonstrated by conditional Tie2-Cre-regulated knockout of FAK in endothelial cells, which results in disruption of endothelial cell development and embryonic death at E10.5-11.5 \(^{200}\). Interestingly, Yano et al. reported that knockdown of FAK and paxillin signaling by introduction of specific siRNA in HeLa cells resulted in increased cell migration, suggesting that these pro-
teins under certain conditions function to negatively regulate cell motility. In addition, suppression of FAK and paxillin expression resulted in impaired assembly of N-cadherin–containing cell–cell adhesions, suggesting a novel role for these proteins in the crosstalk between cell–matrix and cell–cell adhesions. These data implicate FAK and paxillin both as positive and negative regulators of cell migration, perhaps dictated by the cellular contexts.

Paxillin

Paxillin is a 68 kDa protein containing multiple domains, including four tandem LIM (lin-11, isl-1, mec-3) domains in the C-terminus, and an N-terminal leucine-rich domain (LD motif), which functions in protein recognition. LD motifs in the N-terminal region have been identified that support the binding of diverse signaling molecules including FAK, vinculin, integrin-linked kinase (ILK), and Crk. Extensive tyrosine phosphorylation occurs during integrin-mediated cell adhesion, embryonic development, fibroblast transformation and following stimulation of cells by mitogens that operate through the 7-transmembrane family of G-protein-coupled receptors. Five tyrosine residues (Y31, Y40, Y88, Y118, and Y181) serve as main phosphorylation sites of paxillin and phosphorylation of these residues plays an important role in focal adhesion formation and actin cytoskeletal organization. pTyr31 and pTyr118 generate docking sites for SH2 domain-containing proteins such as Crk to facilitate downstream signaling. In addition, two threonine (T396 and T401) and four serine residues (S188, S190, S455, and S479) have been identified as phosphorylated in paxillin. Phosphorylation at these sites is likely to induce conformational changes in paxillin, thereby regulating its interaction with specific binding partners. Paxillin null cells plated on fibronectin display altered focal adhesions, loss of lamellipodia, decreased rates of migration, decreased rates of spreading, effects on FAK, Cas, and MAPK phosphorylation, and inefficient FAK localization.

Integrin-linked kinase (ILK)

ILK is a serine/threonine kinase that binds to integrin β subunits and is suggested to function in diverse integrin signaling pathways. Ser473 in ILK is phosphorylated by Akt/PKB leading to integrin-mediated cell proliferation and survival. ILK is composed of four ankyrin repeats in the N-terminus, and a pleckstrin homology (PH)-like domain in the central part. The ankyrin repeat domain binds to an adaptor protein, PINCH (particularly interesting new cysteine-histidine rich protein) which has LIM domains. Several adaptor molecules including α- and β-parvin with actin-binding properties interact with the C-terminal domain of ILK. Paxillin can also interact with ILK and inhibition of ILK activity leads to loss of paxillin from...
focal adhesions. The complex formation of ILK-PINCH-parvin has been shown to be important for cell adhesion, spreading, focal adhesion assembly, and stress fiber formation. Tan et al. showed that ILK can promote VEGF expression in prostate cancer cells and VEGF-stimulated blood vessel formation. Furthermore, prostate tumor (PC3)-bearing mice treated with an ILK inhibitor showed reduced tumor growth and tumor angiogenesis. Additionally, Kaneko et al. demonstrated that both VEGF-induced chemotactic migration and proliferation in HUVEC was suppressed by the introduction of dominant negative, kinase-deficient ILK. More studies are needed to gain insight into the in vivo role of ILK in tumor progression.

Molecular organization and biology of histidine-rich glycoprotein (HRGP)

Biological properties of HRGP

HRGP was first isolated from human plasma in 1972. HRGP is a relatively abundant heparin-binding plasma protein (100-150 µg/ml in adults). It is synthesized in the liver and stored in platelets. The amino acid sequence of HRGP is conserved between vertebrates such as human, mouse, rat, and rabbit. HRGP interacts with many important factors in the hemostasis and immune systems. It has been shown to associate with heparin, plasminogen, fibrinogen, TSP and with components of the complement cascade, thereby modulating fibrinolytic and thrombogenic activities. Interactions of HRGP with IgG and Fc receptors may affect immune surveillance. Interestingly, gene targeting of HRGP does not appear to disturb development or growth of the mouse but results in enhanced coagulation and fibrinolytic activities.

The 12 kbp human HRGP gene is localized on chromosome 3q27 and consists of 7 exons. The location of the HRGP gene is very close to genes encoding fetuin and kininogen which all belong to the cystatin (cysteine protease inhibitor) superfamily. HRGP is equipped with two N-terminal cystatin modules that are encoded by exons I to III and IV to VI, respectively (Figure 9). The His/Pro-rich central domain encoded by exon VII is unique and is organized in 12 tandem repeats of five amino acids – the GHHPH motif – from amino acids 330 to 389.
Figure 9. Domain structure of HRGP. HRGP has three main domains; the N-terminus with two cystatin-like domain, a histidine-proline-rich (His/Pro-rich) domain in the central part, and the C-terminus. Both the His/Pro-rich domain and the C-terminus are disulfide bonded to the N-terminal part of the protein. Figure modified from Olsson et al., 2004.

The striking structure with multiple histidine and proline residues is affected by changes in the local pH, furthermore, it displays metal ion and heparan sulfate binding capacity. A number of divalent cations, such as Zn$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, and Co$^{2+}$ bind to the His/Pro domain. HRGP binds Zn$^{2+}$ with a $K_D$ in the range of 1–4 μmol/l which corresponds to physiologically relevant levels. The binding of Zn$^{2+}$ is of particular interest since it appears to be required for interactions with components such as heparin and heparan sulfate. Thus, Vanwilde-meersch et al. showed that HRGP335, a 26 amino acid peptide (amino acid residues 335-360) derived from the His/Pro-rich domain, displays full heparin/HS binding capacity in a Zn$^{2+}$-dependent manner. Moreover, it has been reported that plasminogen as well as tropomyosin bind to the His/Pro-rich domain under conditions of elevated Zn$^{2+}$ and/or low pH.

Effects of HRGP on phagocytosis

HRGP affects macrophage protein expression pattern. Long term-treatment (over 8 hours) of mouse inflammatory peritoneal macrophages with HRGP, results in decreased Fcγ receptor expression and impaired phagocytic function. In addition, HRGP regulates macrophage phagocytosis of opsonized sheep erythrocytes and complement activation. Gorgani et al. demonstrated that HRGP binds to apoptotic T cells and mature human monocyte-derived macrophages to potentiate the clearance of apoptotic cells. Furthermore, Jones et al. recently reported that HRGP strongly binds to necrotic cells in a heparan sulfate-independent manner. This interaction is mediated by the...
N-terminal domain of HRGP and facilitates phagocytosis of the necrotic monocytic cells.

**HRGP in regulation of angiogenesis**

Several studies have implicated HRGP in regulation, both stimulation and inhibition, of pathological angiogenesis. Simantov *et al.* showed that HRGP inhibits the interaction of TSP-1 to its receptor CD36, thereby inhibiting the anti-angiogenic effect of TSP-1. The binding of HRGP to TSP-1 is mediated by the N-terminal domain of HRGP. Furthermore, Juarez *et al.* were first to publish that HRGP inhibits tube formation and proliferation of endothelial cells in a His/Pro-rich domain-dependent manner. This was in agreement with our data (see Present investigation). Subsequently, the same group demonstrated that a synthetic peptide (HHPHG motif) derived from the His/Pro-rich domain inhibited angiogenesis and tumor growth at 75 mg/kg/day, allegedly in a manner dependent on cell surface-exposed tropomyosin. However, we could show that a peptide (HRGP335) containing this motif from the His/Pro-rich domain displays only a limited anti-chemotactic effect on endothelial cells. In this context, it is important to understand that HRGP undergoes limited proteolysis in vivo and in vitro, resulting in the release of fragments, one of which corresponds to all or part of the His/Pro-rich domain.

**Role of other cystatins in angiogenesis and tumorigenesis**

That cystatins may play an important role in regulation of angiogenesis and tumor growth is well documented by now. High-molecular-weight kininogen (HK), a cystatin family member, has been shown to possess anti-angiogenic activity. Cleaved high-molecular-weight kininogen (HKa) is generated from HK through kallikrein-mediated proteolysis. The anti-angiogenic activity of HKa is mediated via domain 5 (D5) which is a histidine/glycine rich region. The mechanism of action of the anti-angiogenic activity of D5 which is named kinostatin, involves decreased endothelial cell proliferation and migration. The D5 of kininogen and the His/Pro-rich domain of HRGP seem to be structurally and functionally related including the ability to bind HS and metals. The high degree of sequence homology implies that the His-rich domain in the two molecules exert similar functions. Both the His/Pro-rich domain of HRGP and the D5 derived from high-molecular-weight kininogen also exert anti-microbial effects by inducing breaks in bacterial membranes, in a Zn-dependent manner. FetuinB, yet another cystatin family member has been suggested to suppress tumor growth of mouse models. Moreover, cystatin family members have been reported to also act directly on tumor cells. Thus, Swallow *et al.* demonstrated that α2-HS glycoprotein (also, referred to FetuinA) inhibits TGF-β1 binding to TGF-
\( \beta \) receptors, thereby blocking receptor phosphorylation and activation \(^{254}\). Consequently, the nuclear translocation of Smad2/3 is inhibited. The inhibition of TGF-\( \beta \) signaling leads to suppressed tumor progression of intestinal neoplasias.
Present investigations

Aims

HRGP is a heparin-binding plasma protein, present in the plasma at a concentration of 100-150 μg/ml in adult. Multiple binding partners for HRGP have been suggested including heparan sulfates, divalent cations, and many components in blood coagulation and immune systems. The specific aims were:

- to study biological functions of HRGP in tumor growth and characterize the effects of HRGP on endothelial cells.
- to define the minimal active domain of HRGP and characterize its effect.
- to investigate HRGP330 with emphasis on its effects regarding tumor growth and tumor angiogenesis.
- to define the signaling pathways induced by HRGP and HRGP330.
- to examine the potential regulation of macrophage infiltration by HRGP and HRGP330 in tumor models.

Results

Paper I: A fragment of histidine-rich glycoprotein (HRGP) is a potent inhibitor of tumor vascularization.

The effect of HRGP on angiogenesis was tested in the chicken chorioallantoic membrane (CAM) assay. Treatment of the CAM with recombinant HRGP led to inhibition of blood vessel formation. Interestingly, administration of purified HRGP derived from human plasma as well as recombinant HRGP produced in human 293 cells efficiently arrested growth and vascularization of fibrosarcoma tumors in C57/Bl6 mice. To determine the molecular mechanism in inhibition of blood vessel formation by HRGP we tested its ability to inhibit chemotaxis of endothelial cells. We recorded a dose-dependent inhibition by HRGP of FGF-2- and VEGF-A-induced chemotaxis of endothelial cells but not of FGF-2-induced chemotaxis of mouse fibroblasts. These data imply a certain degree of specificity of HRGP for regulation of endothelial cell chemotaxis.
To initiate the dissection of the molecular mechanism of HRGP in inhibition of endothelial cell function, we examined the consequence of HRGP treatment on activation of FGFR-1 and the downstream Erk pathway. The activation of FGFR-1 and Erk1 and 2 was not influenced by HRGP treatment. Based on the data that HRGP blocked endothelial cell chemotaxis, the potential effects of HRGP on focal adhesions were studied. Interestingly, a 10 min exposure of endothelial cells to HRGP enhanced tyrosine phosphorylation of paxillin in BCE cells. Moreover, immunofluorescent staining of BCE cells with an anti-paxillin antibody showed increased number and intensity of focal adhesions. We therefore tested the effect of HRGP on the ability of endothelial cell to adhere onto different matrix proteins such as collagen I, collagen IV, laminin-1, and vitronectin. The attachment of the cells to collagen I and IV was slightly decreased in the presence of HRGP, but the most noteworthy effect of HRGP was on impaired attachment of endothelial cells to vitronectin. These data indicate that HRGP interferes with focal adhesion function, leading to reduced cell attachment and as a consequence, impaired cell migration. These data implicate $\alpha\beta3$ integrin, the main receptor for vitronectin on endothelial cells, as a target molecule for HRGP.

We then sought to identify which domain of HRGP would be responsible for the anti-angiogenic effect, by testing the ability of truncated, recombinant HRGP variants, to inhibit endothelial cell motility. Remarkably, a recombinant fragment encompassing the His/Pro-rich domain showed inhibition of chemotaxis toward FGF-2. By use of HRGP domain-specific antibodies, we could show that the His/Pro-rich domain was spontaneously released from the parent molecule, in the purified HRGP preparations. Interestingly, a HRGP truncated variant which failed to release the His/Pro-rich domain, lacked the ability to inhibit endothelial cell chemotaxis.

Paper II: Minimal active domain and mechanisms of action of the angiogenesis inhibitor histidine-rich glycoprotein

To further evaluate the potential of HRGP330 in tumor treatment, we treated HRGP330 in a human pancreatic carcinoma (BxPC3) model. We observed that tumor growth and vascularization were decreased by the treatment of HRGP330. Also, the data showed that HRGP330 inhibited VEGF-induced chemotaxis in endothelial cells. We then investigated the molecular mechanisms of action of HRGP330.

Complex-formation between ILK and paxillin is essential for the correct subcellular localization of ILK to focal adhesions and for cell migration. VEGF-stimulated endothelial cells induced complex-formation between the two molecules as shown by immunoprecipitation, immunoblotting, and immunohistochemical analysis, whereas HRGP330 inhibited VEGF-induced
complex formation between ILK and paxillin in focal adhesions. The molecular mechanism was further analyzed showing that HRGP330 inhibited VEGF-induced tyrosine phosphorylation of FAK. In addition, HRGP330 induced tyrosine phosphorylation of a FAK substrate, α-actinin, which results in negative regulation of its binding to actin. Furthermore, immunohistochemical data showed that HRGP330 interfered with VEGF-induced polarization and lamellopodia formation of endothelial cells. Combined, these mechanisms led to arrest endothelial cell motility.

To identify a potential receptor relaying the signal of HRGP/HRGP330, we employed a functional blocking assay using a neutralizing anti-αvβ3 antibody (LM609). The vitronectin-mediated endothelial cell adhesion was inhibited by the treatment with HRGP/HRGP330 and the anti-αvβ3 LM609 antibody. Furthermore, chemotaxis of endothelial cells in response to VEGF was reduced to similar levels by HRGP, HRGP330, and the anti-αvβ3 antibody. These data suggest that HRGP/HRGP330 may act by interfering with αvβ3 integrin signaling.

Paper III: Signal transduction in endothelial cells by the angiogenesis inhibitor HRGP targets focal adhesions

To identify signaling pathways induced as a consequence of treatment of endothelial cells with HRPG or HRGP330, we employed signal transduction antibody arrays. The arrays were probed with total cell lysates from vehicle and HRGP330-treated telomerase-immortalized micro capillary endothelial cells (TIME). Subsequently, the array filter was incubated with an antiphosphotyrosine antibody to identify tyrosine phosphorylated proteins induced by HRGP330. The data showed that a narrow range of signal transduction molecules implicated in cell adhesion/migration were specifically tyrosine phosphorylated as a consequence of HRGP330 treatment, such as FAK and its substrates paxillin and Grb2. These array data were validated in bovine adrenal cortex endothelial cells and TIME cells, showing increased tyrosine phosphorylation of FAK and Grb2 after HRGP330-treatment and furthermore, FAK tyrosine phosphorylation was sustained for up to 2 hours. We next investigated the distribution of tyrosine phosphorylated FAK in TIME cells after 10 min stimulation with HRGP and HRGP330. The immunostaining data showed that the number of focal adhesions containing tyrosine phosphorylated FAK increased by treatment with both HRGP and HRGP330 for 10 min.

The actin cytoskeleton organization appears to be a main target for several angiogenesis inhibitors. We already showed that focal adhesion formation and cell motility were affected by HRGP and HRGP330 and in the present study, we showed that the integrity of the actin stress fibers was disturbed by treatment with HRGP and HRGP330. Interestingly, treatment with
geldamycin, a known FAK inhibitor, restored the HRGP330 interruption of actin filament organization to the basal condition. Actin reorganization is critical not only in endothelial cell migration but also in formation of the lumen-containing vessel. We therefore examined the effect of HRGP/HRGP330 on VEGF-induced tubular morphogenesis of TIME cells in a three-dimensional collagen matrix. In agreement with our data on actin filament disruption, tubular morphogenesis was also severely disturbed by HRGP/HRGP330.

Paper IV: Regulation of tumor-associated macrophage infiltration, VEGF, and MMP production, by the angiogenesis inhibitor histidine-rich glycoprotein

Tumor-associated macrophages (TAM) represent a major component of leukocyte infiltration of many tumors and metastatic lesions. TAM have pleiotropic functions which can influence tumor growth. We wished to test the possibility that HRGP/HRGP330 exerts its anti-angiogenic effects on tumor angiogenesis and growth by regulation of tumor-associated macrophages (TAM) infiltration. To examine the extent of TAM infiltration, sections from human pancreatic carcinomas (BxPC3) grown in mice treated or not with HRGP330 were analyzed by immunofluorescent staining. Infiltrating TAM detected by the macrophage markers, F4/80 and CD68, was reduced in tumors derived from HRGP330-treated mice. Moreover, we investigated the extent of TAM infiltration in a loss-of-function model of HRGP. TAM infiltration was increased in fibrosarcomas (T241) derived from mice lacking HRGP.

TAMs express proteases (e.g. MMPs) which are implicated in the release of a number of pro-angiogenic factors (e.g. VEGF) bound to heparan sulfates, thereby facilitating tumor progression. We examined the expression levels of VEGF in tumors derived from the HRGP gain- or loss-of-function models. VEGF expression was reduced in the tumors derived from HRGP330-treated mice. Furthermore, double staining with F4/80 and VEGF showed that the fraction of TAMs expressing VEGF was decreased in response to the treatment with HRGP330. In contrast, tumors from mice lacking HRGP showed generally increased VEGF expression as well as specifically increased VEGF expression in the TAMs.

MMP-9 facilitates tumor angiogenesis by the degradation of ECM components and liberates VEGF which is sequestered by the ECM. We investigated MMP-9 expression regulated by HRGP in the different tumor models. Tumors derived from HRGP330-treated mice displayed a reduction of MMP-9 expression. Also, the fraction of TAM expressing MMP-9 was reduced in tumors from HRGP330-treated mice. Conversely, fibrosarcomas derived from the HRGP-deficient mice showed an increased level of MMP-9.
expression and the fraction of TAMs expressing MMP-9 was increased in the HRGP-deficient mice.
Concluding remarks

There is a growing body of experimental evidence that anti-angiogenic strategies will contribute to the future, refined cancer therapies that most likely will be tailored to meet the needs of particular tumor types or even individuals. It appears clear that anti-angiogenic cancer therapy offers several advantages over conventional therapy. Since the vascular endothelium is usually quiescent and genetically stable, targeting tumor endothelium may avoid cytotoxic effects which are so noticeable in conventional chemotherapy; furthermore, anti-angiogenic therapy is believed to be less likely to elicit drug resistance. Recently, treatment with Avastin (Bevacizumab) in combination with a specific type of chemotherapy (intravenous 5-fluorouracil [5-FU]-based chemotherapy), for treatment of patients diagnosed with metastatic colorectal cancer, was approved by the US Food and Drug Administration. This was a major breakthrough for the anti-angiogenic field, still, more critical research is required. In addition to the clinical development of anti-VEGF therapy, a variety of therapeutic strategies, such as tyrosine kinase inhibitors, soluble VEGF receptors (VEGF-trap), VEGF-neutralizing aptamers, and a monoclonal anti-αvβ3 integrin antibody (Vitaxin) are being evaluated. To achieve the ultimate therapeutic goal, namely eradication of tumor disease, anti-angiogenic therapy has to carefully assessed with regard to long term effects on the vasculature in non-affected tissue, and moreover, developed to identify the optimal design of combinatorial therapy.

Tumor-associated macrophages (TAM) play key roles in the regulation of tumor angiogenesis. TAMs may promote tumor growth by secreting growth factors and proteases that stimulate tumor cell proliferation, invasion and metastasis, as well as tumor angiogenesis. The effects of TAMs may include release or activation of growth factors, but TAMs may also contribute to a chronic inflammation in the tumor involving a plethora of second messengers acting to adverse the tumor disease. A number of therapeutic approaches have been tested to prevent these tumor-promoting effects of TAMs. However, these therapies may also impinge on another aspect of TAMs, namely in the immune surveillance against the cancer. Clearly, the apparent dual roles of TAMs potentially mediated by different TAM sub-populations are still very poorly addressed.

The biological properties of HRGP derived from in vitro studies should be validated by in vivo confirmations. Tsuchida-Straten et al. have reported
on a knockout mouse with a targeted deletion of the HRGP gene. Interestingly, the HRGP knockout mice were viable and fertile with no particular phenotypes or abnormalities with the exception for slightly enhanced blood coagulation and fibrinolytic activity. Obviously, tumor studies with the knockout mice will provide an important opportunity for us to evaluate HRGP as a true endogenous angiogenesis inhibitor. The tumor challenge study will be indispensable in our pursuit of the development of HRGP-based therapy in tumor treatment. Additionally, our data suggest that HRGP negatively regulates pro-angiogenic activities of macrophages in tumor progression.

It is critical to continue the work with detailed characterizations of the action and in vivo effects of HRGP and HRGP330 to develop this strategy for clinical trial. We hope to further contribute to insights in how HRGP is involved in regulation of immunity, hemostasis, angiogenesis, and other pathophysiological processes.
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Åsa, can I learn how to play the piano from you? Mozart or Chopin?
Sofie, why don’t you stop by Korea? I will guide you.
Irja and Charlotte, I will practice baking the cake. Let’s keep our fingers crossed.
Ingrid, can I see your acting as Lena again?
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Chunsik Lee

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