Galectins as targets for angiostatic cancer therapy

Iris Antoinette Ernestine Schulkens
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Galectins as targets for angiostatic cancer therapy

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Chapter 1

General introduction

Based on:
Angiostatic cancer therapy by targeting galectins in the tumor vasculature.
Iris A Schulkens, Arjan W Griffioen, Victor L Thijssen.

ACS Symposium Series: Galectins and Disease Implications for Targeted Therapeutics.
Introduction

The human vasculature is the biological infrastructure that facilitates the transport of nutrients, gasses and cells throughout the body. It consists of a complex network of blood vessels with an estimated length of approximately 100,000 km. Already early during embryonic development, the first vessels arise from endothelial precursor cells that organize into a primitive network of small capillaries. This de novo formation of blood vessels, or vasculogenesis, is followed by angiogenesis, a process by which new vessels emerge from existing capillaries. Several variants of angiogenesis are known, of which sprouting angiogenesis is the most extensively studied and best understood. Sprouting angiogenesis involves a series of steps which starts with the activation of endothelial cells (EC) in capillary vessels. The EC form the inner lining of all blood vessels and their activation is induced by growth factor signaling through specific receptors on the cell surface. Upon activation, several proteases are activated that degrade both the basement membrane and the extracellular matrix surrounding the EC. In addition, the EC become proliferative and migrate into the surrounding tissue creating new tubular structures. These premature growing vessels attract pericytes and smooth muscle cells for structural support and eventually a functional mature vascular network is formed.

While angiogenesis is important during physiological processes like embryonic development, wound healing and inflammation, it has also been implicated in different pathologies including cancer. Tumors, like most other tissues, rely on a continuous supply of nutrients and oxygen. To grow beyond a few cubic millimeters in size tumor cells have to induce angiogenesis. Under hypoxic conditions most tumors eventually undergo the so-called angiogenic switch which results in the secretion of pro-angiogenesis growth factors. This stimulates tumor angiogenesis which in turn allows expansion of the tumor mass and facilitates tumor metastasis (Figure 1). Not surprisingly, it has been proposed that interfering with tumor angiogenesis could provide an opportunity for cancer therapy. Evidence has accumulated that galectins in the tumor endothelium might provide opportunities for targeted cancer therapy.

Galectins

Galectins are structurally related carbohydrate-binding proteins, which are defined by their affinity for β-galactosides present on glycoconjugates, and by the sequence similarities in the carbohydrate recognition domain (CRD). To date, the galectin protein family consists of fifteen mammalian members, eleven of which are also expressed in humans. Based on the number and organization of the CRDs, galectins can be classified into three subgroups: prototype, tandem-repeat and chimeric galectins (Figure 2). Prototype galectins consist of a single CRD, while tandem-repeat galectins contain two different CRDs which are connected via a linker peptide. Galectin-3 is the sole member of the chimeric galectins and contains one CRD fused to an N-terminal tail of amino acid repeats.
General introduction

Each galectin has its own carbohydrate binding specificity, but interestingly galectins are also known to oligomerize. Prototype galectins, such as galectin-1, can dimerize, while tandem-repeat galectins can bi- or multivalently bind carbohydrates, thereby forming highly organized lattices with glycoconjugates. On the other hand, the chimeric galectin-3 forms disorganized heterogeneous networks with multivalent carbohydrates upon pentamerization. This oligomerization facilitates important functions of galectins, such as cell-cell or cell-ECM interactions. These carbohydrate-mediated functions mainly occur outside the cell, while protein-protein interactions in the cytoplasm or nucleus mediate intracellular function, e.g. in pre-mRNA splicing or apoptotic signaling. Considering the numerous diverging functions of galectins, it is not surprising that the deregulation of these proteins has been implicated in various pathologies, including cancer.

Figure 1. Tumor angiogenesis. Schematic representation of the different steps of the angiogenesis cascade. First, tumor cells under metabolic stress, e.g. hypoxia, secrete growth factors that activate endothelial cells in nearby capillaries. The activated endothelial cells produce proteases which degrade the basal membrane, accompanied by vessel dilatation and pericyte detachment. Next, endothelial tip cells start to move in the direction of the growth factor gradient. The continuous proliferating and migrating endothelial cells form tube-like structures which grow towards the tumor cells. Eventually, the growing sprouts anastomose and a functional vessel is formed by depositing a new basal membrane and attraction of pericytes. This process will eventually allow the tumor to grow and also will facilitate tumor metastasis.

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Chapter 1

Galectins in cancer

Galectin expression is often found to be altered in tumor tissue as compared to normal tissue. The two galectins that are most extensively studied in this context are galectin-1 and galectin-3. Galectin-1 levels are frequently found to be increased in cancerous tissues, irrespective of the tumor type. For example, increased galectin-1 expression was detected in pancreatic cancer\(^{21,22}\), hepatocellular carcinoma\(^{23}\), non-small cell lung cancer (NSCLC)\(^{24}\), and renal cell carcinoma\(^{25}\) as compared to healthy tissue. The alterations in galectin-3 expression levels appear to depend on the cancer type. While increased expression is observed for most digestive tract\(^{22,26,27}\) and urinary system tumors\(^{25,28}\), galectin-3 levels are predominantly decreased in tumors of the reproductive system, such as in prostate\(^{29,30}\) and ovarian cancer\(^{31}\). Furthermore, galectin-9 expression levels are often found to be decreased in tumor versus normal tissue\(^{29,32,33}\). The expression of other galectins in cancer has been less extensively studied.

Ample evidence suggests that altered galectin expression levels can be of prognostic value. In general, increased galectin-1 levels are associated with a more malignant phenotype and poor prognosis. For example, Zhang et al. observed a positive correlation between galectin-1 expression and clinical stage in epithelial ovarian cancer\(^{24}\). The prognostic potential of galectin-1 was shown in e.g. NSCLC, where high galectin-1 expression was associated with decreased survival rates\(^{24,35}\). As is the case for galectin-3 expression alterations in tumor tissue, the prognostic value of galectin-3 is also tumor type dependent. For example, galectin-3 expression negatively correlates with overall survival in NSCLC\(^{24,35}\), while a positive correlation was found in gastric cancer\(^{36}\). Although still limited, an increasing number of studies observed that high galectin-9 levels are generally associated with better patient outcome\(^{33,37-39}\).
The emerging prognostic potential of galectin expression in different cancer types suggests a role for galectins in tumor progression. Indeed, galectins have been shown to facilitate several key-processes during cancer progression, e.g. tumor cell transformation, metastasis, and tumor immune escape\(^2\). For example, evidence suggests that galectin-1 and galectin-3 are involved in the neoplastic transformation of normal cells into tumor cells, by deregulation of cell-growth regulatory mechanisms. Possibly, these effects are mediated by interaction of galectins with oncogenic Ras\(^{40,41}\). Another well-studied phenomenon of galectins in tumor progression is the effect on the regulation of cell survival. Exogenously added galectin-1 and galectin-9 were shown to induce apoptosis in e.g. large cell lymphoma cells\(^{42}\) and melanoma cells\(^{43}\), respectively, whereas anti-apoptotic activity was attributed to galectin-3 in a range of tumor cells\(^{19}\). Apart from direct effects on tumor cell function, galectins can promote tumor progression by facilitation of metastasis. Metastasis involves the detachment of tumor cells from the primary tumor and their attachment to endothelial cells or extracellular matrix (ECM) proteins at distal sites. Galectins can modulate the adhesion of cells to the ECM or to other cells via crosslinking of carbohydrates present on ECM proteins and cell membranes\(^{16}\). Both galectin-1 and galectin-3 have been associated with loss of tissue integrity by decreasing the adhesion of tumors cells to ECM proteins\(^{44-46}\). In contrast, galectin-9 was found to increase tissue integrity in models for melanoma\(^{38}\) and breast cancer\(^{37}\). These results correspond to the observations that intratumoral levels of galectin-1 and galectin-3 are often increased, while galectin-9 levels are generally decreased in tumor tissue. When tumor cells disseminate through the body, immune cells will recognize tumor antigens present on these cells and eradicate the tumor cells. To avoid this, galectins can be secreted from the tumor cells, thereby affecting a variety of inflammatory responses\(^{47,48}\). Moreover, galectins are also expressed by immune and inflammatory cells themselves\(^{48}\). Galectin-1, -3 and -9 all have well-documented immune-suppressive functions, for example by inducing apoptosis of T-cells\(^{49-51}\). In vivo experiments indeed revealed that galectin-1 enables tumors to escape from immune surveillance and facilitates metastasis by negatively regulating T-cell survival\(^{52,53}\). Although galectin-3 and galectin-9 have been found to modulate the activation and survival of several types of immune cells\(^{48}\), a clear role in tumor immune escape has not yet been described.

Apart from expression in tumor cells and immune cells, galectins have been found to be expressed by tumor endothelial cells. Endothelial galectins have been described to promote both tumor metastasis and tumor immune escape, but can also facilitate another key event during tumor progression, i.e. tumor angiogenesis. Since endothelial galectins appear to affect tumor progression at multiple levels, the inhibition of galectins in EC is considered as an attractive anti-cancer strategy\(^{7,8}\).
Galectin expression in the tumor endothelium

In normal, quiescent endothelial cells the mRNA expression of galectins appears to be confined to galectin-1, -3, -8, and -9 of which the latter two have been described to be subjected to alternative splicing. The galectin proteins can be detected at the cell surface but have also been found in the cell cytoplasm and the nucleus. This suggests a role for galectins in different cellular functions in endothelial cell biology which is supported by the observation that expression and localization of galectins changes in activated endothelial cells. For example, we observed decreased numbers of galectin-8 and -9 positive endothelial cells in the vasculature of colon tumor compared with normal colon. Additional data on the expression of these galectins in endothelial cells is limited. Most other studies assessed the endothelial expression and function of galectin-1 and/or galectin-3. We observed increased galectin-1 expression in activated EC of colon carcinoma, breast carcinoma and sarcoma as compared with EC in normal tissue. Clausse et al. demonstrated that the frequency of galectin-1 positive EC in primary prostate tissue sections increased from 7% in non-tumor associated capillaries to 64% in capillaries localized in tumor areas. Galectin-1 expression was also found to be increased in oral squamous cell carcinoma-associated EC compared to adjacent normal tissue, and in head and neck carcinoma and lung cancer. Expression analysis of galectin-3 in tumor EC mainly concerns brain neoplasms. Endothelial galectin-3 expression was shown to change from high in low-grade astrocytomas, to intermediate in anaplastic astrocytomas, to low in glioblastomas. This was confirmed by Strik et al., who observed a reduced number of galectin-3 positive EC in glioblastoma compared with low-grade and anaplastic astrocytoma. In oligodendrogliomas, galectin-3 expression was also inversely related to malignancy. On the other hand, in a murine model of hepatocellular carcinoma, galectin-3 expression was approximately 30-fold increased in tumor-derived EC compared with normal liver-derived EC.

Endothelial galectin expression has also been studied in relation to patient survival. D’Haene et al. evaluated the endothelial expression of galectin-1 and galectin-3 in patients with primary central nervous system lymphomas. While they did not detect any endothelial expression of galectin-1, they did find that elevated endothelial expression of galectin-3 was a prognostic factor for poor survival. In contrast, Deininger et al. found that decreased endothelial galectin-3 levels corresponded to shorter progression free and overall survival in oligodendrogliomas. All these findings suggest that endothelial galectin expression is frequently altered in the tumor vasculature and that it might serve as a prognostic marker. However, the galectin expression levels are differently regulated depending on the tumor type or on environmental conditions.

Environmental triggers of galectin expression

As described above, hypoxia is one of the main triggers that leads to the induction of tumor angiogenesis. Usually, the tumor vascular bed is irregular and chaotic and uneven flow dynamics have been shown to affect endothelial galectin-3 expression. In addition, the inadequate perfusion maintains a hypoxic and angiogenic environment and in various cell types the expression of galectins has been reported to be regulated by hypoxia. For example, Le et
al. showed that hypoxia induced galectin-1 expression, both in several cancer cell lines as well as in a murine tumor model. Moreover, in head and neck squamous cell carcinoma patients, galectin-1 expression correlated with the hypoxia marker CAIX. Furthermore, galectin-3 was described as a novel hypoxia-inducible factor 1 (HIF1)-regulated gene in murine fibroblasts subjected to 1% oxygen. This was confirmed by Zeng et al. who also showed a direct interaction between HIF1alpha and two hypoxia responsive elements in the galectin-3 gene promoter. These data clearly show a direct link between hypoxia and the expression of galectin-1 and -3. However, whether hypoxia also regulates galectin expression in endothelial cells and whether it also involves other endothelial galectins still needs to be resolved.

Other factors that influence the galectin expression in tumor endothelial cells include extracellular matrix components and growth factors. For example, increased galectin-1 expression on the surface of endothelial cells was observed after stimulation with minimally oxidized low density lipoprotein or with LPS and a mixture of cytokines, including IL-1β, TNFα and IFNγ. Treatment of human umbilical vein EC (HUVEC) with the pro-inflammatory cytokine IL-1β also induced a 4-fold increased expression of galectin-3, thereby enhancing rolling of eosinophils and adhesion to EC. In addition, incubation of HUVEC and fibroblasts with conditioned media from prostate and ovary carcinoma cells significantly increases galectin-1 expression. This suggests that the environment of a developing tumor indeed may play a role in regulating endothelial galectin-1 expression. In line with this, Glinskii et al. and others demonstrated increased mobilization of galectin-3 to the cell surface of metastasis-associated endothelium upon interaction with metastatic breast carcinoma cells. Also the interleukin-8-induced adhesion of neutrophils to endothelial cells markedly increased plasma membrane and cytoplasmic galectin-3 expression in the EC, while it reduced nuclear galectin-1. In addition to growth factors and interacting cells, advanced glycation end products (AGEs) have been shown to induce galectin-3 expression in HUVEC and human micro-vascular EC (HMEC). While data on the regulation of galectin-8 in EC is lacking, endothelial expression of galectin-9 mainly involves inflammatory and viral triggers. For example, galectin-9 expression was increased by stimulation of HUVEC with the pro-inflammatory cytokine IFNγ.

Altogether, these data show that multiple tumor-associated triggers can affect the expression of galectins in the tumor endothelium and that changes in endothelial galectin expression can be associated with disease outcome. Thus, analysis of galectin expression in the tumor vasculature could be of diagnostic or prognostic value.

Role of endothelial galectins in tumor angiogenesis

The previous sections show that different galectins are expressed by tumor endothelial cells and that this expression can be regulated by multiple triggers. The functional consequence of this expression regulation has been subject to many studies and has revealed that galectins exert a variety of activities in cell biology. They have been shown to be involved in cellular processes like proliferation, migration and adhesion, cell transformation, apoptosis and mRNA splicing. Consequently, they are likely involved in the progression of various cancer types, as their expression is increased in e.g. melanoma, glioma, colon, prostate and head and
Endothelial galectin-1 might contribute to tumor progression by modifying the antitumor response, e.g. by inducing apoptosis in activated T-cells and reducing the recruitment of lymphocytes and neutrophils to the endothelium. Additionally, endothelial galectin-1 and -3 have been shown to mediate heterotypic cancer cell adhesion, thereby potentially facilitating tumor metastasis. Endothelial galectin-1 and -3 have been shown to mediate heterotypic cancer cell adhesion, thereby potentially facilitating tumor metastasis.

There is also ample evidence that galectins are involved in angiogenesis (Figure 3). We have shown that endothelial galectin-1 expression is required during the proliferation and migration of HUVEC in vitro. Hsieh et al. confirmed increased proliferation, migration and adhesion of HUVEC by galectin-1, which was shown to be mediated by interaction with neuropilin-1 and activation of VEGF-R2 signaling. Moreover, adding recombinant galectin-1 to EC cultures induces tube formation as well as EC proliferation and migration. In fact, galectin-1 secreted by tumor cells can be taken up by EC, resulting in enhanced angiogenesis. A key role of galectin-1 in tumor angiogenesis in vivo was confirmed in galectin-1-null (gal-1-/-) mice. Gal-1-/- mice that were injected with teratocarcinoma cells displayed significantly abrogated tumor growth compared to wild-type mice, which was caused by decreased vascularization of the tumor. Similar results were obtained using other tumor models.

Galectin-3 has also been shown to be involved in angiogenesis. It dose-dependently stimulates the proliferation and tube forming capacity of cultured EC. Stimulation of HUVEC with either exogenous galectin-3 or in the presence of conditioned medium from a galectin-3 secreting cell line resulted in increased tube formation. Moreover, nude mice that were injected with galectin-3 expressing cells or recombinant galectin-3 showed increased vascularization. Yang et al. demonstrated that galectin-3 significantly enhances HUVEC invasion and tube formation, which was thought to be mediated by aminopeptidase N (APN / CD13). APN is located on the endothelial cell surface and binds to galectin-3 in a carbohydrate dependent manner. Knockdown of APN reduced invasion and tube formation, and galectin-3 could no longer induce both processes. The involvement of galectin-3 in angiogenesis in vivo was also revealed in a mouse corneal micro-pocket assay by Markowska et al. Stimulation with galectin-3 resulted in increased angiogenesis, which required both the C- and the N-terminal domain of the protein. Furthermore, VEGF- and bFGF-mediated angiogenesis was reduced in galectin-3-null mice. Integrin αvβ3 was thought to be involved in the angiogenic activity of galectin-3, since anti-αvβ3 integrin antibodies inhibited galectin-3 mediated angiogenesis. More recently, Markowska et al. showed that galectin-3 induces phosphorylation of VEGF-R2 and retains VEGF-R2 on the plasma membrane of EC. While all these findings indicate a role in angiogenesis, others did not find any difference in the occurrence or progression of spontaneous tumor models in galectin-3 deficient mice compared to normal mice. This highlights the necessity for further studies to determine the exact role of galectin-3 in tumor angiogenesis.

Recently, galectin-8 was also reported to have angiogenic properties. Bovine aortic EC (BAEC) and HMEC cultured on matrigel supplemented with galectin-8 showed significantly increased tube formation. Exogenous galectin-8 also stimulated BAEC migration, whereas knockdown of endogenous galectin-8 hampered capillary formation and migration of BAEC.
Furthermore, galectin-8 was shown to induce angiogenesis in vivo in a matrigel plug assay. The angiogenic properties of galectin-8 were mediated by CD166, as it was demonstrated that anti-CD166 antibodies hampered galectin-8 induced BAEC migration and tube formation.\textsuperscript{62} The potential role of galectin-9 in angiogenesis is still unresolved.

![Figure 3. Established roles of galectins in angiogenic processes. Several galectins have been implicated in multiple steps of the angiogenesis cascade (opaque). Galectins for which their role in the specific angiogenic processes has not yet been studied are depicted transparent.](image)

In summary, endothelial galectins appear to be involved in cell function during angiogenesis, both in vitro and in vivo. However, the exact role of all the galectins in tumor angiogenesis is not completely understood and further studies are required. Nevertheless, interfering with endothelial galectin function might provide an opportunity to perform angiostatic cancer therapy.

**Targeting endothelial galectins for diagnosis or therapy**

Several targeting strategies have been applied to block galectin function, including the use of antibodies, carbohydrates, and proteins (Figure 4). These different strategies have shown that targeting galectins can affect tumor progression at multiple levels by interfering with tumor angiogenesis, tumor immune escape and metastasis formation.\textsuperscript{7}

Targeting endothelial cells with galectin blocking antibodies has been shown to efficiently reduce their angiogenic capacity. For example, incubation with polyclonal anti-galectin-1 antibody was shown to inhibit migration of EC in vitro.\textsuperscript{59} In addition, this antibody reduced angiogenesis in vivo in a chorioallantoic membrane (CAM) assay.\textsuperscript{59} Blocking galectin-3 with polyclonal antibody decreased capillary tube formation by HUVEC\textsuperscript{101} and was shown to hamper angiogenesis in vivo in the ischemic rat brain.\textsuperscript{100} These results suggest that targeting galectins with antibodies is a good strategy to interfere with endothelial cell function. However, Fukushi et al. demonstrated that anti-galectin-3 antibody did not affect matrigel-, laminin-, or fibronectin-induced transwell migration of EC.\textsuperscript{106} Apparently the effect of galectin-targeting antibodies is dependent on the context and the type of assay in which they are used. Furthermore, the effect of blocking antibodies might depend on the binding site on the galectin, e.g. near
the carbohydrate binding pocket or at the site responsible for di- or oligomerization.

Another approach used to target galectins involves blocking the carbohydrate recognition domain with competing carbohydrates. An example of a simple competing carbohydrate is lactose. This compound was shown to inhibit galectin-mediated HUVEC migration and capillary tube formation\textsuperscript{62,66,101}. However, lactose binds to multiple galectins with only low affinity, which makes it less appropriate for therapeutic applications. Another more potent disaccharide inhibitor is thiodigalactoside (TDG) which was shown to impair both galectin-1- and galectin-8-mediated tube formation\textsuperscript{62,97}. Thiodigalactoside also inhibits EC proliferation and migration \textit{in vitro}\textsuperscript{62,97}. \textit{In vivo}, intra-tumor treatment with TDG dose-dependently inhibited tumor growth in melanoma and mammary carcinoma mouse models\textsuperscript{97}. Moreover, immunohistochemical staining of sections from TDG-treated wild-type tumors revealed that the number and diameter of the blood vessels was markedly reduced. On the other hand, tumor cells that were depleted of galectin-1 displayed hampered growth in these mice and additional treatment with TDG no longer affected the tumor growth, suggesting that TDG impairs tumor progression by inhibition of galectin-1 in tumor cells. This was supported by the observation that in galectin-1 knock out tumors no significant differences in tumor vascularization were observed after treatment with TDG\textsuperscript{97}. Rabinovich \textit{et al.} designed three synthetic lactulose amines (SLA) derivatives that were found to differentially inhibit the binding of galectin-1 and -3 to the highly glycosylated 90K. In addition, the compounds selectively inhibited EC tube formation on matrigel\textsuperscript{107}. Thus far, the antitumor activity of these compounds has not been tested which is also true for two taloside compounds that bind to the carbohydrate recognition domain of galectin-1 and -3 and that might be used for inhibiting these galectins\textsuperscript{108}.

A more complex galectin-binding carbohydrate that has been shown to have antitumor activity is a modified form of citrus pectin (MCP). Galectin-3-induced endothelial tube formation was considerably reduced by MCP\textsuperscript{101}. In addition, MCP prevents the binding of galectin-3 to HUVEC and blocks the galectin-3-induced chemotaxis of these cells\textsuperscript{109}. Finally, MCP was shown to impair tumor vascularization \textit{in vivo} resulting in hampered tumor growth\textsuperscript{109}.

While targeting galectins with carbohydrates show promising results, a more specific way of galectin-targeting might be achieved by peptides and proteins. In search for peptides with angiostatic properties, the synthetic 33-mer peptide anginex was found to inhibit EC migration, proliferation and sprouting \textit{in vitro}\textsuperscript{110}. In addition, anginex inhibits angiogenesis \textit{in vivo} and hampers tumor growth in mice models\textsuperscript{110,111}. Using yeast-two-hybrid analysis galectin-1 was identified as the main receptor of anginex\textsuperscript{59}. This was confirmed in galectin-1 deficient mice, in which tumor angiogenesis could no longer be inhibited by anginex treatment\textsuperscript{59}. Further studies showed that anginex also inhibits galectin-1-mediated membrane translocation of activated H-Ras in endothelial cells which could account for the inhibitory effects on cell proliferation\textsuperscript{98}. More recently it was suggested that binding of anginex affects the carbohydrate binding affinity and specificity of galectin-1\textsuperscript{112} which might be exploited for future therapeutic applications.

Apart from galectin-binding peptides, dominant negative isoforms of galectins have also been described to possess angiostatic activity. For example, a N-terminally truncated form of galectin-3 (galectin-3C), which retains its carbohydrate binding capacity but lacks the ability
to cross-link carbohydrate-containing ligands on cell surfaces and ECM, acts as a dominant-negative inhibitor of full-length galectin-3 function by competing with its carbohydrate binding ability\textsuperscript{104,113}. Unlike full-length galectin-3, galectin-3C fails to induce HUVEC migration and network formation \textit{in vitro} and angiogenesis \textit{in vivo}\textsuperscript{104}. In tumor models, treatment with galectin-3C inhibits tumor growth although a direct link with angiogenesis was not presented\textsuperscript{113,114}. Nevertheless, the development of additional dominant negative isoforms of e.g. galectin-1 might provide novel therapeutic opportunities. Altogether, different strategies have been developed to interfere with galectin function during angiogenesis (summarized in Table 1 and Figure 4). While pre-clinical data are promising the evidence for therapeutic efficacy in patients is still lacking. At present it is difficult to determine which strategy will be most effective and it cannot be excluded that combining different approaches might lead to the most potent therapy.

Table 1. Stimulatory effects of galectins on different steps of the angiogenic cascade and potential inhibitors.

<table>
<thead>
<tr>
<th>Process</th>
<th>Stimulatory galectins</th>
<th>Inhibitors\textsuperscript{1}</th>
<th>Peptide/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>gal-1, gal-3</td>
<td>knockdown\textsuperscript{2}</td>
<td>Lac</td>
</tr>
<tr>
<td>Migration\textsuperscript{1}</td>
<td>gal-1, gal-3, gal-8</td>
<td>antibody</td>
<td>TDG Ax</td>
</tr>
<tr>
<td>Proliferation</td>
<td>gal-1, gal-3</td>
<td>gal-1</td>
<td>Lac TDG Ax gal-3C</td>
</tr>
<tr>
<td>Sprouting</td>
<td>gal-1, gal-3</td>
<td>gal-3</td>
<td>Ax</td>
</tr>
<tr>
<td>Tube formation\textsuperscript{1}</td>
<td>gal-1, gal-3, gal-8</td>
<td>gal-3</td>
<td>Lac TDG SLA MCP Ax gal-3C</td>
</tr>
<tr>
<td>Angiogenesis in vivo</td>
<td>gal-1, gal-3, gal-8</td>
<td>gal-1, gal-3</td>
<td>Lac TDG SLA MCP Ax gal-3C</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Lac = lactose, TDG = thiodigalactoside, SLA = synthetic lactulose amines, MCP = modified citrus pectin, Ax = anginex.

\textsuperscript{2}Either by siRNA or antisense oligo’s.

\textsuperscript{3}Inhibitory effects influenced by the type of adhesive matrix used in the assay.

Figure 4. Galectin-targeting molecules. Examples of molecules that have been used to block galectins and interfere with their angiogenic activity. N-lactulose-octamethylene diamine is one of the SLAs reported by Rabinovich et al\textsuperscript{107}. 
Apart from direct therapeutic applications, targeting the endothelial galectins could also be used for e.g. specific delivery of anticancer drugs or for molecular imaging of tumor angiogenesis, an emerging diagnostic method\textsuperscript{115}. For example, fluorescently labeled anginex specifically homes to the tumor vasculature\textsuperscript{111}, and anginex-conjugated liposomes specifically bind to and are taken up by cultured HUVEC\textsuperscript{116}. Interestingly, anginex-conjugated paramagnetic liposomes showed increased contrast in MRI, indicating that anginex might be used for diagnostic imaging applications\textsuperscript{116}. Indeed, Kluza and colleagues recently demonstrated that conjugating paramagnetic liposomes to both anginex and RGD-peptide (targeting integrin $\alpha_v\beta_3$) enhanced the uptake by HUVEC and significantly inhibited EC proliferation\textsuperscript{117}. This dual-targeting approach could be used for both therapeutic and diagnostic applications by loading these nanoparticles with e.g. MRI contrast agents. Moreover, treatment of melanoma MA148 tumor-bearing mice with anginex that was conjugated to the cytotoxic drug 6-hydroxypopylacylfulvene (HPAF) inhibited tumor growth significantly more than equivalent doses of either compound alone\textsuperscript{118}. Microvessel density was still reduced by the conjugate, indicating that the conjugate does not affect the angiostatic properties of anginex\textsuperscript{118}. More importantly, the conjugate showed less toxicity compared to the unconjugated HPAF\textsuperscript{118}.

Another indirect application of targeting endothelial galectins appears to involve sensitization to cytotoxic therapies. For example, treatment with galectin-3C was observed to enhance the antitumor activity of bortezomib in a xenograft mouse model of human multiple myeloma\textsuperscript{113}. Targeting galectin-3 with MCP and Lac-L-Leu sensitized murine angiosarcoma cells for the cytotoxic drug doxorubicin\textsuperscript{119}. In addition, mice that were grafted with melanoma cells transfected with anti-galectin-1 siRNA displayed increased sensitivity for the autophagic drug temozolomide\textsuperscript{120}. This is in line with findings of Le Mercier \textit{et al.}, who demonstrated that decreasing galectin-1 expression in a xenograft mouse model of glioblastoma cells by \textit{in vivo} siRNA delivery in the brains, enhanced the therapeutic benefits of temozolomide\textsuperscript{121}. Finally, anginex has also been shown to enhance the effect of different anti-cancer therapies including cytotoxic therapy and radiation therapy\textsuperscript{122,123}. Additional studies suggested that this effect might be related to a transient normalization of the tumor vasculature which temporarily enhances tumor perfusion and oxygenation\textsuperscript{124}. Whether this is also true for other galectin inhibitors still needs to be resolved.

Taken together, galectin-targeted angiostatic therapy can directly affect tumor progression but can also be indirectly exploited for targeted delivery of anticancer drugs, for diagnostic applications, and for sensitization of tumor cells to cancer treatment.
**Aim and outline of the thesis**

Galectins are involved in many key processes during cancer progression, including tumor angiogenesis. Of the fifteen mammalian galectins, galectin-1, -3, -8 and -9 are found to be expressed in the endothelium. The expression of these galectins appears to be differentially regulated upon endothelial cell activation, which makes them attractive targets for angiostatic therapy. For example, galectin-1 expression levels are significantly increased in activated endothelial cells and interfering with galectin-1 has therapeutic value. Additional research has confirmed a role for galectin-1, -3 and -8 in angiogenesis. On the other hand, the function of endothelial galectin-9 remains largely unexplored. Moreover, the underlying pathways that regulate galectin expression and the functional consequences are still not fully understood.

The aim of the work described in this thesis is threefold. Firstly, we set out to assess the prognostic value of galectins in cancer patients. Secondly, we aim to further unravel the expression and function of galectins in endothelial cells. Thirdly, we studied methods to therapeutically target galectins.

Since galectins are often differentially expressed in normal compared to tumor tissue, they are appreciated as prognostic or diagnostic markers in several types of cancer. In chapter 2 we performed extensive galectin expression profiling in early stage non-small cell lung cancer (NSCLC) patients to obtain a more complete insight into the relevance of galectin expression levels in lung cancer. This study confirmed the prognostic value of galectin-1 and, more importantly, identified a galectin-9 splice variant, i.e. gal-9Δ5, as a novel prognostic marker in early stage NSCLC. These findings also exemplify the importance of distinguishing between individual galectin-9 splice variants in prognostic marker analysis.

As described above, galectins have been associated with endothelial cell function during angiogenesis. To facilitate further research on this angioregulatory role we adapted several known angiogenesis methods to study galectins and galectin inhibitors in endothelial cell biology in vitro (chapter 3) and angiogenesis in vivo (chapter 4). Since only limited information was available on galectin-9 in angiogenesis, we set out to explore the function of galectin-9 in the endothelium. In chapter 5 we show that galectin-9 protein expression levels are increased in the endothelium of different tumors. However, only a limited role was found for the dominant galectin-9 splice variant in endothelial cell function and angiogenesis. On the other hand, by extensive galectin-9 expression profiling we identified two novel splice variants which have not been reported before in endothelial cells. The relevance of distinguishing between all these different splice variants for prognostic marker analysis was further exemplified in chapter 6. In this study we performed galectin expression profiling in renal cancer patients. Moreover, we assessed the effect of one particular CRD of galectin-9, i.e. the N-terminal CRD (gal-9N) on endothelial cell function. This research identified gal-9N as a potent angioregulatory protein, both in vitro and in vivo.

The differential expression of galectins in tumor and normal (endothelial) tissues makes them not only valuable as prognostic and diagnostic markers, but also provides us with
promising targets for anti-cancer therapy. Several galectin-interfering strategies have been applied, including the use of the small angiostatic peptide anginex which targets galectin-1. In chapter 7 we hypothesized that the chemokine platelet factor 4 (CXCL4), which structurally and functionally resembles anginex, mediates its angiostatic activity through interaction galectin-1. In this study, we indeed identified galectin-1 as a binding partner for CXCL4. In addition, we describe how CXCL4 affects galectin-1-mediated EC function and signaling. Moreover, we studied the effects of CXCL4 on galectin-1-induced platelet function.

In chapter 8 we initiated research into a new approach to interfere with galectin function. We speculated that, apart from direct targeting with blocking antibodies, carbohydrates or peptides, we might also be able to indirectly interfere with galectin stability and function by e.g. modulation of the cellular redox state. Therefore, we assessed the endothelial expression and function of metallothioneins (MTs), proteins which are known to influence the redox balance in cells. We detected a broad repertoire of MTs in endothelial cells, the expression of which was influenced by different triggers, including hypoxia. Moreover, we describe that the most prevalent isoform, i.e. MT2A, is important for EC function.

Finally, the main findings of the research presented in this thesis are discussed in chapter 9. Apart from placing our findings in a broader perspective, we also discuss the opportunities for future studies.
References


55. Clausse N, van den Brule F, Waltregny D, Garnier F, Castronovo V. Galectin-1 expression in prostate tumor-


78. van den Brule F, Califfe S, Garnier F, Fernandez PL, Berchuck A, Castronovo V. Galectin-1 accumulation in the ovary carcinoma peritumoral stroma is induced by ovary carcinoma cells and affects both cancer cell proliferation and adhesion to laminin-1 and fibronectin. *Lab Invest.* 2003;83(3):377-386.
Chapter 1


Chapter 2

Galectin expression profiling identifies galectin-1 and galectin-9Δ5 as prognostic factors in stage I/II non-small cell lung cancer

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*Authors contributed equally

Chapter 2

Abstract

Approximately 30-40% of the patients with early stage non-small cell lung cancer (NSCLC) will present with recurrent disease within two years of resection. Here, we performed extensive galectin expression profiling in a retrospective study using frozen and paraffin embedded tumor tissues from 87 stage I/II NSCLC patients. Our data show that galectin mRNA expression in NSCLC is confined to galectin-1,-3,-4,-7,-8, and -9. Next to stage, univariable Cox regression analysis identified galectin-1, galectin-9FL and galectin-9Δ5 as possible prognostic markers. Kaplan-Meier survival estimates revealed that overall survival was significantly shorter in patients that express galectin-1 above median levels, i.e. 23.0 (2.9-43.1) vs. 59.9 (47.7-72.1) months (p=0.020) as well as in patients that express galectin-9Δ5 or galectin-9FL below the median, resp. 59.9 (41.9-75.9) vs. 32.8 (8.7-56.9) months (p=0.014) or 23.2 (-0.4-46.8) vs. 58.9 (42.9-74.9) months (p=0.042). All three galectins were also prognostic for disease free survival. Multivariable Cox regression analysis showed that for OS, the most significant prognostic model included stage, age, gal-1 and gal-9Δ5 while the model for DFS included stage, age and gal-9Δ5. In conclusion, the current study confirms the prognostic value of galectin-1 and identifies galectin-9Δ5 as a novel potential prognostic marker in early stage NSCLC. These findings could help to identify early stage NSCLC patients that might benefit most from adjuvant chemotherapy.
Introduction

In non-small cell lung cancer (NSCLC), clinicopathological staging according to the TNM classification is still the main delimiter to classify patients with a distinct outcome. Unfortunately, of the patients diagnosed with early stage disease almost 30% to 40% will present tumor recurrence within two years after surgical resection\(^1\). Since it has been shown that adjuvant chemotherapy can improve the survival of patients with resected stage II-IIIa NSCLC, identification of early stage patients with poor survival is clinically relevant\(^1\).

Galectins are a protein family of which the members are defined by the presence of a conserved carbohydrate recognition domain\(^2\). Thus far, fifteen galectins have been identified, eleven of which are also expressed in different human cells and tissues\(^3,4\). They exert many different functions, with regulation and fine-tuning of the immune system being the best studied. Consequently, deregulation of galectin expression is frequently associated with an inadequate immune response which contributes to different pathologies, including cancer\(^5,6\). In addition, galectins have been found to mediate tumor cell metastasis\(^7-9\) and to induce and maintain tumor angiogenesis\(^10-15\) which further adds to cancer progression. All this has resulted in the recognition of galectins as diagnostic and prognostic markers in different cancer types, including lung cancer. For example, increased galectin-3 expression has been described as an indicator of poor prognosis in NSCLC patients\(^16,17\). Similar observations were reported for galectin-1 expression\(^16-18\). Furthermore, galectin-1 expression is elevated in lung cancer tissue as compared to normal lung\(^19\). More recently, elevated levels of galectin-1 expression were found to promote lung cancer progression and chemoresistance\(^20\) while increased galectin-4 expression was shown to predict lymph node metastasis in adenocarcinoma of the lung\(^21\). All these findings illustrate the prognostic potential of galectins in lung cancer. However, whether galectin expression can also be used to distinguish between early stage NSCLC patients with good or bad prognosis has not been well established. Therefore, the objective of this study was to determine whether measurement of galectin mRNA expression could serve as a predictor of clinical outcome in patients with stage I/II NSCLC using a multivariable model.
Chapter 2

Materials and Methods

Ethical statement
This study was approved by the local internal review board (Maastricht Pathology Tissue Collection, http://www.pathologymumc.nl/research/external-projects/maastricht-pathology-tissue-collection-mptc) and complies with the recommendations guiding physicians in biomedical research involving human subjects as laid down in the Declaration of Helsinki. In accordance with governing ethics, the use of anonymized tissue from the tissue bank did not require specific written consent.

Patients
The current study included tumor samples of patients with stage I/II NSCLC who underwent an anatomic curative resection at the academic hospital Maastricht between 1994 and 2004. Exclusion criteria were 1) Previous other malignancy, 2) Development of an unrelated malignancy during a follow-up of at least 4 years, or 3) Neo-adjuvant therapy.

Specimen characteristics
Resected material was stored at -80°C as part of the Maastricht Pathology Tissue Collection. Only tissues from patients with stage I/II disease and with a tumor area >50% (mean 65.9%, 95%CI: 59.9-71.9), as evaluated in hematoxylin/eosin stained sections by an experienced pathologist (R-JvS), were considered eligible for further investigations.

Study design
We retrospectively analyzed tumor tissue from stage I/II NSCLC patients who underwent curative resection surgery between 1994 and 2004 at the academic hospital Maastricht. In total, 87 patients were included. The patients received no prior treatment and did not have a history of or develop unrelated malignancies up to 4 years following surgery. The follow-up was at least 5 years during which the patients were examined routinely every 3 months the first 2 years and thereafter every 6 months. Clinical endpoints included overall survival (OS) and disease free survival (DFS). Overall survival was the time in months from the day of surgery until the day of death from any cause. Disease free survival was the time in months from the day of surgery until the day of tumor recurrence, either locoregional or distant. Candidate variables that were considered for inclusion in models included the mRNA expression levels of each galectin (in $2^{-\Delta Ct}$), age (in years), stage (I or II), gender, histology (squamous or other), smoking status (former or other). The number of patients included was determined by the availability of tumor samples. Within this sample size, approximately 50 events occurred which allowed the inclusion of 5 variables for multivariable analysis to avoid the risk of over-fitting.

Cell cultures
The following cell lines were used: A549 human alveolar carcinoma (ATCC CCL-185), H460 human large cell carcinoma (ATCC HTB-177), H1650 human bronchoalveolar carcinoma (ATCC
Galectin expression profiling in NSCLC

CRL-5883), H1975 human non-small cell lung carcinoma (ATCC CRL-5908), H3255 human non-small cell lung carcinoma (ATCC CRL-2882, discontinued). All cell lines were cultured in RPMI (Invitrogen) supplemented with 10% fetal calf serum, penicillin (50 U/mL) and streptomycin (50 micrograms/mL). Cells were maintained at 37°C and 5% CO₂ in a humidified incubator.

RNA isolation and cDNA synthesis
Total RNA was isolated from 10x10 μm thick frozen tissue sections or from cultured cells using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. In case of tumor tissue, an additional section was taken before and after the series of 10 for H/E staining and evaluation of the percentage of tumor area. Genomic DNA contamination was removed by on column DNAsel treatment. The concentration and purity of the RNA was analyzed using the NanoDrop ND-1000 (NanoDrop Technologies). Subsequently, cDNA synthesis was performed with the iScript cDNA Synthesis Kit (Biorad) using 0.5 to 1.0 micrograms of total RNA.

Real-time qPCR
qPCR was performed on an iQ5 Multicolour Real-Time PCR Detection System (BioRad) or the CFX96 (BioRad) using the iQ SYBR Green PCR master mix (BioRad) using 400 nmol/L of the appropriate primers which have been described before23. To distinguish between the different galectin-9 splice variants the following primers were used: gal-9FL forward GCAGACAACACCTCCCG, gal-9FL reverse CCCAGAGCACAGGTTGATG, gal-9Δ5 forward ATCAGTTCCAGCCTCCC, gal-9Δ5 reverse CCCAGACACAGGTTGATG, gal-9Δ5/6 forward CTACATCAGCTCCAGACCCA, gal-9Δ5/6 reverse CCCAGACACAGGTTGATG. qPCR for these splice variants was performed using Sensimix (Quantace) at Tm = 61°C. All primers were synthesized by Eurogentec.

Western blot
Western blot was performed according to standard protocols. In brief, 5 to 10 10 μm thick cryosections were suspended in 60 μL Laemmli sample buffer (Biorad) supplemented with 1:20 β-mercapto-ethanol. Samples were boiled for 5 minutes and immediately separated by gel electrophoresis on a 15% polyacrylamide gel and transferred onto PVDF membranes (Millipore). Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences) for 1 hour and incubated overnight at 4°C with either rabbit anti-galectin-1 antibody (Peprotech) or goat anti-galectin-9 antibody (R&D systems). Loading of the gels was checked by α-actin detection using mouse anti-α-actin (1:10000, MP Biomedicals). The membranes were washed three times with PBS/0.1% tween and subsequently incubated with the appropriate secondary IRDye antibodies (LI-COR Biosciences) at room temperature for 1 hour. Finally, membranes were washed with PBS/0.1% tween and rinsed with PBS after which images were acquired using the Odyssey infrared imaging system (LI-COR Biosciences).

Immunohistochemistry
Immunohistochemistry for galectin-1 and galectin-9 was performed on paraffin embedded tissue sections according standard procedures using rabbit anti-galectin-1 (1:500; Peprotech)
and goat anti-galectin-9 (1:500; R&D systems) antibodies of which the specificity was determined before\textsuperscript{23}. Stainings were visualized using the StreptABComplex/HRP kit (Dako). The sections were counterstained with hematoxylin (Merck), dehydrated, and mounted in Depex (BDH prolabo). Blinded scoring of galectin-1 and galectin-9 was performed on three different compartments of the tumor, i.e. the tumor cells, the tumor stroma, and the tumor endothelial cells. For scoring of galectin-1 and galectin-9 the frequency of staining was determined using the following scale: 0 = no or hardly any cells positive, 1 = small fraction of cells positive, 2 = approximately half of the cells positive, 3 = more than half of the cells positive, 4 = all or the majority of cells positive. All stainings were scored by at least two independent persons.

\textbf{Statistical analysis}

Bivariable Pearson correlation coefficients were calculated to study the relationships between galectin mRNA expression levels ($2^{-\Delta C_{t}}$), clinical parameters, and/or immunohistochemical staining scores. To identify the most important predictors for patients' outcome a two-step approach was used. First, univariable associations between OS or DFS with clinical parameters or each galectin were examined using Cox regression analysis. Next, multivariable Cox regression analysis with forward selection was performed on the most significant galectin predictors identified in the univariable analysis, i.e. gal-1 (categorical), gal-9FL (categorical), gal-9\textDelta 5 (categorical), together with age (continuous) and stage (categorical), with either OS or DFS as the outcome. The analysis included the Kaplan-Meier survival estimates with the Log rank test were performed to determine median OS or DFS. Median mRNA expression levels were used as cut-off value to divide the patients into a high expression group (above median) and low expression group (below median). Confidence intervals for median survival were calculated according to Bonnet \textit{et al}\textsuperscript{24}. All statistical computations were performed in SPSS20.0.0.

\textbf{Results}

\textbf{Expression of galectin mRNA in early stage NSCLC and lung cancer cell lines}

We performed extensive galectin gene expression analysis in a retrospective study on resected tumor tissues derived from 87 patients diagnosed with early stage (stage I/II) NSCLC. The median age of the patients was 65.5 years (range 37.4 - 85.5) and follow-up was at least 5 years during which 47 patients (54.0\%) presented recurrent disease and 50 patients died (57.5\%). The median overall survival (OS), defined as time between day of surgery until day of death, was 48.7 months (95\% CI 33.1 - 64.2 months). Disease free survival (DFS), defined as time between day of surgery until day of locoregional or distant recurrence, was 33.3 months (95\% CI 34.8 - 49.6 months). The overall demographic and standard prognostic variables of the patient group are listed in Table 1.
Galectin expression profiling in NSCLC

Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>87</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>65.5 (37.4 - 85.5)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>65 (74.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>22 (25.3%)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Squamous</td>
<td>43 (49.4%)</td>
</tr>
<tr>
<td>Adeno</td>
<td>32 (36.8%)</td>
</tr>
<tr>
<td>Large cell</td>
<td>12 (13.8%)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>Stage IA</td>
<td>11 (12.6%)</td>
</tr>
<tr>
<td>Stage IB</td>
<td>44 (50.6%)</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>6 (6.9%)</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>26 (29.9%)</td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>40 (46.0%)</td>
</tr>
<tr>
<td>Positive</td>
<td>47 (54.0%)</td>
</tr>
<tr>
<td>Smoke status</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>2 (2.3%)</td>
</tr>
<tr>
<td>Current</td>
<td>21 (24.1%)</td>
</tr>
<tr>
<td>Former</td>
<td>59 (67.8%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>5 (5.7%)</td>
</tr>
<tr>
<td>Median OS (months; 95% CI)</td>
<td>48.7 (39.6 - 53.6)</td>
</tr>
<tr>
<td>Events (death)</td>
<td>50 (57.5%)</td>
</tr>
<tr>
<td>Median DFS (months; 95% CI)</td>
<td>33.3 (34.8 - 49.6)</td>
</tr>
<tr>
<td>Events (recurrence)</td>
<td>47 (54.0%)</td>
</tr>
</tbody>
</table>

To get insight in the prognostic value of galectin expression in stage I/II NSCLC we first determined which galectins are expressed in NSCLC tumor tissue. qPCR analysis with previously validated primers targeted against all known human galectins revealed that of six galectins, i.e. galectin-1,-3,-4,-7,-8, and -9, mRNA expression could be detected (Figure 1A). Because extensive splicing has been reported for galectin-9, we also determined the mRNA expression of the most common galectin-9 splice variants, i.e. galectin-9 full length (FL), galectin-9 with a deletion of exon 5 (∆5), and galectin-9 with a deletion of exons 5 and 6 (∆5/6). All three variants were detectable with gal-9∆5 as the dominant variant (Figure 1A, inset). Protein expression of the different galectin family members was confirmed by screening immunohistochemical stainings available in the human protein atlas (Figure 1B). Protein expression of the different galectin-9 splice isoforms was further confirmed by Western blot analysis (Figure 1C). Furthermore, expression profiling on different lung cancer cell lines confirmed that expression was confined to galectin-1, -3, -4, -7, -8, and -9 (Supplementary Figure 1). This corroborates with a study by Lahm et al. who analyzed the expression of multiple galectins in a broad panel of cancer cell lines. All these findings show that galectin-1, galectin-3, and galectin-8 are the most abundantly expressed galectins while the expression of galectin-4, galectin-7, and galectin-9 is relatively low, both in tumor tissues and in different lung cancer cell lines.
Chapter 2

Relationship between galectin mRNA expression and clinical parameters in early stage NSCLC

Analysis of the relationship between the different galectin mRNA expression levels showed a significant positive correlation between total galectin-9 and the specific galectin-9 splice variants, i.e. gal-9FL (r = 0.48), gal-9Δ5 (r = 0.85), and gal-9Δ5/6 (r = 0.52). Within these splice variants there was a significant correlation between gal-9FL and gal-9Δ5 (r = 0.44) as well as between gal-9Δ5 and gal-9Δ5/6 (r = 0.53). Significant correlations were also observed between galectin-3 and gal-9FL (r = 0.34) and between gal-4 and gal-9Δ5/6 (r = 0.31). No significant correlations were found between the mRNA expression levels of the other galectins. Regarding the relationship between galectin mRNA expression and the clinical parameters, i.e. age, stage, gender, histology, and smoking status, we observed a weakly significant correlation between gender and gal-9Δ5 (r = 0.24) and between age and respectively gal-1 (r = 0.26), gal-9 (r = -0.25) and gal-9Δ5 (r = -0.30). No significant correlation between galectin mRNA expression and the remaining parameters, i.e. histology, stage, and smoking status, was found.

Figure 1. Galectin mRNA expression profile in tumor tissues obtained from early stage non-small cell lung cancer patients (n=87). A) The inset shows the expression of the three galectin-9 splice variants. B) Images of immunohistochemical staining of the galectins with detectable mRNA expression in NSCLC 27. C) Western blot analysis of galectin-9 isoform expression in NSCLC tumor tissue from 5 different patients. Three bands at expected molecular weights of galectin-9FL, galectin-9Δ5 and galectin-9Δ5/6 were observed at varying intensities.
Association between galectin mRNA expression and prognosis in early stage NSCLC

Next, univariable Cox regression analyses were performed to select the markers with the strongest association with OS (Table 2) and DFS (Table 3). This identified stage, gal-1, gal-9FL and gal-9∆5 as possible prognostic factors for both OS and DFS in early stage NSCLC patients.

Table 2. Univariable analyses of standard clinical variables and galectin mRNA expression levels in relation to overall survival in 87 patients with early stage NSCLC.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.03</td>
<td>1.00 to 1.06</td>
<td>0.064</td>
</tr>
<tr>
<td>Gender</td>
<td>0.71</td>
<td>0.37 to 1.40</td>
<td>0.325</td>
</tr>
<tr>
<td>Stage*</td>
<td>1.91</td>
<td>1.09 to 3.35</td>
<td>0.024</td>
</tr>
<tr>
<td>Histology*</td>
<td>0.76</td>
<td>0.44 to 1.33</td>
<td>0.340</td>
</tr>
<tr>
<td>Smoking status*</td>
<td>1.30</td>
<td>0.70 to 2.42</td>
<td>0.402</td>
</tr>
<tr>
<td>Gal1*</td>
<td>1.94</td>
<td>1.10 to 3.41</td>
<td>0.022</td>
</tr>
<tr>
<td>Gal3*</td>
<td>0.60</td>
<td>0.34 to 1.05</td>
<td>0.071</td>
</tr>
<tr>
<td>Gal4*</td>
<td>0.68</td>
<td>0.39 to 1.18</td>
<td>0.170</td>
</tr>
<tr>
<td>Gal7*</td>
<td>0.96</td>
<td>0.53 to 1.74</td>
<td>0.899</td>
</tr>
<tr>
<td>Gal8*</td>
<td>0.78</td>
<td>0.45 to 1.36</td>
<td>0.383</td>
</tr>
<tr>
<td>Gal9*</td>
<td>0.76</td>
<td>0.44 to 1.33</td>
<td>0.333</td>
</tr>
<tr>
<td>Gal9FL*</td>
<td>0.54</td>
<td>0.30 to 0.99</td>
<td>0.045</td>
</tr>
<tr>
<td>Gal9∆5*</td>
<td>0.48</td>
<td>0.26 to 0.87</td>
<td>0.016</td>
</tr>
<tr>
<td>Gal9∆5/6*</td>
<td>0.73</td>
<td>0.41 to 1.32</td>
<td>0.304</td>
</tr>
</tbody>
</table>

*Stage I vs. stage II, *b) Squamous vs. others, *c) Former smoker vs. others, *d) Above vs. below median mRNA expression

Subsequently, Kaplan-Meier analyses were used to estimate median OS and DFS in patients that expressed a specific galectin below or above the median mRNA expression level. Patients that expressed galectin-1 above median levels had a significant shorter OS and DFS (Figure 2A and Table 4). The univariable Cox model also identified two splice variants of
galectin-9, i.e. gal-9FL and gal-9∆5, to be possibly associated with both OS and DFS. Indeed, patients with either gal-9FL or gal-9∆5 expression levels below the median had significant shorter OS as well as shorter DFS (Figure 2B+C and Table 4). None of the other galectins was significantly associated with OS or DFS.

Table 4. Kaplan-Meier estimates of median OS and DFS in early stage NSCLC patients with galectin expression below or above the median level.

<table>
<thead>
<tr>
<th>Median OS (95% CI) in months</th>
<th>Median DFS (95% CI) in months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low* expression</td>
<td>High expression</td>
</tr>
<tr>
<td>Gal-1</td>
<td>59.9 (47.7-72.1)</td>
</tr>
<tr>
<td>Gal-3</td>
<td>28.0 (5.3-50.6)</td>
</tr>
<tr>
<td>Gal-4</td>
<td>33.2 (10.1-56.3)</td>
</tr>
<tr>
<td>Gal-7</td>
<td>57.3 (36.6-78.0)</td>
</tr>
<tr>
<td>Gal-8</td>
<td>40.7 (21.6-59.8)</td>
</tr>
<tr>
<td>Gal-9</td>
<td>42.6 (20.4-64.8)</td>
</tr>
<tr>
<td>Gal-9FL</td>
<td>23.2 (-0.4-46.8)</td>
</tr>
<tr>
<td>Gal-9∆5</td>
<td>32.8 (8.7-56.9)</td>
</tr>
<tr>
<td>Gal-9∆5/6</td>
<td>39.3 (16.8-61.8)</td>
</tr>
</tbody>
</table>

HR = hazard ratio; CI = confidence interval

Finally, multivariable Cox regression analysis was performed with forward selection on the most significant factors identified in the univariable analyses, i.e. stage, age, galectin-1 galctin-9FL and galectin-9∆5. These analyses showed that for OS, the most significant prognostic model included stage, age, gal-1 and gal-9∆5 while the model for DFS included stage, age and gal-9∆5 (Table 5).

Table 5. Multivariate Cox regression analysis with forward selection.

<table>
<thead>
<tr>
<th>Overall survival</th>
<th>Variables in the Equation</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
<th>overall p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>age</td>
<td>1.05</td>
<td>1.01 to 1.09</td>
<td>0.009</td>
<td>0.008</td>
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<tr>
<td>Step 2</td>
<td>stage I_II, age</td>
<td>2.41</td>
<td>1.32 to 4.43</td>
<td>0.004</td>
<td>0.001</td>
</tr>
<tr>
<td>Step 3</td>
<td>stage I_II, age</td>
<td>2.10</td>
<td>1.14 to 3.87</td>
<td>0.018</td>
<td>0.000185</td>
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<tr>
<td></td>
<td>stage I_II, age</td>
<td>1.06</td>
<td>1.02 to 1.10</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gal9∆5</td>
<td>0.52</td>
<td>0.28 to 0.97</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>Step 4</td>
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<td>1.30 to 4.49</td>
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<td>0.000099</td>
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<td></td>
<td>stage I_II, gal1, gal9∆5</td>
<td>0.46</td>
<td>0.24 to 0.85</td>
<td>0.013</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disease free survival</th>
<th>Variables in the Equation</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
<th>overall p-value</th>
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<td>Step 1</td>
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<td>0.003</td>
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<td>gal9∆5</td>
<td>0.47</td>
<td>0.25 to 0.85</td>
<td>0.013</td>
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<tr>
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<td>1.12 to 3.87</td>
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<td>1.01 to 1.10</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>stage I_II, gal9∆5</td>
<td>0.54</td>
<td>0.29 to 1.00</td>
<td>0.051</td>
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</tr>
</tbody>
</table>

HR = hazard ratio; CI = confidence interval
Localization and distribution of galectin-1 and galectin-9 protein expression in early stage NSCLC tissue

To get more insight in the localization and distribution of galectin-1 and galectin-9 protein expression we performed immunohistochemical stainings on a representative subset of tumors (n=45). Galectin-1 was widely expressed in most tumor tissues. The expression in the tumor cells varied between tumors as well as within tumors, with some tissues showing no positive tumor cells while in other tissues the tumor cells were strongly positive. Most tissues showed positive staining in the stroma as well as in the tumor endothelial cells (Figure 3A). Galectin-9 staining was less prominent as compared to galectin-1. In fact, positive tumor cells were only infrequently observed although some tissues appeared to display a gradient with increasing galectin-9 levels in the tumor cells closer to the stromal tissue (Figure 3B). Both the stroma and the tumor endothelial cells stained positive more frequently (Figure 3B).
Chapter 2

Subsequent Pearson correlation analysis of the staining scores showed that there was a significant inverse correlation between the score of galectin-1 and galectin-9 in the tumor cells (corr. coef. -0.36) while there was a positive correlation between galectin-1 and galectin-9 protein score in the tumor endothelial cells (corr. coef. 0.44). However, neither the galectin-1 or galectin-9 protein staining scores were associated with OS or DFS. Furthermore, there was no correlation between IHC scores and mRNA levels.

Discussion

We evaluated the prognostic significance of galectin mRNA expression in patients with stage I/II non-small cell lung cancer. Univariable Cox regression analyses were used to select a set of the most prognostic clinical parameters and galectins. These were subsequently used in a multivariable analysis to generate a model that could serve to predict OS or DFS in patients with stage I/II NSCLC. The main finding of this study is that for predicting OS, the most significant prognostic model included stage, age, gal-1 and gal-9∆5 while the model for DFS included stage, age and gal-9∆5.

Galectins have previously been associated with lung cancer progression\textsuperscript{16,17,29}. Our observation that patients that express galectin-1 above median levels have a significant shorter overall is in agreement with these studies\textsuperscript{16,17} as well as with studies in other types of cancer\textsuperscript{30}. The prognostic value of galectin-1 was confirmed in the multivariable analysis. Galectin-3, which has also been associated with poor disease outcome in lung cancer patients\textsuperscript{16,17}, did not reach statistical significance in our patient group. This corroborates with two more recent
Galectin expression profiling in NSCLC

On the other hand, it has been suggested that cellular localization of galectin-3, i.e. nuclear vs. cytoplasmic might be of prognostic value for recurrence\textsuperscript{33}. We only measured galectin-3 mRNA expression levels and did not determine the cellular localization of galectin-3 protein expression in our patient group. Thus, we cannot exclude that these parameters could be of prognostic value in stage I/II NSCLC patients.

A novel finding of the current study was the identification of a specific gal-9 splice variant, i.e. galectin-9Δ5 as a prognostic marker in NSCLC. Using multivariable Cox regression analysis we now observed that low galectin-9Δ5 expression was associated with poor OS and DFS in early stage NSCLC patients. These observations are in line with other reports in which galectin-9 expression was inversely correlated with cancer progression and patient survival in a number of different cancer types, including skin cancer, liver cancer, and breast cancer\textsuperscript{34-36}. More recently, Jiang \textit{et al.} identified galectin-9 expression as an independent prognostic factor in a retrospective study on 305 patients with gastric cancer. Again, low galectin-9 expression was associated with poor survival\textsuperscript{37}.

Galectin-9Δ5 is one of the three most frequently identified galectin-9 variants. These splice variants encode protein isoforms that vary in the length of the linker region between the two CRD domains which affects multimer formation and valency\textsuperscript{38,39}. Previous data suggest that the different galectin-9 isoforms have a diverging role in tumor cells, e.g. they differently affect the adhesion of cells to the ECM and to the endothelium\textsuperscript{40}. In general, altered galectin-9 expression has been linked to abnormal cell adhesion, growth and migration\textsuperscript{39}. Others have described that galectin-9 can influence cell survival as well as homo- and heterotypic cell aggregation\textsuperscript{9,34,40-43}. Loss of galectin-9 expression could compromise tissue integrity allowing tumor cells to intravasate into circulation and metastasize. Indeed, in breast cancer low galectin-9 expression was a better predictor of distant metastasis compared to lymph node status\textsuperscript{42}. Similar observations were made in melanoma and cervical squamous cell carcinoma\textsuperscript{34,44}. However, these effects depend on multiple parameters, including the specific galectin-9 variant, the type of cell and the adhesion matrix component to which the cells bind\textsuperscript{45}. Whether and how all these parameters influence lung cancer progression requires further studies. Possibly, galectin-9 can act as a chemoattractant for lung cancer cells, similar as described for eosinophils\textsuperscript{46,47} or endothelial cells\textsuperscript{26}. Together with our observation that stromal galectin-9Δ5 expression remains elevated in lung tumors this chemoattracting activity indicates that galectin-9Δ5 might act as a guidance cue for metastatic tumor cells to migrate towards the site of intravasation, i.e. the vasculature. This could promote tumor metastasis especially if loss of galectin-9 in tumor cells results in loss of tissue integrity\textsuperscript{45}. Finally, it has been reported that in animal models and cancer patients, tumor cells can release galectin-9 containing exosomes that can induce T-cell apoptosis\textsuperscript{48,49}. Whether tumor endothelial cells also secrete galectin-9 containing exosomes needs to be further investigated, but such a mechanism could contribute to tumor progression by providing a way to escape immune surveillance.

Immunohistochemical assessment of galectin-1 and galectin-9 protein expression showed differences in the localization and distribution within the tumor tissue. These observations are in line with previous findings in different tumors where both galectin-1 and galectin-9 proteins
could be detected in different compartments of the tumor, including tumor cells, tumor stroma and tumor endothelial cells\textsuperscript{16,23,30,42,44}. Nevertheless, protein expression had no prognostic value in our patient group. Most likely, this is related to the fact that immunohistochemical staining represents a more qualitative evaluation rather than a quantitative analysis. Thus, actual protein expression levels could not be accurately quantified by IHC staining. Furthermore, no galectin-9 antibodies are available that recognize specific splice variants. This suggests that in case of early stage NSCLC patients, determining galectin mRNA levels is of more value for prognosis estimates as compared to immunohistochemical staining.

The main limitation of the present study is the relatively low sample size of 87 in relation to the large number of parameters that was analyzed. The sample size allowed the inclusion of only 5 covariates in the regression model to minimize the risk of over-fitting. In addition, we only included early stage NSCLC patients. Thus, additional studies using larger patient groups and also including later stages of NSCLC, i.e. stage III/IV, might provide more insight in the prognostic value of galectin mRNA expression profiling.

In summary, extensive galectin expression profiling confirmed the prognostic value of galectin-1 and identified gal-9Δ5 as a potential novel prognostic markers in early stage NSCLC. Identification of such markers is important to identify patients that will benefit from adjuvant chemotherapy. In addition, our findings exemplify the relevance of profiling individual splice variants of galectin-9. It remains to be determined whether splice variant-specific profiling has a similar benefit in other cancer types, including those in which overall galectin-9 expression is a prognostic marker.

**Acknowledgements**
The authors would like to thank Dr. F. Galindo Garre for statistical advice and E. Aanhane for technical assistance.
Supplementary Figure

Supplementary Figure 1. Galectin mRNA expression profile in different NSCLC lines. The inset shows the expression of the three galectin-9 splice variants.
References

25. Spitzenberger F, Graessler J, Schroeder HE. Molecular and functional characterization of galectin 9 mRNA.


Chapter 3

Examination of the role of galectins and galectin inhibitors in endothelial cell biology

Iris A Schulkens, Esther A Kleibeuker, Kitty C Castricum, Arjan W Griffioen, Victor L Thijssen


Abstract

The growth of new blood vessels is a key event in many (patho)physiological processes, including embryogenesis, wound healing, inflammatory diseases, and cancer. Neovascularization requires different, well-coordinated actions of endothelial cells, i.e. the cells lining the luminal side of all blood vessels. Galectins are involved in several of these activities. In this chapter we describe methods to study galectins and galectin inhibition in three key functions of endothelial cells during angiogenesis, i.e. endothelial cell migration, endothelial cell sprouting, and endothelial cell network formation.
**Introduction**

Blood vessels are the infrastructure of an organism as they facilitate the transport of e.g. nutrients and oxygen throughout the body. The vascular infrastructure is formed during embryogenesis by two main processes, i.e. vasculogenesis and angiogenesis. Vasculogenesis refers to the early *de novo* formation of a primitive vasculature by angioblasts. During further development, new vessels are continuously sprouting from existing capillaries in this vasculature by a process called angiogenesis. Angiogenesis is a complex process that involves different well-coordinated and step-wise activities of endothelial cells (EC), i.e. the cells that cover the luminal side of blood vessels. The angiogenesis cascade is initiated by activation of EC by pro-angiogenic growth factors like vascular endothelial growth factor (VEGF). Once activated, supportive cells (pericytes) detach from the capillary and supportive structures (basal membrane and the underlying extracellular matrix) are degraded. Next, the endothelial cells sense the direction of the angiogenic stimulus and start to proliferate and migrate into that direction. The growing EC sprouts form primitive tubular structures which are eventually stabilized by deposition of a new basal membrane and attraction of pericytes. This allows the EC in these newly formed capillaries to return to a quiescent state.

As evident from their pleiotropic functions in cell biology, several galectins are involved in different steps of the angiogenesis cascade. This is not restricted to physiological angiogenesis during e.g. wound healing and embryogenesis, but extends to different pathologies that are characterized by dysregulated angiogenesis, including cancer. Galectins have been shown to activate EC and to promote angiogenic signaling in EC. In addition, galectins have been associated with EC adhesion, migration and proliferation during angiogenesis. Thus, galectins are now recognized as regulators of angiogenesis and even as targets for angiostatic therapy. In this chapter, we provide methods to assess the effect of galectins on endothelial cell migration, network formation and sprouting *in vitro*. Apart from studying the direct effects of galectins, these methods can also be used to determine the effects of galectin-blocking compounds on endothelial cell function.
Chapter 3

Materials

General
a. Endothelial cells (see note 1).
b. Recombinant galectins and/or galectin inhibitors.
c. Phosphate buffered saline (PBS).
d. 0.2% gelatin in PBS.
e. Humidified 5% \( \text{CO}_2 \) incubator at 37°C/99.5°F.
f. Laminar flow cabinet.
g. Pipetboy with 1.0 - 10 mL sterile pipettes.
h. 10-1,000 μL pipettes with sterile tips.

Special equipment
a. Inverted microscope equipped with a camera. We use a Leica DMI3000B microscope equipped with an automated xyz-stage and a Hitachi 1.4 Mb GiGE color camera.

Migration
a. Flat bottom 96-well plates (Costar) (see note 2).
b. A 96-well pin tool scratcher (see note 3).
c. UniversalGrab 6.2 software (DCIlabs) for image acquirement.
d. Image analysis software, e.g. ScratchAssay 6.2 (DCIlabs), ImageJ or Photoshop.

Network formation
a. Flat bottom 96-well plates.
b. BD Matrigel basement membrane matrix (see note 4).

Sprouting
a. Flat bottom 24-well plates.
b. Non-adhesive square Petri dishes (10x10 cm).
c. Methocel medium: RPMI with 20% methocel and 10% heat-inactivated human serum.
d. PBS with 10% heat-inactivated human serum.
e. BD Matrigel basement membrane matrix (see note 4).
Methods

Migration
1. Coat a flat bottom 96-well plate with 0.2% gelatin/PBS for at least 30 minutes at 37°C/99.5°F or 2 hours at room temperature. Aspirate gelatin before seeding the cells. Alternatively, the wells can be coated with the recombinant galectin of interest in PBS (optimal concentration should be determined experimentally).
2. Seed cells and grow to confluence in 2-3 days (see note 5).
3. Scratch the confluent monolayer with a 96-well pin tool (see note 3).
4. Aspire/drain the culture medium.
5. Carefully wash cells with 100 μL PBS.
6. Apply the appropriate medium containing recombinant galectin or galectin inhibitors (see note 6). Always include proper controls, e.g. sunitin or bFGF for inhibition or stimulation of migration, respectively.
7. Take images of the scratch at t = 0, t = 2, t = 4, t = 6 and t = 8 hours (see note 7 and Figure 1A).
8. Measure wound width or scratch area with automated ScratchAnalysis software. Alternatively use ImageJ (use straight line selection tool to measure wound width or free hand selection tool for measuring scratch area) or Photoshop (use ruler for wound width measurement or wand tool for scratch area). Calculate percentage of wound closure or remaining wound width/area (% of t = 0).

Network formation
1. Coat each well of a flat bottom 96-well plate with 40 μL Matrigel (see note 4) and incubate at 37°C/99.5°F for 30 minutes.
2. Apply the required number of cells in 50 μL medium per well (see note 8). Add recombinant galectin or galectin inhibitors to the medium.
3. Incubate overnight in a humidified incubator at 37 °C/99.5°F, 5% CO₂.
4. Acquire images.
5. Endothelial cell network formation can be quantified by counting the number of branch points, number of meshes (see Figure 1B).

Sprouting
1. Harvest cells and resuspend to a final concentration of 40,000 cells/mL in methocel medium (see note 9).
2. Distribute 25 μL drops with a multi-channel pipet on the lid of a non-adhesive square petri dish (see note 10).
3. Add 10 mL PBS to the bottom of the petri dish to prevent evaporation of the drops.
4. Turn the lid with drops carefully upside down on and place on the bottom dish containing PBS.
5. Incubate overnight in a humidified incubator at 37°C/99.5°F, 5% CO₂.
6. Harvest the spheroids by rinsing the plate twice with 6 mL PBS containing 10% heat-inactivated human serum.

7. Collect the spheroids in a 15 mL tube and centrifuge for 1 minute at 250 g.

8. Aspirate the PBS and carefully flick the tube to prevent the spheroids from sticking together.

9. Resuspend 60 spheroids per condition in 200 μL Matrigel (see note 4) and transfer the suspension to a 24-well plate (see note 11).

10. Incubate the plate for 15 minutes at 37°C/99.5°F until the Matrigel is solidified.

11. Apply 500 μL medium, containing recombinant galectin proteins and/or galectin inhibitors on top of the Matrigel (see note 12).

12. Incubate in a humidified incubator at 37°C/99.5°F, 5% CO₂ for 16-24 hours and take pictures of the spheroids (see note 13 and Figure 1C).

13. Analyze sprout length and sprout number per spheroid using ImageJ.

---

**Figure 1. In vitro angiogenesis assays.** Representative pictures of migration (A), network formation (B), and sprouting (C) of non-treated (control) and galectin inhibitor-treated HUVEC. The first panels represent the starting conditions at t = 0. The second and third panels indicate typical results at the end point of the experiment of non-treated and galectin inhibitor-treated cells, respectively. The last panels illustrate different ways of analyzing the respective results: A) For analysis of migration, wound width (white arrow), or wound area (in black) can be measured at different time points and compared with t = 0. B) Tube formation can be quantified by counting the number of meshes (M) and branch points (white dots). For clarity only part of the image is scored. C) Sprouting is analyzed by measuring the sprout length (white lines) and counting the number of sprouts.
Notes

1. Different sources of endothelial cells are available\textsuperscript{10}. Human umbilical vein endothelial cells (HUVEC) are frequently used and these cells can be isolated in house or purchased from a commercial source. Apart from HUVEC, we also use the EC lines EC-RF24 (HUVEC origin) and HMEC (Human microvascular endothelial cells). For network formation and sprouting assays we generally use HUVEC while migration assays can be performed with any source of EC. If required, cells can be transfected with galectin-targeting siRNA or galectin expression constructs according to standard protocols. All EC are cultured in RPMI1640 (Lonza) supplemented with 10% fetal bovine serum (FBS), 10% human serum, 1% L-glutamine and 1% penicillin/streptomycin. At confluence, passage the cells 1:3.

2. We prefer the use of Costar flat bottom plates since plates from other suppliers sometimes contain markings on the back of the plates due to the production process. These markings can interfere with the scratch analysis.

3. A pin tool scratcher is preferred since this gives low variability and high reproducibility in wound width. We use the Peira HTSScratcher. However, it is also possible to apply a scratch manually using a small pipetting tip, e.g. 10 uL tips. Take a fresh tip for each well to avoid increasing scratch width due to cells that stick to the tip.

4. Matrigel is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains several extracellular matrix proteins that facilitate endothelial cell adhesion and migration. However, standard Matrigel also contains several growth factors that stimulate the angiogenic activity of endothelial cells. Therefore, depending on the research question, e.g. test effect of inhibitors on galectin-induced network formation or sprouting, it is better to use growth factor reduced Matrigel. Note that Matrigel stock solutions should be kept at temperatures below 4\textdegree C/39.2\textdegree F to prevent solidification. Thawing should therefore be performed on ice in a fridge (This takes approximately 1 hour).

5. It is important to start with a confluent monolayer of cells in order to induce unidirectional migration. For RF24 cells seed 15,000 cells/well, for HUVEC/HMEC use 5,000 cells/well.

6. Evaluation of inhibitory activity can be performed in normal EC culture medium. To evaluate stimulatory activity, low serum (0.5% FBS and/or 0.5% human serum) can be used. Furthermore, we have observed a biphasic activity of galectin-1 depending on the concentration\textsuperscript{6}. Therefore, use a broad concentration range to optimize your assay.

7. For analysis it is important that the images of the scratch within the time series are taken at the exact same position. If no appropriate software and automated xyz table are available to automate this, make sure to mark the culture plate in such way that the area in the well where the images are taken can be easily found. For example, prior to plating the cells, use a scalpel to make a scratch on the backside of each well in the plate. Make sure that the scratch is perpendicular to the direction of the ‘wound’. The perpendicular scratch can then be used to identify the region where the wound width is measured.

8. The optimal number of cells used in this assay should be individually determined for each cell line. Too high cell numbers will result in robust branches or confluent areas at the
center of the well. Too few cells will cause incomplete network structures.

9. Dilute to 40,000 cells/ml to obtain 1,000 cells/25 μL drop. Prepare 60 drops (60,000 cells in 1,500 μL methocel medium) per test condition since not all drops will successfully form a spheroid.

10. Use reverse pipetting technique to avoid air bubbles disturbing the spheroid formation.

11. When transferring the spheroid suspension to a 24-well plate, make sure the whole well is covered by pipetting the suspension “swirl-wise”, starting in the middle of the well. Avoid air bubbles.

12. As a positive control for induction of sprouting, add 100 nM of the γ-secretase inhibitor dibenzazepine (DBZ) to the medium. As a positive control for inhibition of sprouting 10 μM sutent can be added to the medium.

13. For reliable analysis of sprouting at least 10 spheroids per treatment condition should be photographed and analyzed.
References

Chapter 4

Examination of the role of galectins during \textit{in vivo} angiogenesis using the chick chorioallantoic membrane assay

Esther A Kleibeuker, Iris A Schulkens, Kitty C Castricum, Arjan W Griffioen, Victor L Thijssen

Abstract

Angiogenesis is a complex multi-process involving different activities of endothelial cells. These activities are influenced \textit{in vivo} by environmental conditions like interactions with other cell types and the micro-environment. Galectins play a role in several of these interactions and are therefore required for proper execution of \textit{in vivo} angiogenesis. In this chapter we describe a method to study galectins and galectin inhibitors during physiologic and pathophysiologic angiogenesis \textit{in vivo} using the chicken chorioallantoic membrane (CAM) assay.
Introduction

Angiogenesis is a complex multi-process involving different activities of endothelial cells (see previous chapter). The function of endothelial cells can be influenced by environmental conditions like changing flow dynamics, interactions with other cell types, and interactions with specific extracellular matrix components. Thus, while in vitro assays can provide insights in the effects of molecules like galectins and/or galectin inhibitors on endothelial cell function, further assessment of their role in angiogenesis in vivo is important. A commonly used assay to study angiogenesis in vivo is the chick chorioallantoic membrane (CAM) assay. The CAM is a highly vascularized extra-embryonic membrane that mediates exchange of gas and nutrients during embryonic chick development. It is formed between embryonic day of development (EDD) 3-10 of the 21 day gestation period by fusion of the allantois mesodermal layer -extending out of the embryo- with the mesodermal layer of the chorion. Within the resultant double layer a dense vascular network develops up to EDD11 after which endothelial cell proliferation drops rapidly allowing further maturation of the vascular bed.

Apart from the rapid vascular development there are numerous advantages that warrant the use of the CAM assay for in vivo angiogenesis studies. First, the assay is low in cost, reproducible, reliable and fairly simple to perform. Furthermore, there is a variety of methods for the application to test compounds onto the CAM and several methods are available to monitor the subsequent response in the vasculature. For example, we have used the CAM assay to study the effects of galectin-1 and galectin-1 inhibition on angiogenesis. The CAM assay can also be used to test the effects of other treatment modalities like radiotherapy or photodynamic therapy. Finally, the model does not require a sterile work environment and since the immune system of the chicken embryo is not fully developed until ± EDD18 the CAM assay can also be used for grafting xenograft cells and tissues. On the other hand, nonspecific reactions can occur due to contamination with egg shell itself or due to the use of reactive carrier vehicles. In addition, CAM development is sensitive to alterations in environmental conditions like temperature, oxygen tension and osmolarity. This indicates that experiments using the CAM assay should be carefully executed. In this chapter we will describe a method for the topical application of soluble compounds (galectins and/or galectin inhibitors) on the CAM in order to study their effects on angiogenesis in vivo. In addition, we provide a method to graft tumor cells onto the CAM which can provide information on the role of galectins in tumor growth and tumor angiogenesis.
Materials

We use fertilized eggs of white leghorn chicken that are purchased from a local commercial supplier. The eggs can be stored for several days at 4°C/39.2°F but after more than one week the quality and viability of the eggs decreases affecting the quality of the data. Please be aware that depending on the legislation of your country regarding animal use in research experiments, a license might be needed to perform the described experiments.

Special equipment
a. Fan-assisted humidified (egg) incubator, 37.5°C/99.5°F (see note 1). We use a FIEM MG140/200 Rural which allows us to switch between tilting racks and non-tilting racks (see note 2). The incubator should be well humidified throughout the whole experiment to prevent dehydration of the CAM. We achieve this by putting water basins on the floor of the incubator (see note 3).
b. Fiber optic illuminator. We use a Schott KL 1500 Electronic Light Source (see note 4).
c. Stereo microscope equipped with a camera. We make use of a Leica M125 stereomicroscope with 12.5:1 zoom which is equipped with a Leica KL1500 LED ring illumination system and a Leica 5 Megapixel DFC425 CCD camera.

Incubation of the chicken eggs
a. 70% Ethanol.
b. Sterilized fine tweezers.
c. Scotch ‘Magic’ adhesive tape (see note 5).

Application of galectins/galectin inhibitors onto the CAM
a. Non-latex elastic rings (see note 6).
b. Saline.
c. Test compounds, i.e. galectins and/or galectin inhibitors.
d. 10-100 μL pipette with sterile filter tips.

Data acquisition and analysis
a. Fridge or cold room.
b. 20 mL syringe.
c. 33 Gauge injection needle.
d. Contrast solution (4 grams of zinc oxide in 50 mL pure vegetable oil).
e. Image analysis software (HetCAM, DClabs) or Adobe Photoshop/MS Office.

Grafting tumor cells onto the CAM
a. Approximately 5x10⁶ cells (see note 7).
b. Matrigel (see note 8).
c. Soft paper tissues.
Methods

A schematic drawing of the chicken gestation period is shown in Figure 1A. A typical CAM assay takes approximately 10 days and a CAM tumor graft experiment takes 17 days. Thus, depending on the type and frequency of treatment, careful planning of the experiments is important. The time schedules that are used in our lab for a normal CAM experiment as well as for a tumor graft CAM experiment are shown in Figure 1B.

Incubation of the chicken eggs
1. Transfer the eggs from the cold storage to room temperature for at least twelve hours prior to the incubation (see note 9).
2. Clean the shell of each egg with a tissue soaked in 70% ethanol.
3. Place the eggs horizontally on a 90° tilting rack, which rotates minimally 6 times per 24 hours. Place the rack in a pre-warmed and humidified fan-assisted egg incubator at 37.5°C/99.5°F (see notes 1-3). The starting day of the incubation is regarded as EDD0.
4. On EDD3, put the eggs in an upright position and make a small hole in the narrow end of the shell with fine tweezers. This will translocate the air compartment in the egg to the top of the egg. Seal the hole with adhesive tape using as little tape as possible (see note 10). Stop the rotation of the racks and place the eggs back in the incubator, with the sealed hole at the top.
5. On EDD6, check the eggs for fertilization. Point the fiber optic light source towards one side of the egg. Vasculature should become visible at the opposite side of the egg. If not, the egg is not fertilized and can be discarded.
6. Create a window of ±1 cm³ in the top of the shell with fine tweezers (see note 11). The CAM vasculature can now be observed through the window.
7. Proceed with direct application of galectins/galectin inhibitors onto the CAM or with grafting tumor cells onto the CAM.
Figure 1. Time schedule for the CAM assay. A) Schematic representation of the CAM assay schedule during embryonic chicken development from embryonic day of development (EDD) 0 until EDD20, i.e. the day before hatching. The EDD during which extensive angiogenesis takes place in the CAM are shown in bold. The images show the CAM vasculature at different EDD. B) Scheme of a standard CAM assay (upper panels) and of the tumor graft assay on the CAM (lower panel).
Application of galectins/galectin inhibitors onto the CAM

1. On EDD6, carefully place a sterilized non-latex plastic dental ring through the window on top and in the center of the CAM. Seal the window with adhesive tape (see note 10) and place the egg back in the incubator for at least 2 hours. This allows the ring to settle down on the CAM (see note 12).
2. Prepare the treatment solution by diluting the appropriate galectin with or without the specific inhibitor in saline. The total amount of solution depends on the number of eggs, the diameter of the ring, and the duration/frequency of the treatment. (see note 13).
3. Take an egg out of the incubator. Open the sealed window and check if the embryo is still alive. Apply 50-80 μL of galectin/galectin inhibitors within the ring without touching the CAM itself. Reseal the window and place the eggs back in the incubator (see note 10).
4. Repeat the addition of galectin/galectin inhibitors depending on your required treatment schedule. Usually, treatment is performed on a daily basis until EDD9.

Data acquisition

1. On EDD10, place the eggs at 4°C/39.2°F for 30 min. to induce hypothermia (see note 14).
2. Prepare contrast solution by mixing 4 grams of zinc oxide with 50 mL of pure vegetable oil in a 50 mL tube. Shake vigorously and leave it on a roller platform for 20 minutes.
3. Fill a 20 mL syringe with the zinc oxide/oil mixture. Make sure to remove any air bubbles.
4. Open the shell of a hypothermic egg as far as possible without disrupting the CAM.
5. Carefully inject ± 1 mL of the contrast solution directly under the CAM where the ring is located.
6. Use the microscope with camera to acquire images of CAM vasculature within the ring area (see note 15).
7. If necessary, the treated CAM area can be collected for further analysis, e.g. gene expression, immunohistochemistry. Following acquisition of images, isolate the CAM area under the ring using fine tweezers and small surgical scissors. Wash the freshly isolated CAM in PBS and transfer it to the desired fixation buffer or liquid nitrogen. Further processing of the tissue is not described in this chapter.
8. Finally, euthanize the chicken embryo by transferring the egg to -20°C/-4°F for 24 hours.

Data analysis

Several methods have been published to analyze the CAM images. Nowadays, software-based image analysis is often used for rapid, objective and extensive image analysis. The software uses specific algorithms to recognize and skeletonize the vascular bed from which different vascular parameters can be extracted like vessel length, vessel branchpoints, vessel endpoints, total vessel area etc. (Figure 2A). We use HetCAM software (DCI labs, Belgium) but other software packages might be used as well. However, we are aware that such software is expensive and not always available. Therefore, we here describe a widely accepted morphometric method to analyze images of the CAM, using software that is available in most research labs (Figure 2B) (see note 16).
1. Open the desired CAM image in a graphics editing program like Adobe Photoshop (or any comparable software package).

2. Set the image to grayscale to enhance the contrast between the vessels and the background. If necessary, use the image autocontrast function to improve contrast. Note that this should be performed for all images within a single experiment and that this should not be used to obscure or remove any unwanted data.

3. Place the CAM image in a graphic design program like Adobe Illustrator or a presentation program like PowerPoint.

4. Project 5 concentric rings over the CAM image and count the cross-sections between the vessels and the rings. The sum of these counts is a morphometric measurement for vessels density in the CAM.

Grafting tumor cells onto the CAM

While the methods described above provides information on the direct effects of galectin/galectin inhibitors on angiogenesis, a lot of galectin research is performed in the context of tumor biology. Consequently, it is important to determine how galectin expression in tumor cells or treatment with galectin-targeting compounds affects tumor growth and tumor angiogenesis. This can be readily studied using the CAM assay since it is possible to graft (human) tumor cells onto the CAM, most of which will rapidly grow into well vascularized tumors.
1. On EDD6, harvest the tumor cells. Count the cells and aliquot them in separate tubes, each tube containing 5x10⁶ cells and spin them down. Discard the medium.
2. On ice, mix 5x10⁶ cells with 50 μL Matrigel (see note 7).
3. Carefully ‘damage’ a small area of the CAM with a soft tissue.
4. Transfer the Matrigel/cell mix onto the damaged area.
5. Close window and place egg back into incubator.
6. Check growth of tumor daily and measure size and width using a ruler (see note 17).
7. If necessary, start treatment on EDD10 by applying the appropriate galectin inhibitor topically onto the tumor or by direct injection into the tumor tissue.
8. Measure size on a daily basis until EDD14 or maximally until EDD17 (see note 17).
9. At the end of the experiment, harvest, photograph and weigh the tumor and subsequently place it in the appropriate fixative for further processing (Figure 3).
10. Finally, euthanize the chicken embryo by transferring the egg to -20°C/-4°F for 24 hours.

Figure 3. Tumor grafts on the CAM. A) Image of a HT29 tumor on the CAM at EDD14. The tumor cells were grafted on EDD6. B) HT29 tumor after resection. The tumor is well vascularized indicating adequate tumor angiogenesis. C) Image of hemotoxilin/eosin staining on a paraformaldehyde fixed and parafin embedded HT29 tumor graft. Both the CAM as well as nest of tumor cells (TC) surrounded by tumor stroma (S) are clearly visible. D) Image of vessel staining (CD31, brown) on a paraformaldehyde fixed and parafin embedded HT29 tumor graft.
Notes

1. Temperature setting depends on the type of incubator. For a still-air incubator (no fan): 38.5°C (101.3°F) measured at the top of the eggs. For a fan assisted incubator: 37.5°C (99.5°F) measured anywhere in the incubator.

2. If the incubator has no tilting racks, manual rotation of the eggs is also possible. Turn the eggs through 180 degrees by hand at least twice a day.

3. Humidity should be maintained at ±50% during incubation. Excessive humidity could result in an increased rate of infections in the eggs.

4. The fiber optic illuminator is used to check successful fertilization on EDD3. However, if no such device is available successful fertilization can also be readily checked following opening of the egg shell.

5. Other brands of tape can be used but we have good experience with the Scotch Magic tape because it is not too sticky which makes it easy to repeatedly open and close the window in the egg shell.

6. Non-latex rings are commercially available or they can be custom-made. It is important that the weight of the ring is as low as possible to avoid aspecific responses in the vasculature due to occlusion of vessels by the ring. We have found that orthodontic dental elastic bands (non-latex, Ø 9.5 mm) are a good and cheap alternative. Varying the diameter of the rings allows larger area's to be treated but also requires more compound. In addition, with increasing diameter the weight of the ring also increases. A diameter of ± Ø 9.5 mm typically allows application of 50-80 μL solution.

7. The number of cells required for adequate grafting depends on the specific cell line and should be tested. However, we have found that increasing the cell number increases the success of grafting and most cell lines tested in our lab show successful grafting when 5 million cells are used.

8. We have successfully used both normal and growth factor reduced Matrigel. The latter is preferred if the angiostimulatory effect of cells is tested since normal Matrigel already contains more stimulatory factors by itself.

9. We use anywhere between 8 - 10 eggs per treatment condition. For experienced users a total number of about 80 eggs per experiment is manageable which thus allows for 8 to 10 different groups per experiment.

10. The adhesive tape prevents the CAM from dehydration. However, the tape also prevents gas exchange through the egg shell and should therefore be kept to a minimum.

11. The egg shell should be removed carefully since debris of the shell can induce a response in the CAM.

12. Instead of a ring to define the therapeutic area, some people prefer to use for example gelatin or methylcellulose discs\textsuperscript{13,14}. Also other absorbent materials might be used as long as these do not induce a response in the CAM.

13. In general, 50-80 μL of compound is applied daily from EDD6 until EDD9. The solution can be prepared fresh daily or stored at 4°C/39.2°F. Note that long term storage at 4°C/39.2°F of
galectins in solution can affect protein stability and activity. Furthermore, solutions stored at 4°C/39.2°F should be allowed to return to room temperature before applying them to the CAM.

14. Movements of the embryo are very likely to disturb you while taking pictures of the CAM. The induced hypothermia will result in less movements, by slowing down the metabolism of the embryo. However, if the aim is to measure blood flow, hypothermia should not be applied.

15. The magnification will determine the level of detail that can be analyzed. At 25-fold magnification (2.5 x 10) mainly the larger, mature vessels will be visible while at 100-fold magnification (10 x 10) detailed images of the capillary bed can be obtained. We usually acquire images at both magnifications in order to distinguish between both vessel types.

16. This quantification method is laborious, less accurate and more sensitive to subjective errors. In addition, it will only provide information about the vascular density. Nevertheless, it is a cheap method and available to everyone.

17. Be aware that not all tumors will grow on top of the CAM. We have observed that some tumors will grow just underneath the CAM.
Chapter 4

References

Chapter 5

Endothelial LGALS9 splice variant expression in endothelial cell biology and angiogenesis

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Chapter 5

Abstract

Galectins are carbohydrate binding proteins with versatile functions in tumor progression. Galectin-9, encoded by LGALS9, has been associated with metastasis and immunosuppression. We previously reported on regulation of LGALS9 expression during endothelial cell activation. Here, we show increased galectin-9 protein levels in the endothelium of different tumors, including carcinomas of lung, liver, breast and kidney. Endothelial cells were found to express five LGALS9 splice variants, two of which have not been reported before. Splicing was found to be confined to exons 5, 6 and 10. Transfection of human microvascular endothelial cells (HMEC) with galectin-9Δ5, a specific LGALS9 splice variant, induced a small but significant increase of proliferation, while migration was not affected by any LGALS9 splice variant. Application of recombinant galectin-9Δ5 protein dose-dependently reduced proliferation and migration of HMEC as well as human umbilical vein endothelial cells in vitro. Enhanced sprouting and migration of HUVEC towards a galectin-9Δ5 gradient was observed. Interestingly, galectin-9Δ5 was found to induce a small inhibitory effect on angiogenesis in vivo. Collectively, these data show that endothelial cells regulate the expression and splicing of LGALS9 during angiogenesis. The function of the dominant splice variant, i.e. galectin-9Δ5, in endothelial cell biology depends on the concentration and environmental context in which it is presented to the cells.
Introduction

Galectin-9 is member of a family of glycan-binding proteins that are characterized by the presence of one or two conserved carbohydrate recognition domains (CRDs), i.e. galectins. Galectin-9 belongs to the subgroup of tandem-repeat galectins which have two CRDs that are joined by a linker domain. The protein of approximately 39.5 kD protein is encoded by LGALS9 which consists of 11 exons. Expression of galectin-9 is accompanied by extensive posttranscriptional splicing which can regulate the length of the linker region between both CRDs. Apart from the full length variant, which consists of all exons (gal-9FL or gal-9L), two other LGALS9 splice variants are frequently described in the literature, i.e. gal-9Δ5 (or gal-9M, lacking exon 5) and gal-9Δ5/6 (or gal-9S, lacking exons 5 and 6). Splicing of the linker encoding exons has been shown to influencing galectin-9 valency and function.

Regarding its function in cancer, galectin-9 contributes to different aspects of tumor growth and aggressiveness, including metastasis and immunomodulation. In fact, an inverse correlation between LGALS9 expression and disease outcome has been reported in several cancers, including breast, liver, skin, and cervix. Moreover, it has been suggested that galectin-9 might have clinical benefit during cancer treatment. This requires a better understanding of LGALS9 expression in different compartments of the tumor, including the tumor vasculature. Previously, we reported on LGALS9 expression in normal and tumor associated endothelial cells, i.e. the cells that line the luminal side of all blood vessels. Interestingly, altered endothelial expression of LGALS1, LGALS3 and LGALS8 has been associated with enhanced angiogenic activity in tumors. Furthermore, pro-angiogenic activity has been attributed to galectin-1, galectin-3 and galectin-8. Here, we studied the expression of LGALS9 as well as the function of galectin-9 in endothelial cell biology and angiogenesis.
Materials and methods

Isolation of primary cells and cell culture
Primary human umbilical vein endothelial cells (HUVECs) were isolated as described previously. Quiescent HUVEC were directly harvested from the umbilical cord without further culturing. Activation of HUVEC was achieved by culturing the cells for at least three days in culture medium supplemented with 20% human serum. This has already been shown to induce sufficient EC activation causing alterations in galectin expression. HUVEC and immortalized human microvascular endothelial cells (HMEC-1) were cultured at 37°C/5% CO₂ in RPMI (Gibco) containing 10% fetal calf serum (Invitrogen) and 10% human serum supplemented with L-glutamine (Invitrogen) and penicillin/streptomycin (Invitrogen).

Tumor endothelial cells were isolated from freshly resected kidney tumor as described previously. Cells were stored at -80°C until RNA isolation.

RNA isolation and cDNA synthesis
RNA was isolated using the RNeasy kit (QIAgen) according to the supplier’s protocol. Isolations were subjected to on-column DNaseI digestion (QIAgen). The purity and concentration of the RNA samples was determined by spectrophotometry (ND-1000, Nanodrop technologies). cDNA synthesis was performed on 500 ng total RNA using the iScript cDNA synthesis kit (BioRad) according to the supplier’s protocol.

Real-time PCR
Real time PCR was performed on the CFX96 real time PCR detection system (BioRad). In short, 1.5 μL of cDNA was used in a total reaction volume of 25 μL containing 1x SensiMix (Quantace) and 400 nmol/L of LGALS9 splice variant specific primers (Supplementary Table 1). A standard 2-step amplification protocol was used with Tm = 61°C followed by a melting curve analysis. Measurements were performed on at least 3 different HUVEC isolations or in triplicate for cell lines. To compare expression levels between different conditions, the ΔΔCt method was used as described previously.

Cloning of galectin-9 splice variants
Endothelial LGALS9 splice variants were cloned from HUVEC cDNA into the pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen). LGALS9 flanking primers were used for the cloning PCR (forward primer: 5’-ATGGGCTTCAAGCGTGTTCC; reverse primer 5’-CTATGCTCAGCAGTGTCAG). Ligation products were transformed into OneShot TOP10 E. Coli (Invitrogen) and grown overnight on LB plates containing ampicillin. Positive clones were selected by insert PCR with M13 primers. MidiPreps were performed with the QiaPrep Spin Midiprep Kit (Qiagen), according to the supplier’s protocol. To obtain eukaryotic expression, all LGALS9 splice variants were directionally cloned into pcDNA3.1 using HindIII/Xhol digestion and subsequent ligation with T4 DNA Ligase (New England Biolabs). Successful cloning was confirmed by restriction enzyme digestion and sequence analysis which was performed by BaseClear (Leiden, the Netherlands).
Transfection
For gain of function experiments, HMEC were seeded in 96-well plates coated with 0.2% gelatin/PBS at a density of 1.5x10^4 cells/well. The next day, the subconfluent cells were transfected with the appropriate constructs using lipofectamine LTX and PLUS reagent (Invitrogen) according to the supplier’s protocol. 72 hours following transfection, functional assays, i.e. migration and proliferation, were performed. In addition, expression of galectin-9 variants was confirmed by Western blot as described below.

In vitro proliferation and migration assays
To assess the effect of endogenous galectin-9 expression on endothelial cell proliferation and migration, HMEC were transfected with LGALS9 splice variants and used in functional assays 72 hours post transfection. To assess the effect of exogenous galectin-9 variants, HUVECs or HMEC were incubated with a concentration range of exogenous recombinant galectin-9D5 (R&D systems) for 48 hours (proliferation) or during the scratch assay (migration). The CellTiter-Glo assay (Promega) was used to assess the proliferation rate of endothelial cells. In short, cells in a 96 well plate were washed and incubated with 100 ml CellTiter-Glo reagent for 10 minutes on a shaker. Next, luminescence levels were determined on a microplate reader (Tecan). Endothelial cell migration was assessed by creating a scratch in a confluent layer of endothelial cells, followed by monitoring of its closure. Images of each scratch were automatically acquired at t = 0 and t = 8 hours with a 1.4 Mb GiGE color camera (Hitachi) on a DMI3000B microscope equipped with an automated xyz-stage (Leica) using Universal Grab software (version6.3, DCIIabs). Scratch area was determined using Scratch Assay software (version6.2, DCIIabs). Proliferation experiments following HMEC transfection were performed in quadruplicate with four replicates of treatment conditions within each experiment. Migration experiments following HMEC transfection were performed in quintuplicate and within each experiment there were at least three replicates of all treatment conditions. Proliferation and migration with recombinant galectin-9 on HMEC and HUVEC were performed in triplicate with three replicates per treatment condition within each experiment.

Sprouting
For sprouting, HUVEC were resuspended to a final concentration of 40,000 cells/mL in methocel medium (RPMI with 20% methocel and 10% heat-inactivated human serum). Next, 25 μL drops were pipetted onto the lid of a non-adhesive square petri dish which was then inverted and incubated overnight in a humidified incubator at 37˚C, 5% CO₂. The next day, the spheroids were harvested in PBS. For each experimental condition approximately 40 spheroids were resuspended in 200 μL Matrigel and transferred to a 24-well plate. Following solidification of the Matrigel 500 μL medium was applied and spheroids were allowed to sprout in a humidified incubator at 37˚C, 5% CO₂ for 16-24 hours. Finally, pictures of the spheroids were taken and sprout length and sprout number per spheroid was analyzed using ImageJ. All experiments were performed in triplicate and per experiment at least 20 spheroids per condition were analyzed.
Transwell migration
For transwell migration, 7,500 HUVEC were seeded in HTS Fluoroblock inserts (8 micrometer pore size, Falcon). Inserts were placed in 24-well plates containing culture medium supplemented with the appropriate compounds. Cells were allowed to migrate overnight. Next, cells were fixed with 1% paraformaldehyde in 0.1%BSA/PBS. Following three wash steps with PBS, cells were stained with phalloidin red in 0.1% Triton/PBS overnight at 4°C. Images were acquired with LAS v3.7 software (Leica) using an inverted fluorescent microscope (DM-IL Leica) equipped with a DFC345 FX camera (Leica). The number of cells was scored in 5 random selected areas by two independent observers. Experiments were performed in triplicate.

Galectin-9 secretion
To assess the secretion of galectin-9 by endothelial cells, transfected HMEC were grown for 48 hours in culture medium after which the cells were washed with PBS and continued to grow in serum-free medium. After 24 hours, the serum-free medium was collected for Western blot analysis.

Western Blotting
For Western blot analysis, cells were lysed in the culture plates using cell lysis buffer (Cell Signaling) supplemented with protease inhibitors according to the supplier’s protocol. Protein concentrations were determined using the BCA protein assay (Pierce). Subsequently, 30 μg of protein was suspended in Laemlli sample buffer (Biorad) supplemented with 1:20 β-mercapto-ethanol. Samples were boiled for 5 minutes and immediately separated by gel electrophoresis on a 15% polyacrylamide gel and transferred onto PVDF membranes (Millipore) following standard protocols. Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences) for 1 hour and incubated overnight at 4°C with goat anti-galectin-9 antibody (1:500, R&D systems), which recognizes the N-terminal part of galectin-9. Equal loading of the gels was confirmed by α-actin detection using mouse anti-α-actin (1:10000, MP Biomedicals). The membranes were washed three times with PBS/0.1% tween and subsequently incubated with the appropriate secondary IRDye antibodies (IRDye 800 anti-goat and IRDye 800 anti-mouse, LI-COR Biosciences) for 1 hour at room temperature. Finally, membranes were washed two times with PBS/0.1% tween and rinsed with PBS. Images were obtained by scanning the membranes with the Odyssey infrared imaging system (LI-COR Biosciences).

Immunohistochemistry
Galectin-9 immunohistochemistry was performed on tissue-micro arrays of 4 different cancer types (Super Bio Chips); kidney (CL), liver (CSA), lung (CCA) and breast (CBA) according to the supplier’s protocol. Each array contains 50 tumor sections and 9 matched control sections, i.e. sections made from adjacent non-malignant tissue. As a primary antibody, we used a polyclonal goat anti-human galectin-9 antibody (1:500, R&D systems). Galectin-9 was visualized by DAB staining of a polyclonal HRP-conjugated rabbit-anti-goat secondary antibody (DAKO 1:500). Blind scoring was performed by two independent observers who first assessed the overall
Galectin-9 splice variant expression in the tumor endothelium

staining intensity on a single array. Next, we used a 3-step scoring scale to score individual sections: 0 for absent or very low staining, 1 for moderate staining and 2 for strong staining. Scoring was performed for both tumor cells (their non-malignant counterparts) and endothelial cells.

**Chick chorioallantoic membrane assay**
Fertilized chicken eggs (Het Anker, the Netherlands) were incubated and rotated at a relative air humidity of 65% and a temperature of 37.6°C in a hatching incubator with an automatic rotator for 3 days. On embryonic day 3 (EDD3), a small opening of approximately 3 mm in diameter was made in the eggshell at the top of the egg and sealed with tape. The eggs were then incubated for 3 days with the opening facing upwards. On EDD6, the eggs were opened further to allow easier access to the chorioallantoic membrane. For the topical application of recombinant galectins, polyethylene rings were deposited on the CAM. From embryonic EDD6 to EDD9, recombinant galectins were applied daily within the polyethylene rings. Finally, on EDD10, pictures of the chorioallantoic membrane were taken using an Optech LFZ stereo microscope (Optech) after injection of approximately 1 mL contrast agent (zinc oxide in pure vegetable oil) under the chorioallantoic membrane and the ring. Quantification of different vascular parameters was obtained using HetCAM software (Peira, Belgium). At least 8 eggs per condition were used.

**Statistical analyses**
All data are presented as mean +/- sem unless indicated otherwise. For testing, the Mann-Whitney rank sum test was used. P-values < 0.05 were considered statistically significant and all statistical analyses were performed using SPSS (SPSS Inc., Gorinchem, the Netherlands).

**Results**

**Galectin-9 protein levels are increased on the tumor endothelium of solid tumors**
We have previously reported on galectin-9 expression in normal and tumor endothelium. To get more insight in endothelial galectin-9 expression, we performed immunohistochemical staining on tissue sections from 4 types of solid tumors and their normal counterparts, i.e. lung, liver, breast, kidney. Interestingly, all four tumor types displayed increased galectin-9 protein levels in the tumor endothelial cells as compared to the normal endothelium (Figure 1A). Subsequent scoring indicated that endothelial galectin-9 staining in most tumors was significantly increased compared to normal endothelium (Figure 1B). Tumor cell expression did not change as compared to normal epithelium, except for kidney (Figure 1C). Thus, endothelial cells show elevated galectin-9 protein levels in a tumor micro-environment pointing towards a role for galectin-9 in tumor angiogenesis.
Endothelial cells express five LGALS9 splice variants

To get more insight in the function of galectin-9 in endothelial biology we first determined LGALS9 expression in endothelial cells. Since LGALS9 transcripts are subject to extensive splicing we set out to identify characterize all LGALS9 splice variants in endothelial cells. To that end, a PCR was performed on HUVEC cDNA with primers flanking the open reading frame of the full length LGALS9 transcript, i.e. the transcript containing all 11 exons (Gal-9FL). The PCR products were cloned into the pCR2.1 T/A cloning vector and clones were screened for different insert lengths by insert PCR and restriction enzyme digestion (Supplementary Figure 1). This approach identified 5 different LGALS9 splice variants which were further characterized by sequence analysis. This identified three transcripts that have already been described, i.e. gal-9FL (Full length, containing all 11 exons), gal-9Δ5 (lacking exon 5) and gal-9Δ5/6 (lacking exons 5 and 6). In the two additional splice variants the splicing of exon 5 or exons 5/6 was accompanied by splicing of exon 10 resulting in gal-9Δ5/10 and gal-9Δ5/6/10, respectively. Exclusion of exon 10 from the mature transcript caused a frame-shift, resulting in a premature stop codon in exon 11 and a truncated C-terminal CRD (Figure 2A and Supplementary Figure 1).

To confirm that the cloned variants indeed compromise the entire endothelial repertoire of LGALS9 splice variants, real-time PCR was performed on HUVEC cDNA with splice-variant specific primers (Supplementary Table 1). All primer combinations were analyzed for specificity and sensitivity (Supplementary Figure 2). In accordance with the cloning data, the real-time
PCR analysis indicated that LGALS9 expression in endothelial cells is restricted to 5 transcripts, i.e. gal-9FL, gal-9Δ5, gal-9Δ5/6, gal-9Δ5/10, and gal-9Δ5/6/10 (Figure 2B). Real-time PCR also indicated that gal-9Δ5 is the most abundantly expressed splice variant in quiescent endothelial cells. Gal-9Δ5/6/10 expression was the lowest while the remaining three splice variants were expressed at comparable levels. None of the other potential splice variants varying in exons 5, 6 and 10 were expressed at detectable levels. The latter was also observed when LGALS9 splice variant expression was assessed in endothelial cells that were derived from freshly resected tumors as described previously [27] (Supplementary Figure 3). To evaluate splicing of other exons we also performed an in silico analysis to search for consensus splicing donor/acceptor sites within LGALS9. As expected, consensus splicing sites were only found for all known exons (Supplementary Table 2). In addition, both the ASPic and SpliceNest databases [28,29] predicted alternative splicing of only exons 5, 6 and 10 in sufficiently long protein-coding transcripts of LGALS9. Altogether, these data suggest that alternative splicing of LGALS9 transcripts is restricted to exons 5, 6 and 10.

Since most of the studies that reported on LGALS9 expression in cells and tissues of different origin did either not discriminate between different splice variants or determined the expression of only a few variants we determined the complete LGALS9 splice variant expression profile in a panel of human cell lines of different origin. These data show that the expression and relative abundance of LGALS9 splice variants varies between these cell lines, suggesting diverging activities in cell biology (Table 1).

**Table 1.** LGALS9 expression in human cell lines of different origin.

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+ = splice variant detected, - = splice variant not detected, ++ = dominant splice variant.
LGALS9 splice variant expression is altered following endothelial cell activation

To determine whether the expression of individual splice variants was differentially affected by endothelial cell activation, we assessed the expression in quiescent and activated HUVEC by real-time PCR. In line with previous findings we observed that endothelial cell activation in vitro results in a significant decrease of overall LGALS9 mRNA expression levels (Figure 2C). This was predominantly caused by a decrease in gal-9Δ5 levels, the most abundantly expressed splice variant. Compared to the overall LGALS9 expression, the relative mRNA expression levels of the splice variants lacking exon 6, i.e. gal-9Δ5/6 and gal-9Δ5/6/10, increased upon endothelial cell activation (Figure 2C).

Galectin-9 modulates endothelial cell function

The observation that tumor endothelial cells in vivo have elevated galectin-9 levels while endothelial cell activation in vitro is accompanied by a decreased expression urged us to investigate the role of galectin-9 in angiogenesis. To that end, we cloned and expressed the specific LGALS9 splice variants in human microvascular endothelial cells (HMEC), which have low levels of endogenous galectin-9 mRNA (Figure 3A+B). Subsequent functional analyses revealed a modest increase in endothelial cell proliferation which reached statistical significance for gal-9Δ5 while here was no effect on migration (Figure 3C+3D).
Next, we determined whether exogenous application of galectin-9 could modulate endothelial cell function, similar as described for other galectins. First, we established whether endothelial cells can secrete galectin-9 in the supernatant. Western blot analysis of supernatants collected from HMEC transfected with different galectin-9 splice variant expression vectors suggested that secretion of detectable protein levels is restricted to gal-9Δ5 and gal-9Δ5/6 (Figure 4A). Since gal-9Δ5 is the dominant endothelial splice variant which is also available as a recombinant protein, we further focused on the effects of this variant. First, we performed a proliferation assay using different galectin-9Δ5 concentrations. In contrast to the experiments with increased endogenous expression we observed that exposure of HMEC to galectin-9Δ5 did not affect proliferation, except for a high concentration of 500 nM (Figure 4B). The same was observed for HMEC migration in the presence of galectin-9Δ5 (Figure 4C). On the other hand, in primary isolated human umbilical vein endothelial cells, we did observe effects on cell proliferation and migration at lower galectin-9Δ5 concentrations (Figures 4D + 4E).
Figure 4. Effect of exogenous galectin-9Δ5 on endothelial cell proliferation and migration. A) Western blot showing detectable galectin-9 protein in the supernatant of HMEC transfected with galectin-9Δ5 and galectin-9Δ5/6. Blank represents cells treated with transfection reagent only. Mock represents cells transfected with empty vector. B+D) Relative proliferation rate of HMEC (B) and HUVEC (D) following treatment with recombinant gal-9Δ5 for three days (n=3). * p < 0.05. C+E) Relative inhibition of HMEC (C) and HUVEC (E) migration upon treatment with recombinant gal-9Δ5 (n=3). * p < 0.05.

Interestingly, when sprouting in a 3D environment was analyzed we observed that adding galectin-9Δ5 directly to Matrigel did not affect sprout formation while applying the protein topically in the medium resulted in increased sprout formation (Figure 5A). This suggests that galectin-9Δ5 might serve as a chemotactant for HUVEC. Indeed, in a transwell migration assay more cells migrated towards galectin-9Δ5 containing medium as compared medium without galectin-9Δ5 (Figure 5B). All these in vitro data suggest that the contribution of galectin-9Δ5 to endothelial cell function depends on the concentration and context in which the protein is presented to the cell. To get a better insight in the overall role in angiogenesis in vivo, we finally determined the effects of exogenous galectin-9Δ5 using the chick chorioallantoic membrane (CAM) assay. Here, application of recombinant galectin-9Δ5 induced a significant reduction in number of vessels endpoints at 100 and 500 nM concentrations (Figure 5C).
Figure 5. Galectin-9Δ5 acts as chemoattractant and inhibits in vivo angiogenesis. A) Analysis of endothelial cell sprouting in the presence and absence of Galectin-9Δ5 (100nM). Left panels show representative spheroids incubated for 24 hours in matrigel with no galectin-9Δ5 (top), galectin-9Δ5 in matrigel (middle) or galectin-9Δ5 in medium topically applied (bottom). The panel shows the relative change in sprout number and sprout length as scored in 20 spheroids per condition in three independent experiments. * p < 0.05. B) Transwell migration of HUVEC towards galectin-9Δ5 (100 nM). The images show phalloidin red/dapi staining of cells that migrated over a membrane with 0.8 micrometer pores (black dots). Diagram shows the relative change in number of cells that migrated over the membrane towards medium with or without galectin-9Δ5. Five microscopic fields were scored per condition in three independent experiments. * p < 0.05. C) In vivo analysis of galectin-9Δ5 activity on angiogenesis using the chicken chorioallantoic membrane assay. Bottom panels show representative images of CAMs after treatment with different gal-9Δ5 concentrations (n=9 eggs/condition). Diagrams show the quantification of vessel length, branch points and end points as determined with the HetCAM software. * p < 0.05.
Discussion

We have previously shown that endothelial cells predominantly express galectin-1, -3, -8 and -9\textsuperscript{15}. While galectin-1, -3, and -8 have been linked to endothelial cell biology\textsuperscript{17,19,22,23,30}, little is known regarding the function of galectin-9 in endothelial cells. Here, we report on the expression and function \textit{LGALS9} splice variants in endothelial biology. To our knowledge, this is the first study in which the expression of all splice variants of \textit{LGALS9} is assessed in endothelial cells. Alternative splicing of \textit{LGALS9} has been reported in cells and tissues of different origin.

Three main variants have been described in which splicing appears to be confined to the exons encoding the linker between the two CRDs, i.e. exon 5 and 6\textsuperscript{31-33}. Endothelial expression of these 3 \textit{LGALS9} splice variants was first reported by Spitzenberger \textit{et al.}\textsuperscript{32} and later confirmed by others\textsuperscript{15,34}. Here, we described two additional splice variants which lack exon 10 next to exon 5 or exons 5/6. Thus far, expression of gal-9Δ5/10 has only been reported by Lipkowitz \textit{et al.} in cells of non-endothelial origin, i.e. colon epithelial cells and peripheral blood lymphocytes\textsuperscript{35}. The variant lacking all three exons, i.e. gal-9Δ5/6/10, has not been described before to our knowledge. Further expression profiling of the \textit{LGALS9} splice variants in a panel of cell lines revealed that the splicing is cell type specific, which is in agreement with a previous study\textsuperscript{36}. In addition, our current data, together with the \textit{in silico} data provided by splicing prediction algorithms, suggest that the splicing of \textit{LGALS9} is confined to exons 5, 6, and 10. The regulatory mechanisms underlying this extensive splicing still need to be resolved. In addition, the functional consequence of splicing is still poorly understood. Exclusion of exon 5 and 6 affects the length and rotational freedom of the linker domain between the two CRDs which influences higher-order multimer formation and gal-9 valency\textsuperscript{4,5}. Thus, while both the N- and C-terminal CRDs already vary in the glycoconjugate specificity and affinity\textsuperscript{37,38}, the carbohydrate binding might be further regulated by splicing. In addition, exon 6 appears to contain a myristoylation site which potentially confers a co-translational modification that has been associated with signal transduction and membrane targeting\textsuperscript{39}. Whether exon 6 lacking variants indeed have a different cellular distribution compared to the other splice variants remains to be determined.

Finally, the linker contains protease sensitive sites allowing cleavage of the protein into two single CRDs\textsuperscript{40,41} while exclusion of exon 10 gives rise to a truncated C-terminal CRD. Although the latter has been observed for gal-8, another tandem-repeat galectin\textsuperscript{42} it is still unknown what the functional consequence of this truncation is. Given the functional importance of bivalent carbohydrate binding by galectins, the loss of the second CRD, either by proteolytic cleavage or by splicing of exon 10 might generate a dominant negative protein. In support of this it has been shown that eosinophil chemoattractant activity differs among the \textit{LGALS9} splice variants\textsuperscript{43} and that it relies on the bivalent activity of galectin-9 as the complete protein is more active compared to the separate CRDs\textsuperscript{44}. Different splice variants have also been shown to differentially affect E-selectin expression in colon carcinoma cells thereby modulating the adhesion of the cells to endothelial cells\textsuperscript{5}. While we did not assess adhesion, we did observe that galectin-9Δ5 can act as chemoattracting agent for endothelial cell. This effect, as well as the modulation of proliferation and migration by galectin-9Δ5, appears to depend on
Galectin-9 splice variant expression in the tumor endothelium

the cellular context since application of exogenous galectin-9Δ5 hampered cell proliferation while endogenous overexpression induced proliferation. In addition, we observed a biphasic effect of exogenous galectin-9Δ5 endothelial cell migration which was also described for galectin-1. Thus, regulation of cellular functions by galectin-9Δ5, and most likely also by other galectin-9 variant depends on local concentration and localization. Nevertheless, the effects of galectin-9Δ5 on angiogenesis in vivo appear to be less as compared to other endothelial galectins like galectin-1, galectin-3 and galectin-8. Whether this is also true for other galectin-9 variants needs further investigation.

Despite the lack of strong angiogenic activity we did observe that endothelial galectin-9 protein levels significantly increased in 4 types of solid tumors, i.e. kidney, liver, lung and breast tumors. This appears to contradict with findings in vitro where galectin-9 protein levels decreased upon endothelial cell activation. Most likely this is due to the presence of a complex tumor microenvironment in tumors with specific growth factors, cytokines and other environmental queues that are not present in the in vitro setting. For example, IFN-γ has been shown to induce galectin-9 expression in endothelial cells and is now also implicated as a tumorigenic factor. Alternatively, the endothelial galectin-9 might have been secreted by other (tumor) cells and consequently taken up by endothelial cells, as shown for galectin-1.

The increased expression of endothelial galectin-9 might be involved in tumor immune escape. Galectin-9 has a well-documented role in the modulation of the activity and the survival of immune cells. While initially discovered as an eosinophil chemoattractant, subsequent studies demonstrated the protein induces apoptosis potently in Th1 cells and in cytotoxic T cells via its receptor Tim-3 although Tim-3 independent signaling of galectin-9 has also been reported. Possibly, increased endothelial galectin-9 expression in the tumor vasculature could contribute to tumor immune escape by providing an immunosuppressive environment. Furthermore, it might be that endothelial galectin-9 inhibits transendothelial migration of leukocytes independent of its apoptotic activity, similar as described for galectin-1. Whether and how these and other immunomodulatory activities are induced by the different endothelial galectin-9 splice variants remains to be established.

Taken together, our data show that galectin-9 protein levels are increased in the tumor endothelium. Furthermore, endothelial cells express five different galectin-9 splice variants which are differentially regulated during endothelial cell activation. The function of galectin-9 in endothelial cell biology and angiogenesis depends on cellular localization, local concentration and the context in which the protein is presented to the cells.

Acknowledgements
The authors would like to thank E. Kleibeuker and K. Castricum for their help with the CAM experiments, M. de Bree for contributing to the cloning and Y. Paulis for helping with the TMA scoring. This work was supported by grants from the Dutch Cancer Society (UM2008-4101; VU2009-4358) to AWG and VLT.
Chapter 5

Supplementary information

Supplementary Table 1. *LGALS9* splice variant-specific primers

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Supplementary Table 2. Splicing donor-acceptor sites in the galectin-9 transcript

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(bp upstream from 3' flanking exon)
Galectin-9 splice variant expression in the tumor endothelium

Supplementary Figure 1. Exclusion of exon 10 results in a frame-shift. A) Gel electrophoresis of insert PCR on bacterial colonies following cloning of galectin-9 PCR products in PCR2.1 TOPO/TA. B) Control digest on different LGALS9 clones showing different insert sizes representing different splice variants. C) Aligned amino acid sequence of the 5 endothelial LGALS9 splice variants. The amino acids encoded by the shift in the open reading frame caused by splicing of exon 10 are underlined. D) A detailed schematic representation of the frame-shift and the subsequent premature stop codon causing a truncation of the second carbohydrate recognition domain.
Supplementary Figure 2. *LGALS9* primer sensitivity and specificity. A) Schematic representation of the LGALS9 splice specific primers. Thin lines represent exons spanned by the specific primer. B) Representative dilution series with primers targeting three specific galectin-9 splice variants as determined by real-time PCR. C) Analysis of primer specificity by real-time PCR. Each panel shows the signals generated with all primers on the individual splice variants of *LGALS9*. While the 'ALL' primers amplify each splice variant, all the other primers are specific for a single splice variant.

Supplementary Figure 3. *LGALS9* splice variant expression in tumor derived EC. Bar graph showing the expression of all possible *LGALS9* splice variants in endothelial cells isolated from two human kidney tumors. N.D. = not detectable.
Galectin-9 splice variant expression in the tumor endothelium

References

Chapter 6

Potent angioregulatory activity of a monovalent galectin-9 isoform

Iris A Schulkens, Esther A Kleibeuker, Kitty C Castricum, Hakon Leffler, Arjan W Griffioen, Victor L Thijssen

In preparation
Abstract

Galectin-9 is a glycan binding protein that consists of two carbohydrate recognition domains (CRDs). Multiple galectin-9 isoforms have been described and recent findings suggest diverging functions of these isoforms in cell biology. Here, we evaluated the prognostic value of galectin-9 splice variants in renal cancer and studied the role of galectin-9 isoforms in endothelial cell function and angiogenesis. Retrospective analysis of galectin-9 splice variant expression revealed a significant increase in gal-9FL and gal-9Δ5 expression in malignant (n=25) compared to normal (n=19) kidney tissues. Kaplan Meier analysis identified increased gal-9Δ5/6 and gal-9Δ5/6/10 expression as markers of better overall and/or disease free survival. In immunohistochemical stainings galectin-9 was mainly confined to endothelial cells in the tumor vasculature. Subsequent analysis showed enhanced angioregulatory activity of monovalent (gal-9N) as compared to bivalent (gal-9Δ5) galectin-9, both in vitro and in vivo. Endothelial cell proliferation was significantly inhibited by both isoforms, albeit to a lesser extent by gal-9N. On the other hand, migration was only significantly inhibited by gal-9N. Furthermore, we observed a stronger stimulatory activity of gal-9N on endothelial cell transwell migration and sprouting as compared to gal-9Δ5. This was dependent on the environmental context. In vivo angiogenesis was significantly inhibited by gal-9N and not by gal-9Δ5. These data show prognostic value of galectin-9 splice variants expression profiling in renal cancer. Furthermore, we identify gal-9N as a potent angioregulatory protein. These findings stress the importance of distinguishing between different galectin-9 isoforms in angiogenesis and cancer biology.
Introduction

Galectins are proteins characterized by one or two conserved domains that can recognize and bind specific carbohydrate groups on a variety of substrates including extracellular proteins, glycoproteins and glycolipids. In recent years it has become evident that many members of the galectin family are involved in many key processes of tumor progression. For example, they have been linked to cellular processes that facilitate tumor cell transformation, e.g. enhanced proliferation and inhibition of apoptosis. In addition, tumor cells have been shown to employ galectins to create an environment that stimulates tumor growth by facilitating immune escape and tumor metastasis and by promoting tumor angiogenesis. Regarding the latter, we and others have shown that tumor cells can enhance angiogenesis by secretion of galectin-1, while at the same time endothelial cells require galectin-1 for proper function during tumor angiogenesis. Angiostimulatory activity has also been ascribed to galectin-3 and galectin-8. More recently, we have described a role for galectin-9 in angiogenesis.

Galectin-9 is a tandem repeat galectin, i.e. it consists of two carbohydrate recognition domains (CRD) covalently connected by a short linker peptide. The length of this linker, and thereby the flexibility and valency of the protein, is regulated by posttranscriptional splicing of two exons, i.e. exon 5 and 6. Consequently, three galectin isoforms are frequently reported in literature, i.e. gal-9FL (full length galectin-9), gal-9Δ5 (lacks exon 5) and gal-9Δ5/6 (lacks exons 5 and 6). In an effort to unravel the function of galectin-9 variants in angiogenesis we recently screened for galectin-9 splicing in endothelial cells. Apart from the common variants lacking exon 5 or exons 5/6, two additional transcript variants were identified from which exon 10 has also been spliced. Interestingly, splicing of exon 10 results in a frame shift and a premature stop codon giving rise to a truncated galectin-9 protein which lacks the C-terminal CRD. Interestingly, the linker peptide has also been found to be susceptible to proteolytic digestion which could also result in the separation of both CRD domains. Since it has been suggested that the galectin-9 CRDs can exert different functions (e.g. on T-cells), we studied the potential function of the galectin-9 N-terminal CRD in endothelial cell biology.
Materials and methods

Patient material
Tissue samples (n = 44) were retrospectively collected of 25 patients with clear cell kidney cancer (CC-RCC) and of 19 normal kidneys. None of the patients received cancer-related therapy prior to the radical or partial nephrectomy. Hematoxylin and eosin staining of tissue sections was used to confirm nuclear Fuhrman grading. Patient characteristics are summarized in Table 1.

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Isolation of primary cells and cell culture
Primary human umbilical vein endothelial cells (HUVECs) were isolated as described previously. HUVEC, immortalized HUVEC line EC-RF24, and immortalized human microvascular endothelial cells (HMEC-1) were cultured at 37°C/5% CO₂ in RPMI (Gibco) containing 10% fetal calf serum (Invitrogen) and 10% human serum supplemented with L-glutamine (Invitrogen) and penicillin/streptomycin (Invitrogen).

RNA isolation and cDNA synthesis
RNA was isolated using the RNeasy kit (QIAgen) according to the supplier’s protocol. Isolations were subjected to on-column DNasel digestion (QIAgen). The purity and concentration of the RNA samples was determined by spectrophotometry (ND-1000, Nanodrop technologies). cDNA synthesis was performed on 500 ng total RNA using the iScript cDNA synthesis kit (BioRad) according to the supplier’s protocol.

Real-time qPCR
Real time qPCR was performed on the CFX96 real time PCR detection system (BioRad). In short, 1.5 µL of cDNA was used in a total reaction volume of 25 µL containing 1x SensiMix (Quantace) and 400 nmol/L of LGALS9 splice variant specific primers. A standard 2-step amplification
The angioregulatory role of gal-9N

protocol was used with Tm = 61°C followed by a melting curve analysis. To compare expression levels between different conditions, the ΔΔCt method was used as described previously19.

Immunohistochemistry

Images of galectin-9 staining in kidney cancer tissues were acquired from previous stainings as described14. In brief, a tissue microarray (Super Bio Chips; kidney (CL2)), containing 50 tumor samples and 9 matched controls, was stained with a primary galectin-9 antibody (1:500, R&D systems according standard procedures. The slide was counterstained with hematoxylin and images were acquired on an Olympus BX50F (Olympus) microscope equipped with a Leica DC300 (Leica Microsystems) camera.

In vitro migration assay

Endothelial cell migration was assessed by creating a scratch in a confluent layer of endothelial cells, followed by monitoring of its closure in time. Images of each scratch were automatically acquired at t = 0 and t = 8 hours with a 1.4 Mb GIGE color camera (Hitachi) on a DMI3000B microscope equipped with an automated xyz-stage (Leica) using Universal Grab software (version6.3, DCIlabs). Scratch area was determined using ImageJ.

To assess the effect of exogenous galectin-9 variants, HUVECs or HMEC-1 were incubated with a concentration range of recombinant galectin-9Δ5 (R&D systems) or the N-terminal CRD of galectin-9 (gal-9N, produced as described previously20) for the duration of the experiment (8 hours). Migration experiments with recombinant galectin-9 on HMEC-1 and HUVEC were performed in triplicate with three replicates per treatment condition within each experiment.

In vitro proliferation assay

The CellTiter-Glo assay (Promega) was used to assess the proliferation rate of endothelial cells. In short, 7,500 cells were seeded in a 96 well plate and incubated with recombinant galectin-9Δ5 (R&D systems) or the N-terminal CRD of galectin-9 (gal-9N, produced as described previously20). After 48 hours, cells were washed and incubated with 100 μl CellTiter-Glo reagent for 10 minutes on a shaker. Next, luminescence levels were determined on a microplate reader (Tecan). Proliferation experiments with recombinant galectin-9 on HMEC-1 and HUVEC were performed in triplicate with three replicates per treatment condition within each experiment.

Sprouting

For sprouting, HUVEC were resuspended to a final concentration of 40,000 cells/mL in methocel medium (RPMI with 20% methocel and 10% heat-inactivated human serum). Next, 25 μL drops were pipetted onto the lid of a non-adhesive square petri dish which was then inverted and incubated overnight in a humidified incubator at 37°C, 5% CO₂. The next day, the spheroids were harvested in PBS. For each experimental condition approximately 40 spheroids were resuspended in 200 μL Matrigel (BD Biosciences), with or without 100 nM recombinant galectin, and transferred to a 24-well plate. Following solidification of the Matrigel, 500 μL medium with or without 100 nM recombinant galectin was applied topically and spheroids were allowed
to sprout in a humidified incubator at 37°C, 5% CO₂ for 16-24 hours. Finally, pictures of the spheroids were taken and sprout length and sprout number per spheroid was analyzed using ImageJ. All experiments were performed in triplicate and per experiment at least 20 spheroids per condition were analyzed.

**Transwell migration**

For transwell migration, 7,500 HUVEC were seeded in HTS Fluoroblock inserts (8 micrometer pore size, Falcon). Inserts were placed in 24-well plates containing culture medium supplemented with 100 nM of the specific recombinant galectins. Cells were allowed to migrate overnight. Next, cells were fixed with 1% paraformaldehyde in 0.1%BSA/PBS. Following three wash steps with PBS, cells were stained with phalloidin red in 0.1% Triton/PBS overnight at 4°C. Images were acquired with LAS v3.7 software (Leica) using an inverted fluorescent microscope (DM-IL Leica) equipped with a DFC345 FX camera (Leica). The number of cells was scored in 5 random selected areas by two independent observers. Experiments were performed in triplicate.

**Chick chorioallantoic membrane assay**

Fertilized chicken eggs (Het Anker, the Netherlands) were incubated and rotated at a relative air humidity of 65% and a temperature of 37.6°C in a hatching incubator with an automatic rotator for 3 days. On embryonic day 3 (EDD3), a small opening of approximately 3 mm in diameter was made in the eggshell at the top of the egg and sealed with tape. The eggs were then incubated for 3 days with the opening facing upwards. On EDD6, the eggs were opened further to allow easier access to the chorioallantoic membrane (CAM). For the topical application of recombinant galectins, polyethylene rings were deposited on the CAM. From embryonic EDD6 to EDD9, recombinant galectins were applied daily within the polyethylene rings. Finally, on EDD10, pictures of the chorioallantoic membrane were taken using an Optech LFZ stereo microscope (Optech) after injection of approximately 1 mL contrast agent (zinc oxide in pure vegetable oil) under the CAM and the ring. Quantification of different vascular parameters was obtained using HetCAM software (Peira, Belgium). At least 8 eggs per condition were used.

**Statistical analyses**

All data are presented as mean +/- SEM unless indicated otherwise. The Mann-Whitney rank sum test was used for comparison of the in vitro migration, proliferation and transwell migration experiments and for comparison of gene expression levels in qPCR. The student t-test was used for comparison of CAM data and the sprouting assay. Survival comparisons were performed using Kaplan-Meier survival estimates with the Log-Rank test. P-values < 0.05 were considered statistically significant and all statistical analyses were performed using SPSS (SPSS Inc., the Netherlands).
Results

We have recently reported on the prognostic value of specific galectin-9 splice variants in cancer\textsuperscript{21}. These findings pointed towards distinct activity of specific galectin-9 splice variants in different pathologies. To further substantiate this we performed quantitative PCR analysis comparing mRNA expression levels between normal and cancerous renal tissues. This revealed that total galectin-9 mRNA levels were increased in kidney cancer tissue as compared to normal tissue. This could be attributed to two variants, i.e. gal-9FL and gal-9Δ5, while all other detected variants did not change significantly (Figure 1A). Subsequent Kaplan Meier survival analyses indicated that patients that express gal-9Δ5/6/10 below median levels had significantly shorter overall survival while decreased gal-9Δ5/6 nearly reached statistical significance (OS, Figure 1B). With regard to disease free survival both splice variants did reach statistical significance (DFS, Figure 1C). These data not only suggest a diagnostic and prognostic value for galectin-9 in renal cancer, they also confirm the importance of distinguishing between different galectin-9 splice variants\textsuperscript{21}. Subsequent immunohistochemical staining of kidney tumor tissues confirmed our previous observation that galectin-9 protein expression was frequently confined to the vasculature of renal cell cancer tissue (Figure 1D).

![Figure 1. Galectin-9 splice variant expression in renal cancer patients. A) mRNA expression of all human galectin-9 splice variants in normal (white bars, n=19) and cancerous renal tissue (black bars, n=25). B-C) Kaplan-Meier plots of overall survival (B) and disease free survival (C) in renal cancer patients with low (dotted line) or high (solid line) expression of galectin-9Δ5/6/10 (left panel) or galectin-9Δ5/6 (right panel). D) Images of immunohistochemical galectin-9 stainings of four representative kidney tumors.](image-url)
Indeed, we recently reported on a role for gal-9Δ5 in angiogenesis\textsuperscript{14}. Furthermore, we described that endothelial cells express two splice variants that lack exon 10 which results in a premature stop that gives rise to a galectin-9 isoform that lacks the C-terminal CRD domain. To extend these findings we now studied the function of the galectin-9 N-terminal CRD monomer (gal-9N) in endothelial cell function and angiogenesis. First, we assessed the effect of exogenous recombinant gal-9N on endothelial cell function. In accordance with previously published data for gal-9Δ5\textsuperscript{14}, the gal-9N isoform did not affect HMEC proliferation while HUVEC proliferation was significantly decreased albeit less potent as compared to gal-9Δ5 (Figure 2A). Whereas previous experiments did not reveal a clear effect of gal-9Δ5 on EC migration\textsuperscript{14}, we found that gal-9N significantly inhibited wound closure in both HUVEC and HMEC-1 (Figure 2B). Surprisingly, sprouting experiments showed that gal-9N significantly increased the number and length of HUVEC sprouts (Figure 3A). Furthermore, no difference in activity was observed between gal-9N present in the culture medium or in the Matrigel. This was different from gal-9Δ5 (Figure 3A) which only stimulated sprouting when added topically resulting in a gradient by diffusion into the Matrigel\textsuperscript{14}. This suggests that gal-9N is a more potent chemoattractant which was further studied in a transwell migration assay. Both gal-9Δ5 and gal-9N stimulate HUVEC transwell migration and although the latter appeared somewhat more potent this did not reach statistical significance (Figure 3B).

![Figure 2](image-url)

**Figure 2. Effect of exogenous gal-9N and gal-9Δ5 on EC proliferation and migration.** A-B) Relative proliferation (A) and inhibition of wound closure (B) of HUVEC (left panel) and HMEC-1 (right panel) following exogenous...
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Figure 3. Chemoattractive activity gal-9N and gal-9Δ5 in HUVEC sprouting and transwell migration. A) Analysis of HUVEC sprout number, sprout length and total sprout length after application of 100 nM galectin-9N (black bars) or galectin-9Δ5 (grey bars) either in the Matrigel or in the culture medium (n=3). * p< 0.05. B) Analysis of number of cells migrated through a transwell membrane towards a gradient of galectin-9N (black bar) or galectin-9Δ5 (grey bar) (n=3).

Next, the effect of gal-9N on angiogenesis *in vivo* was studied using the chick chorioallantoic membrane (CAM) assay. Whereas we previously observed a small inhibitory effect of gal-9Δ5 only at high concentrations (500 nM)\(^4\), gal-9N significantly decreased most measured parameters already at lower concentrations (Figure 4A-E). Consequently, gal-9N was a more potent inhibitor of *in vivo* angiogenesis as compared to gal-9Δ5.
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Figure 4. Effect of exogenous gal-9N and gal-9Δ5 on in vivo angiogenesis in CAM model. A-D) Analysis of the skeleton area (A), number of branch points (B), number of endpoints (C), and total vessel length (D) following topical application of increasing concentrations of galectin-9N (black bars) or 500 nM galectin-9Δ5 (grey bar). E) Representative images of the CAM treated with PBS, galectin-9N or galectin-9Δ5.

Discussion

Galectins have emerged as important regulators of blood vessel growth. We and others have shown that several galectins, including galectin-1, -3, and -8, can induce, facilitate and maintain endothelial cell function during angiogenesis. More recently, we reported on a potential role of galectin-9 in endothelial cell biology and shown the prognostic value of specific galectin-9 splice variants in NSCLC, stressing the importance of distinguishing between different galectin splice variants in cancer. To exemplify this, we now studied the expression of all known human galectin-9 splice variants in normal and malignant kidney tissue. We found that galectin-9 mRNA expression in normal kidney tissue is mainly confined to gal-9FL, gal-9Δ5, gal-9Δ5/6, and gal-9Δ5/10. The expression of the most dominant isoforms, i.e. gal-9FL and gal-9Δ5, was significantly increased in renal cancer. Surprisingly, we previously found a decreased expression of galectin-9 protein in renal cancer cells. Although this appears contradictory, we also observed that the galectin-9 expression in the vascular/stromal compartment was increased. Similar changes in galectin-9 protein expression were also reported in skin cancer. Furthermore, several studies report that patients that show lower galectin-9 expression have a
poor outcome in different types of cancer\textsuperscript{24,25}. In line with this, we observed a positive correlation between galectin-9 expression and survival in renal cancer patients. Patients that express gal-9Δ5/6 or gal-9Δ5/10 above median levels show better disease free survival, which identifies these two splice variants as new prognostic markers in renal cancer. Overall it appears that increased galectin-9 expression protects against tumor progression. Since galectin-9 is not only expressed by endothelial cells, but also by immune tissues including spleen, thymus, and lymph nodes\textsuperscript{16}, the increased expression of galectin-9 in renal cancer tissue could possibly be attributed to the infiltration of inflammatory cells. Although we did not specifically look into the contribution of immune cells to the expression of galectin-9 in the patient samples, these cells could provide an anti-tumor response and thereby enhance patient survival.

Similar to our findings in other cancer types\textsuperscript{14}, increased expression of galectin-9 protein was often observed in the tumor vasculature, suggesting a role for galectin-9 in angiogenesis. However, the dominant galectin-9 isoform in endothelial cells, i.e. gal-9Δ5, appears to fulfill only a minor role for in endothelial cell function\textsuperscript{14}. Since alternative splicing as well as proteolytic cleavage of the linker sequence\textsuperscript{17} might result in the separation of both galectin-9 CRDs, we assessed the angioregulatory activity of the N-terminal galectin-9 CRD (gal-9N). We found that gal-9N significantly inhibits HUVEC proliferation and migration \textit{in vitro} as well as angiogenesis \textit{in vivo}. Except for proliferation, the inhibitory activity of gal-9N was more potent as compared to galectin-9Δ5. Possibly, the single galectin-9 CRD acts as a dominant negative isoform by competing for carbohydrate binding sites. On the other hand, gal-9N did stimulate endothelial sprouting and transwell migration. Furthermore, gal-9N appeared to be a more potent chemoattractant for EC as compared to gal-9Δ5. These results appear to contradict the previous observations and do not point towards a dominant negative role for galectin-9N in EC function. In fact, these data indicate that gal-9N might exert chemoattractive activity on EC. Interestingly, Nishi \textit{et al.} showed that thrombin mediated cleavage of the galectin-9 linker peptide reduced the chemoattraction of eosinophils\textsuperscript{17}. Apparently, the activity depends on the environmental context in which the different isoforms are presented or sensed by cells. This is supported by findings of others. For example, galectin-9 is a potent inducer of T-cell apoptosis by binding to Tim-3 on these cells\textsuperscript{27}, while binding to e.g. VCAM-1 (vascular cell adhesion molecule-1) was found to impair adhesion of tumor cells to the endothelium\textsuperscript{28}. In addition, Nagahara \textit{et al.} observed increased numbers of CD8+ T-cells upon galectin-9 treatment in tumor bearing mice, while simultaneously apoptosis of CD4+ T-cells was induced\textsuperscript{29}. The specific CRD domains can also exert distinct functions. This was demonstrated by Bi \textit{et al.}, who showed that specific CRDs differentially regulate T-cell death induction\textsuperscript{18}. The specificity of the C-terminal CRD was found to be the primary determinant of receptor recognition, T-cell death signaling, and susceptibility to cell death. In line with this, Li \textit{et al.} showed a more potent role for gal-9C in T-cell death compared to gal-9N, while gal-9N was more effective in the activation of dendritic cells\textsuperscript{30}. All these data illustrate that both galectin-9 CRDs, either in the tandem repeat configuration or as single domains, display distinct activities on different cell types. For future studies it would thus also be interesting to evaluate the effect of the C-terminal galectin-9 CRD on endothelial cell function. Especially since the structure of this domain is evolutionary homologues to galectin-1.
and galectin-3, two other galectins with angiostimulatory activity. Interestingly, proteolytic cleavage of the N-terminal fragment of galectin-3 significantly potentiated several steps in tumor progression, including the induction of angiogenesis. Whether this points towards a shared mechanism of proteolytic activation of galectin CRDs in angiogenesis should be further investigated.

In summary, our current data and observations in the literature suggest that expression profiling of galectin-9 splice variants might provide prognostic value in different types of cancer. Furthermore, the regulation of endothelial cell function and angiogenesis by galectin-9 involves complex modulation of galectin-9 isoform expression by splicing and protein processing. Together, this demonstrates the importance of distinguishing between all galectin-9 variants, not only for diagnostic and prognostic purposes, but also to unravel the contribution of each specific isoform to cellular functions in physiological and pathophysiological processes, including cancer. Further research into the mechanisms and regulatory cues that govern this functional diversity is required for the development of effective galectin-9-targeted treatment strategies against e.g. cancer.

**Acknowledgements**
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Chapter 7

Galectin-1 is a functional binding partner for the CXC-chemokine platelet factor 4 implicated in angiogenesis and platelet function

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In preparation
Abstract

The CXC-chemokine platelet factor 4 (PF4/CXCL4) is one of the first proteins described to have angiostatic activity. However, the mechanisms of action are still poorly understood. Since we previously identified galectin-1 as the receptor for the angiostatic CXCL4-like peptide anginex, we hypothesized that this glycan-binding protein could also serve as a functional binding partner for the endogenous CXCL4. Here, we confirmed the physical interaction between galectin-1 and CXCL4 and we suggest that the angiostatic activity of CXCL4 is mediated through galectin-1. We found that the interaction affects the affinity, and possibly specificity, of carbohydrate binding by galectin-1. Furthermore, the effect of the interaction on endothelial cells and platelets was studied. We observed that CXCL4 neutralizes the stimulatory effect of galectin-1 on HUVEC proliferation and migration. Concomitantly, signaling through pErk and pMEK was reduced. In addition, CXCL4 increased the internalization of galectin-1 in endothelial cells. Regarding platelet function, CXCL4 potentiated both platelet activation and aggregation induced by galectin-1. The current findings provide a direct functional link between two protein families, i.e. galectins and cytokines, which regulate coagulation, angiogenesis and inflammation. This contributes to our understanding of how these processes are regulated and has implications for the development of drugs targeting these processes.
Introduction

Platelet factor 4 (PF4/CXCL4) is a 7.8 kD heparin-binding protein classified as the first member of the chemokine family, a group of cytokines with chemotactic properties. It is predominantly synthesized by megakaryocytes and stored in the alpha-granules of platelets. CXCL4 can exert a wide range of functions including regulation of hematopoiesis, coagulation, inflammation and angiogenesis. Regarding the latter, i.e. blood vessel growth, different mechanisms and several binding partners have been proposed that mediate the CXCL4 activity. For example, CXCL4 directly interacts with angiogenic growth factors like bFGF and VEGF, preventing their dimerization which is required for receptor activation. CXCL4 was also found to interact with integrins resulting in hampered endothelial cell adhesion and migration. In addition, it has been suggested that the angiostatic activity of CXCL4 involves CXCR3B, an alternative splice variant of the chemokine receptor CXCR3. Despite these insights there are still several unresolved issues regarding CXCL4 function. For example, Sulpice et al. reported that only 10% of human umbilical vein endothelial cells express CXCR3B at low levels and that an anti-CXCR3 antibody could not block the angiostatic effects of CXCL4. In addition, CXCL4 displays similar activity in mice as compared to humans while no CXCR3B splice variant has been found in mice. These findings suggest the existence of additional receptors that govern CXCL4 activity.

Previously, we identified galectin-1 as the receptor for the CXCL4-like angiostatic peptide called anginex. Galectin-1 is a member of the conserved galectin protein family which is characterized by a high binding affinity for β-galactoside-containing complex N-glycans. The protein can be found both intra- and extracellularly and is involved in many different cellular functions, including adhesion, proliferation, survival, and mRNA processing. Consequently, galectin-1 has been implicated in many biological processes similar as CXCL4, i.e. hematopoiesis, coagulation, inflammation and angiogenesis. Based on this, together with the observed binding of anginex, we hypothesized that galectin-1 could also serve as a functional binding partner for CXCL4. Here, we report that galectin-1 and CXCL4 indeed interact and that this affects the carbohydrate binding of galectin-1. Moreover, this interaction was found to modulate the function of galectin-1 on both endothelial cells and platelets.
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Materials and methods

Isolation of primary cells and cell culture
Primary human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords as described previously. The cells were cultured on 0.2% gelatin-coated flasks in a humidified incubator at 37 °C/5% CO₂ in RPMI1640 (Lonza) containing 10% fetal calf serum (FBS; PAA) and 10% human serum (HS) and supplemented with L-glutamine (ICN) and penicillin/streptomycin (Lonza). Native HUVEC were harvested 2 hours after isolation, whereas activated HUVEC were obtained by culturing cells for 3 days in high-serum medium. Immortalized endothelial cell lines HMEC-1 (human microvascular endothelial cells) and EC-RF24 (immortalized HUVEC) were cultured in the same medium, without human serum.

Proliferation
For proliferation, 5,000 HUVEC cells were allowed to adhere overnight in a gelatin-coated 96-well plate in culture medium containing 10% FBS and 10% HS at 37 °C/5% CO₂. Subsequently, the cells were incubated with galectin-1 (kind gift from Professor L. Baum), CXCL4 or anginex (both synthesized and provided by Professor K. Mayo) for 3 days in medium supplemented with 1% FBS and 1% HS. For quantification, CellTiter-Glo reagent (Promega) was used according to the manufacturer’s instructions and luminescence was measured using a microplate reader (Tecan). All experiments were performed in quadruplicate and within each experiment there were three replicates of all treatment conditions.

Migration
The migratory capacity of HUVEC treated with galectin-1, CXCL4 or anginex was assessed using a wound healing assay. For this, 7,500 HUVEC cells were grown to confluence in culture medium containing 10% FBS and 10% HS for 3 days in gelatin-coated 96-well plates at 37 °C/5% CO₂. Then, the cells were silenced overnight in medium supplemented with 1% FCS and 1% HS, followed by application of a scratch using a guided 96-well pin tool (Peira). After washing with PBS, the compounds were added to the cells in fresh silencing medium. Wound width was recorded at t=0 and t=8 hours with a Leica DMi3000B microscope equipped with an automated xyz-stage and a Hitachi 1.4 Mb GiGE color camera, using Universal Grab software (version 6.3, DCIlabs). Scratch areas were analyzed manually using ImageJ (version 1.41), and wound closure was expressed as a fold change to the control wells. All experiments were performed in quintuplicate and within each experiment there were three replicates of all treatment conditions.

Galectin-1 internalization
Galectin-1 recombinant protein was labeled with Oregon Green 488 (OG488) as previously described. 15,000 HUVEC were allowed to adhere overnight in a gelatin-coated 96-well plate in medium containing 10% FBS and 10% HS. Next, cells were silenced for 24 hours in medium containing 1% FBS and 1% HS and subsequently incubated with 0.1 μM galectin-1 OG488 in the
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presence or absence of CXCL4 (10 μM), anginex (1 μM) or erlotinib (5 μM) in silencing medium. Pictures of galectin-1 internalization were acquired after 24 hours with LAS v3.7 software (Leica) using an inverted fluorescent microscope (DM-IL Leica) equipped with a DFC345 FX camera (Leica).

RNA isolation and cDNA synthesis
Total RNA was isolated using the RNeasy kit (QiAgen), according to the manufacturer’s instructions. Genomic DNA contamination was removed by on-column DNase treatment. The purity and concentration of the RNA was determined using the Nanodrop ND-1000 (Nanodrop technologies). cDNA synthesis was performed on at least 100 ng RNA using the iScript cDNA synthesis kit (BioRad) according to the supplier’s protocol.

Real-time qPCR
Real-time quantitative PCR was performed on a CFX96 real time PCR detection system (BioRad). In short, a total reaction volume of 25 μL was used containing 1.5 μL cDNA, 1x iQ SYBR Green PCR mix (BioRad) and 400 nmol/L of the appropriate forward and reverse CXCR3B primer. The following primers were used: CRCR3B forward 5’-AAGTCATATCCAGAGCCC-3’; CXCR3B reverse 5’-CTGTGTAAAGGCCTGGCAG-3. The primers were synthesized by Eurogentec. A standard 2-step amplification protocol was used with Tm = 60 °C followed by a melting curve analysis. The measurements were performed on 4 different HUVEC isolations, 2 HMEC-1 and EC-RF24 cultures, and 1 Colo205 sample. The expression of the target genes was normalized to the expression of three reference genes (cyclophilinA, beta-actin and beta-2-microglobulin) using the ΔCt method as described previously20.

Spot blot
Spot blot analysis was performed with 10 μL drops containing 3 μg of the specific proteins (except for CXCL4, all cytokines were purchased from R&D systems) that were spotted onto a nitrocellulose membrane. The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences) for 1 hour, washed with PBS/0.1% Tween, and incubated with 1 μg/ml recombinant galectin-1 in PBS/0.1% Tween for 1 hour. After washing with PBS/0.1% Tween, the membrane was incubated with rabbit anti-galectin-1 antibody (1:1000, Peprotech) for 1 hour. Following three washing steps, the secondary antibody (goat anti-rabbit IRDye 800CW, 1:10000, LI-COR Biosciences) was applied for 1 hour. Finally, the membrane was washed two times with PBS/0.1% Tween and rinsed in PBS. Images were obtained by scanning the membrane with the Odyssey infrared imaging system (LI-COR Biosciences).

Gel-shift analysis
For gel-shift analysis, 2 μg of the specific proteins were combined in a total volume of 10 μL in PBS and incubated together with 2 μL DSS (Disuccinimidyl suberate, Thermo Scientific) for 2 hours on ice to fix existing protein interactions. Where indicated, 25 mg/mL heparin or 5 mM lactose (Sigma Aldrich) was added. The reaction was quenched with 1 μL Tris buffer pH 7.5 for
15 minutes at room temperature. Subsequently, the protein mixture was suspended in Laemmlli sample buffer (Biorad) supplemented with 1:20 β-mercapto-ethanol, boiled for 5 minutes and subjected to SDS-PAGE followed by Western immunoblotting using anti-galectin-1 antibody (1:1000, Peprotech).

**H-Ras signaling**
RF24 cells were plated at a density of 250,000 per well in gelatin-coated six-wells plates and grown for 24 hours in the presence of 10% FBS. Following washing with PBS, the cells were incubated for 24 hours in culture medium in the absence and presence of 10 μmol/L recombinant CXCL4. Cells were then lysed using M-PER lysis buffer (Pierce) and proteins (20 μg) were subjected to SDS-PAGE followed by Western immunoblotting using anti–phospho-Erk1/2, anti-Erk1/2, anti–phospho-Mek1/2 and anti-MEK1/2 antibodies (1:1000, Cell Signaling).

**SDS-PAGE gel electrophoresis and Western blotting**
The protein samples were separated by gel-electrophoresis on a 15% polyacrylamide gel and transferred onto PVDF membranes (Millipore) following standard protocols. Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences) for 1 hour and incubated overnight at 4 °C with a primary antibody. The membranes were washed three times in PBS/0.1% Tween and subsequently incubated with the appropriate secondary IRDye-labeled antibody (LI-COR Biosciences) for 1 hour. Finally, the membranes were washed two times with PBS/0.1% Tween and rinsed in PBS. Images were obtained by scanning the membrane with the Odyssey infrared imaging system (LI-COR Biosciences).

**Surface plasmon resonance**
Binding kinetics between galectin-1 and CXCL4 were performed using the BIAcore 1000 biosensor system (BiaCore) as described previously. In short, galectin-1 was covalently attached to CM5 Sensor Chips (BiaCore) using amine-coupling chemistry according to the manufacturer’s instructions. Next, binding measurements were performed at 25 °C using HBS-EP buffer containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.005 % surfactant P20 adjusted to pH 7.4. Interactions between galectin-1 and CXCL4 were analyzed by injection of different CXCL4 concentrations (20 μL at a flow rate of 30 μL/minute). Flow cells were regenerated by injection of 20 μL regeneration buffer (10 mM glycine-HCl, pH 2.0). Association-rate (K_a) and dissociation-rate (K_d) constants were obtained by analysis of the sensorgrams using the Biaevaluation software, version 3.2. All measurements were performed at least in duplicate at all concentrations and the experiment was performed in duplo.

**Fluorescence anisotropy**
Fluorescence anisotropy was performed as described previously. For these experiments, the oxidation resistant mutant galectin-1, i.e. galectin-1 C3S (kind gift from Professor H. Leffler), was used to avoid the necessity to add β-mercaptoethanol, which may disturb interacting glycoprotein ligands. In brief, 40 μL reaction mixture containing different concentrations of
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gal-1C3S and 0.1 μM of fluorescein labeled saccharide probe either with or without 8 μM CXCL4 was incubated under slow rotary shaking for 5 minutes in black 96-well half-area polystyrene microplates. All dilutions were prepared in HEPES buffer with pH 7.4 to prevent precipitation of CXCL4. Fluorescence anisotropy was measured with excitation at 485 nm and emission at 520 nm using a Polar Star plate reader with PHERAsar software (BMG Labtech). Binding curves were generated by plotting anisotropy against increasing concentrations of galectin-1. Anisotropy was measured without galectin-1 (A₀ representing free probe), at experimental conditions (Aₚ) and at saturating concentrations of galectin-1 (Aₚ₉₉). The experiments were performed at least in duplicate. For the inhibition assay, increasing concentrations of the glycoprotein asiolofetuin (ASF), an inhibitor of the galectin-1-probe interaction, were added to fixed low concentrations of gal-1C3S (0.1 μM) and tdga-probe (0.1 μM) in the presence or absence of 8 μM PF4 (n=1). Data plotting, non-linear regression analysis and curve construction was performed with Prism 5 software (Graphpad).

Hemagglutination assay

In a 96-well round bottom plate (Greiner) 25 μL of recombinant galectin-1 in PBS was mixed with 25 μL of CXCL4 or β-lactose in TSB buffer. Next, 25 μL of 3% fixed rabbit red blood cells (Fitzgerald Industries International) in PBS was added and air bubbles were removed to prevent disturbance of the agglutination area. After 1 hour incubation at room temperature the plate was scanned and the agglutination diameter (d) was measured using Adobe Photoshop CS4. The agglutination area (A) was calculated by A = π*(d/2)². The relative agglutination area was calculated as: relative A = A/A_blank-1. All experiments were performed at least in triplicate.

Preparation of platelet-rich plasma and washed platelets

Blood was drawn from healthy volunteers, who claimed not to have used aspirin or other nonsteroidal anti-inflammatory drugs for at least one week, using a non-vacuum blood collection system. The blood was collected in 0.38% sodium citrate buffer and centrifuged at 160 g for 15 min at room temperature. The platelet rich plasma (PRP) was collected and diluted 20 times in HEPES tyrode buffer pH 7.3 (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgSO₄, and 5 mM D-glucose) containing 2% GPRP (glycyl-L-prolyl-L-arginyl-L-proline) for platelet activation experiments.

For platelet aggregation experiments, washed platelets (WP) were prepared by addition of 10% ACD (2.5% trisodium citrate, 1.5% citric acid, and 2% D-glucose) to the PRP followed by centrifugation at 330 g for 15 min at room temperature. The platelet pellet was resuspended in HEPES tyrode buffer pH 6.5 containing 10 ng/mL prostaglandin (PGI₂, diluted in TSB pH 9.0). Finally, the platelets were centrifuged for 25 min at 330 g and the pellet was resuspended in HEPES tyrode buffer pH 7.3. Platelets were counted using CELL-DYN Sapphire (Abbot Laboratories) and adjusted to 2x10⁹ platelets/L for aggregation experiments.
Platelet activation
Platelet activation was determined by flowcytometric analysis of GPIIbIIIa (CD61, integrin α₃β₃) expression on platelets. Different concentrations of galectin-1 and CXCL4 were incubated together for 1 hour on ice. Subsequently, 45 μL of PRP was stimulated with 5 μL of protein mixture and immediately incubated with FITC-labeled PAC-1 (anti-CD61) antibody (BD Biosciences) for 10 min at room temperature. Expression levels were determined using a FACSCalibur flowcytometer (BD Biosciences). The data was analyzed using CellQuest Pro software (BD Biosciences). At each galectin-1 concentration, the deltaMFI was calculated as: ΔMFI = MFI_{(x \mu M gal-1 + y \mu M CXCL4)} – MFI_{(x \mu M gal-1 + 0 \mu M CXCL4)} . Experiments were performed at least in quadruplicate.

Platelet aggregation
For platelet aggregation, different concentrations of galectin-1 and CXCL4 were incubated together for 1 hour on ice and then added to 500 μL WP. Aggregation was measured at 37°C under continuous stirring and monitored for 12 min using a lumiggregometer (Kordia 490). The area under the curve (AUC) was measured manually using ImageJ and corrected for the AUCblank. The relative AUC was calculated as a percentage of the maximal aggregation for each experiment, i.e. the aggregation at 6 μM gal-1 + 6 μM CXCL4: relative AUC = AUC_{(x \mu M gal-1 + y \mu M CXCL4)} / AUC_{(6 \mu M gal-1 + 6 \mu M CXCL4)} \times 100\%. At each galectin-1 concentration, the deltaAUC was calculated as: ΔAUC = AUC_{(x \mu M gal-1 + y \mu M CXCL4)} – AUC_{(x \mu M gal-1 + 0 \mu M CXCL4)} . Experiments were performed in quintuplicate.

Statistics
The data are shown as mean values±SEM. The Mann-Whitney rank sum test was used to calculate statistically significant differences in EC proliferation, EC migration, H-Ras signaling, MFI for platelet activation, and relative AUC for platelet aggregation. The Wilcoxon signed ranks test was used to compare deltaMFI and deltaAUC values. Statistical computations were performed in SPSS 20.0.0. (SPSS Inc.) and p- values ≤ 0.05 were considered statistically significant.

Results
We hypothesized that galectin-1 could serve as a functional binding partner for CXCL4 and that this interaction modulates the functional activity of either protein. To determine whether both proteins interact, we first performed a spot blot analysis. This revealed that galectin-1 indeed binds immobilized CXCL4. The same was observed for the known galectin-1 binding proteins anginex and anti-galectin-1 antibody, while no interaction was found with negative control proteins, i.e. BSA and anti-ICAM-1 antibody (Figure 1A). To irrefutably confirm the interaction, gel-shift analysis was performed. In line with the spot blot results, adding either CXCL4 or anginex to galectin-1 in the presence of the crosslinking agent DSS caused a band-shift of ~8 kD or 4 kD, respectively, corresponding to the size of both proteins (Figure 1B). No shift
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Table 1. Rate and affinity constants for galectin-1 binding

<table>
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<th>Analyte</th>
<th>$k_a$ (Ms$^{-1}$)</th>
<th>$k_d1$ (s$^{-1}$)</th>
<th>$k_d2$ (s$^{-1}$)</th>
<th>$K_i$</th>
<th>$K_r$</th>
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<tr>
<td>Anginex</td>
<td>$6.5 \times 10^3$ (±1.7)</td>
<td>$4.2 \times 10^{-2}$ (±0.4)</td>
<td>$5.9 \times 10^{-4}$ (±1.9)</td>
<td>$6.4 \pm 1.7$ μM</td>
<td>$90.0 \pm 6.7$ nM</td>
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<tr>
<td>PF4</td>
<td>$1.02 \times 10^4$ (±0.05)</td>
<td>$9.2 \times 10^{-2}$ (±1.2)</td>
<td>$5.0 \times 10^{-4}$ (±0.5)</td>
<td>$9.0 \pm 0.8$ μM</td>
<td>$483 \pm 28$ nM</td>
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<td>BSA</td>
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Since both proteins are known to bind carbohydrates, we next performed hemagglutination assays to evaluate the effect of the interaction on their carbohydrate binding capacity. Maximal red blood cell agglutination was observed at ~0.5 μM galectin-1 with a half maximal effective concentration (EC50) of 0.05±0.03 μM (Figure 2A). Subsequent titration of β-lactose to a fixed concentration of galectin-1 (2 μM) inhibited the agglutination (Figure 2A). Maximal agglutination by CXCL4 was reached at ~1 μM CXCL4 with an EC50 of 0.17±0.03 μM (Figure 2B). Interestingly, the maximal agglutination area induced by CXCL4 was larger compared to galectin-1. This suggests that different binding moieties are involved. As expected,
the CXCL-4 mediated agglutination was neutralized by heparin (data not shown). An inhibitory effect could also be achieved by titration of galectin-1, suggesting that galectin-1 scavenges CXCL4 thereby neutralizing its agglutinating activity (Figure 2B). To determine whether the interaction affects the agglutination activity of galectin-1, increasing concentrations of CXCL4 were added to a fixed concentration of galectin-1. This revealed an additive effect of combining CXCL4 and galectin-1 (Figure 2C). BSA was used as a control protein and had no effect on the carbohydrate binding capacity of galectin-1 (data not shown).

Figure 2. Effect of galectin-1/CXCL4 interaction on hemagglutination. A) Hemagglutination by galectin-1 in the absence and presence of β-lactose. B) Hemagglutination by CXCL4 in the absence or presence of increasing concentrations of galectin-1. C) Effect of CXCL4 on agglutination by galectin-1.

The effect of the interaction on the carbohydrate binding activity of galectin-1 was further studied using fluorescent anisotropy. Increasing concentrations of galectin-1 were combined with a fluorescein-tagged high affinity saccharide probe (thiodigalactoside amide; tdga-probe)\(^{25}\). This resulted in an increased anisotropy curve consistent with a \( K_d \) of 0.88±0.05 \( \mu \)M (Figure 3A). In the presence of a fixed concentration of CXCL4, the carbohydrate binding affinity increased almost 10-fold to a \( K_d \) of 0.09±0.01 (Figure 3A). In line with previous results with anginex\(^{21}\), the \( A_{\text{max}} \) (anisotropy of the galectin-probe complex) was also increased in the presence of CXCL4, which indicates a reduced mobility of the fluorescein tag (Figure 3A). As expected, the increased binding capacity could be neutralized by addition of heparin (Figure 3A). To confirm that the interaction enhances the carbohydrate affinity of galectin-1, similar experiments were performed with a low affinity probe (lacto-N-triose; LNT-probe). In the absence of CXCL4, no increase in anisotropy with increasing concentrations of galectin-1 was observed (Figure 3B). However, in the presence of CXCL4 the LNT-probe did bind to galectin-1 with a \( K_d \) in the range of the high-affinity tdga-probe, i.e. 0.48±0.18 \( \mu \)M (Figure 3C). At galectin concentrations higher than 1.25 \( \mu \)M the anisotropy levels decreased again, probably due to competition of the free galectin for the probe. The enhanced carbohydrate binding affinity was further confirmed by a competition experiment in which increasing concentrations of an unlabeled inhibitor of the galectin-1-probe interaction, i.e. the glycoprotein asialofetuin (ASF), was added to a fixed concentration of galectin-1 (the \( EC_{50} \) of the corresponding slope in the
Galectin-1 is a functional binding partner for CXCL4

absence of ASF). In the presence of CXCL4, the inhibitory potency of ASF was more than 600-fold increased (Figure 3C). Altogether, these results show a modulation of the binding affinity and possibly the binding specificity of galectin-1 by CXCL4.

![Figure 3. Effect of galectin-1/CXCL4 interaction on fluorescence anisotropy. A) Effect of CXCL4 on the binding of galectin-1 to the high-affinity tdga-probe in the absence or presence of heparin. B) Effect of CXCL4 on the binding of galectin-1 to the low-affinity LNT-probe. C) Inhibition assay with ASF and galectin-1 in the absence or presence of CXCL4.]

Next, we set out to study the functional consequence of the interaction between CXCL4 and galectin-1 on endothelial cells and blood platelets since both proteins have been associated with the function of these cells\(^2,11,15\). First, we determined the effect of combining recombinant galectin-1 and CXCL4 on HUVEC proliferation and migration. As expected, CXCL4 alone significantly reduced proliferation, albeit less potent as compared to equimolar concentrations of anginex alone (Figure 4A). Furthermore, the stimulatory effect of galectin-1 alone was completely abolished by CXCL4 (Figure 4A). Comparable results were obtained when assessing HUVEC migration (Figure 4B). These effects could not be attributed to the previously described endothelial receptor CXCR3B\(^7\), since we could hardly detect mRNA expression of CXCR3B in quiescent or activated endothelial cells of different origin (Figure 4C). This is in line with previous results\(^5\), suggesting that the activity of CXCL4 in EC is probably not mediated through CXCR3B. Interestingly, it has been shown that surface galectin-1 promotes proangiogenic signaling via Ras and Akt\(^17,18,26\). Possibly, binding of CXCL4 to galectin-1 interferes with this angiostimulatory mechanism. Therefore we determined the effect of CXCL4 on the cellular localization of galectin-1. This revealed increased internalization of fluorescently labeled galectin-1 in the presence of CXCL4 (Figure 4D). Moreover, Ras signaling in EC was inhibited upon CXCL4 treatment (Figure 4E). These data show a link between CXCL4 and galectin-1 in endothelial cell function.
The functional consequence of CXCL4/Gal-1 interaction was further explored in blood platelets since these are the prime source of CXCL4 and a recent study suggests that exposure of platelets to galectin-1 binding results in platelet activation\(^1\). Indeed, galectin-1 showed a concentration dependent induction of platelet activation, which reached a MFI of 155.4±25.5 at 6 μM galectin-1 (Figure 5A). A small concentration dependent induction was also observed for CXCL4, albeit with a maximum MFI of only 2.8±0.1 (Figure 5A). Apparently, the platelet activation by CXCL4 alone is negligible compared to galectin-1 alone. Next, different concentrations of CXCL4 and galectin-1 were combined. There appeared to be a concentration dependent potentiation of platelet activation by CXCL4 at increasing concentrations of galectin-1, which reached statistical significance at 6 μM galectin-1 combined with 6 μM CXCL4 (Figure 5B). Similar effects were observed with regard to platelet aggregation (Figures 5C+D).

**Figure 4.** Effect of galectin-1/CXCL4 interaction on endothelial cell function. A) HUVEC proliferation. B) HUVEC migration. C) CXCR3B mRNA expression in different cells. D) Internalization of fluorescently labeled galectin-1 in the absence or presence of CXCL4 (10 μM), anginex (1 μM), or erlotinib (5 μM). E) Effect of CXCL4 on pErk and pMEK signaling. * p < 0.05 vs. untreated, # p < 0.05 vs. corresponding galectin-1 treatment.
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Figure 5. Effect of galectin-1/CXCL4 interaction on platelet function. A) Platelet activation by galectin-1 and CXCL4. * p < 0.05 vs. untreated. B) Additional platelet activation by CXCL4 at different galectin-1 concentrations. * p < 0.05 vs. 0 μM CXCL4 at each galectin-1 concentration. C) Platelet aggregation by galectin-1 and CXCL4. * p < 0.05 vs. untreated. D) Additional platelet aggregation by CXCL4 at different galectin-1 concentrations. * p < 0.05 vs. 0 μM CXCL4 at each galectin-1 concentration.

Discussion

We set out to explore whether galectin-1 can serve as a functional binding partner for CXCL4. The results presented in this study show that CXCL4 can indeed bind to galectin-1. This interaction affects the affinity and possibly the specificity for carbohydrate binding of galectin-1. Furthermore, our current data suggest that the interaction might have functional consequences on both galectin-1 and CXCL4 activity.

An important finding of the current study is the identification of galectin-1 as a novel binding partner for CXCL4. While the angiostatic activity of CXCL4 was described almost 25 years ago, the underlying mechanisms are still poorly understood. Apart from interacting with angiogenic growth factors such as bFGF and VEGF, it has been suggested that CXCL4 as well as its variant CXCL4L1 exerts its effects via binding to the chemokine receptor CXCR3. However, Sulpice et al. could not prevent the angiostatic activity of CXCL4 by blocking CXCR3, which was
explained by the low percentage of CXCR3-expressing HUVEC. The latter is in agreement with our observation that CXCR3B expression in HUVEC is low, and even further decreases upon EC activation. Thus, it is unlikely that CXCR3 is the main receptor mediating CXCL4 activity. More recently, it was shown that part of the angiostatic activity could be mediated via binding to integrins, predominantly αvβ3, αvβ5 and α5β1. Interestingly, several integrins are known to interact with galectin-1 for which we now show that it can bind CXCL4. It is thus tempting to speculate that the effects of CXCL4 on integrins are mediated via galectin-1. However, further research is needed to confirm this hypothesis.

Apart from integrins, galectin-1 is known to have several other binding partners, both intra- and extracellularly. Intracellularly, galectin-1 engages in direct protein-protein interactions while extracellular binding is usually carbohydrate-mediated. As evident from our interaction studies, CXCL4 binds to galectin-1 via direct protein-protein interaction, with similar kinetics as anginex, a CXCL4-like peptide. Furthermore, gel-shift analyses in the presence of lactose as well as the fluorescent polarization experiments indicate that CXCL4 does not compete for the carbohydrate binding pocket of galectin-1. In fact, while hemagglutination data suggest an additive effect, fluorescent polarization experiments indicate that binding of CXCL4 to galectin-1 potentiates the binding affinity and possibly alters the carbohydrate specificity. The latter is in agreement with previous studies using anginex and its derivative DB16, which reported that direct protein-protein interactions can modulate galectin-1 glycan binding. Interestingly, it has been shown that carbohydrate interactions can affect the tertiary and quaternary structure of galectin-1. The current data suggest that modulation of the protein structure by interaction with small proteins can also induce altered carbohydrate affinity. To our knowledge, we are the first to identify an endogenous protein that exerts such a function. However, the functional consequence of this modulation remains elusive. Our in vitro data show that the angiostimulatory effects of galectin-1 can be completely reversed by CXCL4. On the other hand, the angiostatic effect of CXCL4 could not be neutralized by adding increasing amounts of galectin-1. Both observations can be explained by the relatively low concentration of galectin-1 compared to CXCL4, 0.5 vs. 10 μM, respectively. These concentrations were based on previous studies showing significant responses in endothelial cells. Possibly, an effect of galectin-1 on CXCL4 could be observed at lower CXCL4 concentrations. Interestingly, more potent CXCL4-variants have been described which would allow experiments to be performed at equimolar concentrations, providing that the interaction of galectin-1 with these variants is established. In addition, future studies using gal-1−/− mice should be performed to validate whether the angiostatic effect of CXCL4 is mediated through galectin-1, as published previously for anginex. Additional studies with gal-1−/− endothelial cells would also be valuable to address whether the observed reduction in pMEK and pErk by CXCL4 is mediated via galectin-1. We and others have already shown the importance of galectin-1 in inducing and maintaining MAP kinase signaling. It was shown that galectin-1 is required for membrane translocation of activated H-Ras, thereby maintaining downstream signaling via MEK/Erk, which could be prevented by treatment with anginex. Based on our current results it is tempting to speculate that CXCL4 induces a similar effect. This is supported by the observation that CXCL4 inhibits
Galectin-1 is a functional binding partner for CXCL4. On the other hand, the reduced signaling might also be explained by decreased surface receptor activity. Galectin-1 is known to prolong VEGFR2 activity by increasing surface retention\textsuperscript{17,41}. We observed increased internalization of galectin-1 upon CXCL4 treatment, which could indicate a more rapid uptake of galectin-1-binding receptors, resulting in reduced downstream signaling. Whether this involves VEGF receptors needs further investigation.

Apart from an angioregulatory activity, galectin-1 as well as other galectins have recently emerged as regulators of platelet function\textsuperscript{42}. It was shown that galectin-1 can induce both platelet activation and aggregation. This was evidenced by a dose-dependent increase in surface exposure of P-selectin and integrin α\textsubscript{IIb}β\textsubscript{3}\textsuperscript{15}. More recently, the activity of galectin-1 was linked to increased “outside-in” signaling through binding to integrin α\textsubscript{IIb}β\textsubscript{3}\textsuperscript{43}. We also observed an enhanced platelet activation and activation upon galectin-1 treatment. Interestingly, we found that addition of CXCL4 could potentiate both platelet responses to galectin-1. This is in agreement with findings by Capitanio et al., who described that CXCL4 potentiates the platelet-stimulatory effects of thrombin, arachidonic acid and ADP\textsuperscript{44}. Furthermore, they described that this potentiating effect only occurred in narrow concentration ranges, which is in line with our current results. Apparently, platelet agonists can employ CXCL4 to enhance their stimulatory activity. Whether this is a result of direct interactions with CXCL4 and whether this is also true for other platelet-secreted molecules needs to be established. Regarding the latter, a recent study showed that different galectins can induce the platelet release of specific pro- and anti-angiogenesis molecules like VEGF and endostatin via specific signaling pathways\textsuperscript{45}. Together with our current findings, this suggests the existence of a complex mechanism to regulate the cross-talk between platelets and the endothelium during coagulation, angiogenesis and inflammation.

In summary, we describe that galectin-1 is a functional binding partner for the endogenous angiogenesis inhibitor CXCL4. The interaction modulates the carbohydrate binding affinity of galectin-1 and might even affect binding specificity. Furthermore, CXCL4 neutralizes the stimulatory activity of galectin-1 on endothelial cell proliferation and migration, accompanied by reduced MEK/Erk phosphorylation and increased galectin-1 internalization. CXCL4 also augments both platelet activation and aggregation induced by galectin-1. All these data suggest that galectins and chemokines can physically interact, thereby affecting their specific functions. Since both protein families are involved in similar processes including coagulation, angiogenesis and inflammation, our current findings have major implications on the understanding of how these processes are regulated and interconnected. Furthermore, our data provide leads for the development of drugs that affect the function of not only galectin-1 but possibly of other galectin family members as well.
Chapter 7

References

Galectin-1 is a functional binding partner for CXCL4.


Chapter 8

Expression, regulation and function of human metallothioneins in endothelial cells

Iris A Schulkens, Kitty C Castricum, Ester M Weijers, Pieter Koolwijk, Arjan W Griffioen, Victor L Thijssen

Abstract

Metallothioneins (MTs) are small cysteine-rich proteins which are involved in e.g. metal homeostasis, metal detoxification and protection against oxidative stress. In addition, several MTs have been shown to regulate expression of pro-angiogenic growth factors like VEGF. Detailed information about the expression and regulation of specific metallothionein isoforms in endothelial cells is limited. We therefore performed extensive mRNA expression profiling of all known human metallothioneins in endothelial cells. We found that the basal endothelial expression is restricted to MT1E, MT1X, MT2A, and MT3. Physiological activation of endothelial cells by exposure to serum increased the expression of MT1E and MT2A and induced the expression of MT1M. Furthermore, exposure to zinc or copper induced the expression of most MT1 isoforms, while hypoxia specifically increased the expression of MT1E, MT1M, MT1X, and MT3. Finally, knockdown of the dominant MT isoform in endothelial cells, i.e. MT2A, resulted in decreased proliferation and sprouting as well as in increased migration of HUVEC. Together these findings provide a link between metallothioneins and angiogenesis.
Introduction

Metallothioneins (MTs) are a family of small proteins (±6 kD) that contain a high percentage of cysteines (±30%) which allow the binding of heavy metals like zinc and copper. Thus far, four isoforms have been identified (MT-I–IV), comprising of 8 MT-I genes each encoding functional MT (MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1M, MT1X) and a single gene for the other isoforms (MT2A, MT3, MT4). Metallothioneins can bind multiple metals and this binding confers stability to the protein. Because of this binding capacity, MTs play a role in metal homeostasis by controlling the levels of free metal that are available for proteins involved in cellular processes, like zinc-finger proteins and p53. Animal studies have shown that MT-I/II knockout mice are more sensitive to dietary zinc restriction and are more susceptible to toxic metals, illustrating that MTs can serve as a cellular protection mechanism against toxic metals by scavenging e.g. zinc, cadmium, mercury, or platinum.

Metallothioneins can also protect cells against oxidative stress by virtue of their free radicals scavenging capacity and their inhibitory effect on the activity of ROS-producing enzymes, like inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COS-2). At the same time, MTs have been shown to be involved in neovessel formation, i.e. angiogenesis, both in vitro and in vivo. By inducing angiogenesis, MTs could provide a possible feedback mechanism to counteract oxidative stress. MTs can induce the expression of hypoxia inducible factor 1 alpha (HIF1α), an important pro-angiogenesis transcription factor. Furthermore, Miyashita et al. observed that knockdown of MT-I expression decreases proliferation, migration, and network formation of endothelial cell in vitro. As a result, effective angiogenesis in vivo following MT-I knockdown is hampered. This was confirmed by Zbinden et al., who found decreased vessel density and less EC migration in matrigel plugs implanted in MT-I/II knockout mice as compared to wild-type animals.

All these findings suggest that MTs are involved in neovascularization. Surprisingly, little is known regarding the expression of MTs in endothelial cells. Apart from Miyashita et al., an increase in MT protein levels in rat liver endothelial cells following exposure to cadmium was observed by Caperna et al. The same was reported by Mckim et al. who also used a MT-II specific probe to show increased mRNA expression levels.

The aim of this study was to gain insight in the expression and function of MTs in endothelial cell biology. Therefore, we performed an extensive MT profiling analysis in human endothelial cells. We assessed the effect zinc and copper on MT expression and determined how hypoxic conditions affect endothelial MT expression. Finally, we determined the effect of MT2A knockdown, the most prominent MT isoform, on endothelial cell function.
Chapter 8

Materials and Methods

Endothelial cell culture and reagents
Primary HUVEC were isolated from umbilical cords as described previously\textsuperscript{13}, and cultured in RPMI1640 (Lonza) supplemented with 10% fetal bovine serum (PAA), 10% human serum (VU university medical center), 2 mM L-glutamine (ICN), 100 U/mL penicillin (Lonza), and 100 mg/mL streptomycin (Lonza). Quiescent HUVEC were harvested 2 hours after isolation, whereas activated HUVEC were obtained by culturing cells for 3 days in high-serum medium.

Primary human microvascular endothelial cells (MVEC) were isolated from foreskin as described previously\textsuperscript{14}, and cultured in Medium 199 supplemented with 10% heat-inactivated newborn calf serum (Lonza), 10% heat-inactivated human serum, 2 mM L-glutamine (Lonza), 100 U/mL penicillin (Lonza), 100 mg/mL streptomycin (Lonza), 5 U/mL heparin, and 3.75 μg/mL endothelial cell growth factor (ECGF, crude extract from bovine brain).

Immortalized human microvascular endothelial cells (HMEC-1)\textsuperscript{15} were cultured in HUVEC medium without penicillin/streptomycin, whereas immortalized EC-RF24 cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum and 1% L-glutamine. All endothelial cells were cultured in gelatin-coated flasks or culture plates at 37°C and 5% CO\textsubscript{2} in a humidified incubator. Where indicated, cells were treated with 100 μM Zn(II)SO\textsubscript{4} or Cu(II)SO\textsubscript{4} for 24 hours.

Hypoxia experiments
Cell culture under hypoxic conditions was performed as described previously\textsuperscript{16}. In brief, MVEC were first stimulated with 10 ng/mL vascular endothelial growth factor (VEGF, Invitrogen) for 24 hours. Subsequently, both MVEC and HUVEC were cultured for 24 hours in 1% O\textsubscript{2}, 5% CO\textsubscript{2} and 37°C inside a custom designed hypoxic workstation (T.C.P.S.). All media and buffers were pre-incubated for 4 hours before use, to prevent re-oxygenation during hypoxic culture.

Transfections
HUVEC were reversely transfected with siRNA in gelatin-coated 96-well plates, using HiPerfect transfection reagent (QIAGen) according to the supplier’s instructions. Briefly, 20 nM siRNA was incubated together with 0.75 μl HiPerfect transfection reagent in 25 μl serum free medium to allow complex formation. After 20 minutes, the appropriate amount of cells in 25 μl was added to the complexes. Cells were either transfected with siRNA targeting siMT2A (FlexiTube siRNA SI00650699, QIAGen), or a non-relevant scrambled siRNA (Eurogentec) as a control. The medium was replaced 4 hours after transfection.

Migration
To assess the migratory capacity of MT2A knockdown EC, 20000 HUVEC cells were reverse transfected with siRNA in a gelatin-coated 96-well plate and grown to confluence in two days. Then, the wells were scratched using a guided 96-well pin tool (Peira). Following washing with PBS, fresh medium was added to the wells. As a positive control for inhibition of migration, 10 μM anginex was added to the medium. Images of the scratch were taken at t=0 and t=8 hours.
with a Leica DMI3000B microscope equipped with an automated xyz-stage and a Hitachi 1.4 Mb GiGE color camera, using Universal Grab software (version 6.3, DCILabs). Scratch areas were determined using ImageJ (version 1.41), and wound closure was expressed as a fold change to the control wells. All experiments were performed in triplicate and within each experiment there were three replicates of all treatment conditions.

Proliferation
The proliferative capacity of MT2A knockdown EC was assessed by reverse transfection of 7500 HUVEC cells with siRNA. Four hours after transfection the medium was replaced and the cells were incubated for 48 hours at 5% CO₂ and 37 °C. As a positive control for inhibition of proliferation, 10 μM anginex was added to the medium. As a measure of proliferation, ATP-content was evaluated using CellTiter-Glo luminescent cell viability assay (Promega). Briefly, 30 μL CellTiter-Glo reagent was added to the medium and incubated on a shaker for 10 minutes. Next, 100 μL was transferred into an opaque white 96-well plate and luminescence was measured using a microplatereader (Tecan). All experiments were performed in triplicate and within each experiment there were three replicates of all treatment conditions.

Sprouting assay
For evaluation of endothelial cell sprouting, HUVEC (24 hours following transfection) were resuspended in 20% metocellulose, 10% human serum and 70% RPMI (Lonza) to a concentration of 40000 cells per mL. Droplets of 25 μL cell suspension were incubated overnight on an inverted lid of a non-adhesive culture plate. On the next day, 60 hanging drop spheroids per condition were embedded in 200 μL growth factor reduced matrigel (BD Bioscience) in a 24-wells plate after which 500 μL of HUVEC culture medium was added. Pictures of at least 30 spheroids per condition were taken after 48 hours and images were analysed using ImageJ, measuring both the length and number of sprouts per spheroid. Total sprout length was calculated by multiplying average sprout length and number. Experiments were performed on three different HUVEC isolations.

RNA isolation, cDNA synthesis and real time qPCR
Total RNA from cultured cells was isolated using the RNeasy Mini kit (QiAgen), according to the manufacturer’s instructions. Genomic DNA contamination was removed by on-column DNAse treatment. The purity and concentration of the RNA was determined using the Nanodrop ND-1000 (Nanodrop technologies). At least 100 ng RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad) according to the supplier’s protocol. Real-time quantitative PCR was performed on a CFX96 (BioRad) using the 2x iQ SYBR Green PCR mix (BioRad) and 400 nM of the forward and reverse primer as previously described13. Primers (Table1) were designed and tested as described before17 and synthesized by Eurogentec. The expression of each target gene was normalized to the expression of three reference genes (cyclophilinA, beta-actin and beta-2-microglobulin)18.
Statistical analysis

The data are shown as mean values±SEM. The Mann-Whitney rank sum test was used to calculate statistically significant differences in mRNA expression and EC proliferation. Two-way ANOVA was used to analyze the migration data over time. Statistical computations were performed in SPSS 20.0.0. (SPSS Inc.) and p-values ≤ 0.05 were considered statistically significant.

Results

We aimed to determine the complete MT expression profile in endothelial cells. First, primers were designed that specifically detect all functional MT isoforms. Because of the high degree of homology between MT1 isoforms, a pan-reverse primer was designed in a homologous region of MT1 while isoform specific primers were designed to target the more variable 5’ UTR (Table 1 + Supplementary Figure 1). For all other family member, i.e. MT2A, MT3 and MT4, both the forward and reverse primers were targeted against unique parts of the coding region. To ensure comparable amplification kinetics and to allow simultaneous analysis of all isoforms, primers were designed to anneal/elongate at ±60°C with amplicon lengths of approximately 150 nucleotides. The latter was confirmed by gel electrophoresis (Figure 1A) while dilution series showed comparable amplification kinetics over a broad range of concentrations for all primer combinations (Figure 1B + Table 2). There was no cross-reactivity between the different isoform/primer combinations (data not shown). All these data show that the primers can be used to specifically detect and compare MT expression in cells of human origin.

Table 1. Human MT primers

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<tr>
<th>Target mRNA</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Tm (ºC)</th>
<th>Amplicon length (nt)</th>
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W = A or T nucleotide; nt = nucleotides; *MT2A forward primer can also bind MT1F
Metallothionein expression and function in endothelial cells

Next, the MT expression profile was determined by qPCR in human endothelial cells of different origin. First, we studied the expression of MTs in quiescent HUVEC. In these cells MT1E, -1X, -2A and -3 were expressed at detectable levels (Figure 2A). Comparable MT expression levels were observed using another source of primary EC, i.e. microvascular endothelial cells (MVEC). These cells also expressed MT1H and MT1M (Figure 2B). We then compared the MT expression in primary EC with immortalized endothelial cells lines, i.e. human microvascular endothelial cells (HMEC-1) and immortalized HUVEC (EC-RF24). Both cell lines showed mRNA expression of MT1E, -1H, -1X, -2A, and -3, while HMEC-1 cells also expressed MT1A and -1M (Figure 2C and 2D). Of all the MTs analyzed, MT1B, -1F, -1G, and MT4 were never expressed at detectable levels in human endothelial cells. Overall, MT1E, -1X, 2A and -3 seem to be expressed in all EC, with MT2A being the most prominently expressed metallothionein.

Table 2. Primer characteristics within linear amplification range

<table>
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<th>Target</th>
<th>Slope</th>
<th>Upper Ct limit</th>
<th>Cross reactivity</th>
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<td>MT4</td>
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ND = not detectable

Figure 1. Metallothionein primer validation. A) Gel electrophoresis of amplicons generated by different MT primers. *Commercially available MT2A primers (QiAgen). All primers generated specific amplicons of the expected size. B) Standard curves of different MT primers.
Methionine mRNA expression in endothelial cells.

To get more insight in endothelial MT regulation we next determined the effect of different environmental stimuli on MT expression. Activation of quiescent HUVEC by culturing the cells under high serum conditions significantly increased the expression of MT1E and MT2A. In addition, the expression of MT1M was induced in activated endothelial cells (Figure 3A). As MTs are described to be involved in the detoxification of heavy metals, HUVEC were also cultured in the presence of zinc or copper, two metals that are scavenged by MTs. Both 100 μM zinc- and copper-sulfate induced the expression of most MT1 genes, except for MT1A and -1B. In general, zinc induced higher expression of MT compared to copper. The expression of MT2A and MT3 was not changed significantly, while MT4 expression remained below detection limits (Figure 3B and 3C).

Apart from the response to metals, metallothionein expression can also be regulated by hypoxia. Therefore, we cultured HUVEC for 24 hours under hypoxic conditions (1% oxygen), and evaluated the effect on MT mRNA expression. MT1M and MT3 expression was shown to be significantly increased in hypoxic HUVEC cells, whereas expression of other MTs remained unchanged. The expression of GLUT-1 and VEGF-A, two known hypoxia-inducible genes, was increased in both cell types (Figure 3D). Comparable results were obtained when MT expression was compared between MVEC cells cultured under normoxic or hypoxic conditions. In addition, the expression of MT1E and MT1X was also significantly increased in these cells (Figure 3D).
Metallothionein expression and function in endothelial cells

Figure 3. Effect of environmental stimuli on endothelial MT expression. A) Metallothionein mRNA expression upon serum activation of quiescent HUVEC (n=7). B+C) Metallothionein mRNA expression in HUVEC upon exposure to 100 μM zinc (n=3) (B) or copper (n=3) (C). D) Metallothionein mRNA expression under hypoxic conditions relative to expression in normoxic HUVEC or MVEC (dotted line) (n=3). * p ≤ 0.05.

Since MT2A is the most predominant metallothionein present in endothelial cells, we finally studied the effect of MT2A expression knockdown on endothelial cell function. Transfection of HUVEC with siRNA targeting MT2A decreased mRNA expression with 53% in HUVEC (Supplementary Figure 2). Analysis of HUVEC proliferation showed a slight but significant reduction 72 hours after transfection with siMT2A as compared to a scrambled control siRNA (Figure 4A). On the other hand, a small and statistically significant increase in migration was observed in siMT2A transfected cells at t=8 hours (Figure 4B). Addition of the galectin-1 inhibitor anginex was used as a positive control for inhibition of HUVEC proliferation and migration (Figure 4A and 4B). Finally, sprouting assays were performed to determine the effect of loss of MT2A on endothelial cell behaviour in a 3D matrix. These experiments showed a clear and significant reduction in sprout number as well as sprout length. Consequently, total sprout length was also significantly reduced (Figure 4C).
Figure 4. Effect of MT2A knockdown on HUVEC proliferation, migration and sprouting. Relative HUVEC proliferation (A) and migration (wound width) (B) following transfection with siMT2A or scrambled control siRNA (n=3). Anginex was used as a positive control. * p < 0.05, ** p < 0.01. The lower panels indicate representative images of the wound width 8 hours after applying the scratch. C) Relative HUVEC sprout length, number, and total length (=number x length) compared to control. * p < 0.05. The lower panels indicate representative images of sprouting spheroids.

Discussion

Although metallothioneins have gained increasing interest in the past three decades, detailed information on the expression and function of the specific MT isoforms is still scarce. In this study, extensive mRNA expression profiling of all known human metallothioneins was performed in endothelial cells. Thus far, the information on specific MT isoform expression in endothelium is limited and to our knowledge, this is the first study that explored the complete MT expression profile in endothelial cells. Miyashita et al. found MT1 to be expressed in endothelial cells, without further specifying which isoforms were expressed. In this study, we confirmed expression of MT1 in endothelial cells of different origin, i.e. HUVEC, HMEC-1 and EC-RF24. We found MT1E and MT1X expression in all studied endothelial cells, whereas e.g. MT1H was mainly expressed in the immortalized cells lines.

Apart from several MT1 isoforms, we also observed expression of MT2A and MT3 in all the different endothelial cell sources. Endothelial expression of MT2A has been described
by others while MT3 expression was suggested to be restricted to the brain and male reproductive organs. Here, we show that MT3 mRNA is expressed by endothelial cells as well, albeit indeed at low levels. Whether such low levels result in detectable MT3 protein levels in endothelial cells needs further investigation. Regarding MT4, we did not detect expression of this isoform in endothelial cells, which is in accordance with the observation that MT4 expression is specific for stratified squamous epithelia.

Because MTs are known for their role in metal homeostasis and detoxification, we also studied the regulation of endothelial MT expression by exogenous zinc and copper. We found that, except for MT1A, -1B and -1F, the expression of all MT1 isoforms was induced by both 100 μM zinc- and copper-sulfate. The expression of MT2A, MT3 and MT4 remained unchanged. Several other studies evaluated the effect of zinc and/or copper on MT expression in different cell types. Hidalgo et al. found a 1.5 to 5-fold increase in MT1 levels in neurons and astrocytes upon exposure to 50 μM zinc or copper. Bobilya et al. also found increased MT expression in bovine pulmonary artery endothelial cells after exposure to 100 μM zinc. Interestingly, Joshi et al. observed induction of the MT1G promoter in zinc- and cadmium-stimulated human aortic endothelial cells, but also upon stimulation with VEGF, suggesting a role for MT1G in angiogenesis. However, we did not see an induction of MT1G when HUVEC were cultured under hypoxia, a condition that did not induce VEGF. Possibly, this is related to the available concentration of VEGF or duration of stimulation. We did observe that hypoxia induced the mRNA expression of MT1M and MT3 in HUVEC, but also of MT1E and MT1X in MVEC. This is in agreement with other reports showing that metallothionein expression is increased under hypoxia. We did not study whether this was accompanied by induction of HIF1α protein, as was described by Xue et al. However, we did see that two other HIF1α regulated genes, i.e. VEGF-A and GLUT-1 were increased providing indirect evidence for a role of HIF1α in the regulation of endothelial MTs during hypoxia. In support of this, Kim et al. demonstrated that MT3 induces VEGF production via HIF1α in brain endothelial cells, linking MTs to HIF1α and suggesting a possible role for MT3 in neovascularization. Altogether, evidence suggests that hypoxia and subsequent induction of angiogenesis might involve regulation of endothelial MT expression. This is further supported by our functional experiments in HUVEC where knockdown of MT2A expression induced small but significant alterations in cell proliferation and migration while the effect on endothelial cell sprouting was more prominent. More pronounced effects might be observed following a complete knockout of MT2A expression as was seen for MT1 by Miyashita et al. Nevertheless, with 50% reduction of mRNA levels, we already found that HUVEC proliferation and sprouting were decreased whereas migration increased. The more potent effect in sprouting might be related to the fact that this 3D assays also relies on matrix degradation. Since matrix metalloproteinases (MMP) involved in this process depend on metal ions as cofactors, interfering with metallothioneins might directly affect MMP activity. For example, MT2A expression has been linked to expression of MMP-9, an MMP which is involved vascular remodeling. Future studies should explore the exact role of MT2A as well as of the other expressed MT isoforms in endothelial cell function.
In summary, we performed extensive metallothionein expression profiling in endothelial cells. The basal endothelial expression appears to be restricted to MT1E, MT1X, MT2A, and MT3, with MT2A as the dominant isoform. Furthermore, the expression of nearly all MT1 isoforms was increased by exposure to zinc or copper, while hypoxia specifically induced expression of MT1E, MT1M, MT1X, and MT3. Finally, siRNA mediated knockdown of MT2A decreased proliferation as well as sprouting of HUVEC, and increased EC migration. All these findings provide a link between metallothioneins and angiogenesis which should be further explored.

Acknowledgements
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Supplementary Figures

**Supplementary Figure 1.** Alignment of all MT1 isoform sequences using WebLogo (Crooks GE, Hon G, Chandonia JM, Brenner SE WebLogo: A sequence logo generator, 2004, Genome Research, 14:1188-1190.). The conserved region of the reverse primer and the variable region of forward primers are indicated in boxes. The ATG-start codon and TGA-stop codon are underlined.

**Supplementary Figure 2.** Knockdown of MT2A mRNA expression in HUVEC following transfection with siMT2A as assessed by qPCR.
References


Chapter 9

General discussion and perspectives
General discussion and perspectives

The human vasculature consists of a complex network of blood vessels that facilitates the transport and exchange of nutrients, gasses and cells throughout the body. The sprouting of novel blood vessels from pre-existing capillaries, i.e. angiogenesis, is important during physiological processes like embryonic development and wound healing, but is also induced by tumors to supply tumor cells with nutrients and oxygen and to facilitate metastasis. Therefore, interfering with tumor angiogenesis is considered as a promising strategy for anti-cancer therapy. Accumulating evidence reveals the importance of galectins in tumor angiogenesis as well as in other key events during tumor progression, e.g. tumor cell transformation, heterotypic cell adhesion, metastasis, and immune escape. This suggests that galectins could be of prognostic value for cancer patients and potentially could serve as targets for cancer treatment.

Regarding galectins and angiogenesis, we and others have previously shown that the expression of galectins in the endothelium is confined to galectin-1, -3, -8, and -9. While the role of galectin-1 and -3 in endothelial cells has been extensively described, data on endothelial galectin-8 and 9 are still scarce. Moreover, the underlying pathways that regulate galectin expression and the functional consequences of expression regulation are still not fully understood.

The research described in this thesis was aimed to 1) Assess the prognostic value of galectins in cancer patients, 2) Further unravel the expression and function of galectins in endothelial cells, and 3) Study methods to therapeutically target galectins.

To assess these aims, several known angiogenesis methods were adapted to study the role of galectins as well as of galectin inhibitors in endothelial cell biology in vitro (Chapter 3) and angiogenesis in vivo (Chapter 4).

Prognostic potential of galectins

Ample evidence has shown that galectins can be used as prognostic or diagnostic markers in several types of cancer. Galectin levels can be detected both in serum and in tumor tissue. For example, serum levels of galectin-3 are significantly increased in osteosarcoma patients compared to healthy controls, which is associated with cancer stage. Moreover, elevated tumor galectin-3 expression was shown to negatively correlate with overall survival (OS) in colorectal cancer and in non-small cell lung cancer (NSCLC) patients. In contrast, a positive correlation between tumor galectin-3 levels and OS was found in e.g. neuroblastoma, breast cancer, melanoma and renal carcinoma. These findings suggest that the prognostic value of galectin-3 is dependent on the specific tumor type. This appears different from galectin-1, of which increased expression is usually associated with poor outcome, including in patients with lung cancer. Indeed, elevated galectin-1 levels were found to promote lung cancer progression and chemoresistance, illustrating the prognostic potential of galectin-1 in lung cancer. However, whether galectin expression could also be used to distinguish between early stage NSCLC with good or poor prognosis has not been well established. This is relevant since approximately 30-40% of NSCLC patients with early stage disease will still present tumor...
General discussion

recurrence within 2 years after resection. It is important to identify these patients, since it has been shown that patients with resected early stage NSCLC will likely benefit from adjuvant chemotherapy. Therefore, we performed extensive galectin gene expression analysis on tumor tissues from patients with stage I/II NSCLC (Chapter 2). Our data confirmed the prognostic value of galectin-1 in these patients. Galectin-1 mRNA expression above median levels was associated with significantly shorter overall survival and disease free survival. Galectin-3, of which elevated expression has been associated with patient outcome had no prognostic value in early stage patients. This is in agreement with other studies that also included later stage patients.

On the other hand, we found that the expression of a specific galectin-9 splice variant, i.e. gal-9Δ5, positively correlated with patient survival (Chapter 2). This corroborates with other studies that found decreased expression of galectin-9 to correlate with decreased OS and increased metastatic potential in different types of cancer. However, we are the first to assess distinct splice variants and the importance of distinguishing between different galectin-9 splice variants was further exemplified in renal cancer (Chapter 6). In this cancer type we identified increased gal-9Δ5/6 and gal-9Δ5/6/10 expression as novel markers for better patient survival. Together, these findings suggest that there is a different splice variant expression profile in different tumor types. This further suggests that the environmental context dictates the requirement of specific galectins. Possibly, the extracellular matrix components that present specific glycans to which galectins bind play a role. Indeed, Nobumoto et al. showed that the adhesion of melanoma and colon cancer cells to glycosylated ECM components such as collagen type I and IV, fibronectin, laminin, and vitronectin, was inhibited by full length galectin-9, thereby decreasing the metastatic potential. On the other hand, galectin-1 was found to stimulate adhesion of tumor cells to laminin or fibronectin. Apart from the glycoproteins in the ECM, other environmental factors that might influence specific galectin expression are inflammatory cytokines. For example, increased endothelial galectin-1 expression was observed after stimulation with a mixture of cytokines, including IL-1β, TNFα and IFNγ. Endothelial galectin-9 was also shown to be increased by IFNγ. Another environmental factor that can influence galectin expression is the oxygen level in the tumor. Galectin-1 and -3 expression are known to be stimulated in response to hypoxia. Whether this is also true for galectin-9 has not yet been established. Recently, Croci et al. showed that the increased galectin-1 expression upon stimulation with cytokines and hypoxia might be the result of an altered glycoprofile of a cell. Immunosuppressive cytokines (IL-10 and TGF-β1) and hypoxia were shown to favor a galectin-1 permissive glycophenotype, whereas proinflammatory signals (IFN-γ and IL-17) reduced the exposure of galectin-1 ligands in EC. Whether and how such alterations in the glycoprofile affect the binding and subsequent signaling of other galectins and their splice variants in EC needs further investigation. Nevertheless, these findings suggest that the enzymes involved in protein glycosylation might also be of prognostic value.

Overall, the studies presented in this thesis further illustrate the prognostic potential of galectins in NSCLC and renal cell carcinoma. Moreover, we show the importance of distinguishing between different galectin splice variants. The expression and function of galectins appears to
depend on the environmental context. Therefore, further research is warranted to reveal the regulatory mechanisms of alternative galectin expression and splicing in tumor cells and the tumor endothelium.

**Expression and function of galectins in endothelial cells and angiogenesis**

As described above, the endothelial expression of galectins appears to be confined to galectin-1, -3, -8, and -9. Although the roles of galectin-1, -3, and to a lesser extend galectin-8, in angiogenesis have been well-studied, only limited studies focused on the role of galectin-9. Here, we describe that galectin-9 expression was often confined to the endothelial cells in the tumor vasculature (Chapter 5 and 6), suggesting a role in angiogenesis. Since we have previously shown the relevance of distinguishing between different galectin-9 splice variants (Chapter 2 and 6 + Heusschen et al.), we determined the endothelial expression of all possible galectin-9 isoforms that vary in excision of exons 5, 6 and 10. We observed that quiescent endothelial cells express 5 galectin-9 isoforms, i.e. gal-9 full length, gal-9Δ5, gal-9Δ5/6, gal-9Δ5/10, and gal-9Δ5/6/10, with gal-9Δ5 being the most prominently expressed (Chapter 5). Until now, three main variants had been described in which splicing occurs in exon 5 and 6, encoding the linker between two carbohydrate recognition domains (CRDs). Deletion of exon 5 and 6 affects the length and rotational freedom of the linker peptide between two CRDs, which influences multimerization and galectin-9 valency. In this thesis we describe two additional splice variants in endothelial cells, lacking also exon 10, i.e. gal-9Δ5/10 and gal-9Δ5/6/10 (Chapter 5). Excision of this exon results in a frame-shift and a premature stop codon, and concomitantly in a truncated C-terminal CRD. Thus far, only one other study showed the existence of gal-9Δ5/10 in cells of non-endothelial origin. To our knowledge, we are the first to describe a variant lacking all three exons. The functional consequence of these splicing events remains poorly understood.

We showed that upon endothelial activation, the expression of the isoforms is differentially regulated. Total galectin-9 levels are decreased, which is mostly caused by a significant decrease in the abundant gal-9Δ5 levels. Interestingly, the expression of splice variants lacking exon 6, i.e. gal-9Δ5/6 and gal-9Δ5/6/10, appears to be increased, suggesting diverging functions for different splice variants.

Thus far, only few studies reported on the functions of specific galectin-9 isoforms. For example, Chabot et al. described increased eosinophil chemoattractant activity for gal-9FL compared to gal-9Δ5. In addition, different splice variants have been shown to differentially affect E-selectin expression in colon carcinoma cells, thereby modulating the adhesion to endothelial cells. We now show that endogenous overexpression of the individual isoforms in endothelial cells yielded only small effects on proliferation and migration of these cells. Apparently, the differential regulation of the dominant gal-9Δ5 variant that occurs during endothelial cell activation plays only a minor role in endothelial cell function (Chapter 5). As discussed previously, this might be related to a lack of appropriate glycans through which galectin-9 exerts its function. Indeed, exogenous gal-9Δ5 also only moderately affected EC function in vitro and angiogenesis in vivo. Nevertheless, gal-9Δ5 showed a biphasic effect on EC
migration, where low concentrations stimulated migration, while high concentrations inhibited wound closure. This corresponds to previous observations of galectin-1 on EC migration\(^{40}\). Thus, under the proper environmental conditions, e.g. within a tumor, gal-9\(\Delta5\) might have angioregulatory functions. In addition, other splice variants might have even more pronounced functions in angiogenesis. Since the \(\Delta10\) isoforms contain a truncated C-terminal CRD, these variants might act as dominant-negative proteins by occupying carbohydrates on cells surfaces or the extracellular matrix, while lacking the capacity to form bivalent interactions. Potentially, this prevents these carbohydrates from being crosslinked by other galectins, thereby affecting e.g. cell migration. On the other hand, we have shown that the \(\Delta10\) isoforms, unlike gal-9\(\Delta5\) and -\(\Delta5/6\), are not secreted by endothelial cells, suggesting an intracellular role for these isoforms in e.g. posttranscriptional splicing or intracellular signaling. Alternatively, the truncated splice variants might heterodimerize with other galectins, thereby affecting galectin function as was previously shown for the artificially conjugated galectin-1 and -9\(^{42}\). However, evidence for galectin heterodimerization is still lacking and should be further explored.

Since alternative splicing as well as proteolytic cleavage of the linker sequence\(^{47}\) might result in separation of both galectin-9 CRDs, we also assessed the angioregulatory activity of the N-terminal CRD (gal-9N) (Chapter 6). Endothelial cell migration \textit{in vitro} and angiogenesis \textit{in vivo} were more potently inhibited by gal-9N compared to gal-9\(\Delta5\). This supports the hypothesis that the truncated isoforms could serve as dominant negative proteins. On the other hand, endothelial cell sprouting and transwell migration were promoted by gal-9N. Thus, gal-9N appeared to be a more potent chemoattractant for EC than gal-9\(\Delta5\). Possibly, separation of the two CRDs causes a switch from a direct in to a more indirect angioregulatory function, i.e. chemoattractant. In contrast, Nishi \textit{et al.} found that thrombin-mediated cleavage of the galectin-9 linker peptide reduced the chemoattraction of eosinophils\(^{47}\). This opposing activity further suggests that the function of galectins and galectin isoforms is dependent on the cell type and the environmental context, as discussed above. Furthermore, previous studies demonstrated that specific CRD domains can also exert distinct functions. For example, the C-terminal CRD of gal-9 (gal-9C) was found to be responsible for the mechanism of induction of T-cell death, while gal-9N and the linker peptide contributed to the potency of the response\(^{42}\). Therefore, it would be interesting to investigate the function of gal-9C in endothelial cells. As the structure of the C-terminal galectin-9 domain is evolutionary homologous to galectin-1 and -3\(^{48}\), which have well-known angiostimulatory properties, it is tempting to speculate on a possible role of gal-9C in angiogenesis.

Since our current results showed only a limited role for galectin-9 in angiogenesis, we might hypothesize that the elevated galectin-9 levels in the tumor endothelium have more prominent effects on immunomodulation. For galectin-1 it has already been shown that, apart from a role in angiogenesis, it can also have immune-suppressive functions, e.g. by inducing apoptosis of activated T-cells\(^{49}\) and reducing the recruitment of lymphocytes and neutrophils to the endothelium\(^{50,51}\). Indeed, galectin-1 was found to facilitate tumor immune escape in mouse models by interfering with T-cell function\(^{52,53}\). Blocking galectin-1 resulted in rejection of the tumor and potentiation of a tumor-specific Th1 immune response\(^{53}\). Galectin-9 has
also predominantly been studied in the context of the immune system and tumor immune escape. It has been shown to potently induce apoptosis of cytotoxic T-cells and Th1 cells via binding to the Tim-3 receptor on these cells\textsuperscript{54,55}. In addition, galectin-9 was found to suppress the differentiation of naïve T-cells to Th17 cells, while differentiation to immune-suppressive regulatory T-cells (Tregs) was promoted\textsuperscript{56}. Therefore, increased galectin-9 levels in the endothelium might contribute to tumor immune escape by shifting the balance towards an immunosuppressive environment. Recently, we postulated a model in which a decrease in intratumoral galectin-9 levels results in loss of tissue integrity, thereby facilitating tumor cell invasion and metastasis, while increased endothelial galectin-9 expression possibly maintains an immune-suppressive barrier between circulating immune cells and the tumor and facilitates metastasis by mediating heterotypic cell adhesion\textsuperscript{57}. Future research is needed to test this model in e.g. galectin-9 knockout mice. It would be interesting to study how tumors develop in this model and whether immune cells are indeed better capable of invading the tumor, thereby facilitating tumor immune escape.

Taken together, the data presented in this thesis contribute to a better understanding of the role of galectin-9 in EC function and broaden the insights into the function of galectins in general in angiogenic processes (Figure 1). Furthermore, we demonstrate the importance of distinguishing between all galectin-9 variants, not only for diagnostic and prognostic purposes, but also to unravel the diverging functions of different isoforms in physiological and pathophysiological processes. Further research into the mechanisms that regulate this functional diversity and into the function of other splice variants is needed for the development of effective galectin-9-targeted anti-cancer therapies.

![Figure 1. New and established roles of galectins in angiogenic processes. Several new functions of galectin-9 (domains) have been added to the already established roles of galectins in multiple steps of the angiogenesis cascade (opaque). Galectins for which their role in the specific angiogenic processes has not yet been studied are depicted transparent.](image-url)
Therapeutic targeting of galectins

Due to the differential expression of galectins in tumor endothelium compared to the normal vasculature, these proteins are considered as promising targets for angiostatic cancer therapy. Over the past two decades, several strategies have been applied to interfere with galectin function, including the use of antibodies, carbohydrates and peptides/proteins (Chapter 1). For example, we developed a cytokine-like angiostatic peptide, i.e. anginex, blocking the function of galectin-1\(^8,58,59\). Based on the structural and functional similarities between anginex and the chemokine platelet factor 4 (PF4/CXCL4), we hypothesized that galectin-1 could also serve as a receptor for endogenous CXCL4, thereby facilitating its angiostatic properties. In this thesis we indeed proved binding between galectin-1 and CXCL4 (Chapter 7). We show that CXCL4 neutralizes the stimulatory effects of galectin-1 on EC proliferation and migration, with concomitant reduction in pErk and pMEK signaling. This result is supported by the observation that CXCL4 significantly inhibits MAP kinase signaling pathways downstream of bFGF and VEGF receptors\(^50,61\). Future studies should reveal whether decreased galectin-1-mediated membrane translocation of activated H-Ras also underlies our observations with CXCL4, as was previously established for anginex\(^40\). As we demonstrated increased galectin-1 internalization in endothelial cells in the presence of CXCL4, another possible explanation for the reduced galectin-1-mediated EC function and signaling could be the increased uptake of galectin-1 binding receptors. In support of this, galectin-1 was previously shown to prolong VEGFR2 residency on the EC surface\(^37,62\). Possibly, by interacting with CXCL4, galectin-1 can still bind to but not activate VEGFR2 and other galectin-1 binding receptors on the EC surface. This could lead to increased internalization of these receptors and concomitant uptake of galectin-1. Further research is required to unravel the functional relevance of the interaction between CXCL4 and galectin-1 in endothelial cells.

Apart from their role in endothelial cell biology, accumulating evidence reveals that galectins are involved in the regulation of platelet function\(^63\). Galectin-1 has been shown to bind to \(\alpha_{IIb}\beta_3\) integrin on the platelet surface, thereby triggering platelet activation via “outside-in” signaling\(^64\). Subsequently, platelets release the contents of their \(\alpha\)-granules, e.g. growth factors, which can promote tumor progression and angiogenesis. Recently it was shown that different galectin family members differentially regulate the release of proangiogenic molecules from platelets. Moreover, releasates from platelets stimulated with galectin-1, -3 and -8 promoted endothelial cell proliferation and tube formation, thereby amplifying the direct angiogenic effects of these galectins\(^65\). Additionally, platelets are known to form heterotypic aggregates with tumor cells, resulting in increased metastatic potential of the cancer cells\(^66-68\) and evasion from immune system\(^69,70\). Possibly, tumor galectin-1 expression could facilitate this aggregation. As galectin-1 is also known to induce P-selectin expression on platelets\(^71\), galectin-1 might promote stable adhesion of the cell-aggregates to the endothelium, thereby facilitating metastasis. In this thesis, we show that CXCL4 potentiated both the platelet activation and aggregation induced by galectin-1 (Chapter 7). CXCL4 might increase the affinity of galectin-1 for integrin \(\alpha_{IIb}\beta_3\), possibly resulting in enhanced platelet activation. Alternatively, since CXCL4
itself is also known to bind to several integrins\textsuperscript{72}, CXCL4 might serve as a co-stimulatory ligand for integrin $\alpha_{IIb}\beta_3$ on platelets. Studies using gal-1\textsuperscript{−/−} mice should be performed to validate whether the angiostatic and platelet effects of CXCL4 are indeed mediated via galectin-1. Furthermore it would be interesting to study whether a more potent angiostatic CXCL4 variant, i.e. CXCL4L\textsuperscript{73,74}, also interacts with galectin-1 and induces similar or even stronger effects in endothelial cells and platelets.

Different models can be proposed to explain how the interaction between galectin-1 and CXCL4 affects the function of either protein. Possibly CXCL4 modulates the carbohydrate binding capacity of galectin-1, thereby affecting galectin-1 function. Indeed, our experiments show that binding of CXCL4 alters the affinity, and possibly specificity, of carbohydrate binding by galectin-1. This is in line with previous findings, where anginex and its derivative DB16 were found to modulate glycan-binding by galectin-1\textsuperscript{75,76}. To our knowledge, we are the first to describe an endogenous small protein that, upon binding to a galectin, modulates the carbohydrate binding capacity. Interestingly, it has been described that binding of lactose, a low-affinity carbohydrate, to one CRD of a galectin-1 dimer decreases the lactose binding capacity of the other CRD. This process is called negative cooperativity and is mediated via small structural changes of the CRDs upon carbohydrate binding\textsuperscript{77}. Possibly, CXCL4 binding also alters the CRD structure thereby affecting the carbohydrate binding affinity of galectin-1 via either positive or negative cooperativity. This could result in the binding of galectin-1 to different receptors, providing a fine-tuning mechanism to regulate galectin-1 function.

Alternatively, galectin-1 could simply act as a chaperone protein which facilitates the transport of CXCL4. Upon release from the $\alpha$-granules of platelets, CXCL4 might bind to circulating galectin-1, which then guides the angiostatic protein to e.g. the site of injury or inflammation.

Finally, another possible explanation is that galectin-1 affects CXCL4 function, e.g. by blocking the interaction of CXCL4 with the angiogenic growth factors bFGF and VEGF, or by interfering with integrin binding. By binding to CXCL4, galectin-1 might regulate the effective concentration of this and possibly also of other cytokines in the blood stream. Since galectin-1 seems to bind preferentially to proteins with extensive $\beta$-sheet structures (e.g. anginex and CXCL4) it is tempting to speculate that it can also bind other angioregulatory cytokines that contain $\beta$-sheet structures, such as interleukin-8 and RANTES. Interestingly, the cytokine-like anginex was found to bind to other galectins as well, including galectin-9. More specifically, anginex interacted with gal-9N, but not with gal-9C\textsuperscript{76}, which could present another functional aspect of different galectin-9 splice variants. This could involve a more general mechanism for the fine-tuning of (angioregulatory) cytokine concentrations by galectins which needs further investigation. Nevertheless, the current insights on the interaction of galectin-1 with CXCL4 could provide leads for the development of drugs that target galectins. Possibly, CXCL4 or CXCL4-derivatives could be used to target not only galectin-mediated angiogenesis, but also affect immunomodulation. Since the CXCL4-like peptide anginex has shown promising results as an anti-angiogenesis and anti-cancer therapeutic\textsuperscript{78}, further research on CXCL4 as a potential
galectin-targeting agent should be encouraged.

The importance of the structural integrity of the galectin CRD for proper activity also opens alternative avenues to interfere with protein activity. Galectins, which were previously known as S-type lectins because of their sulfhydryl dependency confined to cysteine residues, are known to lose activity in the absence of reducing agents\textsuperscript{79,80}. Normally, the highly reducing environment in the cytoplasm maintains galectin protein stability and activity. Although speculative, we thus hypothesized that by interfering with the redox balance in a cell the galectin functions can be modulated.

Metallothioneins (MTs) are small cysteine-rich proteins which are mainly known for their role in metal homeostasis, detoxification of toxic metals, and the protection against oxidative stress\textsuperscript{81,82}. However, MTs have also been shown to influence the cellular redox state\textsuperscript{83}, and few studies suggest MTs to be involved in angiogenesis\textsuperscript{84,85}. Surprisingly, little is known about the expression of MT isoforms in endothelial cells. Therefore we performed extensive mRNA expression profiling of all known human MTs in EC (Chapter 8). We found a broad MT expression repertoire in endothelial cells that was influenced by different environmental conditions including metal exposure and hypoxia. Moreover, we observed decreased EC proliferation and sprouting as well as mildly increased migration after knockdown of the most prevalent isoform, i.e. MT2A. Future research should reveal whether MT2A could be a valuable target for angiostatic therapy and whether interfering with MT expression indeed affects galectin expression and activity. Moreover, interfering with the cellular redox state will probably not only affect galectin function, but also the stability and activity of other proteins. Therefore, the feasibility of this approach should be thoroughly tested.

Altogether, the studies presented in this thesis further contribute to our understanding of the expression and function of endothelial galectins in (tumor) angiogenesis. We describe novel galectin-9 splice variants in endothelial cells and emphasize the importance of distinguishing between different splice variants for prognostic marker analysis. Furthermore we provide evidence for an angioregulatory role for galectin-9 with divergent functions for different isoforms. Moreover, we provided a new functional link between two protein families, i.e. galectins and chemokines, which are involved in similar processes including inflammation, coagulation and angiogenesis. Interconnection of these processes could have major implications for the development of galectin-targeting drugs as anti-cancer therapy. Finally, we suggest a novel approach of interfering with galectin function, i.e. by modulation of the cellular redox state with metallothioneins, potentially affecting galectin protein stability and activity. Still, many questions and open issues remain to be resolved, but the current findings significantly contribute to the insights on the prognostic value of galectins in cancer and provide important leads for the development of galectin-targeting drugs.
General discussion


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Summary

Galectins as targets for angiostatic cancer therapy
Summary

Galectins are a family of proteins that are characterized by the presence of a conserved carbohydrate recognition domain (CRD). Via this CRD, galectins bind to certain carbohydrates present on glycoproteins and glycolipids. In line with the numerous diverging cellular functions of galectins, the deregulation of these proteins is shown to contribute to various pathologies, including cancer. Indeed, multiple studies confirmed the prognostic and therapeutic value of certain galectins in several types of cancer. It has been shown that galectins facilitate several key steps during tumor progression, such as tumor cell transformation, metastasis and tumor immune escape. In addition, several galectins have been implicated in the sprouting of novel blood vessels from pre-existing capillaries, i.e. angiogenesis. Tumors induce angiogenesis to be able to grow beyond a few cubic millimeters in size. This process provides the tumor with a continuous blood supply, which facilitates the transport of nutrients, cells and gasses. Consequently, the tumor mass can be expanded and a route for metastasis of tumor cells is formed. Therefore, the inhibition of angiogenesis is considered as a promising anti-cancer therapy. Endothelial cells (EC), which form the inner lining of blood vessels, are genetically stable cells and are easily accessible for therapeutics via the blood stream. These characteristics make them attractive targets for angiostatic therapy. Recently, evidence has accumulated that galectins in the tumor endothelium might provide opportunities for the inhibition of angiogenesis. Of the fifteen mammalian galectins, only galectin-1, -3, -8 and -9 are found to be expressed in the endothelium. This expression appears to be differentially regulated upon endothelial cell activation, suggesting a role for galectins in tumor angiogenesis. The knowledge with regard to the expression and function of galectins in endothelial cell biology and angiogenesis as well as the opportunities to target galectins are summarized in chapter 1.

In this thesis, we aimed to assess the prognostic value of galectins in cancer patients. In addition, we set out to further unravel the expression and function of galectins in endothelial cells. Finally, we explored methods to therapeutically target galectins.

To address these aims, several known angiogenesis methods were adapted to study the role of galectins as well as of galectin inhibitors in endothelial cell biology in vitro (Chapter 3) and angiogenesis in vivo (Chapter 4).

In chapter 2, we evaluated the prognostic value of all human galectins in patients with stage I/II non-small cell lung cancer (NSCLC). It is important to identify early stage NSCLC patients with poor survival, since 30-40% of the early stage patients will present tumor recurrence within two years after surgical resection and these patients are likely to benefit from adjuvant chemotherapy. Extensive galectin mRNA expression profiling confirmed the prognostic value of galectin-1 in these patients, where high galectin-1 expression significantly correlated with shorter overall survival (OS) and disease free survival (DFS). This corroborates with other studies which described elevated galectin-1 expression levels to be associated with poor patient outcome. In addition, we assessed the expression of the three main galectin-9
splice variants, and identified galectin-9Δ5 as a novel potential prognostic marker in early stage NSCLC. The observation that different galectin-9 splice variants appear to have different prognostic value suggests diverging functions of the isoforms. Together with the observation that galectin-9 was abundantly expressed in the tumor endothelium, this prompted us to assess the expression of all possible galectin-9 isoforms in endothelial cells, as described in chapter 5. The galectin-9 transcript is subject to extensive post-transcriptional splicing varying in the exclusion of exons 5 and 6, which encode for the linker region between the two CRDs, and exon 10, which encodes the C-terminal CRD. Apart from the three main galectin-9 splice variants (gal-9 full length, gal-9Δ5 and gal-9Δ5/6), we found two additional splice variants, i.e. gal-9Δ5/10 and gal-9Δ5/6/10, lacking also exon 10, which results in a truncated C-terminal CRD. These splice variants were shown to be differentially regulated during endothelial cell activation. The function of the most abundant isoform, i.e. gal-9Δ5, in endothelial cell biology and angiogenesis appeared to depend on cellular localization, local concentration and the context in which the protein was presented to the cells. Overall, gal-9Δ5 appeared to have only a minor inhibitory effect on angiogenesis.

In chapter 6, we further examined the prognostic value of all known galectin-9 splice variants, including the two novel isoforms that were described in chapter 5, in renal carcinoma patients. Here we identified increased gal-9Δ5 and gal-9Δ5/6/10 as novel markers for better patient survival, again stressing the importance of distinguishing between different splice variants. The latter isoform contains a truncated C-terminal CRD, which potentially affects protein function. Therefore, we compared the effects of recombinant gal-9N and gal-9Δ5 on endothelial cell function. As compared to gal-9Δ5, gal-9N appeared to be a more potent inhibitor of endothelial migration in vitro and angiogenesis in vivo, whereas endothelial cell sprouting and transwell migration were more potently stimulated. As was mentioned before, the environmental context appears to be an important determinant of galectin-9 function. Further research into the regulation and functional diversity of different splice variants is warranted for the development of effective galectin-targeted cancer therapies.

Apart from being valuable as prognostic or diagnostic markers, galectins are increasingly appreciated as potential targets for angiostatic cancer therapy. Previously, we identified galectin-1 as the receptor for the angiostatic peptide anginex. The structural and functional similarities between anginex and the endogenous angiostatic chemokine platelet factor 4 (CXCL4), prompted us to hypothesize that galectin-1 could also serve as a functional binding partner for CXCL4. This hypothesis was addressed in chapter 7, where we indeed proved binding between CXCL4 and galectin-1. As a consequence of the interaction, the carbohydrate binding affinity of galectin-1 is altered. However, the functional relevance of this modulation remains elusive. We showed that CXCL4 neutralizes the stimulatory effects of galectin-1 on proliferation and migration of endothelial cells. Whether this is a result of e.g. decreased H-Ras signaling or increased uptake of galectin-1-binding receptors needs further investigation. Interestingly, CXCL4 also affected galectin-1-induced effects in blood platelets. Both platelet activation and
aggregation induced by galectin-1 were potentiated by CXCL4. Possibly, the observed effects on endothelial cell and platelet function can be explained by the altered carbohydrate binding affinity of galectin-1 upon interaction. Alternatively, by binding to galectin-1 the transport of CXCL4 to e.g. distant sites of inflammation might be facilitated, or galectin-1 might interfere with CXCL4 function. Evidently, further research on CXCL4 as a potential galectin-targeting agent should be encouraged.

In general, research on galectin-targeting therapies focuses on interfering with galectin function by using blocking antibodies, carbohydrates or peptides. However, the importance of reducing environments for the correct protein folding and functioning of galectins opens alternative avenues to interfere with protein activity. In chapter 8 we initiated research into a new approach to possibly interfere with galectin function by modulation of the cellular redox state. Metallothioneins (MTs) are small cysteine-rich proteins which are involved in e.g. metal homeostasis, metal detoxification and regulation of the redox balance. In addition, evidence suggests MTs to be involved in angiogenesis, both in vitro as in vivo. Since little is known regarding the expression and function of MTs in EC, we performed extensive expression profiling of all human MTs in endothelial cells. We detected a broad repertoire of endothelial MTs, the expression of which was influenced by different triggers, including hypoxia. In addition, we revealed a role for the most prevalent MT-isoform, i.e. MT2A, in endothelial cell function in vitro. Future research should reveal whether MT2A could be a valuable target for angiostatic therapy and whether interfering with MT2A indeed affects galectin-1 activity.

The research described in this thesis further increased the knowledge on the prognostic value of galectins in cancer and emphasized the importance of distinguishing between different splice variants. Furthermore, we identified novel galectin-9 isoforms with potentially diverging functions in angiogenesis. Moreover, we propose several galectin-interfering strategies which might provide leads for the development of galectin-targeting drugs as angiostatic cancer therapy.
Samenvatting

Galectines als targets voor anti-angiogene kankertherapie
Samenvatting


Het in dit proefschrift beschreven onderzoek had als doel om de prognostische waarde van galectines in kankerpatiënten verder te bepalen. Tevens hebben we de expressie en functie van galectines in endotheelcellen verder onderzocht. Ten slotte hebben we methodes onderzocht om galectines therapeutisch te kunnen targeten.

Om deze onderzoeksdoelen te bereiken werden verschillende reeds bekende angiogenese technieken aangepast om de rol van galectines en galectineremmers in endotheelcelbiologie in vitro (hoofdstuk 3) en angiogenese in vivo (hoofdstuk 4) te kunnen bestuderen.

In hoofdstuk 2 beschrijven we ons onderzoek naar de prognostische waarde van alle humane galectines in patiënten met vroege stadia (I/II) van niet-kleincellige longkanker. Het is belangrijk om binnen deze patiënten diegenen te identificeren waarbij de tumor terugkeert na operatieve verwijdering, omdat deze patiënten waarschijnlijk baat hebben bij adjuvante chemotherapie. Door de galectine-expressie uitgebreid in kaart te brengen hebben wij de prognostische waarde van galectine-1 in deze patiënten bevestigd. Wij zagen namelijk dat hoge galectine-1
Samenvatting

expressie significant correleert met zowel kortere algehele overleving als kortere ziektevrije overleving. Dit komt overeen met andere studies waarin verhoogde galectine-1 expressie werd geassocieerd met een slechtere overleving van de patiënt. Daarnaast hebben we de expressie van drie galectine-9 splicevarianten onderzocht, en hebben we galectine-9Δ5 geïdentificeerd als een nieuwe potentiële prognostische marker in vroege stadia van niet-kleincellige longkanker. Verhoogde expressie was geassocieerd met een betere overleving van de patiënt. Uit bovenstaande resultaten bleek ook dat de prognostische waarde van verschillende galectine-9 splicevarianten van elkaar verschilt, wat zou kunnen betekenen dat deze varianten verschillende functies hebben. Omdat galectine-9 ook tot expressie komt in het tumor endotheel, hebben we vervolgens de expressie van alle mogelijke galectine-9 varianten in endotheelcellen geanalyseerd, zoals beschreven in hoofdstuk 5. Uit dit onderzoek blijkt dat het galectine-9 transcript niet alleen onderhevig is aan post-transcriptionele splicing van exon 5 en 6, die coderen voor een peptidedomine dat twee suikerbindende doeminen verbindt, maar ook van exon 10, dat codeert voor het C-terminale suikerbindende domein. Posttranscriptionele splicing van dit exon resulteert in een getrunceerd C-terminaal suikerbindend domein. Naast de drie reeds bekende galectine-9 splicevarianten (gal-9 full length, gal-9Δ5 en gal-9Δ5/6) vonden we dan ook twee additionele varianten, die naast exon 6 en/of exon 5 ook exon 10 missen (gal-9Δ5/10 en gal-9Δ5/6/10). Wij hebben aangetoond dat de expressie van deze splicevarianten verschillend is en ook verandert tijdens endotheelcelactivatie. Verder onderzoek naar de meest voorkomende vorm, gal-9Δ5, toonde aan dat de functie hiervan afhankelijk was van de cellulaire lokalisatie, de lokale concentratie en de context waarin het eiwit aan de cel wordt gepresenteerd. Daarnaast bleek dat gal-9Δ5 maar een klein remmend effect op angiogenese heeft.

In hoofdstuk 6 beschrijven we het verdere onderzoek naar de prognostische waarde van alle galectine-9 splicevarianten, inclusief de twee nieuwe isovormen, in nierkankerpatiënten. Hierbij hebben we verhoogde gal-9Δ5/6 en gal-9Δ5/6/10 expressie geïdentificeerd als nieuwe markers voor betere overleving, wat opnieuw het belang van het onderscheiden van verschillende splicevarianten benadrukt. Bij de laatste variant, gal-9Δ5/6/10, is het suikerbindend domein aan de C-terminus niet compleet, wat mogelijk de functie van het eiwit beïnvloedt. Daarom hebben wij ook de effecten van het N-terminale domein (gal-9N) op endotheelcellen vergeleken met een galectine-9 waarin beide domeinen nog werkzaam zijn (gal-9Δ5). Vergeleken met gal-9Δ5 vertoonde gal-9N een sterkere remming van endotheelcelmigratie in vitro en angiogenese in vivo. Daarentegen werden endotheelcelsprouting en transwell migratie beter gestimuleerd door gal-9N. Ook hieruit blijkt weer dat de omgeving en context belangrijk zijn voor de functie van galectine-9. Verder onderzoek naar de regulatie en functionele diversiteit van verschillende splicevarianten is noodzakelijk voor de ontwikkeling van effectieve kankertherapieën die gericht zijn op galectine-9.

Naast hun waardevolle bijdrage als prognostische en diagnostische markers worden galectines ook steeds meer beschouwd als targets voor het remmen van angiogenese bij
kankerpatiënten (anti-angiogene therapie). In eerder onderzoek hebben we al beschreven dat galectine-1 een target is voor het anti-angiogene peptide anginex. De structurele en functionele overeenkomsten tussen anginex en plaatjes factor 4 (CXCL4), een natuurlijk voorkomend eiwit met anti-angiogene activiteit, leidden tot de hypothese dat galectine-1 ook een target voor CXCL4 zou kunnen zijn. Het onderzoek naar deze hypothese is beschreven in hoofdstuk 7. We tonen aan dat CXCL4 inderdaad aan galectine-1 bindt. Als gevolg van deze interactie verandert de mate waarin galectine-1 aan bepaalde suikers kan binden. De exacte functionele relevantie van deze veranderde suikerbinding blijft tot op heden onduidelijk. We laten zien dat CXCL4 de stimulerende effecten van galectine-1 op endotheelcelproliferatie en -migratie kan neutraliseren. Of dit een gevolg is van bijvoorbeeld verminderde H-Ras signalering of verhoogde opname van galectine-1-bindende receptoren vereist verder onderzoek. Naast een effect op endotheelcellen, blijkt CXCL4 ook invloed te hebben op de door galectine-1 geïnduceerde effecten op bloedplaatjes. Zowel de door galectine-1 geïnduceerde plaatjesactivatie als plaatjesaggregatie werden versterkt door CXCL4. Mogelijk kunnen de effecten op endotheelcellen en bloedplaatjes worden verklaard door de veranderde binding aan suikers door galectine-1 na de interactie met CXCL4. Daarentegen zou het ook kunnen dat door het binden aan galectine-1 simpelweg het transport van CXCL4 wordt gefaciliteerd, bijvoorbeeld naar plaatsen van ontsteking. Anderzijds zou het ook mogelijk zijn dat galectine-1 juist de functie van CXCL4 verstoort. Hieruit blijkt duidelijk dat er meer onderzoek moet worden gedaan naar de vraag of CXCL4 ingezet kan worden om galectine-1 te remmen als anti-angiogene therapie.

In het algemeen richten anti-galectine-therapieën zich op het verstoren van de functies van galectines door gebruik te maken van antilichamen, suikers of peptides. Het veranderen van de reducerende omgeving die nodig is voor de correcte vouwing en het correcte functioneren van galectines zou echter ook mogelijkheden kunnen bieden om de eiwitactiviteit te verstoren. In hoofdstuk 8 hebben we een begin gemaakt met het onderzoek naar een nieuwe manier om in te grijpen in de functies van galectines, namelijk door het beïnvloeden van de redox status van een cel. Metallothioneïnes (MTs) zijn kleine, cysteïne-rijke eiwitten die betrokken zijn bij o.a. de cellulaire huishouding van metalen, metaалontgiftiging en de regulatie van de cellulaire redoxbalans. Ook zijn er studies die een rol van bepaalde MTs in angiogenese beschrijven. Aangezien er zeer weinig bekend is over de expressie en functie van MTs in endotheelcellen, hebben wij de expressie van alle humane MTs in endotheelcellen bepaald. Daarbij hebben we een breed repertoire van endotheliale MTs gedetecteerd, waarvan de expressie bleek te veranderen onder invloed van o.a. hypoxie. Daarnaast hebben we een rol gevonden voor de meest voorkomende MT-isoform (MT2A) in endothelcelfuncties in vitro. Verder onderzoek moet uitwijzen of MT2A een waardevol target voor anti-angiogene therapie zou kunnen zijn en of het verstoren van MT2A inderdaad de activiteit van galectine-1 beïnvloedt.

Kortom, in dit proefschrift hebben we de kennis over de prognostische waarde van galectines in kanker verder uitgebreid en hebben we het belang van het onderscheiden
van verschillende splicevarianten onderstreept. Daarnaast hebben we nieuwe galectine-9 splicevarianten met mogelijk verschillende functies in angiogenese geïdentificeerd. Ook hebben we verschillende methodes aangedragen om de functies van galectines te verstoren. Dit alles kan mogelijk bijdragen aan het ontwikkelen van medicijnen die galectines targeten als anti-angiogene kankertherapie.
About the author
Curriculum Vitae

Iris Schulkens was born on May 20th 1986 in Maastricht, The Netherlands. She graduated from secondary school (Gymnasium) at the Trichter College in Maastricht in 2004. She continued her education at Maastricht University, where she started with the Bachelor Molecular Life Sciences and graduated Cum Laude in 2007. Subsequently, she started the Master Clinical Molecular Sciences at Maastricht University. During her Master studies she performed an internship at the Angiogenesis Laboratory (Maastricht University) under the supervision of prof.dr. Arjan Griffioen and dr. Judy van Beijnum, where she validated the angiogenic function of genes that were shown to be overexpressed during tumor angiogenesis. After receiving an award for the top 3% best students in 2008/2009, she obtained her Master of Science degree Cum Laude in 2009. She moved to Amsterdam in 2009 to start as a PhD student in the Angiogenesis Laboratory at the VU University. The research was performed under the supervision of her promotor prof. dr. Arjan Griffioen and copromotor dr.ir. Victor Thijssen and the results are presented in this thesis. In September 2014, she started working as a research technician at the department of Radiotherapy, under the supervision of prof.dr. Ben Slotman, prof.dr. Arjan Griffioen and dr.ir. Victor Thijssen, where she continues the ongoing research described in this thesis.
List of publications


Dankwoord
Dankwoord

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