Identifying Proteins Involved in Glioma Angiogenesis: a Proteomics Approach

Dana Mustafa

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Identifying Proteins Involved in Glioma Angiogenesis: a Proteomics Approach

Identificatie van vaatnieuwvorming-gerelateerde eiwitten in gliale tumoren: een "proteomics" benadering

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Dana Adel Mahmoud Mustafa
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إلى قلبين أحبا بلا حدود ...فأعطا بكل وفئ ...إلى الغلا... أمي و أبي

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Introduction

Approximately half of tumors encountered in the brain represent metastases from neoplasms located elsewhere in the body. A large part of the other half represents meningiomas arising from the membranes covering the brain. The remaining group consists of so-called primary brain tumors, which are tumors arising from the cellular components of brain tissue itself. Most of these tumors are called gliomas because they putatively arise from glial cells. Gliomas are the most frequently encountered subtypes of primary brain tumors. Glial tumor cells still display signs of glial differentiation to some extent [1]. Relative to the major cancers affecting humans like lung-, breast -, colonic - and prostate cancer, glial neoplasms are only a minority. The morbidity and mortality of this group is, however, highest of all. Hence, the impact of these tumors on the well-being of the patients and the economic consequences thereof warrants research efforts comparable to those undertaken in the major cancer groups. In several respects gliomas differ from other tumors. They hardly ever metastasize. Further, neoplastic glial cells infiltrate brain tissue diffusely by mechanisms of migration. Because of the diffuse infiltrative character there are no clear-cut tumor borders and therefore, radical surgery is never possible. Another peculiarity of gliomas is their continuous metamorphosis: over time the tumors change their histological appearance. This goes along with increasing genetic instability. The tumors become more cellular; the cells more pleomorphic and finally necrotic areas appear. The metamorphosis goes along with an increase in blood vessels and a change in the structure of the blood vessel walls. Although gliomas are among the most vascularized tumors, it is surprising that anti-angiogenesis therapies have been relatively unsuccessful so far. To improve this situation it is necessary to increase the specificity of the therapeutic targets in the glioma vascularization.

Glioma neovascularization

Neovascularization and tumor cell invasion are essential processes for glioma development and growth[2]. Neovascularization is particularly important to the growth and progression of malignant gliomas and proliferation and hyperplasia of the cellular components of blood vessels are used as indicators of the degree of malignancy of glial tumors [3]. The high level of neovascularization offers targets for anti-angiogenic therapy with a potential impact on the proliferation of these tumors. Neovascularization is a complex process regulated by multiple stimulatory and inhibitory factors modulating the migration and/or proliferation of microvascular cells with the objective of formation of neovasculature from pre-existing vessels. It involves well-coordinated steps including the production and release of angiogenic factors, proteolytic degradation of extracellular matrix (ECM) components to

allow the formation of capillary sprouts, proliferation and directional migration of microvascular cells, finally resulting in the composition of new vessels [4]. Generally, neovascularization falls apart into angiogenesis and vasculogenesis. These respective processes are witnessed in development and also in tumor neovascularization.

Angiogenesis or sprouting angiogenesis is the process characterized by the formation of new blood vessels which sprout from pre-existing vessels. In a non-neoplastic context, this process occurs both during development and in postnatal life [5, 6]. This pattern of neovascularization, localized at the abluminal site of the vessels, is initiated by proteolytic degradation of the basement membrane, after which endothelial cells migrate into the extracellular matrix (ECM) and proliferate. Non-sprouting angiogenesis (also called intussusceptive angiogenesis) is an alternative mode of sprouting angiogenesis characterized by the protrusion of opposing microvascular walls (luminal sites) into the capillary lumen creating a contact zone between endothelial cells [7, 8]. Vasculogenesis is the in situ differentiation of primitive endothelial progenitors known as angioblasts into endothelial cells that aggregate into a primary capillary plexus. Vasculogenesis is predominantly responsible for the development of the vascular system during embryogenesis, but may also occur in postnatal neovascularization [9-11]. The de novo vessel formation takes place by the action of single endothelial cells (ECs) or endothelial precursor/progenitor cells (EPCs) [12], or non-canalized endothelial cell chains (NCECCs) [13] either shed from vessel walls or mobilized from bone marrow. Yet another mechanism of neovascularization which is restricted to tumors is that in which tumor cells take over host vessels (cooption) [13], or form perivascular cuffs around host vessels [14]. After this initial phase additional vascular growth and development of complex vascular beds, including continuous remodeling and adaptation, occurs predominantly by intussusceptive vascular growth (IVG) [7]. An important characteristic of IVG is that it is achieved by an exceedingly low rate of EC proliferation. As compared to sprouting angiogenesis, in IVG blood vessels are generated more rapidly in an energetically and metabolically more advantageous manner, as extensive cell proliferation, basement membrane degradation, and invasion of the surrounding tissue are not required [7].

Structural characteristics of blood vessels in glioma

The development of blood vessels in tumors differs significantly from physiological angiogenesis. The differences include the appearance of aberrant vascular structures in tumors, delayed maturation of the vessels, abnormal blood flow, altered endothelial cell-pericyte interactions and increased permeability. Endothelial cells in tumor vessels are mitotically active, leaky and their many functions and capabilities are disrupted or

altered. It is generally believed that tumor angiogenesis implements normal blood vessels which are recruited and transformed into vascular structures of abnormal distribution and cellular composition. There are indications that despite there are no morphological differences, ECs in high-grade gliomas differ in antigen expression from ECs in low-grade gliomas [15]. Rapid changes in antigen expression by the ECs have been observed already in early passages of cultured ECs. In the early passages there were marked phenotypical and functional differences between the ECs derived from low-grade- high-grade gliomas and normal brain [15]. In order to retrieve most genuine information from ECs it is necessary to use primary tissue materials, that is, in situ models for these investigations. Numerous factors, such as angioproteins and their Tie receptors [16], PDGF-B [17], monocyte chemotactic protein 1 [18], ephrins and Eph-B receptors [19, 20] are likely candidates for the activation of angiogenesis and mediation of endothelial-endothelial and endothelial-pericyte interactions [8]. Tumoral angiogenesis also depends on differential expression of organ-specific cytokines [21-23]. The capillary index of anaplastic gliomas is highest among that of other human tumors [24]. The degree of microvascular proliferation is variably correlated with anaplasia of the tumor cells and indices of tumor cell proliferation. In glioblastoma multiforme, the most common and most malignant glioma, angiogenesis usually takes the form of glomeruloid-like and sarcomatous structures [25]. Increased angiogenesis in high-grade astrocytomas is paralleled by an increased number and density of vessels. There is elongation of vessels and glomeruloid structures are more frequently seen [26]. The level of Ang1 expression under tetracycline control is correlated with the formation of the glomeruloid bodies in xenografts. Ang1 inhibition by blocking of its cognate receptor Tie2 had the opposite effect [27].

The neoplastic vascular structures are often, but not invariably, associated with necrosis and likely originate in hypoxic tumor areas [28]. Not only the physiological response to hypoxia but also genetic alterations, contribute to angioneogenesis. The presence of hypoxic regions within an expanding tumor mass leads to upregulation of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), through increased activity of the transcriptional complex HIF-1 (hypoxia-inducible factor-1) [29, 30]. HIF-1 mediated gene expression may be directly or indirectly modulated by alterations in oncogenes/tumor suppressor genes that occur during astrocytoma development, including PTEN, TP53, p16(CDKN2A), p14ARF, EGFR, and PDGFR [31-34]. Genetic alterations are also believed to influence the HIF-independent expression of pro- and anti- angiogenic factors, such as basic fibroblast growth factor (bFGF) and thrombospondin-1 (TSP-1), respectively [35]. Genetic events that occur during the progression of infiltrating astrocytomas promote angiogenesis, both by modulating hypoxia induced gene expression and by regulating of pro- and anti- angiogenic factors.

Glioma neovascularization and the Blood Brain Barrier

Capillaries of the normal brain consist of a continuous endothelium in which cells are joined by well developed and complex tight junctions; there are no fenestrae, and there are very few plasmalemmal vesicles. It is generally accepted that the maintenance of the blood brain barrier (BBB) is the result of the presence of the endothelial tight junctions, the lack of transcytotic vesicles, the basement membrane, and the tight junctions present among the astrocytic foot processes [36]. In glioma, an exceptionally high degree of vascularization is seen, but the microvessels characteristically lose their normal BBB properties and leak fluid into the neuropilema. The vasculature in gliomas is highly disordered, with numerous vascular shunts, irregular vascular diameters, wide inter-endothelial junctions, large numbers of fenestrated and transendothelial channels, and discontinuous or absent basement membranes [37, 38]. Cerebral edema is the consequence of the disfunction of this vasculature. Ultrastructural investigations of glioma blood vessels have revealed the opening of intermicrovessel endothelial cell tight junctions [5, 39, 40]. The tight junctions are formed by a group of molecules that comprise the proteins occludin, claudin and junctional adhesion molecules. In gliomas it has been noticed that the proteins forming the tight junctions are down-regulated [41-43]. Basically, these proteins are the morphological correlates for the BBB. A loss of tight junction proteins appears to be a central event in the opening of the BBB in gliomas.

Pro- and anti-angiogenic factors

For the process of blood vessel formation in gliomas a complex interplay between tumor cells, endothelial cells, and their surrounding basement membranes is involved. Several growth factors are involved. Over the last decades progress in the understanding of tumor angiogenesis has been made by the revelation of the hypothesized growth factors and their receptors acting on tumor and endothelial cells by paracrine/autocrine loops [44-46]. The several growth factors are distinguished in three classes [47]. The first class consists of factors specifically acting on endothelial cells, e.g., the families of Vascular Endothelial Growth Factor (VEGF) and of the angiopoietins [48]. The second class are direct-acting factors which activate a broad range of target cells besides endothelial cells, e.g., various cytokines, chemokines and angiogenic enzymes [49]. The third class consists of indirectly acting factors with an effect on angiogenesis resulting from the release of substances from macrophages, endothelial or tumor cells, e.g., tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β). Several of these growth factors exhibit angiogenic activity in the adult central nervous system (CNS) under reactive as well as neoplastic conditions. Several angiogenic stimulating factors have been identified in gliomas and

other tumors, including VEGF [50], platelet-derived growth factor (PDGF) [34, 51-53], basic fibroblast growth factor (bFGF) [54-57], TGF-beta [58, 59], and epidermal growth factor (EGF) [60]. These growth factors may influence glioma neovascularization by directly stimulating endothelial cell proliferation, by mediating the expression of key proteases on endothelial cells necessary for neovascularization, or by regulating the expression of VEGF, and interactions among themselves. Tumor neovascularization is believed to be mediated by these soluble factors released from tumor cells and such angiogenic factors may become useful markers of monitoring glioma activity or targets of anti-angiogenic therapy.

Angiogenesis appears to be a balance between angiogenic and anti-angiogenic factors. Some endogenous inhibitors of angiogenesis are known, e.g., angiostatin [61], endostatin [62], antithrombin [63], prolactin [64], thrombospondin [65], Troponin [66], IFN-α [67], IFN-γ [68], pigment epithelium-derived factor (PEDF) [69], CXCL10 (IP-10) [70], Platelet factor 4 [71], interleukin-12 [72], interleukin 4 [73], vascular endothelial growth inhibitor (VEGI) [74], tissue inhibitor of metalloproteinases (TIMP) [75], plasminogen activator inhibitor 1 (PAI1) [76], retinoic acid [77], Angiopoietin-2 (Ang-2) [78], 2-methoxyoestradiol [79]. Anti-angiogenic factors may act either directly or indirectly on endothelial cells [80].

Anti-angiogenic treatment

The promising results obtained with anti-angiogeneic treatment in animal studies have raised high hopes for applications to brain tumor patients. Theoretically, anti-angiogenic agents are powerful weapons against cancer. However, there are serious limitations in their successful application regarding the rather disappointing results obtained in clinical trials so far. Precise understanding of the process of angiogenesis, particularly identification of tumor vessels-specific targets, should lead to new regimens for more efficient anti-angiogenic therapy [81]. In the past decade, more than eighty molecules that displayed anti-angiogenic activity in preclinical studies were tested in clinical trials, but most of them failed to demonstrate any measurable anti-tumor activity and none have been approved for clinical use [24]. Continued advances in understanding the mechanism of the angiogenic process at the biochemical and molecular levels has lead to the discovery of many proangiogenic factors as well as their inhibitors, and anti-angiogenic factors. The off-spin of research in angiogenesis has reached the stage of clinical trials [82]. Current strategies of inhibition of tumor angiogenesis [49, 80] imply the inhibition of pro-angiogenic factors; the application of natural anti-angiogenic substances like angiostatin or endostatin; the inactivation of endothelial cells and the inhibition of molecules which support invasiveness of new blood vessels in surrounding tissue. Several anti-angiogenic drugs are already being used in clinical trials of gliomas [83]. Unfortunately, phase 1 and 2 trials on treatment of

glioblastoma with vatalanib, a small molecule TKI against VEGFR2 yielded disappointing results [84, 85]. Nevertheless, bevacizumab and a VEGFR blocker were effective when applied in combination with standard chemotherapeutic agents [86, 87]. The results were indicative of the antiangiogenic agents enhancing the efficacy of cytotoxic drugs, presumably by normalization of the blood vessels [88]. The permeability of the vessels is normalized because the action of VEGF and VEGFRs is reduced, leading to less edema and restoration of the normal intravascular flow [89-91]. There are also results in animal models suggesting that VEGFR2 blockade with monoclonal antibodies leads to better tissue oxygenation through vessel wall normalization and thus the radiotherapy sensitivity of tumor cells [6, 92, 93]. Identical results were obtained in a trial for the treatment of colonic cancer [94]. Indeed, reduction of interstitial cerebral edema in patients suffering from brain tumors was also obtained in a trial on treatment of glioblastoma [87]. The normalization, however, seems to be a temporary phenomenon and therefore, optimal timing of various treatment modalities is essential for success [95]. The effects of radiation therapy are rather complex and antagonistic: on the one hand, radiation causes apoptosis of endothelial cells [96], while on the other hand it induces VEGF expression which is essential for the survival of endothelial cells [97-99]. Therefore, treatment with radiation in combination with VEGF inhibitors is indicated.

Since neovascularization involves multiple processes mediated by a wide of range of angiogenic inducers, including growth factors, chemokines, angiogenic enzymes, endothelial specific receptors and adhesion molecules, there probably is no single strategy that will be successful all by itself in eradicating solid tumors like gliomas. Therefore, more efficient therapeutic methods, including specific antiangiogenic drugs, targeted drug delivery systems, and the combination of antiangiogenic agents with immunotherapy, chemotherapy or radiotherapy should be explored to render positive results in the future. In addition, as the understanding of the regulatory mechanisms underlying neovascularization improves, we should be in a better position to elaborate novel treatment strategies taking into account the presence of different EC phenotypes such as individual ECs/EPCs, sprouting and non-sprouting patterns. With the development of better tumor model assessment system successful clinical application of antiangiogenic therapies may be achieved.

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Chapter | 2

Introduction II
Combining laser microdissection and proteomics techniques

Dana A.M. Mustafa, Johan. M. Kros, Theo. M. Luider.

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Introduction

Over the last years significant progress in the analysis of the entire genome has triggered efforts to further analyze normal and abnormal protein expression patterns. There is, for instance, an eagerness to discover more and better diagnostic markers for specific diseases so called biomarkers. High expectations of the use of better biomarkers for the purpose of improving diagnosis and enabling targeted or personalized treatment. Human tissues are usually composed of rather complex mixtures of different cell types each has its unique repertoire of proteins. Many techniques have been used for the isolation of pure cell populations and each technique has its advantages and limitations. Immunohistochemistry is an established and relatively easy applicable technique for localizing protein expression. A drawback of immunohistochemistry is the lack of quantitative assessments of proteins. Another method to obtain information about particular cell populations is growing cell cultures in order to amplify target cells. Despite the technical feasibility of this technique, the biological characteristics of the original cells may not reflect the in vitro environment [1]. Alternatively, by using xenografts a better mimicking of the normal situation is reached, but again this method only reflects the real situation of cells in vivo to some extent [2]. Another way of separating cell populations for further investigation is flow cytometry, which has successfully been applied in the study of many disease processes. Flow cytometric analysis is applied to cell suspensions and specific markers for selection of cell population are required. To the best of our knowledge, the combination of flow cytometry and subsequent mass spectrometry has not yet been described for the analysis of solid tissues.

In order to select for specific cell populations in heterogeneous tissues, several micro-dissection techniques have been described. Most techniques involve the use of a needle to scrap off cells of interest under direct microscopic visualization [3, 4]. This method, however, tends to be slow, tedious and highly operator dependent [2]. In 1992, Shibata and coworkers described a new method of cell isolation. They used a specific pigment placed over small numbers of cells in a tissue section, which served as an umbrella preventing the covered cells of being destroyed by ultraviolet light. Ultraviolet light was used to destroy the DNA/RNA of the uncovered cells [5]. Shortly later, laser capture microdissection under direct microscopic visualization was developed by Liotta and coworkers in the National Cancer Institute (NCI). This way of target cell isolation permits rapid, reliable laser microdissection to collect specific cell populations from a section of a complex, heterogeneous tissue [6]. For this approach a tissue section is placed in a holder of an inverted microscope. A transparent, thermoplastic polymer coating [e.g. ethylene vinyl acetate (EVA)] is placed in contact with the tissue. The EVA polymer is positioned over microscopically selected cell clusters and subsequently the polymer is precisely activated by a near-infrared laser pulse steered by the investigator. The laser activation of the polymer results in specific binding to

the targeted area. With the removal of the EVA and the tissue that was bound to it from the section the selected cell aggregates are isolated for molecular analysis [7]. LCM is compatible with a variety of cellular staining methods and tissue preservation protocols [8]. Dependent on the laser microdissection device, the collection caps used are positioned in different ways. For instance, the caps in the PixCell II (Arcturus Engineering, Mountain View, CA) technique make contact with the tissue sections and therefore, strict requirements for preparations are needed. The P.A.L.M laser microdissector (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) provides a powerful separation, in which cutting UV- laser microbeam microdissection (LMM) is combined with laser pressure catapulting (LPC) [9]. A specific glass slide covered with a polyethylene naphthalate (PEN) membrane will aid in stabilizing the morphological integrity of the captured area [10] (Figure 1). In this method, the collecting caps do not make any contact with the tissue sections anymore which increases the flexibility with respect to the section preparation [11]. Both LCM techniques are specific enough to dissect single cells. The P.A.L.M can dissect smaller sections of tissue as compared to the PixCell system. The two methods of microdissection yield RNA retrievals of comparable quality and quantity, but they have not been directly compared with regard to recent developments in protein retrieval by mass spectrometric applications [12]. The collection of large quantities of cells by LCM is a time consuming procedure requiring the microscopical visualization of the cells of interest in a stained tissue sections before lasering. The software and the hardware of the different types of laser microdissection are still underdevelopment.

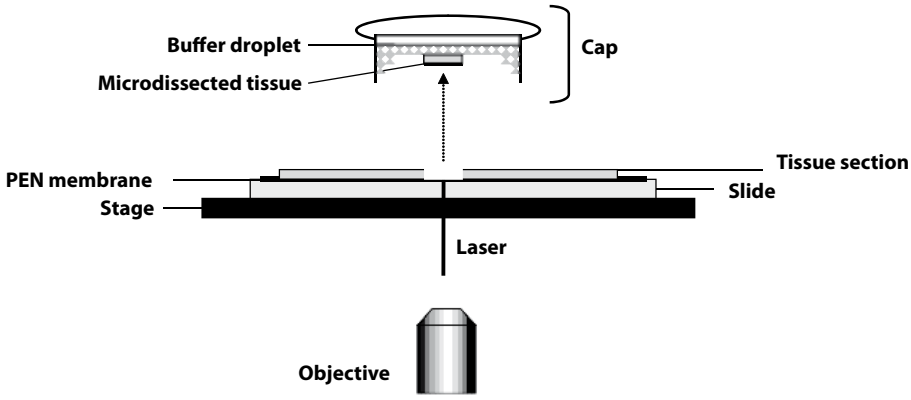

Figure 1
Schematic representation of the principle of the P.A.L.M laser capture microdissection.

LCM and two-dimensional gel electrophoresis (2-D PAGE)

A new development is the application of LCM for protein retrieval of specific cell types for further analysis by proteomic techniques. So far, several approaches have been performed on cells obtained by laser microdissection. In 2000, Emmert-Buck and coworkers applied two-dimensional gel electrophoresis (2-D PAGE) to 50,000 microdissected epithelial cells [13]. They compared tumor cells and normal control tissue from two patients with oesophageal cancer [13]. Staining the gels with silver yielded the visualization of 675 distinct proteins and isoforms. Seventeen differentially expressed protein spots were further analyzed by mass spectrometry. This resulted in the identification of two specific proteins, cytokeratin 1 and annexin I. It was assumed that these proteins were present in an abundance range of 50,000 to 1,000,000 copies per cell [13]. Using colon cancer as a model, Lawrie and coworkers also showed the feasibility of investigating protein expression by combining the technologies of LCM and proteome analysis like 2-D PAGE and mass spectrometry [14].

To overcome the limitation of LCM in producing relatively low numbers of cells, an extra step has been added to the separation method. In addition to the 2 D-PAGE from the microdissected cells, an extra 2 D-PAGE from the whole section of the same set of samples can be useful. The comparison of silver stained 2-D gels created from microdissected epithelial cells of ovarian cancer and the 2-D gels created from the whole section of the same ovarian samples, facilitated the discovery of 23 differentially expressed proteins between low malignant potential and invasive ovarian cancers [15]. In-gel digestion of the specific gel spots followed by MS/MS analysis resulted in the identification of glyoxalase I, RhoGDI, and a 52 kDa FK506 binding protein [15]. In another study based on 2-D PAGE, 315 protein spots were identified by collecting 100,000 cells by laser capture microdissection of normal and cancer ductal units from breast tissue sections [16]. Subsequent measurement of the spots by mass spectrometry resulted in the identification of 57 differentially expressed proteins between the two groups of samples [16].

The relative low number of microdissected cells to be analyzed requires the loading of equivalent amounts of protein on the gels. Shekouh and coworkers (18) followed a strategy to increase the accuracy of 2-D PAGE from LCM samples. The samples were first separated by one-dimensional sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, stained with silver and subsequently subjected to densitometry. Evaluation of the staining intensity was used to normalize the samples. The 2-D PAGE silver stained images from 50,000 microdissected adenocarcinoma cells were compared with the images from whole sections of pancreatic samples. Spots of interest were subjected to MALDI-TOF/TOF mass spectrometry, resulting in the identification of S100A6 as an over-expressed protein in the

pancreatic cancer cells [17]. The same methodology has been used to understand the mechanism of a specific molecule such as (HER-2/neu) in breast cancer [18]. Breast cancer tissue was used for the microdissection of about 50,000 – 70,000 cells from three HER-2/neu-positive tumors and three HER-2/neu-negative tumors. This led to the detection of about 500 – 600 protein spots in each gel. The comparison of these two groups allowed the identification of cytokeratin 19 (CK19) as an over-expressed protein in HER-2/neu-positive breast cancer patients [18]. In another study the 2-D PAGE of 10,000 microdissected cells of hepatocellular carcinoma (HCC) samples was compared with that of normal surrounding tissue. The investigators visualized about 868 spots of which 20 were considered as differentially expressed proteins. The digestion of these proteins into peptides was followed by the application of ESI-MS/MS, which allowed the identification of 11 proteins. Four out of these 11 proteins were considered as novel candidates of hepatitis B-related HCC markers [19]. This approach of separating the microdissected cells on 2-D PAGE followed by in-gel proteins digestion and mass spectrometry measurements for the identification of biomarkers has been applied to a wide range of cancers, using various numbers of microdissected cell. There is a range of 10,000 to 100,000 cells harvested by LMD for the successful application of 2D electrophoresis (Table 1).

LCM and differential in-gel electrophoresis (DIGE)

In 2002, Zhou and coworkers described a new technique called differential in-gel electrophoresis (DIGE) [20]. Two pools of proteins are labelled with 1-(5- carboxypentyl)-1_-propylindocarbocyanine halide (Cy3) N-hydroxy-succinimidyl ester and 1-(5- carboxypentyl)-1_-methylindodi-carbocyanine halide (Cy5) N- hydroxysuccinimidyl ester fluorescent dyes [20]. The labelled proteins are mixed and separated in the same 2-D gel. This strategy improves the sensitivity of detection and enlarges the range of candidate proteins for detection. Molecular weight- and charge- matched cyanine dyes enable multiplex labelling with different samples run on the same gel. The same investigators described a powerful tool for the molecular characterization of cancer progression and identification of cancer-specific protein markers by combining 2-D DIGE with mass spectrometry. They compared the 2-D DIGE of about 250,000 microdissected cells from oesophageal carcinoma with normal epithelial cells from the oesophagus. The cancer cell lysate yielded 1,038 protein spots while the normal epithelial lysate yielded 1,088 protein spots. In-gel digestion of the differentially expressed protein spots was followed by capillary HPLC tandem mass analysis to achieve further identification. This way, tumor rejection antigen (gp96) was found to be up-regulated in oesophageal squamous cell cancer [20]. Applying the same procedure to smaller numbers of microdissected cells from biopsy samples with gastric metaplasia appeared to be successful as well [21]. Approximately 1,200 spots were identified from

30,000 microdissected cells. Twenty- eight of these spots were over expressed in the metaplasia samples as compared to the normal surface cells [21]. However, subsequent MALDI-TOF measurements of the spots did not result in the identification of proteins. The same procedure was applied to 50,000 microdissected cells resulting in the identification of 32 proteins in breast epithelial cancer cells [22], of which thirteen had not been associated previously with the tumors [22]. One technical aspect of the 2-D DIGE method needs special attention: the nature of the fluorescent dyes and their ability to bind to lysine residues only [20]. Proteins with high percentages of lysine residues can be labelled more efficiently as compared to proteins containing little or no lysine. By developing a new generation of dyes reacting with cysteine residues, the sensitivity of DIGE has been improved [23]. Although cysteine is less abundant than lysine in proteins in general, cysteine labelling can be carried to saturation. Lysine labelling must be limited to 1-3% of all the residues to prevent loss of solubility when bulky hydrophobic dyes are coupled to the polar lysine residues [23]. Greengauz-Roberts and coworkers applied the saturated labelling for cysteine residues to study about 5,000 cells obtained by LCM of metaplasia and cancer cells. A total of 1,471 distinct protein features were observed from the relatively small number of cells. Ninety-six of these spots were further identified. Using MALDI-MS and MS/MS measurements in addition to the specific position of the protein in the gel resulted in the identification of 42 proteins in cancer samples [24]. Sitek and co-workers described a novel approach to analyze glomerular proteins from mice and human samples using DIGE saturation labelling [25]. Only ten glomeruli (0.5 µg) picked by LCM from a slide of a human kidney biopsy appeared to be sufficient to visualize 900 spots using DIGE technique [25]. 2-D DIGE holds several advantages over the conventional 2-D gel. One of the most important advantages is the improvement of the reproducibility of 2-D DIGE method. The gel-to-gel differences are minimalized because the separation of the pooled samples takes place in the same gel. Therefore, the comparison of protein expression from two cell populations or samples can be more accurately assessed and easier to be identified. The quantitative differences of protein contents are also better measured by the application of fluorescent dyes. In addition, 2-D DIGE enables a higher throughput analysis of 2D gels by its feasibility to automatic gel imaging. Importantly, the labelling of proteins by fluorescent dyes did not affect the protein identification by mass spectrometry, because only small percentages of the molecules of each protein are labelled. Importantly, for 2-D DIGE the number of microdissected cells, which are required for protein identification is less as compared to the other 2 D electrophoresis techniques (Table 1).

LCM and different labelling techniques

The comparison of the proteome of two different samples (for instance, normal and tumor cells) is facilitated by labelling. In 2004, Li and coworkers described a method for qualitative

and quantitative protein analysis by combining LCM with isotope-coded affinity tag (ICAT) labelling technology and two-dimensional liquid chromatography coupled with tandem mass spectroscopy (2D-LC-MS/MS) [26]. Approximately 50,000 – 100,000 cells of hepatocellular carcinoma (HCC) and non-HCC hepatocytes were microdissected and a total of 644 proteins in HCC hepatocytes were qualitatively determined, and 261 differential proteins between the two groups were quantified [27]. In 2004, $^{16}0/^{18}0$ isotopic labelled peptides were generated from 10,000 microdissected cells of ductal carcinoma of the breast. The approach allowed the identification of 76 proteins [28]. By using reverse phase LC-ESI-MS/MS Zang and coworkers were able to identify proteins that were significantly up-regulated in the breast tumor cells [28]. Separating the radioactive labelled peptides on the high resolution 54 cm serial immobilized pH gradient isoelectric focusing (IPG-IEF) 2D-PAGE gel provided a precise estimate of the abundance ratio for proteins from two samples [29]. The radio-iodination of 3.8 microgram renal carcinoma proteins and 3.8 microgram normal kidney proteins with both ^{125}I and ^{131}I followed by mass spectrometric identification resulted in 29 differentially expressed proteins [29]. Applying the same methodology of radioactive labelling to a pool of microdissected breast cancer cells provided a sensitive method to identify some differentially expressed proteins in correlation with the presence of progesterone receptor (PR) in estrogens receptor (ER)-positive breast cancer [30].

Combining LCM and different separation methods

Previously it was shown that the number of detected and identified peptides and proteins increases significantly by coupling MALDI MS [31] and ESI MS [32] to a peptide or protein separation system. In 2003, Wu and co-workers described a method for discovering biomarkers from microdissected homogeneous cells from breast cancer cell lines [33]. Following capturing the cells, the peptide digest was fractionated by reversed phase HPLC and analysed by ion trap mass spectrometry [33]. HPLC Fractionation of about 10,000 endothelial cells from a breast cancer cell line (SKBR-3) followed by ESI mass spectrometry resulted in the identification of low -expressed proteins in the cell line. Capillary isoelectric focusing combined with the reverse phase nano-LC in an automated and integrated platform provides systematic resolution of complex peptide mixtures generated from limited protein quantities [34]. This method separated the mixture of peptides based on differences in isoelectric points and hydrophobicity and it eliminates peptide loss and analyte dilution [34]. This separation method coupled to ESI-tandem MS assisted in the detection of 6,866 peptides, leading to the identification of 1,820 proteins from 20,000 microdissected cells of glioblastoma [34]. In order to increase the number of identified proteins from LCM of brain samples, Gozal and co-workers added an extra separation step

[35]. After collecting cells by LCM, the total protein content was extracted and resolved on an SDS gel. Gels were cut out into multiple pieces followed by trypsin digestion. Peptides were subjected to highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS). This methodology resulted in identifying hundreds to thousands of proteins [35].

LCM and gel-free mass spectrometry

There are possibilities of measuring the peptide digest of cells harvested by LCM directly by mass spectrometry, without an initial separation step on 2-D PAGE (known as "gel-free mass spectrometry"). Guo and co-workers directly analysed endometrial epithelium cells obtained by LCM using MALDI-TOF/MS [36]. A total of 16 physiologic and malignant endometrial samples including four proliferative and four secretory endometria, and eight endometrioid adenocarcinomas were used for this study. Approximately 2,000 cells appeared to be sufficient to confirm over-expression of two proteins, calgranulin A and chaperonin 10 in the epithelial cells of endometrial adenocarcinoma samples [36]. In another study, the direct analysis of 125 trophoblast and stroma cells of placental tissue resulted in the detection of significant expressed protein differences between these two cell types [37]. Also, differentially expressed proteins between breast cancer and normal samples can be detected by direct MALDI-TOF/MS measurements of 2,000-2,400 LCM cells [38]. In a recent study the possibility of identifying over 1,000 proteins from 3,000 micro-dissected cells by the combination of advanced nanoLC and high resolution FTMS was demonstrated [39].

LCM and Protein chip technology

Currently, there are two approaches to produce arrays capable of generating protein network information. The first method is the forward phase array, in which each spot on the slide represents a specific antibody. The array is incubated with only one test sample [40]. The second method is the reverse phase array in which each spot represents an individual test sample, and the array is composed of multiple, different samples, which then can be tested under the same experimental conditions. In addition, when the arrays are probed separately with two different classes of antibodies, it is possible to specifically detect the total and phosphorylated forms of the protein of interest [40]. By combining LMD technique to protein chip technology, Melle and coworkers identified annexin V as a specific protein in head and neck tumors, and heat shock protein 10 as a biomarker for patients with colorectal cancer [41, 42]. The protein lysates from 3,000 – 5,000 microdissected cells were

analyzed on strong anion exchange arrays and weak cation exchange arrays, followed by separation steps (e.g. 2D-gel or reverse phase chromatography and SDS-PAGE), mass spectrometry measurements and MS/MS analysis [41, 42]. Validation by immunohistochemistry confirmed the findings. In other studies Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Analysis (SELDI-TOF) was applied to microdissected cells because of its sensitivity to smaller amounts of material than other techniques such as 2D gel [43]. Using 30,000-50,000 cells of prostate carcinoma specimens the unique expression of prostate carcinoma–associated protein, called PCa-24 in the epithelial cells, was reached [43]. Protein microarrays hold several technical challenges [44]. Their application offers the advantage of scalability, flexibility and automatic processing [44]. Arrays may also enable the control of key parameters such as temperature, pH and cofactor concentration, which are not easily afforded by cell-based systems.

Perspectives of LCM and mass spectrometry analysis

The application use of laser capture microdissection of (relatively) pure populations of cells to be used for further analysis of their proteome is an important addition to the arsenal of techniques in bioscience. However, this technique is still time consuming and yield relatively small numbers of cells. To overcome this problem alternative steps of processing tissues are needed. Sample collection and reproducible preparation is crucial. During the microdissection procedure, special attention should be taken to prevent waist and contamination of target material. For instance, material may not drop from, or stick to, the cap of the tubes used. Another consideration is to minimize the steps of transferring the collected material from one tube into the other. Therefore, the use of low protein binding tubes is recommended.

2-D PAGE is a well-established technique which has been used in combination with LCM in many studies so far. The need of relative large numbers of cells blocks the possibility to measure large numbers of samples as indicated in Table 1. In addition, the relative low reproducibility is proven by sound statistical analysis. 2-D DIGE improves this reproducibility and also lowers the amount of microdissected tissue.

Recently, the improvement of resolution and detection limits of modern mass spectrometers, particularly FTMS, opened a new research field to analyze small numbers of microdissected cells (in the range of 200 to 5,000). FTMS has specific characteristics, unrivalled high mass resolution (in the order of 100,000 to 1,000,000), high mass accuracy (below 1 ppm), dynamics (three to four orders of magnitude) and good signal to noise ratio [45]. These features facilitate combining this technique with LCM. For instance, by MALDI-

FTMS, peptide digests of no more than 150 cells taken from biological samples (e.g. glioma vessel tissue) resulted in informative mass spectra (Figure 2). It is expected that techniques like FTMS soon will be implicated in the practice of routine laboratories for the detection of disease-related proteins in clinical specimens.

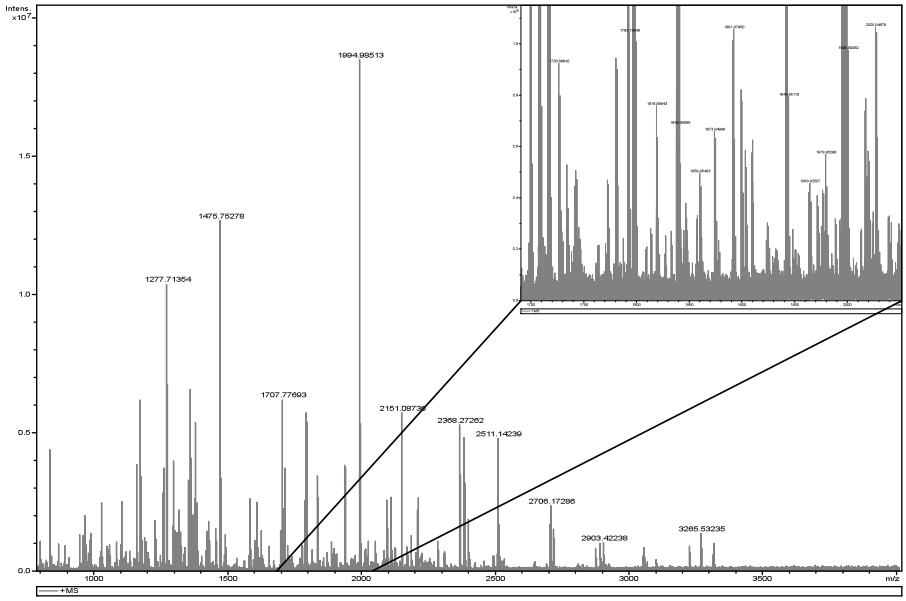

Figure 2

MALDI FTMS spectrum obtained from 150 microdissected cells from a frozen glioma tissue sample. The spectrum contains approximately thousand monoisotopic peaks between 700-3,000 m/z at relative high peak intensities. The small box represents a magnification of a small part of the spectra, between 1,700-2,000 m/z. It shows the very high numbers of peaks obtained from measuring a very small number of cells. The peaks can be identified by different sequencing MS techniques; some examples of identified peptides are indicated in the spectrum.

Table 1

Overview of different methods to combine laser microdissection and different proteomics techniques

Separation technique	Number of microdissected cells/sample	Number of visualized proteins	Identification technique	Number of significant differentially identified proteins	Number of samples/ study	Tissue used	Reference
2-D PAGE, silver staining	50,000	approximately 675 distinct proteins including isoforms	mass spectrometry and immunoblot analysis	n=2; cytokeratin 1 and annexin I	2 cancer samples and 2 normal samples	esophageal cancer	[13]
2-D PAGE, silver staining	1–5 µg of total cellular protein.	not determined	mass spectrometry data from all the protein spots cut from the gels	n=3; cytokeratin 8 cytokeratin 18 and β-actin	2 cancer samples and 2 normal samples	colon cancer	[14]
2-D PAGE, silver staining	50,000	23 differentially expressed proteins were discussed	ESI-MS identification from gels made of whole sections	n=3; FK506 binding protein, glyoxalase I, and RhoGDI	3 invasive OV and 2 noninvasive (LMP) OV.	ovarian cancer	[15]
2-D PAGE, silver staining	100,000	315 protein spots	MS identification from gels made of whole sections	n=57 observed proteins. n=2 after confirmation	6 samples of DCIS and 6 samples of normal ductal/ lobular units.	breast cancer	[16]

2-D PAGE, silver staining	50,000	800 protein spots	MALDI-TOF/TOF	n=1; calcium-binding protein, S100A6	4 cancer samples and 4 normal samples	pancreas cancer	[17]
2-D PAGE, silver staining	50,000–70,000	500–600 protein spots	MALDI-TOF mass spectrometer	n=7; cytokeratin 19, tropomyosin 3, aldolase A, glyoxalase I, cathepsin D chain 3, albumin and MnSOD	3 HER-2/*neu*-positive samples and 3 HER-2/*neu*-negative samples	HER-2/*neu*-positive breast cancer cells	[18]
2-D PAGE, silver staining	10,000	868 protein spots	nano-flow ESI-MS/MS	n=11 proteins, four of them were novel markers	10 hepatic cancer cells samples	hepatic cancer cells. hepatitis B positive cells	[19]
2-D DIGE, lysine specific dyes	250,000	1,038 - 1088 protein spots	Capillary LC tandem mass analysis	n=1; tumor rejection antigen (gp96)	One sample contained normal and one sample contains cancer cells	esophageal carcinoma	[20]
2-D DIGE, lysine specific dyes	30,000	1,200 protein spots	MALDI-TOF measurements	No further identifications	One sample contained gastric mucosa and one SPEM.	gastric metaplasia samples	[21]

2-D DIGE, lysine specific dyes	50,000	not applicable	MALDI-TOF and/or immunoblotting for protein identification	n=32	Five samples contained malignant and normal breast tissue	breast epithelium cell	[22]
2-D DIGE, cysteine specific dyes	5,000	~ 1,000 protein spots	MALDI-MS and MS/MS measurements	n=40	cultured oncogene-transduced epithelial cells and precancerous versus cancerous tissue,	Gastric adenocarcinoma	[24]
2-D DIGE, cysteine specific dyes	between 100-10 glomeruli which equals to 0.5-3 µg protein.	between 1400-900 protein spots	nano LC-ESI-MS/MS	n=23 between mice glomeruli and mice cortex.	3 different protein extracts from human glomeruli and 3 independent isolated glomeruli and cortex from 3 mice	kidney glomeruli.	[25]
(IPG-IEF) 2D-PAGE gel	3.8 µg proteins	not applicable	Mass spectrometry	n=29	2 samples contained renal cell carcinoma and normal kidney tissues	renal carcinoma	[29]

(IPG-IEF) 2D-PAGE gel	Approximately <180 ng per multiplex protein sample per 54-cm gel	not applicable	Mass spectrometry	Quantitative differences in 6 progesterone receptor proteins	12 ER1/PR2 and 12 ER1/PR1 tumors were grouped into four pools.	breast cancer	[30]
HPLC system	10,000	not applicable	ESI mass spectrometry followed by MS/MS	n= 9	3 slides from the same cell culture.	breast cancer cell line (SKBR-3)	[33]
$^{16}O/^{18}O$ isotopic labelling peptides	10,000	not applicable	The reverse phase of LC-ESI-MS/MS on the ion trap mass spectrum	n=76	2 samples with invasive ductal carcinoma of the breast.	ductal carcinoma of the breast	[28]
gel free method	30,000-50,000	not applicable	SELDI-TOF/ MS	n=1; prostate carcinoma–associated protein (PCa-24)	17 prostate carcinoma that contained normal tissue and BPH tissue and 7 BPH samples	prostate cancer	[43]
gel free method	~2,000	not applicable	MALDI-TOF/MS	n=2; calgranulin A and chaperonin 10	8 endometrioid adenocarcinomas, 4 proliferative endometria and 4 secretory endometria	endometrial cancer	[36]

gel free method	150	not applicable	MALDI-TOF/MS	No protein identifications. unique peptide pattern of ~35 peptides for trophoblast and stroma cells	one placenta sample contained trophoblasts and surrounding stroma cells.	placenta samples	[37]
gel free method	2000–2400	not applicable	MALDI-TOF/ TOF mass spectrometry	No protein identifications. 9 differentially expressed peptides	6 invasive ductal breast carcinoma contained cancer and normal cells	breast cancer	[38]
gel free method	3,000	not applicable	nano LC-FTICR mass spectrometry	n=1,003 proteins identified	2 replicate samples of breast cancer epithelial cells	breast cancer	[39]
ProteinChip technology	3,000 – 5,000	not applicable	Isolation by two-dimensional gel electrophoresis and tandem mass spectrometry analysis	n=1; annexin V	57 head and neck tumor samples and 44 mucosa samples	head and nick cancer	[42]
ProteinChip technology	3,000 – 5,000	not applicable	Isolation by reverse-phase chromatography and SDS-PAGE then identified by MS/MS analysis	n=1; heat shock protein 10	39 colorectal tumor samples, 40 normal mucosa samples and 29 adenoma samples	colorectal cancer	[41]

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Chapter | 3

Identification of glioma neovascularisation-related proteins by using MALDI-FTMS and nano-LC fractionation to microdissected tumor vessels

Dana A.M. Mustafa; Peter C. Burgers; Lennard J. Dekker; Halima Charif; Mark K Titulaer; Peter A.E. Sillevis Smitt, Theo M. Luider; Johan M. Kros.

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Abstract

The identification of angiogenesis-related proteins is important for the development of new anti-angiogenetic therapies, and such proteins are potential new biomarkers for gliomas. The aim of this study was to identify proteins which are exclusively present in glioma neovasculature and not in the vasculature of normal brain. We combined advanced proteomic techniques to compare the expression profiles of microdissected blood vessels from glioma with blood vessels of normal control brain samples. We measured the enzymatic generated peptide profiles from these microdissected samples by matrix-assisted laser desorption\ionization Fourier transform mass spectrometry (MALDI-FTMS). Subsequently, the samples were fractionated by nano-LC prior to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF). This combined approach enabled us to identify four proteins which appeared to be exclusively expressed in the glioma blood vessels. Two of these proteins, fibronectin and colligin 2, were validated on tissue sections using specific antibodies. We found that both proteins are present in active angiogenesis in glioma, other neoplasms and reactive conditions in which neo-angiogenesis takes place. This work proves that gel-free mass spectrometric techniques can be used on relatively small numbers of cells generated by microdissection procedures to successfully identify differentially expressed proteins.

Introduction

Gliomas are the most common primary brain tumors, the incidence in the USA is about ~25,000 new cases per year [1]. The diagnosis of these tumors and the decisions regarding therapy are based almost exclusively on histopathology [2, 3]. Diffuse gliomas are highly infiltrative and heterogeneous. Gliomas are among neoplasms with highest degree of vascularisation [4]. The growth of gliomas largely depends on their blood supply, the elimination of which would result in the destruction of these tumors [4]. Despite the elucidation of many genetic aberrations of gliomas over the last decades [5, 6], only few useful biomarkers or therapeutic targets have been found so far [7]. In a previous study, we identified glioma-related proteins by using two-dimensional polyacrylamide gel electrophoresis (2D PAGE) followed by matrix-assisted laser absorption/ionization-time of flight-mass spectrometry (MALDI-TOFMS) analysis. By using specific antibodies raised to *I*-CaD on tissue sections of glioma it was shown that this protein is exclusively expressed in the neovasculature [8] and that it is a potential serum marker for glioma [7]. Rapid and major developments in proteomic technology and methodology over the last decade has opened a new stage in the identification of proteins [9]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) recently became available as a flexible tool in the search for disease markers [10]. Moreover, the recently introduced technique of matrix-assisted laser desorption\ionization Fourier transform mass spectrometry (MALDI-FTMS) provides a powerful technique for accurate peptides mass measurements [11]. This technique has successfully been used for studies in protein interactions and post-translational modifications of proteins [12]. The addition of a technique for pre-fractionation of test samples, such as nano-liquid chromatography prior to mass spectrometry, increases the number of identified proteins significantly [13].

The use of laser microdissection has become an important tool in biological research to isolate relatively pure cell populations from heterogeneous frozen tissue samples [14]. This technique became widely used for tracing genotypical aberrations including aberrant RNA and protein expression of subsets of cells and tissues [15]. Although primary brain tumors have been subjected to direct-tissue profiling and imaging mass spectrometry techniques [1, 16], to the best of our knowledge, laser microdissection of brain blood vessels has never been used before in proteomic analysis.

The aim of this study was to identify proteins which are specifically expressed in glioma vasculature, but not in the normal blood vessels of the brain. To this end, microdissected hypertrophied glioma vessels and normal blood vessels of the brain were used. The peptides of the enzymatically digested proteins derived from the small numbers of cells obtained by microdissection, were measured by MALDI-FT mass spectrometry. The identification of differentially expresses peptides was achieved by combining nano-LC fractionation of

samples with offline MALDI-TOF/TOF and MALDI-FTMS measurements. For validation of our findings specific antibodies were used.

Material and methods

Sampling

Ten fresh-frozen samples of glioblastoma located in the cerebral hemispheres and 10 samples of normal control hemispheric brain were taken form the files of the Department of Pathology, Erasmus MC, Rotterdam (Table 1). Sections of 5 µm from each sample were made, counterstained and examined by the neuropathologist (JMK) to verify the presence of proliferated tumor vessels (Figure 1). The control samples of normal brains were subjected to the same procedure for the identification of the blood vessels.

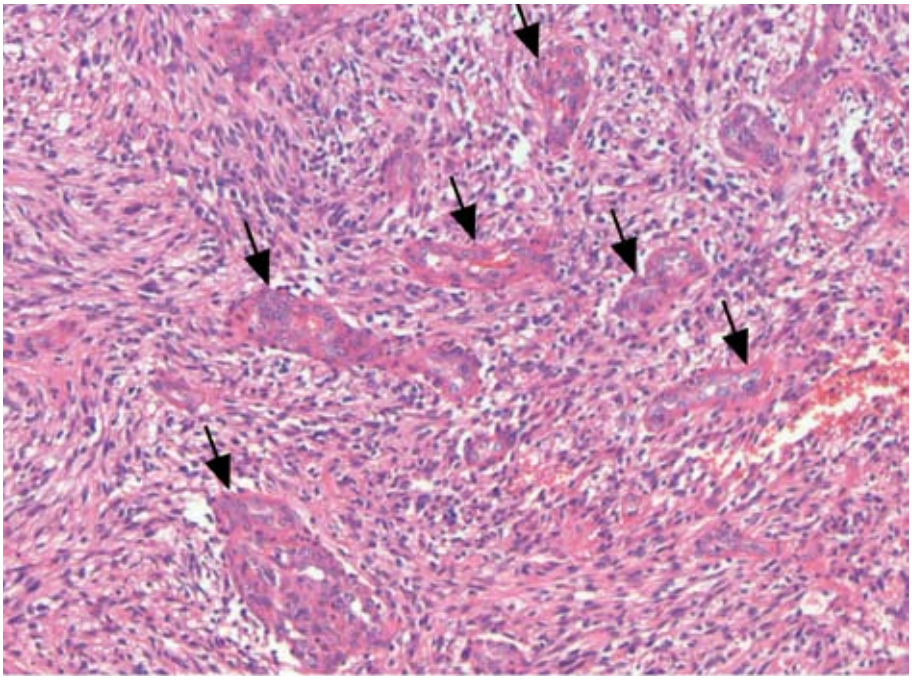


Figure 1
Hypertrophied vessels in high-grade glioma
Hypertrophied blood vessels in a glioma tissue sample (arrows; H&E stain, x 250).

Table 1
Clinical data

Glioma samples	Gender	Age (yrs)	Tumor location
G1	m	57	Ri F
G2	m	57	Le T
G3	m	55	Ri F
G4	m	51	Ri F
G5	m	51	Le T
G6	m	48	Le F
G7	m	47	Ri O
G8	m	36	Le P
G9	m	32	Bi F
G10	f	30	Ri F
Normal brain samples	**Gender**	**Age (yrs)**	**Cause of death**
N1	f	76	pneumonia
N2	f	62	Cirrhosis + hepatocellular carcinoma
N3	m	62	Ischemic cardiac disease
N4	f	60	nasopharyngeal carcinoma
N5	m	48	SAB / aneurysm
N6	f	48	SAB / aneurysm
N7	f	39	SAB / aneurysm
N8	m	34	Brain stem abscess
N9	m	28	hypertensive stroke
N10	m	24 (weeks)	intra-uterine infection

F = frontal; P = parietal; T = temporal; O = occipital
Ri = right; Le = left
f = female; m = male
SAB = subarachnoidal hemorrhage

Laser Capture Microdissection

Cryosections of 8 μm were made from each sample and mounted on polyethylene naphthalate (PEN) covered glass slides (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) as described previously [17]. The slides were fixed in 70% ethanol and stored at -20°C for not more than 2 days. After fixation and immediately before microdissection, the slides were washed twice with Milli-Q water, stained for 10 seconds in haematoxylin, washed again twice with Milli-Q water and subsequently dehydrated in a series of 50, 70, 95 and 100% ethanol solution and air dried. The P.A.L.M. laser microdissection and pressure catapulting (LMPC) device, type P-MB was used with PalmRobo v2.2 software at 40x

magnification. Estimating that a cell has a volume of 10x10x10µm, we microdissected an area of about 190,000 μm^2 of blood vessels and another area of the same size of the surrounding tumor tissue from each sample, resulting in approximately 1,500 cells per sample. A total of 40 samples were collected, viz., 10 glioma vessels, 10 fields of glioma tissue surrounding the glioma vessels, 10 normal vessels and 10 fields of normal tissue surrounding the normal vessels. As a negative control, a corresponding area of the PEN membrane only was microdissected and analysed in the same way as the other samples. This negative control experiment was performed in 3-fold.

The microdissected cells were collected in the caps of P.A.L.M. tubes in 5 µl of 0.1% RapiGest buffer (Waters, Milford, MA, USA). The caps were cut and placed onto 0.5 ml Eppendorf protein LoBind tubes (Eppendorf, Hamburg, Germany). Subsequently, these tubes were centrifuged at 12,000 g for 5 minutes. To make sure that all the cells were covered with buffer, another 5 µl of RapiGest was added to the cells. After microdissection, all samples were stored at -80°C.

Sample Preparation

After thawing the samples, the cells were disrupted by external sonification for 1 minute at 70% amplitude at a maximum temperature of 25°C (Bransons Ultrasonics, Danbury, USA). The samples were incubated at 37°C and 100°C for 5 and 15 minutes respectively, for protein solubilisation and denaturation. To each sample, 1.5 µl of 100 ng/ µl gold grade trypsin (Promega, Madison, WI, USA) in 3 mM Tris-HCL diluted 1:10 in 50 mM NH_4HCO_3 was added and incubated overnight at 37°C for protein digestion. To inactivate trypsin and to degrade the RapiGest, 2 µl of 500 mM HCL was added and incubated for 30 minutes at 37°C. Samples were dried in a speedvac (Thermo Savant, Holbrook, NY, USA) and reconstituted in 5 µl of 50% acetonitrile (ACN) / 0.5% trifluoroacetic acid (TFA) / water prior to measurement. Samples were used for immediate measurements, or stored for a maximum of 10 days at 4°C.

MALDI-FTMS Measurements and Data Analysis

MALDI-FTMS measurements

Samples were spotted onto a 600/384 anchorchip target plate (Bruker Daltonics, Leipzig, Germany) in duplicate. Half a microliter of each sample was mixed on the spot with 1 µl of a 2,5-dihydroxybenzoic acid (DHB) matrix solution (10 mg/mL in 0.1% TFA) / water and the mixture was allowed to dry at ambient temperature. The MALDI-FTMS measurements were performed on a Bruker Apex Q instrument with a 9.4 T magnet (Bruker Daltonics, Bremen, Germany). For each measurement, 450 scans of 10 shots each were accumulated with 60% laser power. Mass spectra were acquired in the mass range of 800 to 4,000 Da. FTMS spectra were processed with a Gaussian filter and 2 zero fillings.

MALDI-FTMS external and internal calibration

A standard peptide calibration mix (Bruker Daltonics, Leipzig, Germany) which contains angiotensin I and II, substance P, Bombesin, Renin Substrate, ACTH clip 1-17, ACTH clip 18-39 and Somatostatin 28 was used for external calibration. To obtain better mass accuracies, an additional post-acquisition internal calibration step in DataAnalysis v3.4, built 169 software (Bruker Daltonics, USA) was performed. Ubiquitous actin peptide masses (m/z 1198.70545, 1515.74913, 1790.89186, 2215.06990 and 3183.61423) were used for internal calibration. To assess the accuracy of the measured masses, the peptides derived from keratin [Q8N175] present in the samples were compared to the calculated masses (1165.58475, 1234.67896, 1365.63930, 1381.64814, 1390.68085, 1707.77211, 1797.01161 and 2096.04673).

Data Analysis

Mono-isotopic peaks with S/N > 3 were annotated with the SNAP algorithm using the pre-release version of DataAnalysis software package (v3.4, built169). The peak lists were saved in a general text format, which was used as an input for a home made script in the R-program, (www.r-project.org). With this script a matrix file was generated, indicating the presence or absence of each peptide mass in the different mass spectra [18, 19]. If a specific peptide appeared at least in 5 samples for each group and never appeared in the other groups, (Fisher's exact p-value < 0.01) it was considered as a group specific peptide. In this way, a list of differentially expressed peptides was generated. These masses of the differentially expressed peptides were submitted to the MASCOT search engine (Matrix Science, London, UK) using the SWISS-PROT (40.21) database, allowing 1 ppm peptide mass tolerance and one missed trypsin cleavage site. In addition, we performed Hierarchical Clustering based on masses and the group of samples using the matrix file in the Spotfire software (Spotfire, Somerville, MA, USA).

Sample Preparation for Nano-LC

Sample G8 was selected for fractionation (Table 1). One, 4 and 8 frozen sections were made, respectively. These sections from the entire tumor sample including the vessels were prepared as described above. Each section contained about 2,000,000 cells of which an estimated 10% were blood vessel derived cells. Twenty µl RapiGest buffer was added (Waters, Milford, MA, USA) to the frozen sections followed by 1 minute sonification, 5 minutes at 37°C and finally 15 minutes at 100°C. For each section 1 µl of 100 ng/ µl gold grade trypsin (Promega, Madison, WI, USA) in 3 mM Tris-HCL was added and samples were incubated overnight at 37°C. Finally, 50 mM HCL was added. For comparison, 8 sections from normal brain sample N5 were prepared in exactly the same way.

In addition, an area of about 900,000 μm^2 of blood vessels from each of the glioma samples and the normal control samples were microdissected and pooled, resulting in one sample of glioma blood vessels and one sample consisting of control blood vessels. Pooling of the

samples was necessary because the nano-LC procedure requires far more tissue than obtained by microdissection. Twenty µl RapiGest buffer was added and the samples were stored at -80°C. All the samples were subjected to the nano-LC fractionation immediately after preparation.

Fractionation by Nano-LC

Fractionation was performed using a C18 Pep Map column (75 µm i.d. x 150 mm, 3µm, Dionex, Sunnyvale, CA, USA). Five µl of the sample was loaded onto the trap column (300 µm i.d. x 5mm, 5µm, Dionex, Sunnyvale, CA, USA). Fractionation was performed for 130 minutes with a gradient of buffer A (100% H_2O, 0.05% TFA) and buffer B (80% ACN, 20% H_2O and 0.04% TFA); 0 to 15 min, 0% buffer B, 15.1 min 15%, 75 min 40%, 90 min 70%, 90.1-100 min 95%, 100.1 min 0% and 130 min 0%. Fifteen second fractions of the sample were spotted automatically onto 384 prespotted anchorchip plates (Bruker Daltonics, USA) containing α- cyano-4-hydroxycinnamic acid (HCCA) matrix, using a robotic system (Probot Micro Fraction Collector, Dionex, Sunnyvale, CA, USA). To each fraction, 1 µl water was added. Finally, we used a 10mM $(NH)_4H_2PO_4$ in 0.1% TFA/water solution to wash the pre-spotted plate for 5 seconds to remove salts. The plates were subsequently measured by automated MALDI-TOF/TOF (Ultraflex, Bruker Daltonics, Germany) using WARLP-LC software. This software obtains MS spectra of each individual spot and subsequently performs MS/MS on each peptide. The best spots for performing the MS/MS measurements were determined automatically by the WARLP-LC software. A file containing the MS and the MS/MS peak lists was submitted to the MASCOT search engine (Matrix Science, London, UK) using the SWISS-PROT (40.21) database allowing 150 ppm parent mass tolerance, 0.5 Dalton fragments tolerance and one missed trypsin cleavage site. In addition, identification was confirmed by exact mass measurements on the MALDI-FTMS, adding 1 µL DHB solution to the fractionated spot and allowed to dry.

Backward database searching

By *in silico* digestion of the identified proteins, theoretical peptides were generated which were sought in the mono-isotopic peaks of the MALDI-FTMS. The accession number for all of the identified proteins was entered into the peptide cutter program (www.expasy.org/tools/peptidecutter), choosing trypsin as enzyme for digestion and allowing one trypsin missed cleavage site. All the possible tryptic fragments from each protein were compared with the peptide masses obtained by MALDI-FTMS within 0.5 ppm (the internal calibration). The distribution of the matched peptides over the four groups was checked manually.

Immunohistochemical staining

The expression of fibronectin and colligin 2 in glioma blood vessels was confirmed by immunohistochemistry using specific antibodies against these proteins on parafin sections

of the samples. We first confirmed our results using the ten glioma samples and the ten normal brain samples which were used in our proteomics approach. To investigate the expression variation between the two groups, an additional six samples of glioma and four samples of normal brain were examined. In addition, a series of other gliomas, carcinomas, vascular malformations, other reactive conditions in which neo-angiogenesis takes place and tissues with notorious neo-angiogenesis were also tested for the presence of these proteins. Immunohistochemical staining was performed following the manufacture procedure (alkaline phosphatase technique), using rabbit polyclonal antibody for fibronectin at a 1:1,000 dilution (DakoCytomation, Glostrup, Denmark) and mouse monoclonal antibody for colligin 2 at a 1:500 dilution (Stressgene, Victoria, B.C., Canada). Five µm paraffin sections were mounted onto poly-L-lysin coated microslides, deparaffinized in xylene for 15 minutes and rehydrated through graded alcohol, then washed with water. The sections were washed with phosphate-buffer saline (PBS) and incubated with the antibody for 30 minutes. After washing the sections with PBS, the corresponding antigen was added and incubated 30 minutes at room temperature. New Fuchsin Alkaline Phosphatase Substrate Solution was freshly prepared and the sections were incubated for about 30 minutes. Afterwards, the sections were washed with tap water, counterstained and cover-slipped with permanent mounting medium.

Results

FTMS measurements

The MALDI-FTMS measurements of the microdissected samples yielded approximately 700 – 1,100 mono-isotopic peaks for almost all spectra. Only one glioma vessel and one normal tissue sample contained less than 100 peaks. However, these spectra were not excluded from our analysis. An accuracy of 3 ppm was obtained by external calibration using a standard peptide calibration mix. After internal calibration the accuracy increased below 0.5 ppm (method described above).

From a comparison of the three control samples and the rest of the samples, it appeared that all spectra contained background signals originating from the PEN membrane of the slides, the buffer and keratin contamination. No peptides resulting from auto-digestion of trypsin were found in the spectra. None of the background signals were found among the list of differentially expressed peptides.

FTMS data analysis

Following our strict criteria, a list of 16 differentially expressed peptides was obtained (Table 2). All 16 peptides were expressed in the glioma vessel group only. The MASCOT data

Table 2
List of differentially expressed peptides

Peptides measured masses	p-value	Number of samples in which these peptides were found:			
		Glioma vessels	Glioma surrounding tissue	Normal brain vessels	Normal brain surrounding tissue
1926.04620 *	0.0004	8	0	0	0
2470.32072 *	0.0050	6	0	0	0
1116.54323	0.0050	6	0	0	0
2157.10653	0.0050	6	0	0	0
2642.21770	0.0050	6	0	0	0
2257.07971 *	0.0136	5	0	0	0
1659.80041 *	0.0136	5	0	0	0
1275.55961 *	0.0136	5	0	0	0
1593.81172 *	0.0136	5	0	0	0
1807.90584 *	0.0136	5	0	0	0
1535.72354 *	0.0136	5	0	0	0
2089.00769	0.0136	5	0	0	0
1731.89535	0.0136	5	0	0	0
2164.00992	0.0136	5	0	0	0
2530.25829	0.0136	5	0	0	0
1849.85488	0.0136	5	0	0	0

* Peptides resulted in protein identification

base search resulted in matching of four out of the 16 peptides to fibronectin precursor protein [P02751]. In order to exclude that matching of the four peptides to fibronectin was just by chance, the following database searches were performed. We added the integers 10, 11, 12, until 30 Daltons to the masses of the 16 peptides which were found for 20 additional searches. By this procedure no proteins were found to match by chance with four peptides. At maximum, only one peptide matched to one protein in the MASCOT database. This virtually ruled out the possibility of randomly finding fibronectin.

Figure 2 shows the result of the unsupervised cluster analysis in two directions; peptide masses and groups of samples in the Spotfire program (Figure 2). A cluster of eight glioma vessel samples is observed. From the two samples which did not cluster, one had a poor spectrum (<100 peaks); this sample clustered with the sample from normal tissue at the top of the heat map which also displayed a poor spectrum. The other one did not cluster with any group. Within the peptide masses, a specific pattern of glioma blood vessels is recognized.

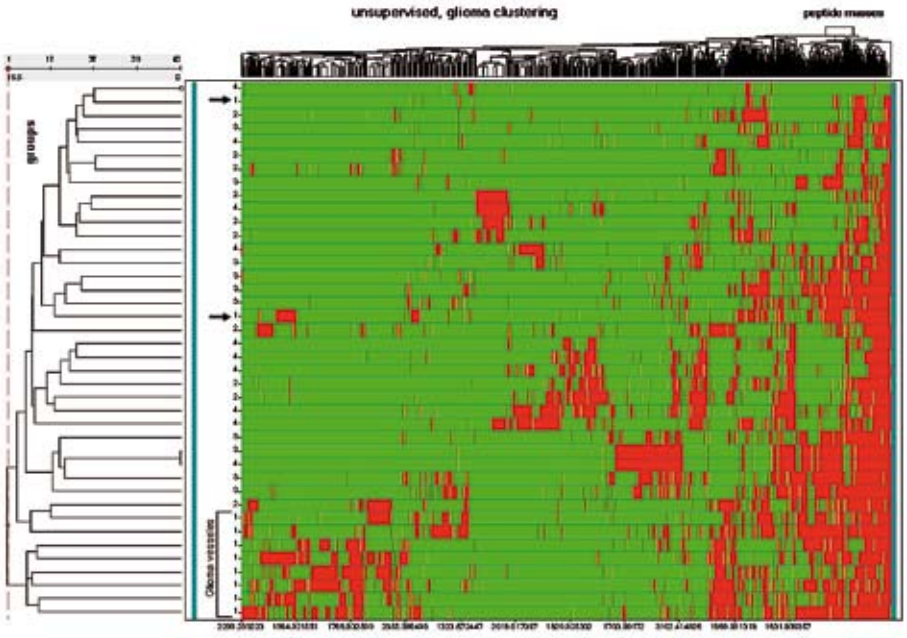


Figure 2

Heat map of unsupervised clustering of the four groups by Spotfire

Result of unsupervised cluster analysis. The peptide masses are displayed on the *x*-axis and the samples ordered by groups are displayed on the *y*-axis. Group 1 = glioma blood vessels; group 2 = normal brain blood vessels; group 3 = glial tumor tissue; group 4 = normal brain tissue. Blocks in red indicate the presence of a specific peptide in the spectrum of the sample. Unsupervised analysis of the data resulted in clustering of eight out of the ten glioma blood vessel samples (group 1). One of the two samples which did not cluster in this group (arrow) appeared to have a spectrum of relatively poor quality and clustered with a sample taken from normal brain tissue, which also was of poor quality, at the top of the heat map. The other glioma sample (arrow) did not cluster. Within the group of glioma blood vessels an exclusive peptide pattern is observed.

Nano-LC Fractionation / MALDI-TOF-MS/MS

Pooling the small number of cells collected by microdissection before nano-LC fractionation resulted in the identification of some highly abundant proteins, among which fibronectin. To identify more proteins, we increased the number of cells by using whole sections of glioma and normal samples. The number of identified peptides was increased and the maximum was reached with the injection of eight sections (Table 3). The capacity of the nanoLC column did not allow further expansion of the number of sections. Fractionation of eight sections led to the significant identification of 189 proteins, with a minimum mowse score of 24 for MS/MS.

Table 3
Results for the various numbers of sections used for fractionation in the nano-LC

Section No.	1 section	4 sections	8 sections	8 sections	15,000 microdissected cells	15,000 microdissected cells
Sample type	Glioma	Glioma	Glioma	Normal brain	Glioma	Normal brain
No. of MS measurements	2307	3328	3383	2985	552	779
No. of MS/MS measurements	734	1194	2160	1752	368	416
No. of identified proteins	32	131	189	140	27	13

The data obtained from MALDI-TOF/TOF after the fractionation procedure were compared to the MALDI-FTMS data, searching specifically for the 16 differentially expressed peptides. Nine out of 16 peptides matched within 200 ppm. To obtain a higher mass accuracy for the peptides, the corresponding spots of these nine peptides were measuregin in the MALDI-FTMS. The exact mass of five out of nine peptides matched within 3 ppm (external calibration) with the masses originally obtained by FTMS. In order to relate these peptides to proteins, the MS/MS data of these peptides were scanned against the database, resulting in a significant matching of four of them (sequence score > 24). Two peptides matched to fibrinogen beta chain precursor [p02675], one peptide to colligin 2 [P50454] and one peptide to acidic calponin 3 [Q15417]. In the MALDI-TOF data set more peptides belonging to these proteins were sought and an additional three peptides belonging to fibrinogen beta chain precursor, and two belonging to colligin 2 protein, were found. We also found an additional 17 peptides from fibronectin, of which nine had a significant MS/MS score.

Backward database searching

The search of the peak list obtained from the *In silico* digestion of fibronectin sequence in the FTMS data resulted in the finding of six extra peptides. Five peptides were found in the glioma vessels group only, and one was also seen in one sample of the normal brain blood vessels (Table 4). The same search for the *In silico* digestion of fibrinogen yielded nine additional peptides of which three were exclusively found in the glioma vessels group and the others in one sample of the normal vessels (Table 5). Searching for the theoretical peptides of colligin 2 and acidic calponin3 did not result in the finding of any extra peptide.

Table 4

Differentially expressed Fibronectin precursor [P02751] peptides

Fibronectin peptides found in FTMS spectra	p-Value	Δ ppm	Number of samples in which these peptides were found:			
			Glioma vessels	Glioma surrounding tissue	Normal brain vessels	Normal brain surrounding tissue
1926.04620 [(a)]	1926.04833	1.11	8	0	0	0
2470.32072 [(a)]	2470.31874	0.80	6	0	0	0
1593.81172 [(a)]	1593.81188	0.05	5	0	0	0
1807.90584 [(a)]	1807.90471	0.63	5	0	0	0
1629.87232 [(b)]	1629.87070	0.99	4	1	0	0
2692.37550 [(b)]	2692.37292	0.97	4	0	0	0
1349.68509 [(b)]	1349.68481	0.21	3	0	0	0
1401.66582 [(b)]	1401.66582	0.01	3	0	0	0
2524.36562 [(b)]	2524.36567	0.03	3	0	0	0
3042.59234 [(b)]	3042.58942	0.96	3	0	0	0

[(a)] i.e., Peptides matching the criteria used in this study

[(b)] i.e., Peptides derived from in silico digestion

Table 5

Peptides derived from in silico digestion of fibrinogen

Fibrinogen peptides found in the FTMS spectra	Exact fibrinogen derived from in silico digestion	Δ ppm	Number of samples in which these peptides were present in:			
			Glioma vessels	Glioma surrounding tissue	Normal brain vessels	Normal brain surrounding tissue
1032.56252	1032.5625	0.02	5	0	0	0
1239.51764	1239.5177	0.05	5	0	0	0
2385.17568	2385.1754	0.12	4	0	0	0
1275.55961	1275.5600	0.3	4	1	0	0
1544.69498	1544.6950	0.01	3	1	0	0
1668.71478	1668.7151	0.2	3	1	0	0
886.38736	886.3876	0.3	2	1	0	0
1951.00371	1951.0031	0.3	2	1	0	0

Immunohistochemistry:

The expression of fibronectin and colligin 2 proteins in glioma blood vessels were confirmed by immunohistochemistry. The proliferated blood vessels present in glioblastoma samples were invariably immunopositive for fibronectin and colligin 2, while the blood vessels in the control brain samples remained negative (Figures 3 and 4). In few capillaries of normal brain some fibronectin was expressed, but to a far lesser extent as compared to the expression observed in the proliferated glioma vessels. The blood vessels in the arachnoidal space were immunopositive for fibronectin, not for colligin 2.

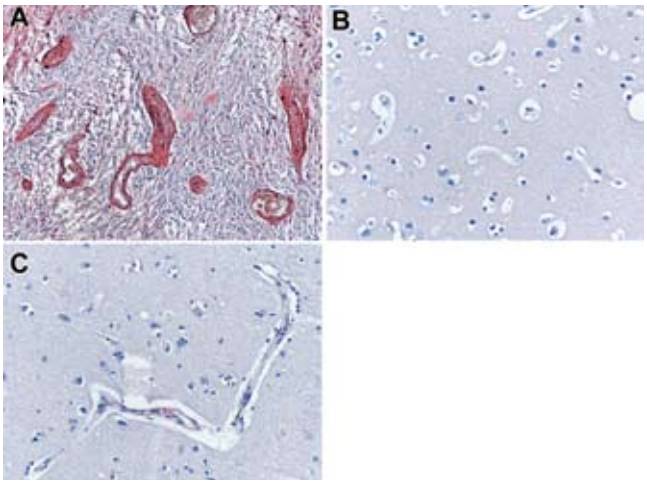


Figure 3
Immunohistochemistry for fibronectin in glioma and normal brain samples
A: Strong immunopositivity for fibronectin protein in the hypertrophied vessels of a glioma sample. B: No immunopositivity for fibronectin protein in blood vessels of normal brain. C: some of the blood vessels of normal brain showed a very faint staining for fibronectin in endothelial cells.

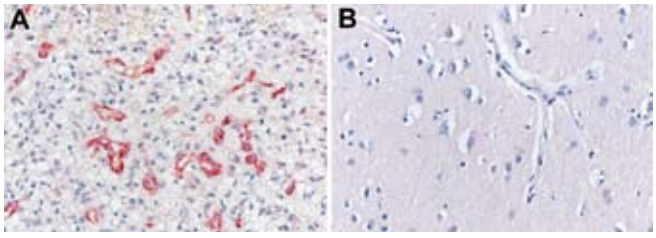
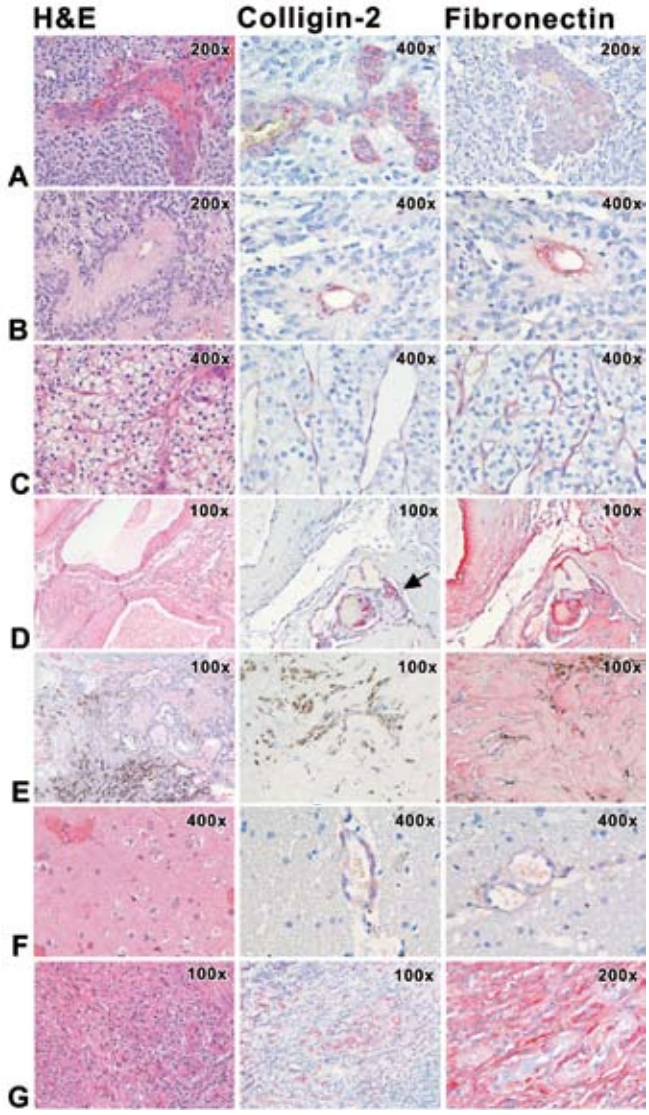


Figure 4
Immunohistochemistry for colligin 2 protein in glioma and normal brain samples
A: Strong positive staining of colligin 2 protein in the hypertrophied vessels of a glioma sample.
B: Absence of immunopositivity for colligin 2 protein in the blood vessels of normal brain.

The results of the immunostaining of various gliomas, carcinomas, vascular malformations and tissues and reactive conditions in which neo-angiogenesis takes place are shown (Figure 5 & Table 6). It appears that both colligin 2 and fibronectin are present in active angiogenesis in tumors, normal tissues and reactive processes. For instance, the vascular malformations (AVM and cavernous hemangioma) remained immunonegative for colligin 2, but in the arteriovenous malformation a spot of active angiogenesis, namely the recanalization of a vessel, was immunopositive (Figure 5D).



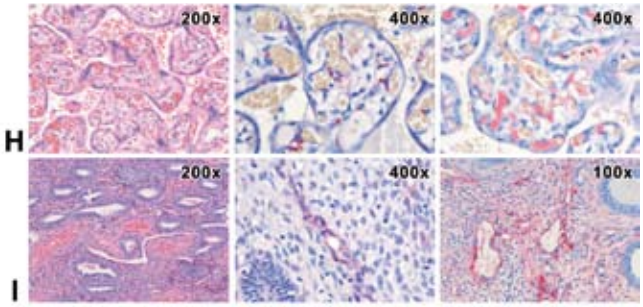


Figure 5
Results of immunostaining of various tissue samples for colligin 2 and fibronectin
A: anaplastic oligodendroglioma; B: ependymoma; C: renal cell carcinoma; D: arteriovenous malformation in brain; E: cavernous angioma; F: contusio cerebri; G: inflammation of skin; H: placenta; I:endometrium.
Staining patterns for both colligin 2 and fibronectin are confined to blood vessels. In case of active blood vessel formation in tumors, in reactive and normal tissues, staining is present. The AVM (D) and the cavernous hemangioma (E) remained largely immunonegative for colligin 2. However, at a single site of recanalization of a thrombosed vessel in the AVM (arrow), positive staining is present.

Discussion

The aim of this study was to identify angiogenesis-related proteins in glioma. To achieve this goal, the vasculature of surgically removed tissue samples of gliomas was compared with normal brain vessels. Tumors consist of complex 3-dimensional structures of heterogeneous mixture of cell types. Laser microdissection provides an efficient and accurate method for obtaining specific cell populations such as the glioma blood vessels in the present study. The hypertrophied vessel walls of glioma vasculature consist of endothelial cells, pericytes and cells expressing smooth muscle actin. These vessels may also contain glial tumor cells (mosaic vessels) [20]. In order to eliminate proteins derived from these tumor cells, we also microdissected glial tumor tissue for comparison. Comparison of the various microdissected tissues is essential for targeting structure-specific proteins. Any peptide present in the blood vessels that was also found in the glioma tissue was eliminated from the list of differentially expressed peptides.

Recent studies showed that the application of MALDI-FTMS holds significant advantages over other types of mass spectrometry [21, 22]. FTMS provides very high mass accuracy, which is considerably increased by its ability to perform an internal calibration [23]. In the present study we achieved an accuracy of ± 3 ppm by external calibration and up to ± 0.5 ppm by internal calibration. One of the advantages of MALDI-FTMS is the very high mass

Table 6
Samples used for immunohistochemistry

Sample type	# of samples	IR for c olligin 2 in blood vessels	IR for fibronectin in blood vessels
Glioma			
Glioblastoma	16 [(a)]	Positive	Positive
Normal brain samples	14 [(b)]	Negative	Negative/ faint
Pilocytic astrocytoma	3	Positive	Positive
Ependymoma	3	Positive	Positive
Myxopapillary ependymoma	2	Positive	Positive
Anaplastic oligodendroglioma	6	Positive	Positive
Renal cell carcinoma	5	Positive	Positive
Vascular malformation			
Arteriovenous malformation (AVM)	5	Negative	Positive
Cavernous hemangioma	2	Negative	Positive
Reactive condition			
Subdural membrane	2	Positive	Positive
Contusio cerebri	2	Positive	Positive
Ischemic infarction of brain	2	Positive	Positive
Inflammation (outside brain)	5	Positive	Positive
Tissues with notorious Neo-angiogenesis			
Placenta	6	Positive	Positive
Endometrium	6	Positive	Positive

IR: Immuno Reactivity
[(a)] i.e., 10 samples used for MALDI-FTMS plus an additional 6 samples
[(b)] i.e., 10 samples used for MALDI-FTMS plus an additional 4 samples

resolution, which in the present study generated relatively complex spectra consisting of 700-1,100 mono-isotopic peaks per spectrum. Yet another advantage is the very high sensitivity and reproducibility of the FTMS [11], which is higher than that of any other mass spectrometric technique currently available. In addition, FTMS provides an excellent signal-to-noise ratio, since the source of noise in MALDI-FTMS is of physical origin and is not a chemical based noise as that generated in the MALDI-TOF [24]. These advantages allow the study of very small numbers of targeted cells.

Although the MALDI-FTMS measurements of microdissected samples enabled us to detect specific peptide patterns for the distinct targeted cell populations, the results were not adequate to directly identify all of the related proteins. The chance of identifying a protein on the basis of accurate peptide masses rises by increasing the number of peptides

generated and detected from that protein. The number of detectable peptides per protein depends on several factors: the size of the protein and its concentration, the chemical properties of both the protein and the derived peptides and the enzyme used in digestion. Last but not least, protein identification by detection of peptides is highly dependent on the accuracy and completeness of the available databases. In the present study we succeeded in identifying the protein fibronectin on the basis of the accurate masses of four peptides generated by MALDI-FTMS. The *in silico* digestion approach appeared to be a valuable tool for confirming the presence of peptides derived from specific proteins in the spectra obtained by MALDI-FTMS. This is because the high peptide mass accuracy of MALDI-FTMS facilitated the match with the calculated masses generated by *in silico* digestion. Nevertheless, a major role in the detection of protein is still played by the nature of a protein, its concentration and its ionization ability.

The identification of peptides based on direct MS/MS measurements is hampered by the complexity of the sample in combination with the relatively low sensitivity for MS/MS in FTMS on ions generated by MALDI. To reduce those effects, we applied nano-LC fractionation prior to MALDI-TOF/TOF. Because the number of cells required for nano-LC fractionation is much higher than that obtained from sample microdissection, we pooled the micro-dissected cells from all samples in one sample of 15,000 cells. There was still a considerable loss of cells during the preparation steps and in the nano LC column. In addition, the overall sensitivity of MALDI-TOF measurements was significantly lower than that of MALDI-FTMS. These factors together led to the identification of the highly abundant proteins of the pooled microdissected cells. The identification of lower abundant proteins is achieved by using high numbers of cells more than harvested by microdissection. The tryptic digest of whole sections allowed the identification of many more proteins both in glioma and normal brain samples, particularly when we used peptide concentrations close to the maximum capacity of the column. Within the spectra that were generated by MALDI-TOF following nano-LC, we specifically sought the peaks that had previously been identified by FTMS, i.e. the 16 differentially expressed peptides. The low percentage of vessels in a section (maximum 10% of the cells) is responsible for producing only few numbers of peptides from their proteins. The detection of blood vessel-specific peptides was probably masked by the high percentage of peptides derived from the surrounding tissue. For that reason, not all of the 16 differentially expressed peptides found in the MALDI-FTMS experiments were detected after fractionation followed by MALDI-TOF/TOF. Yet, MS/MS data were obtained for four peptides, of which the identification was based both on very accurate peptide masses and on their significant MS/MS measurements. Importantly, fractionation also increased the number of peptides generated from an individual protein, thus significantly increased the confidence in the identified proteins (Table 7). However, the number of sequenced peptides was still insufficient to specifically identify splice variants of some

proteins. For instance, none of the fibronectin sequenced peptides were specific for extra domain B fibronectin, which is a splice variant known to be present in angiogenesis [25] The extra-domain B splice variant was not specifically identified, but neither was its presence excluded.

Two of the four proteins identified by the proteomic approach were successfully validated by immunohistochemistry. The faint staining for fibronectin of some of the normal brain blood vessels is in line with the detection of one fibronectin peptide by mass spectrometry in the normal brain vessels. The colligin 2 antibody appeared to be specific for the glioma vessels. The immunohistochemical validation of the findings by mass spectrometry highlights the sensitivity and accuracy of these techniques and illustrates its potential of identifying specific proteins. The additional immunostaining of various lesions and tissues demonstrate that colligin 2 and fibronectin both are expressed in the context of neo-angiogenesis. The expression was not specific for glioma neovascularization, but also found in the proliferating blood vessels in other tumors. Moreover, it is also seen in non-neoplastic tissues in which angiogenesis takes place. Therefore, colligin 2 and fibronectin should be considered as participants in the process of neovascularization in general, without specificity for tissue type.

So far, various growth factors taking part in the process of neo-angiogenesis have been identified in gliomas, such as Vascular Endothelial Growth Factor (VEGF) [26] and Platelet-Derived Growth Factor (PDGF) [27]. Relations have been discovered between some cytokines such as Transforming Growth Factor-beta and tumor blood vessels [28]. Further, endogenous expression of angiogenesis inhibitor factors e.g., angiostatin, endostatin and thrombospondin (TSP)-1 and -2 by glioma tumor vessels have been reported also [29]. Some of these proteins have been used to monitor therapy effects [7]. Despite the gradual unravelling of the roles of these regulatory proteins in the process of tumor neo-vascularisation, no major steps forward in antiangiogenic therapies for gliomas have been recorded. The identification of more tumor vasculature-related proteins may increase the chance of finding targets for anti-angiogenic therapies. Such discoveries may well increase our understanding of the formation of neovasculature in glioma.

In the present study, we identified fibronectin, fibrinogen, colligin 2 and acidic calponin 3 as proteins which are expressed in the glioma vasculature. Fibronectin is a high molecular weight, multifunctional matrix protein which binds to other extracellular matrix proteins such as collagen, fibrin and heparin. Several studies addressed the relation between fibronectin and tumors, among which breast cancer, melanoma [30, 31], and also gliomas. Overexpression of fibronectin in glioblastoma as detected by immunohistochemistry was reported previously [32]. The expression of fibronectin by glioma blood vessels suggests

Table 7
Differentially expressed proteins identified by nano-LC fractionation

Identified proteins and their accession number	Specific peptide masses by FTMS (pre-fractionation)	Calculated mass	Δ ppm	Score	Sequence coverage	Sequence obtained after nano-LC fractionation and MALDI-TOF/TOF measurements	No. of Extra peptides identified after nano-LC fractionation
Fibrinogen beta chain (P02675)	1535.72354 2257.07971	1535.72366 2257.08046	0.13 0.35	52 48	13 %	AHYGGFTVQNEANK GGETSEMYLIQPDSSVKPYR	5
Colligin 2 (P50454)	1659.80041	1659.80126	0.54	39	6 %	LYGPSSVSFADDFVR	1
Acidic calponin (Q15417)	1275.55961	1275.56000	0.31	39	3 %	YDHQAEEDLR	0

that this protein plays a role in the development of glioma vasculature [33]. In a study using suppression subtractive hybridization in which pilocytic astrocytoma were compared to glioblastoma, fibronectin was found to be differentially expressed; the glioblastomas expressed fibronectin while the pilocytic astrocytomas did not [34]. However, we did not find difference in the expression of fibronectin between these two tumor types. Since hypertrophied microvasculature is a hallmark of both, despite their different WHO grades, this finding did not surprise us.

Colligin-2, also called Heat shock protein-47, is a collagen-binding protein that is associated with an increase in the production of procollagen in human vascular smooth muscle cells [35]. Colligin 2 has been related to neo-angiogenesis in oral squamous cell carcinomas [36]. Acidic Calponin, also identified in this study, is a thin filament-associated protein detected in a number of different cells and tissues. It was mentioned among the differentially expressed proteins in human glioblastoma cell lines and tumors [37]. Acidic calponin modulates the contraction of smooth muscle cells. Interestingly, the proteins found in the present study share their prominent role in cell motility. It may very well be that the identification of these proteins is a reflection of their up-regulation in glioma vasculature. During neoplastic angiogenesis, sprouting of pre-existent blood vessels stimulate motility of the activated endothelial cells involved in this process. Further, the putative influx of

angiogenic precursor cells from the bone marrow into glioma may require the activation of motility even more. Further studies may detail the function and interaction of the proteins found in this study.

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Chapter | 4

Specific expression sites of colligin 2 in glioma blood vessels

Dana A.M. Mustafa; Marcel van der Weiden; PingPin Zheng;
Alex Nigg; Theo M. Luider. Johan M. Kros.

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Abstract

The process of neoangiogenesis plays an essential role in tumorgenesis of gliomas. In a previous study using state-of-the-art proteomic techniques, we identified colligin 2 (HSP47) as a glioma blood vessel-specific protein. The aim of the present study was to precisely localize the expression of colligin 2 in the blood vessels present in diffusely infiltrating gliomas and relate the expression pattern to the distinct cellular components of the vessels by using multiple immunolabeling and confocal microscopy. We grouped the glioma blood vessels into morphological categories ranging from normal looking capillaries to vessels with hypertrophic and sclerotic changes. The expression patterns of various markers of endothelial, pericytic and smooth muscle differentiation were correlated with the position of the cells in the vessels and the expression of colligin 2. We found that colligin 2 is expressed in all categories of glioma blood vessels in cells with endothelial and pericytic lineage. In addition, expression of colligin 2 was also found in cells scattered around blood vessels and in few GFAP-positive cells within the blood vessels. There is overlap in the expression of colligin 2 and the collagens type I and IV for which colligin 2 is a chaperon. We conclude that colligin 2 is expressed in all cellular components of glioma blood vessels and may serve as a general marker for active angiogenesis.

Introduction

Gliomas are among neoplasms with the highest degree of vascularization [1]. These neoplasms contain increased numbers of blood vessels relative to normal brain tissue and the vessel walls are variably thickened because of proliferation of their cellular constituents [2]. Along with glial tumor progression, normal looking blood vessels gradually hypertrophy into glomeruloid structures with multiple lumina ultimately degenerating into end-stage vessels with sclerotic walls and obliterated lumina [3]. Many vessels become prone to thrombosis and recanalization of organized thrombi is a frequently observed phenomenon. The newly formed blood vessels are leaky because of defective and aberrant basal membrane formation [4]. While it has long been appreciated that tumor growth and progression are dependent on angiogenesis, the elucidation of the molecular mechanisms that trigger the formation of new blood vessels is still in its early stage [5]. Although many aspects of the angiogenic switch, i.e., the transition of dormant pre-existing blood vessels into an actively sprouting vasculature, are not unravelled yet, a variety of angiogenic regulators have been detected [6] and some have already been tested for the development of anti-angiogenic therapies [7]. Besides the therapeutic approach aiming at destroying glioma vasculature, attempts to normalize the structure and function of the newly formed and dysfunctional blood vessels are also undertaken for reaching better penetration of chemotherapeutics and optimize conditions for effective radiation therapy [8, 9]. In order to manipulate the cerebral microcirculation, knowledge of the interplay of the cells involved and underlying molecular mechanisms is required [10].

The cells in blood vessel walls are characterized either by their position relative to the vascular lumina and/or by their immunohistochemical profiles. In normal blood vessels, endothelial cells are considered to line the lumina and express CD31 [11], CD34 [12], Von Willebrand factor [13] and more. Pericytes (also indicated as smooth muscle cells or mural cells) form an incomplete layer around the endothelial cells [14]. CD105 is a marker for activated endothelial cells taking part in neoangiogenesis not only in gliomas but also in various tumors [15-17]. Most available data on the cells involved in neoangiogenesis concern endothelial cells and factors regulating their proliferation [18, 19]. In recent studies the importance of pericytes and their interaction with endothelial cells for blood vessel formation, stabilization and function was highlighted [20, 21]. There are indications of the existence of various subtypes of pericytes in various organs with different functions and locations in the vessels [22, 23]. Immunohistochemical marker profiles for pericytes are diverse and vary between organs and developmental stages [22]. Because of this diversity, no general pan-pericytic marker is known [5]. A well-known marker for pericytes in cerebral vasculature is α-smooth muscle actin (αSMA) [14]. The marker NG2 is also used for staining of brain pericytes [24] and has been instrumental in proving that pericytic precursor cells

are recruited to sites where vessel growth and repair are occurring [25]. Recently, endosialin was found to be strongly up-regulated in pericytic cells in the developing human brain [21] and glioma [20]. Its expression is closely associated with other perivascular cells [20]. Other markers used to identify pericytes include platelet-derived growth factor receptor beta (PDGFR-β) [21], CD13 and desmin [26], but none is specific for these cells.

In a previous study using state-of-the-art proteomics techniques, colligin 2 was identified as a protein which is expressed in glioma neovasculature but not in normal brain vasculature [27]. In addition, we found that expression of colligin 2 is not limited to glioma vasculature, but is also seen in vasculature of non-glial tumors. It is also seen in non-neoplastic tissues, but only under circumstances of active angiogenesis like wound healing and recanalization of thrombi. Colligin 2 protein (also known as collagen binding stress protein; HSP 47) is localized in the endoplasmic reticulum and specifically binds to collagen type I, collagen type IV and gelatin [28]. It assists in the formation of the rigid triple-helical structures of collagen type I [29] and contributes to the maturation of collagen type IV [30]. Collagen is the major component of the basement membrane and a crucial element of the BBB [31-33]. The aim of the present study was to precisely localize the expression of colligin 2 in the blood vessel walls of gliomas; link the expression to the specific cellular components of the vessel walls in order to obtain indications as to the role of this protein in glioma angiogenesis. To this end, in addition to conventional microscopy of adjacent slides, confocal microscopy was used because of the superior levels of resolution and creating 3-dimensional representations.

Materials and Methods

Patients and tumor samples

Twenty glioblastoma (GBM) samples were taken from the files of the Department of Pathology, Erasmus MC, Rotterdam. In addition, seven samples of autopsy brains of patients without brain tumors were used as controls. Post-mortem times of the control cases were eight hours or less. For immunohistochemical staining paraffin embedded and fresh-frozen samples were used depending on the specifications of the antibodies and type of staining. For application of confocal microscopy, fresh-frozen samples were used. The number of samples, clinical data and tumor types are summarized in Table 1.

Table 1
Clinical data for the patient which had been used in this study

high-grade diffusely infiltrating glioma				
Glioma Samples	**Sex**	**Age**	**Tumor site**	**Diagnosis**
T1	female	30	Ri F	GBM
T2	female	30	Ri F	GBM
T3	female	54	Le F-P	GBM
T4	female	58	Le F-P	GBM
T5	female	63	Le F	GBM
T6	male	36	Le F	GBM
T7	male	44	multifocal	GBM
T8	male	46	Le T	GBM
T9	male	47	Le T	GBM
T10	male	47	Ri T-P	GBM
T11	male	48	Le F	GBM
T12	male	51	Ri F	GBM
T13	male	55	Ri F	GBM
T14	male	56	Le T-P	GBM
T15	male	57	Le T	GBM
T16	male	57	Ri F	GBM
T17	male	57	Le T	GBM
T18	male	62	Le T	GBM
T19	male	62	Le T	GBM
T20	male	68	Le F	GBM
Controls				
Normal Samples	**Sex**	**Age**	**Location**	**Diagnosis**
S1	female	39	Ri F	SAB
S2	female	48	Ri F	SAB
S3	female	60	Ri F	Pneumonia
S4	female	76	Ri F	Pneumonia
S5	male	31	Ri F	AVM
S6	male	34	Ri F	Haemorrhage brain stem
S7	male	70	Ri F	ARDS

Le = left; ri = right; F = frontal; T = temporal; P = parietal; GBM = glioblastoma;
AVM = arteriovenous malformation; SAB = subarachnoidal hemorrhage; ARDS = adult respiratory distress syndrome

Immunohistochemistry

Single staining procedures

Twelve biopsy samples of GBMs and five autopsy control samples were used. The samples were collected in a consecutive way. From each paraffin embedded sample, adjacent slides of five µm sections were stained with various antibodies. Because NG2 and CD105 antibodies do not work on paraffin embedded tissues, adjacent frozen sections were used. Immunohistochemical staining was performed following the manufacturer's instructions (alkaline phosphatase technique). The antibodies and their specifications are summarized in Table 2. Briefly, the paraffin sections were mounted onto poly-L-lysin coated slides, deparaffinized in xylene for 15 minutes and rehydrated through graded alcohol and washed with water. Frozen sections were fixed in acetone for 15 minutes and air dried. The sections were washed with phosphate-buffer saline (PBS) and incubated with the antibody for 30 minutes. After washing the sections with PBS, the corresponding antigen was added and incubated 30 minutes at room temperature. New Fuchsin Alkaline Phosphatase Substrate Solution was freshly prepared and the sections were incubated for about 30 minutes. Afterwards, the sections were washed with tap water, counterstained and cover-slipped with permanent mounting medium.

Double staining procedures

Double immunolabelings were performed combining colligin 2 antibody with the various markers for endothelial cells and pericytes. Ten frozen biopsy samples of GBMs were used for confocal laser microscopy. Adjacent slides of five µm sections were mounted onto non-coated microscope slides, fixed in acetone for 15 minutes and air dried. Sections were incubated with colligin 2 polyclonal antibody for 30 minutes, washed and incubated again with Cy3- conjugated goat-anti-rabbit for 30 minutes. After washing, sections were incubated with the second monoclonal antibody for 30 minutes followed by 30 minutes of labelling with biotin-horse-anti-mouse antibody. Detection was performed by FITC-conjugated-avidin antibody. Nuclei were counterstained with DAPI in a vector sheet (1:1000) and slides were covered and examined with the confocal laser microscope. For all antibodies the staining was always performed single on each sample also, to control for the accuracy of the staining and specificity of the antibodies used. For each antibody, negative controls including the secondary antibodies only were obtained for both single and double stained slides. The specifications of the antibodies are summarized in Table 2.

Confocal laser scanning microscopy

Using the confocal microscope allowed staining of the samples with two different antibodies. In addition, the resolution of the confocal microscope and its ability to produce 3D optical images enabled specific targeting the cellular components of glioma blood

Table 2
Antibodies used in this study

Name	Dilution	Commercial source
monoclonal colligin 2	1:500	Stressgen, Michigan, USA
polyclonal colligin 2	1:100	MBL international, Woburn, Canada
CD31	1:40	Dako, Glostrup, Denmark
CD34	1:30	Dako, Glostrup, Denmark
CD105	1:2000	Dako, Glostrup, Denmark
NG2	1:100	ZYMED laboratories, California, USA
endosialin	1:500	Prof. Isacke, Institute of Cancer Research, London, UK
αSMA	1:40	Biogenex, California, USA
collagen I	1:100	Abcam, Cambridge, UK
collagen IV	1:25	Dako, Glostrup, Denmark
Mib-1 (Ki-67)	1:10	Dako, Glostrup, Denmark
GFAP	1:100	Dako, Glostrup, Denmark
goat-anti-mouse-CY3	1:100	BioLegend, California, USA
biotin-horse-anti-mouse	1:200	Vector, Peterborough, UK
Avidin-FITC	1:50	Jackson Immunoreasearch, Pennsylvania, USA

vessels in a more accurate and reliable way. Confocal images were obtained using a confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc.) equipped with a Plan-Neofluar 40x/1.3 NA oil objective (Carl Zeiss MicroImaging, Inc.). A diode laser was used for excitation of DAPI at 405nm, an argon laser for FITC at 488nm and a HeNe-laser for Cy3 at 543nm. For DAPI an emission bandpassfilter of 420-480nm was used, for FITC a band-passfilter of 500-530nm and for CY3 a longpassfilter of 560nm. The signals were recorded sequentially (multi-track option) to avoid interference and stored in separate channels.

Results

Immunohistochemistry

Normal brain

The endothelial cells of small and medium-sized vessels in the samples of normal brain showed immunopositivity for CD31 and CD34 but not for αSMA (Table 3). The mural cells were variably positive for αSMA. None of the cells of the blood vessels in normal brain were positive for CD105, NG2, endosialin or colligin 2 (Figure 1 and Table 3).

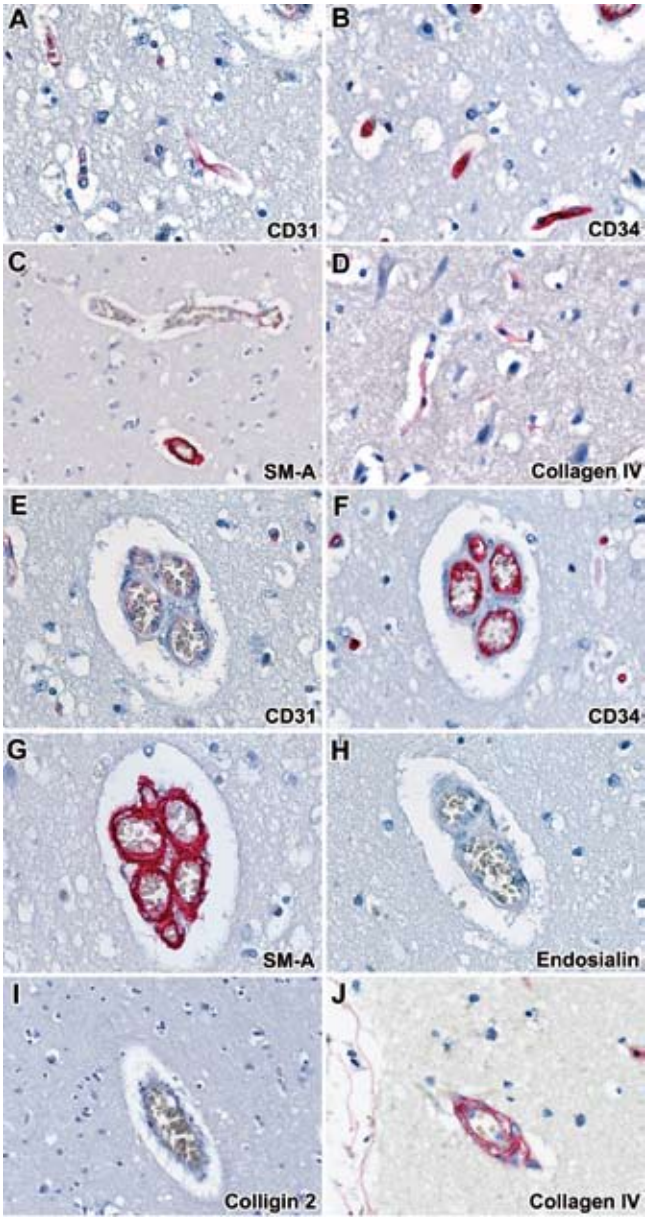

Figure 1

Expression of the various markers in normal brain

(A-D): CD31, CD34 and collagen IV are all expressed in capillaries. αSMA is variably expressed (Panels A, B, D: x 40; panel C: x 20).

(E-J): CD31, CD34, αSMA, collagen IV are expressed in small vessels in normal brain; endosialin and colligin 2 are not expressed (E, F-H, J: x 40; I: x 20).

Table 3
Immunostaining of blood vessels in normal brain

Layers in normal	CD31	CD34	CD105	NG2	endosialin	αSMA	colligin 2
Endothelial cells (lumena-lining)	+	+	-	-	-	-	-
Pericytes (abluminal lining)	-	-	-	-	-	+/-	-

Glioma

The vessels encountered in the glioma samples were divided in small vessels including capillaries, which did not show morphological changes; vessels with hypertrophied walls (either with organized layering as in normal larger blood vessels or with disorganized, haphazardly arranged cellular components); vessels with glomeruloid appearance and vessels with signs of recanalization. The lumina-lining cells of capillaries in glioma were invariably positive for CD31, CD34 and CD105 (Figure 2; Table 4). In addition, there was immunopositivity for αSMA, endosialin and NG2. Expression of colligin 2 was found in all

Table 4
Immunostatining of blood vessels in glioma

Glioma blood vessel subtype	Layers	CD31	CD34	CD105	NG2	endosialin	αSMA	colligin 2
small, normal-looking vessels	single	+	+	+	+	+	+	+
Layered/organized wall hypertrophied vessels	inner	+	+	+	–	+	–	+
	middle	–	–	–	+	–	+	+
	outer	–	–	+	+	+	+	+
Un-layered/ disorganized hypertrophied vessels	inner	+	+	+	–	–	–	+
	middle	–	–	+	+	+	+	+
	outer	–	–	+	+	+	+	+
Glomeruloid vessels	inner	+	+	+	+	+	+	+
	other layers	–	–	+	+	+	+	+
Recanalized vessels other layer	inner	+	+	ND	ND	+/-	+/-	+/-
	other layers	–	–			+/-	+/-	+/-

ND = not determined

small blood vessels (Table 4). In the vessels with hypertrophied walls with organized layering, the endothelium expressed CD31 and CD34 while the intermediate and the external layers expressed αSMA and NG2 (Table 4). The inner (endothelial) and outer layer of these vessels expressed endosialin and CD105 while cells in between these two layers remained negative for these two markers. Colligin 2 was expressed in all cell layers of the hypertrophied blood vessels (Figure 3). In the disorganized hypertrophied vessels, the internal diameter of the lumen appeared irregular while the external diameter of the vessel

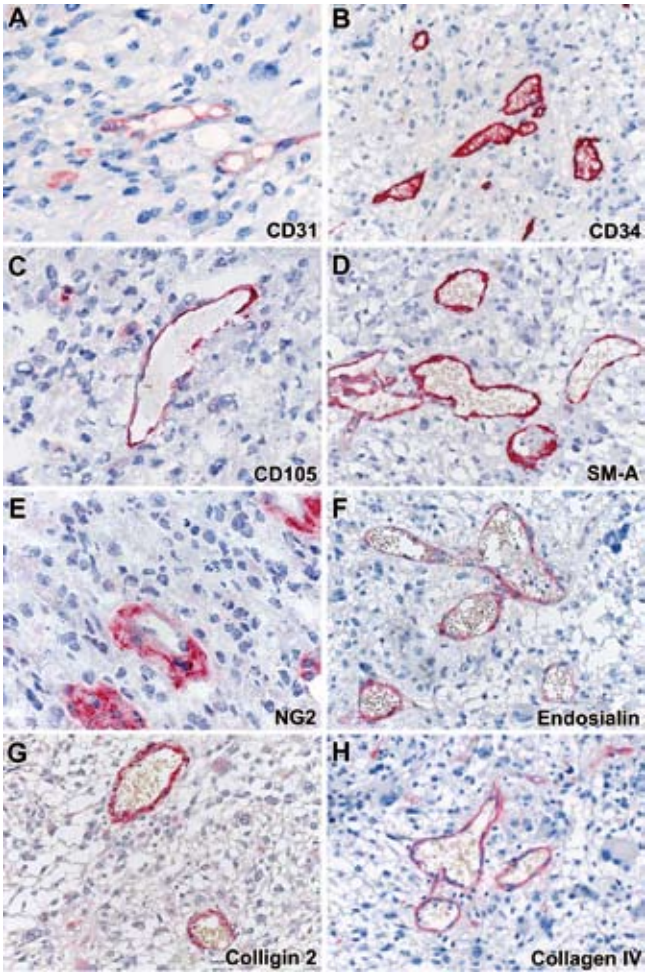

Figure 2
(A-H): Expression of the various markers in capillaries and small vessels in glioma
All capillaries and small vessels are invariably positive for the markers indicated.
(Panels A, C, E: x 40; B, D, F-H: x 20).

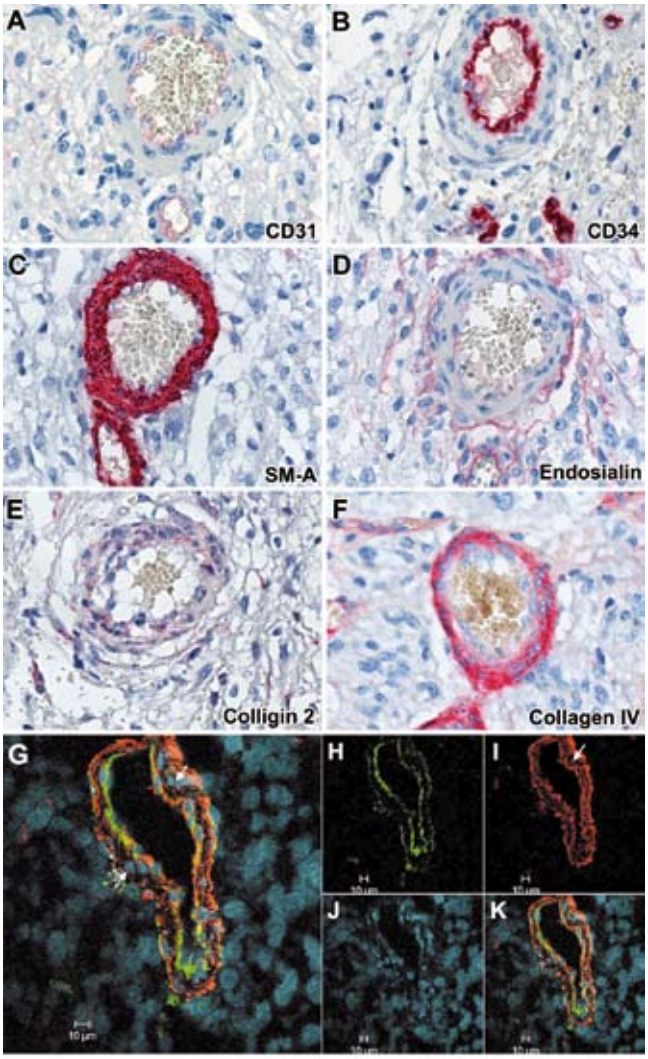

Figure 3

(A-F): Expression of the various markers in layered hypertrophic blood vessels in glioma
CD31 and CD34 are expressed in the endothelial layer. CD105 and endosialin are expressed in the endothelium and the external layer. αSMA, NG2 and collagen IV are expressed in the intermediate and external layers, not in the endothelium. Colligin 2 is expressed in all layers of the vessels. (Panels A-F: x 40). **(G-K): Confocal images of layered hypertrophic blood vessels in glioma for the expression of CD105.** Double immunolabeling for CD105/colligin 2. The endothelail cells as well as the external cells express both CD105 and colligin 2, while the intermediate cells express colligin 2 only (arrows). (Panel H: green = CD105; panel I: red = colligin 2; panel J: blue = DAPI; panel K: merged picture).

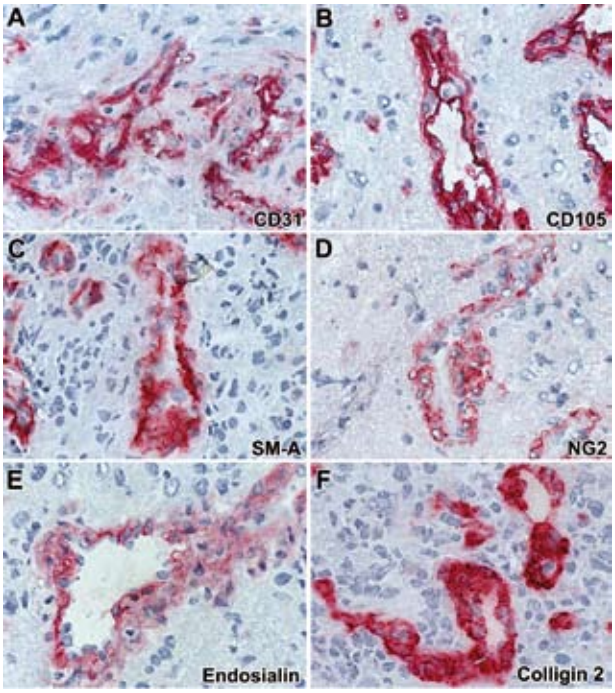

Figure 4
(A-F): Expression of the various markers in hypertrophic vessels without layered structure
CD31 is exclusively expressed in the endothelial layer; CD105 and colligin 2 are expressed in all layers. SM-A, NG2 and endosialin are present in all layers except the endothelial layer. (All panels x 40).

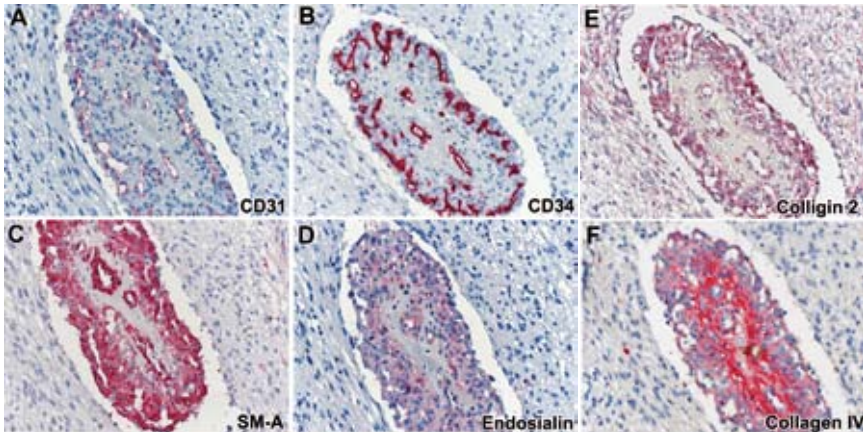

Figure 5
(A-F): Expression of the various markers in recanalized vessels in glioma
CD31 and CD34 are expressed in the endothelial layer. SM-A, endosialin, colligin 2 are expressed by the surrounding cells. Collagen IV is expressed throughout the vessel wall (All panels x 20).

wall varied per segment of the vessel. The endothelium stained positive for CD31, CD34 and CD105 but not for αSMA, NG2 or endosialin (Table 4). The abluminal cells of these vessels stained positive for CD105, αSMA, NG2 and endosialin. Colligin 2 was expressed in all layers of the vessel walls (Figure 4). The lumina-lining cells of the larger recanalized vessels were positive for CD31, CD34 and CD105 (Table 4). The other cells stained positive for αSMA, endosialin and colligin 2. Remarkably, a minority of cells in the recanalized thrombi remained negative for all markers used (Figure 5). In the glomeruloid blood vessels the cells are haphazardly arranged around multiple small lumina. The lumina-lining cells of the glomeruloid blood vessels stained positive for CD31, CD34 and CD105. The cells in abluminal position stained for endosialin, NG2 and αSMA (Table 4). The expression of colligin 2 was positive in all cellular components of these blood vessels (Figure 6).

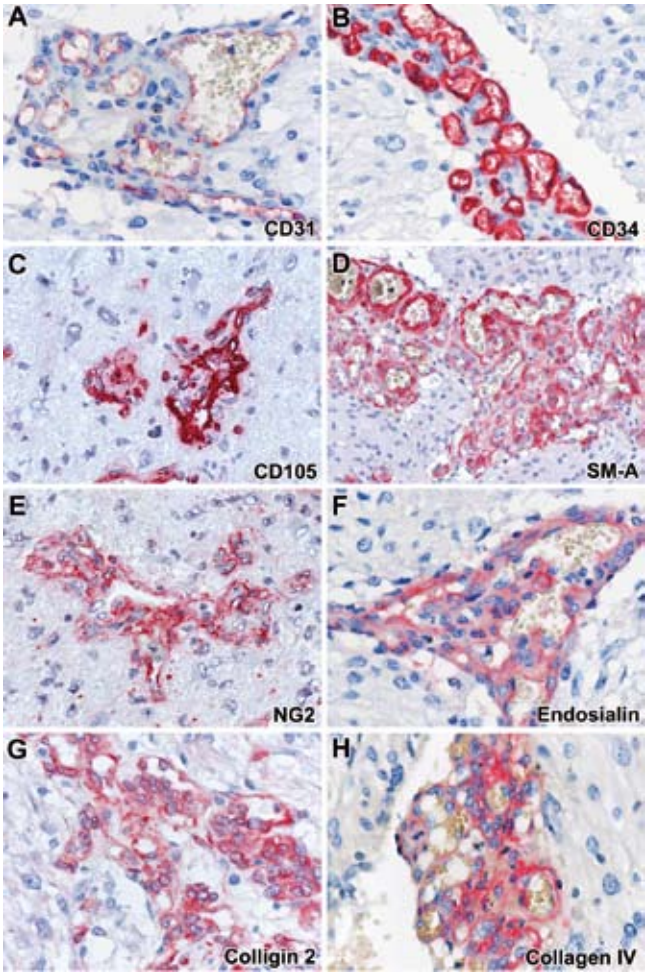

Figure 6
(A-H): Expression of the various markers in glomeruloid blood vessels in glioma
CD31, CD34 and CD105 are expressed in the endothelial layer. SM-A, Ng2, endosialin, colligin 2 and collagen IV are expressed in all components of the vessels.
(Panels A-C, E-H: x 40; D: x 20).

Confocal laser microscopy

The high resolution of the confocal microscope enables to detail the expression of the various markers at the level of individual cells. In all distinguished types of glioma blood vessels the lumina-lining endothelial cells were positive for CD31 and CD34 while none of the other cellular components were positive for these markers (Figure 7). CD105, a marker for activated endothelial cells, was expressed by the endothelial cells in all different types of blood vessels in the glioma samples (Figure 8). The percentage of endothelial cells that expressed colligin 2 varied between the blood vessels in the same sample and ranged between highly expressed and hardly showed any expression. In some endothelial cells expression level of colligin 2 was the same as that of CD31 or CD34, while other cells showed a very low level of expression.

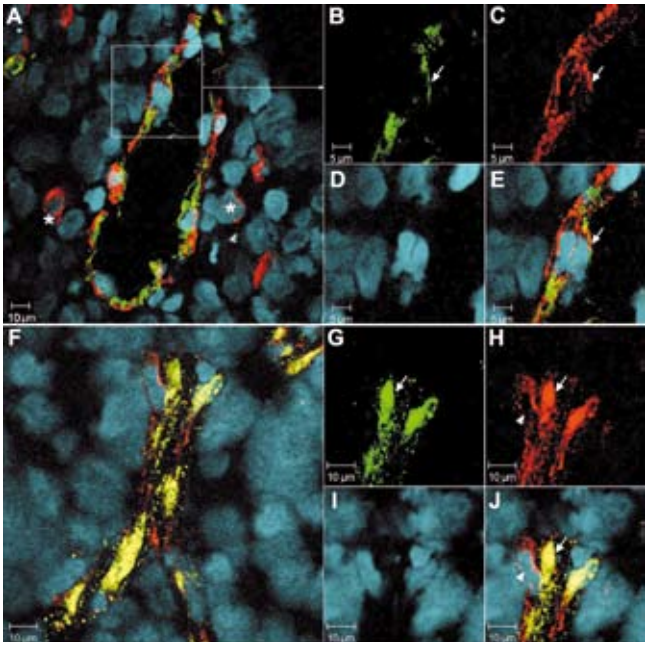

Figure 7
Confocal images of glioma blood vessels visualizing the expression of colligin 2 in the endothelial cells
(A-E): Double immunolabeling for CD31/colligin 2 in a small blood vessel. Some CD31 expressing cells are positive for colligin 2 [35]. Some cells around the blood vessels exclusively express colligin 2 (asterix). (Panel B: green = CD31; panel C: red = colligin 2; panel D: blue = DAPI; panel E: merged picture).
(F-J): Double immunolabeling for CD34/colligin 2 in a hypertrophied blood vessel.
A complete overlap in expression of CD34 and colligin 2 in endothelial cells [35].
Colligin 2 is also expressed by CD34-negative pericytes (Panel G: green = CD34; panel H: red = colligin 2; panel I: blue = DAPI; panel J: merged image).

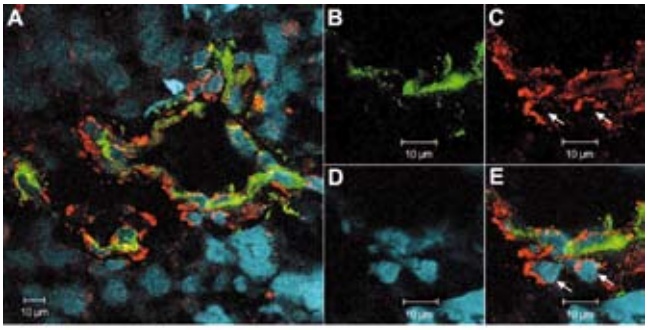

Figure 8
Confocal images of glioma blood vessels for the expression of colligin 2 by activated (CD105-positive) endothelial cells.
(A-E): Double immunolabeling for CD105/colligin 2 in hypertrophied blood vessel. CD105 and colligin 2 are expressed by endothelial cells while the cells around the endothelium express only colligin 2 (arrows). (Panel B: green = CD105; panel C: red = colligin 2; panel D: blue = DAPI; panel E: merged picture).

The pericytic markers NG2, endosialin and αSMA were combined with colligin 2. In all types of blood vessels, NG2 and αSMA were exclusively found in the layers around the endothelium and these cells also expressed colligin 2. Endosialin was expressed around the endothelium as well, but a low percentage of the endothelial cells of the various blood vessel subtypes also expressed this protein (Figure 9). Interestingly, scattered individual colligin 2-positive cells were present around all blood vessel subtypes. These colligin 2 expressing cells lacked expression of any of the endothelial or pericytic cell markers.

Because colligin 2 is the chaperon for the collagen types I and IV, we included these proteins in our investigations. There appeared to be an overlap in expression of colligin 2 and collagen type I and IV. However, not all colligin 2 positive cells showed expression of collagen; for instance, some cells in the glomeruloid blood vessels exclusively expressed colligin 2 and other cells in small blood vessels also expressed colligin 2 exclusively (Figure 10). The endothelial cells remained negative for collagens I and IV. Noticeably, the individual colligin 2-expressing cells found around the blood vessels never expressed either of the two collagens (Figure 10). The proliferation-related marker Mib-1 was combined with colligin 2 and co-expression was seen in some endothelial cells of small and hypertrophied vessels. In the glomeruloid vessels Mib-1 expression was seen in both colligin 2 positive and negative cells (Figure 11). The GFAP-positive astrocytes residing outside the blood vessels remained negative for colligin 2, but a very low percentage of these cells participating in the glioma blood vessel formation were found to co-express colligin 2 (Figure 12).

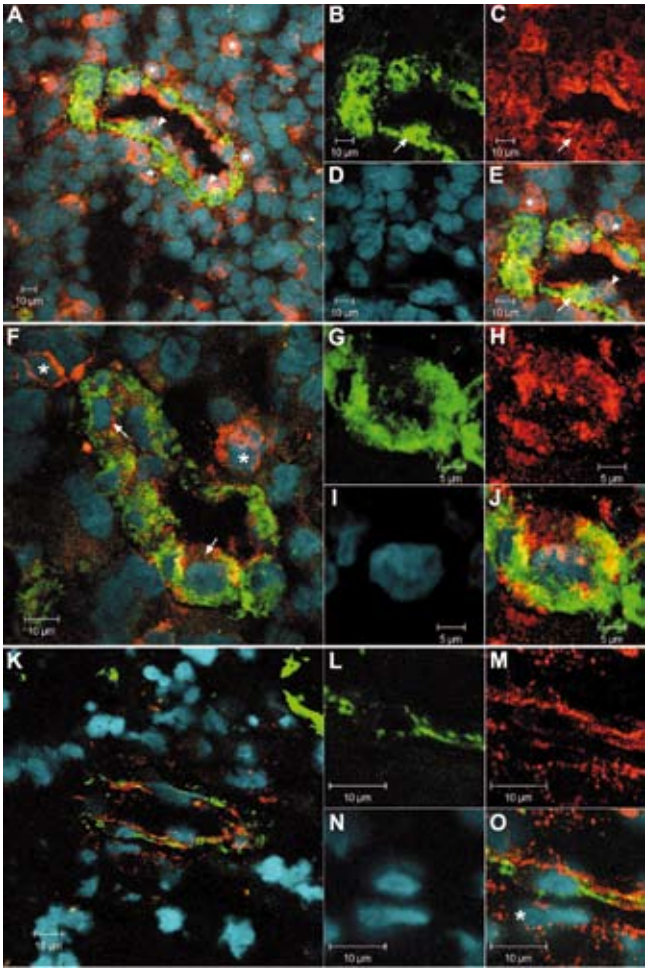

Figure 9

Confocal images of glioma blood vessels for the expression of colligin 2 by the surrounding cells / pericytes

(A-E): Double immunolabeling for NG2/colligin 2 in hypertrophied blood vessel. Cells expressing NG2 show expression of colligin 2. NG2 is not expressed by the endothelial cells. Some cells around the blood vessels exclusively express colligin 2 (asterix). (Panel B: green = NG2; panel C: red = colligin 2; panel D: blue = DAPI; panel E: merged picture).

(F-J): Double immunolabeling for endosialin/colligin 2 in hypertrophied blood vessel. Cells expressing endosialin are positive for colligin 2 as well. Some cells around the blood vessels show exclusive expression of colligin 2 (asterix). (Panel G: green = endosialin; panel H: red = colligin 2; panel I: blue = DAPI; panel J: merged picture).

(K-O): Double immunolabeling for SM-A/colligin 2 in small blood vessel. Expression of endosialin and colligin 2 in the same cells of the vessel wall. Exclusive expression of colligin 2 in cells around the blood vessels (asterix). (Panel L: green = endosialin; panel M: red = colligin 2; panel N: blue = DAPI; panel O: merged picture).

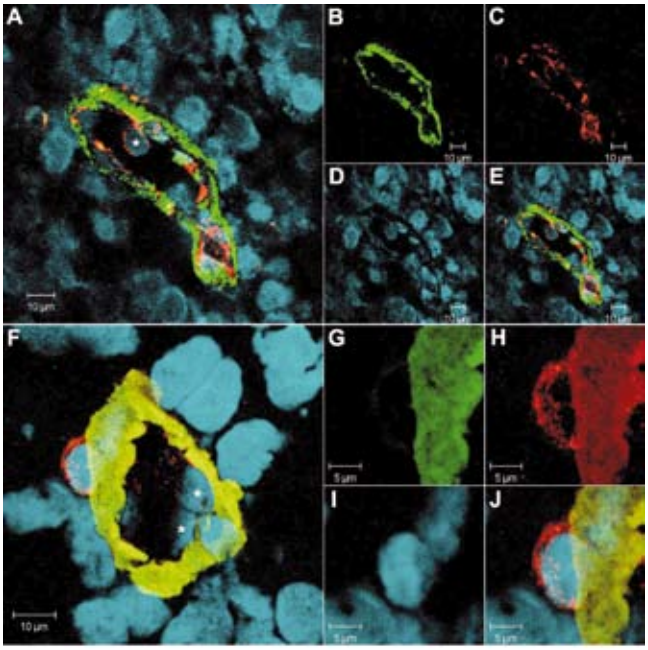

Figure 10

Confocal images of glioma blood vessels for the expression of colligin 2 and collagen type I and IV

(A-E): Double immunolabeling for collagen type I colligin 2 in hypertrophied blood vessel. The endothelial cells express colligin 2 only (stars), while the surrounding cells express both colligin 2 and collagen I. (Panel B: green = collagen I; panel C: red = colligin 2; panel D: blue = DAPI; panel E: merged picture).

(F-J) Double immunolabeling for collagen type IV colligin 2 in a small blood vessel. Double expression of Collagen IV and colligin 2 in the cells around the endothelium, while endothelial cells only express colligin 2. Exclusive expression of colligin 2 in cells around the blood vessels (stars). (Panel G: green = collagen IV; panel H: red = colligin 2; panel I: blue = DAPI; panel J: merged picture).

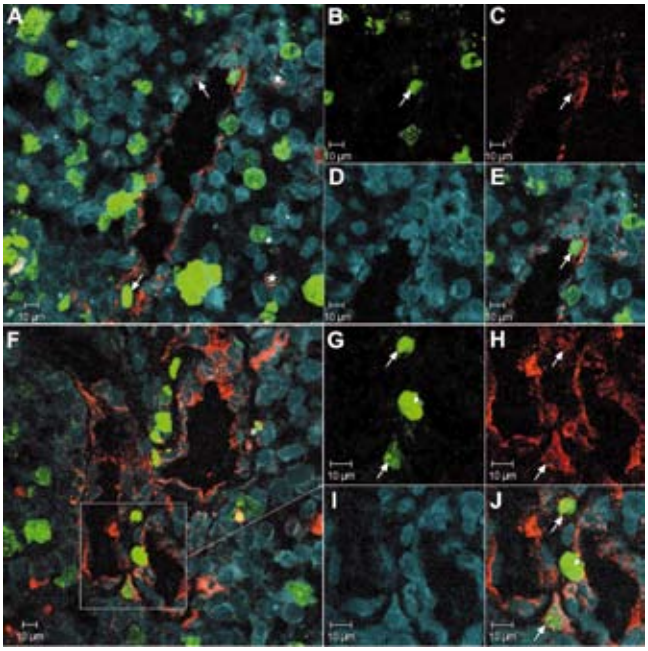

Figure 11
Confocal images of glioma blood vessels for the expression of colligin 2 in Mib-1 positive cells
(A-E): Double immunolabeling for Mib-1/colligin 2 in a small blood vessel. A fraction of colligin 2 positive endothelial cells are Mib-1 positive (arrows). (Panel B: green = collagen I; panel C: red = colligin 2; panel D: blue = DAPI; panel E: merged picture).
(F-J) Double immunolabeling for Mib-1/colligin 2 in glomeruloid vessels in glioma. Some Mib-1 positive endothelium-surrounding cells express colligin 2 (arrows) while others do not (stars). (Panel G: green = Mib-1; panel H: red = colligin 2; panel I: blue = DAPI; panel J: merged picture).

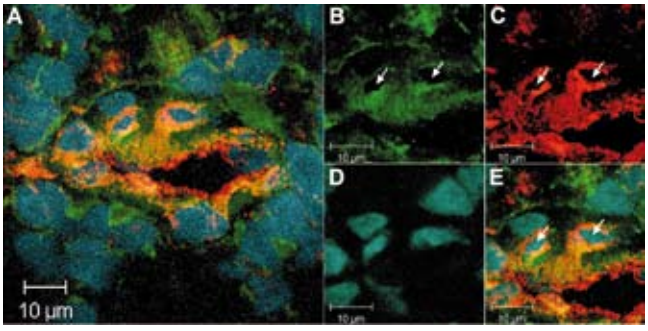

Figure 12
Confocal images of glioma blood vessels for the expression of colligin 2 and GFAP
(A-E): Few GFAP positive cells are present in the blood vessels and express colligin 2 in hypertrophied blood vessel (arrows). (Panel B: green = GFAP; panel C: red = colligin 2; panel D: blue = DAPI; panel E: merged picture).

Discussion

In our previous work we discovered that colligin 2 is expressed in glioma vasculature but not in the blood vessels of normal brain [27]. The aim of the present study was to investigate which subset of vessels and which cellular components of the glioma neovasculature express colligin 2. The results show that colligin 2 was expressed at various stages of glioma blood vessels: in new vascular sprouts, in hypertrophied vessels, in glomeruloid vessels and also in end-stage and thrombosed vessels in which recanalization is taking place. Moreover, the expression of colligin 2 was discovered in capillaries and some larger vessels which had not yet undergone any morphological change. Because colligin 2 was expressed in active angiogenesis but was not seen in blood vessels of normal brains it may be considered as an early marker for angiogenesis in glioma vasculature which remains present throughout the life cycle of the vessels.

At the level of single cells, colligin 2 is present in the lumina-lining endothelial cells showing co-expression of the endothelial markers CD31, CD34 and CD105 (Table 5). Since CD105 is a marker for activated endothelial cells, colligin 2 should be linked with endothelial activation and active angiogenesis. Colligin 2 is also expressed by pericytic or mural cells characterized by immunopositivity for αSMA, NG2 and endosialin (Table 5). In normal human development it has been shown that endothelial cells are driven and guided by migrating pericytes during organization of the growing vessel walls [21]. The immunohistochemical profiles which are the basis for the distinction of the various cells in the glioma blood vessels like endothelial cells or pericytes are less consistent than suggested in the literature so far. The lumina-lining cells of normal brain vasculature invariably express CD31 and CD34 while the expression of the pericytic marker αSMA varies between vessels of comparable sizes (Table 4). Alpha SMA is expressed in all types and sizes of blood vessels of glioma while its

Table 5
Confocal laser microscopy of glioma blood vessels

Endothelial cells		Pericytes	
Positive	**Negative**	**Positive**	**Negative**
CD31	NG2	NG2	CD31
CD34	αSMA	endosialin	CD34
CD105		αSMA	CD105 +/-
colligin 2		colligin 2	
endosialin +/-			

+/-: Shows varies results depending on the type of blood vessel.

expression was limited to only few blood vessels in the normal brain samples. The markers NG2, endosialin and colligin 2 were all exclusively found in glioma blood vessels while never in the blood vessels in the normal brain samples. As reported in the literature, endosialin is expressed in pericytes of glioma tissue [20]. The present results confirm the specificity of endosialin for glioma vasculature but also show that endosialin is present in some endothelial cells (Figure 9). NG2 showed to a lesser extent overlap in expression with endothelial markers and appeared, therefore, to be more specific for pericytic cells. The exclusive expression of colligin 2 and endosialin in morphologically normal blood vessels in glioma is illustrative of a shift in protein expression patterns prior to morphological changes. Interestingly, using confocal microscopy we found a subset of cells in brain tissue close to, but not in contact with, glioma blood vessels to express colligin 2. These cells did not express any of the endothelial / pericytic markers used in this study. It may well be that these cells are migrating vascular precursor cells on their way to merge into the vessel walls, or, alternatively, become a blood vessel *de novo,* compatible with the expression of colligin 2 as reflecting an early stage of vascular development. Further exploration as to the lineage and origin of these cells is indicated.

Colligin 2 (also known as CBP2 or heat shock protein 47) is a collagen-binding stress protein localized in the endoplasmic reticulum [28]. In the normal situation the expression of HSP47 precedes that of collagen and in various cell types and tissues co-expression of the proteins is present [36]. The expression of HSP47 and several types of collagen is induced under pathological conditions [37]. Colligin 2 is essential for the maturation of collagen by assisting in the correct folding and supporting the transportation of collagen to the basal membrane. The absence of colligin 2 causes defective maturation of the microfibrills of collagen type I and IV and impaired formation of the basal membrane [30]. Colligin 2 knockout mice did not survive beyond 11.5 days post fertilization and displayed abnormally orientated epithelial tissues and ruptured blood vessels [29]. Inactivation of the expression of colligin 2 seriously affects the function of the basal membrane of blood vessels [38]. Our results confirm the anticipated overlap in expression of colligin 2 with collagen types I and IV in the glioma blood vessels, corroborating the significance of colligin 2 for the formation of the basal membrane. The fact that expression of colligin 2 precedes the expression of collagen may well explain absent co-expression in some of the vascular cells examined in this study.

Resistance of brain tumors to therapy may in part be due to the abnormal functioning of tumor vasculature caused by pathological changes of the BBB. Normally, astrocytic endfeet and basal membrane substance of the cerebral blood vessels both contribute to the BBB. The majority of blood vessels in glioma loses contact with astrocytic endfeet and loses normal anatomy and function. Disruption of the BBB was noticed in our investigations.

In glioma vasculature, glial cells may participate in glioma angiogenesis (so-called "mosaic vessels") [39], but normal formation of endfeet for a normal BBB function is absent. In this study, by double labeling of colligin2 and GFAP, we excluded the expression of colligin 2 by astrocytes in glioma tissue, but we encountered scattered astrocytes in the glioma blood vessels which expressed colligin 2. This finding indicates that some astrocytes or astrocytic tumor cells that contribute to glioma blood vessels switch their protein expression repertoire to take part in angiogenesis. This finding is important when considering vascular cell populations for therapeutic intervention. The properties and functions of the glial cells in the mosaic vessels need further investigation.

In conclusion, we confirmed that colligin 2 is expressed in glioma vasculature and we found that its expression is by the distinct cell compartments of the tumor vasculature, whether they are activated endothelial cells or cells with immunoprofiles of pericytes. In addition, scattered cells without the immunophenotype of endothelial or pericytic cells were also immunopositive for colligin 2, as were some GFAP-positive cells in some of the vessel walls. Because colligin 2 is detected in a spectrum from morphologically normal to severely disfigured glioma blood vessels it potentially may become in use as a marker for active angiogenesis or serve as a link to targeted anti-angiogenic strategies.

Acknowledgement

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Chapter | 5

Overexpression of colligin 2 in glioma vasculature is associated with overexpression of heat shock factor 2

Dana A.M. Mustafa, Anieta M. Sieuwerts, Ping Pin Zheng, Johan M. Kros.

Submitted for Publication

Abstract

In previous studies we found expression of the protein colligin 2 (heat shock factor 47 (HSP47), *SERPINH1*) in glioma neovasculature while not in normal brain tissue. Generally, the regulation of heat shock gene expression in eukaryotes is mediated by heat shock factors (HSF). In mammals, three heat shock transcription factors, HSF-1, -2, and -4, have been isolated. Here we investigated the relation between the expression of colligin 2 and these heat shock factors at the mRNA level using real-time reverse transcriptase PCR (qRT-PCR) in different grades of astrocytic tumorigenesis, viz., low-grade glioma and glioblastoma. Endometrium samples, representing physiological angiogenesis, were included as controls. Since colligin 2 is a chaperon for collagens, the gene expression of collagen I (*COL1A1)* was also investigated. The blood vessel density of the samples was monitored by expression of the endothelial marker CD31 (*PECAM1*). Because NG2-immunopositive pericytic cells are involved in glioma neovascularization, the expression of NG2 (*CSPG4*) was also measured.
We demonstrate overexpression of *HSF2* in both stages of glial tumorigenesis (reaching significance only in low-grade glioma) and also minor elevated levels of *HSF1* as compared to normal brain. There were no differences in expression of *HSF4* between low-grade glioma and normal brain while *HSF4* was downregulated in glioblastoma. In the endometrium samples, none of the HSFs were upregulated. In the low-grade gliomas *SERPINH* appeared to be slightly overexpressed with a parallel 4-fold upregulation of *COL1A1*, while in glioblastoma there was over 5-fold overexpression of *SERPINH1* and more than 150-fold overexpression of *COL1A1*. In both the low-grade gliomas and the glioblastomas overexpression of *CSPG4* was found and overexpression of *PECAM1* was only found in the latter. Our data suggest that the upregulated expression of colligin 2 in glioma is accompanied by upregulation of *COL1A1*, *CSPG4, HSF2* and to a lesser extent, *HSF1*. Further studies will unravel the association of these factors with colligin 2 expression, possibly leading to keys for therapeutic intervention.

A wide range of physiological and pathological stresses trigger the heat shock gene transcription. Cells respond to elevated temperatures and to chemical and physiological stresses by an increase in the synthesis of heat shock proteins (HSP). HSP are a highly conserved family of proteins which function as molecular chaperones or proteases [1, 2]. Molecular chaperones form a class of proteins that control the proper folding of nascent polypeptides into the correct 3D structure. During stress responses the role of HSPs is critical in preventing the appearance of intermediates that lead to misfolding or otherwise damaging molecules [3]. HSPs assist in the recovery from stress either by repairing damaged proteins (protein refolding), or protein degrading, thus restoring protein homeostasis and promoting cell survival. The regulation of heat shock proteins is mediated by heat shock transcription factors (HSF). Under normal conditions, HSFs reside in the cytoplasm, but are activated upon stress and relocalize to the cell nuclei [4]. Activated HSFs form a trimmer with high-affinity binding to DNA; it binds to heat shock elements (HSE) in the promoters of the heat shock genes [4]. The activation results in the expression of heat shock proteins (HSPs). In vertebrates and plants, there are at least four members of the HSF gene family (HSF1-4), [1], while in human cells, three HSFs (HSF-1, -2, and -4) have been characterized [2, 5]. The expression of HSF1 and HSF2 is ubiquitous. However, the factors that induce their activation differ. While HSF1 is activated by heat shock and other forms of stress, HSF2 activity has been associated with development and differentiation. The expression of HSF4 appears to be tissue-specific and is restricted to heart, skeletal muscle or brain [5]. The simultaneous expression of the different HSFs in particular tissues would enable differential responses to various forms of stress. In the context of tumors, expression patterns of HSPs may be tumor-specific and therefore, they may well become therapeutic targets [6-8].

In previous studies, we found specific overexpression of colligin 2 in glioma neovasculature as compared to the normal vasculature of the brain [9, 10]. Here we investigate whether there is a correlation between the expression of colligin 2 and any of the HSF genes (HSF1, HSF2 and HSF4) at the mRNA level in low- and high-grade glioma. We measured the relative transcription levels of colligin 2 (*SERPINH1*), *HSF1*, *2* and *4* by real time RT-PCR in four glioblastoma (GBM) samples, four samples of low-grade glioma (LGG), four samples of proliferating endometrium and four samples of normal brain tissue (Figure 1)**.** Prior to isolation all tissues were assessed by a pathologist to ensure their origin and quality. The blood vessel density of the samples was monitored by expression of the endothelial marker CD31 (*PECAM1*). Because NG2-immunopositive pericytic cells are involved in glioma neovascularization, the expression of NG2 (*CSPG4*) was also measured. The expression sites of the HSFs were visualized by confocal microscopy. A set of four reference genes were used for data normalization (*GUSB*, *HMBS*, *HPRT1* and *NOXA1*). For statistical testing the mean values were used. Comparisons between groups were made by using the Kruskal Wallis test.

qRT-PCR revealed a significant 5.6-fold increase in mRNA levels for colligin 2 in glioblastoma and mildly elevated levels in low-grade glioma (Figure 1). In parallel, significant increases in mRNA levels of collagen type I were found in the gliomas. Of the heat shock factors, *HSF2* was overexpressed in glioma which reached significance in low-grade glioma. There was only minor overexpression of *HSF1* while *HSF4* was underexpressed in GBM (Figure 1). The cells with nuclear expression of HSF1 were also immunopositive for colligin 2 (Figure 2). In GBM both *PECAM1* (1.8-fold) and *CSPG4* (2.3-fold) were overexpressed while only *CSPG4* was overexpressed (3.4-fold) in LGG. We also observed 2.2-fold upregulation of the expression of colligin 2 mRNA in endometrium samples as compared to normal controls (not significant; p=0.0833) with significant 55-fold upregulation of the expression of collagen I mRNA (p=0.0209). Remarkably, none of the HSFs was upregulated in endometrium as compared to normal brain controls (*HSF1*: p=0.1489; *HSF2*: p=0.2482; *HSF4*: p=0.2482).

This is the first report on the parallel upregulation of colligin 2 and heat shock factors in human glioma. Heat shock responses appear to be implicated in a broad range of pathological conditions including heat shock, oxidative stress, ischemia and reperfusion, inflammation, tissue damage, exposure to heavy metals and infection [1]. In addition, tumor neovascularization is associated with the heat shock response. Mammals have three different HSFs which are considered to be functionally distinct: HSF1 is essential for the heat shock response and is also required for developmental processes; HSF2 and HSF4 are important for differentiation and development [11]. Although deletion of *HSF1* in mammalian cells still allows a basal expression of HSPs, it leads to the abrogation of induction of the response to a variety of stresses [12, 13]. The genes encoding HSF1 and HSF2 are constitutively expressed in most cell lines and tissues under normal growth conditions and both factors are kept in a latent, non-DNA-binding state, indicating that the DNA-binding activity of both HSF1 and HSF2 is negatively regulated [14]. Despite differences in expression, HSF1 and HSF2 both act as positive activators of transcription for all their functions. In contrast, HSF4 lacks activity of a transcriptional activator [15]. HSF4 is highly expressed in the lens and in brain. It may well be a HSE binding trimmer because it lacks an inhibitory domain of trimerization. HSF4 constitutively binds to DNA and regulates the expression of HSP in the absence of stress [16]. HSF4 contains two alternative splice variants: HSF4a and HSF4b [17]. HSF4a isoform acts as a repressor of HSF1 [17] by competitively binding to the heat shock element (HSE). This is the first observation of downregulation of HSF4 in glioblastoma and is suggestive of a role of HSF4 in the regulation of the expression of HSPs including colligin 2. High-resolution chromatin immunoprecipitation on microarray (ChIP-chip) screens have successfully been used for identifying direct target genes for many transcription factors [18]. This approach has also been used for searching target genes for HSF2 [11] and may be used for further unravelling the relation between HSF2, HSF1, HSF4 and colligin 2. In the proliferating endometrium samples we found overexpression of colligin 2 and collagen type I. In contrast

to the glioma samples, no associated upregulation of any of the three HSFs was observed in endometrium. This may well be an important difference between physiological and neoplastic angiogenesis. Obviously, if there is a causal relation between the upregulated HSFs found and the expression of colligin 2, the HSFs may become important for the design of anti-angiogenic therapy.

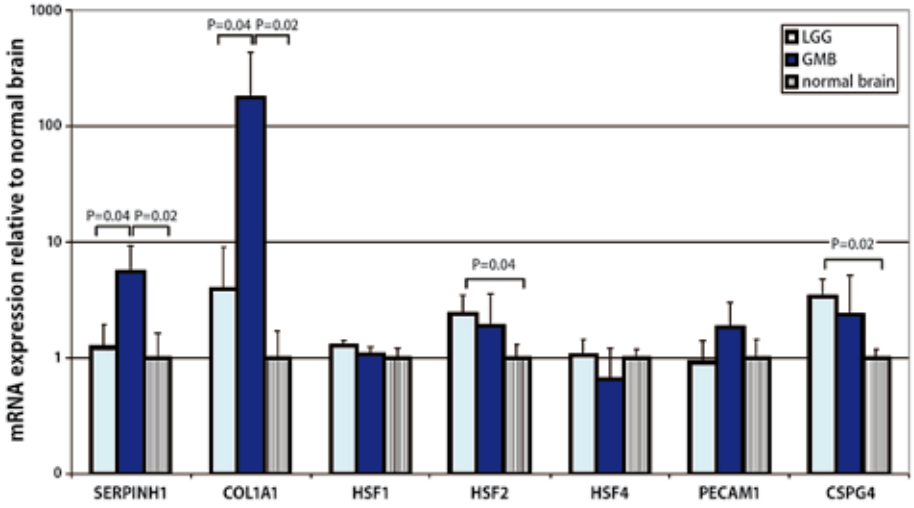

Figure 1

mRNA expression of colligin 2, HSF1, 2 and 3, collagen 1, CD31 and NG2 in low- and high-grade glioma and normal control brain

Data in this figure are the average ± SD of one representative experiment with 4 tissues in each group. Expression data are presented relative to the average mRNA expression levels measured in total RNA isolated from normal brain tissues (n=4). Prior to isolation, all tissues were assessed by a qualified pathologist to ensure the origin and quality of the tissues. Total RNA was isolated with the RNeasy Micro kit (Qiagen BV, Venlo, the Netherlands). cDNA was prepared by use of the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany). The resulting cDNA preparations were analyzed by real-time PCR with TaqMan gene expression assays and TaqMan Universal PCR Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). PCRs were performed in a 20 µL reaction volume in an Applied BioSystems 7900HT Fast Real-Time PCR system. Negative controls included minus RT and H_2O-only samples, which showed to be negative in all cases. Expression of *GUSB*, *HMBS*, *HPRT1* and *NOXA1* was used as a reference to control sample loading and RNA quality, as described previously [19]. LGG = low grade glioma; GBM = glioblastoma; SERPINH1 = mRNA coding for colligin 2; COL1A1 = mRNA coding for collagen 1; HSF = mRNA coding for heat shock factor; PECAM1 = mRNA coding for CD31; CSPG4 = mRNA coding for NG2; RT-PCR = reverse transcriptase – polymerase chain reaction; cDNA = complementary DNA.

Differences in mRNA concentrations were determined by the non parametric Kruskal-Wallis test with $P < 0.05$ being considered statistically significant. All statistical tests were two-sided.

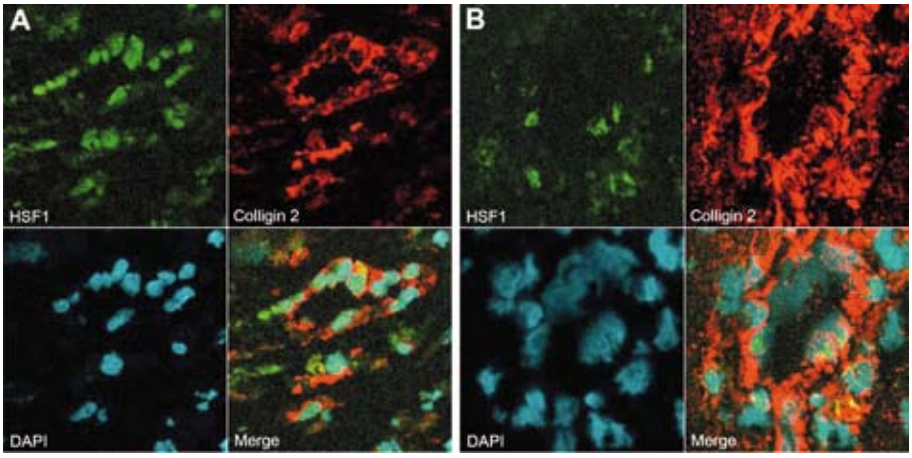

Figure 2

Expression of HSF1 and colligin 2 in blood vessels of glioblastoma

A: Expression of HSF1 and colligin 2 in small blood vessel of glioblastoma. There is immunopositivity in the blood vessels for HSF1 and colligin 2. Around the blood vessels are cells expressing only HSF1.

B: Expression of HSF1 and colligin 2 in proliferated blood vessel of glioblastoma. While not all endothelial cells and pericytes of the proliferated blood vessels express HSF1, all express colligin.

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Chapter | 6

Angiogenesis proteome: a comparison between physiological angiogenesis and angiogenesis in glioblastoma

Dana A.M. Mustafa, Christoph Stingl, Andreas Kremer,
Marcel Stoop, Lennard J. Dekker, Paul Eilers, Peter A.E. Sillevis Smitt,
Johan M. Kros and Theo M. Luider.

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Abstract

Glioblastoma is the most common primary brain tumor with a dismal prognosis. These tumors grow highly infiltrative and show very strong neo-angiogenesis. So far, results of anti-angiogenic therapy have been disappointing. A search for blood-vessel related targets is essential for the development of effective anti-angiogenic strategies. Further, since the degree of angiogenesis correlates with tumor progression, blood vessel-related proteins may also be useful biomarkers for glial tumor progression. In order to discover targets related to glioma blood vessels we compared the protein profiles of the proliferating vessels of glioblastoma to vessels taking part in physiological angiogenesis. Blood vessels of glioblastoma and endometrium in proliferation were laser microdissected, and LTQ Orbitrap mass spectrometry was used to measure the corresponding peptide profiles. Data analysis was carried out at two different levels; a) the level of differentially expressed proteins and b) the level differentially expressed peptides. In total, we were able to identify 35 and 19 differentially expressed proteins for glioma and endometrium blood vessels, respectively. The differentially expressed proteins were used as a starting point to perform an analysis of the molecular pathways involved. In addition, we classified the differentially expressed proteins according to their functions. We also successfully validated the expression of tenascin-C and calponin-1 by immunohistochemistry. The 35 proteins found in the glioblastoma vessels could be related to three networks, among which one was associated with vascular development and angiogenesis. The 19 proteins found specifically in endometrium blood vessels were mapped to four networks among which one involved in cardiovascular development and another associated with the proliferation of endothelial cells.

We conclude that there are essential differences in protein expression profiles between glioblastoma angiogenesis and normal physiological angiogenesis. Additional studies will reveal whether the identified proteins are candidates to be developed as biomarkers or as targets for anti-angiogenic therapies.

Introduction

Most glial tumors develop from low-grade, relatively benign neoplasms into high-grade tumors. The highest grade gliomas are designated the term "glioblastoma", which basically represents a common denominator of all high-grade gliomas irrespective of their lineage. Glioblastoma, or glioblastoma multiforme (GBM), is the most frequently occurring of all primary brain tumors in humans. GBMs are highly infiltrative tumors which show rapid clinical progression. Most patients succumb in less than a year after the diagnosis is made. While the low-grade precursors still have low cell densities, low proliferation parameters and no newly formed blood vessels are visible by routine microscopy, GBMs show regions of high proliferation, high cell density, necrosis and notorious microvascular proliferation [1]. The formation of new blood vessels from pre-existing vasculature is considered to be the most important factor in the development of tumor neoangiogenesis and is a prominent phenomenon in GBM [2, 3]. In spite of the sound rationale for most of the anti-angiogenic, molecularly targeted treatment strategies, all of these therapies have remained with limited success and they should be applied in combination with cytostatic agents [4-7]. Hence, there is a need to develop more effective angiogenesis inhibitors. Another important reason to identify glioma angiogenesis-related proteins is to obtain useful parameters for monitoring glial tumor progression [8, 9]. So far, only few tumor progression markers were introduced and none has sustained as reliably monitoring disease activity and therefore, no putative marker found its way to clinical practice [10-14]

Previously, using various proteomics techniques, we identified several proteins which were specifically upregulated in glioma vasculature while not in normal brain blood vessels [15, 16]. Among these proteins, we found caldesmon, fibronectin, colligin 2 and others. These proteins appeared to be overexpressed in active angiogenesis in carcinomas as well, and were also overexpressed in vascular malformations and tissues in which reactive angiogenesis is taking place [16]. Normal blood vessels are morphologically different from tumor vasculature; they are not activated and they show different protein expression profiles. In order to identify proteins which are specifically expressed in tumor angiogenesis, not only comparisons with expression profiles of blood vessels in normal tissues, but also a comparison with those in tissues in which active angiogenesis takes place, are necessary. Normally, angiogenesis occurs during embryogenesis and development. In addition, it takes place in adults during the menstrual cycle and in the framework of repair or regeneration of tissue during wound healing [17]. The identification of specific tumor-angiogenesis related proteins could ultimately lead to the development of tumor-specific, anti-angiogenic therapies and tumor progression markers.

The rapid development of proteomics techniques during the past years has enabled the identification of novel cancer biomarkers [18]. The discovery and improvement of mass

spectrometers allowed large scale analyses of protein expression profiles which were directly obtained from patients' tissues [19]. In 2005 Hu et al. introduced a commercial Orbitrap mass spectrometer as a tool for proteomics research [20]. The Orbitrap has high resolving power (>150,000), excellent mass accuracy (specified as ~2–5 ppm and operating in the LC/MS mode providing 1 spectrum each second) [21, 22], high sensitivity and a wide dynamic range enhancing the number of protein identifications. In addition, the Orbitrap facilitates the accumulation of a greater number of charges and it is sufficiently fast to enable two consecutive stages of mass spectrometric fragmentation resulting in accurate sequencing and MS/MS in short time scale [23]. The complexity of human biopsy samples is still a considerable obstacle for proteomics analysis [24]. Reducing the complexity can be reached at various levels. Firstly, at the level of the tissue, methods of sample purification applied prior to analysis can improve the accuracy of detection [25]. In order to target specific structures like blood vessels for comparisons of protein expression profiles, laser capture microdissection (LCM) of the tissues should be applied to purify the samples. Secondly, at the level of the applied technology there are methods of enhancing the presentation of the proteins. In several studies it was proven that the use of a fractionation method prior to measuring the peptide digest in any of the mass spectrometers is useful to reduce the complexity of the samples and increases the number of identified proteins [16, 26-28]. For measuring samples originating from small number of cells such as laser microdissected human tissues the combination of a fractionation method with a very accurate mass spectrometer will yield optimal results [16, 26].

The aim of this study was to identify proteins which are differentially expressed between glioma-related, and normal physiological angiogenesis. To this end, we isolated blood vessels from glioblastoma and proliferating endometrium by using laser capture microdissection and measured their protein contents by the LTQ Orbitrap. We identified a number of proteins which were exclusively expressed in each group of blood vessels. Further, the proteins were characterized and the pathways in which they take part were sought. Some of the differentially expressed proteins could be validated by immunohistochemistry.

Materials and Methods

Samples

Ten fresh-frozen samples of glioblastoma located in the cerebral hemispheres were taken from the files of the Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands. We used surgical samples taken from five males and five females. The ages ranged between 41 and 86 years (median 67.5 years). In addition, 10 fresh-frozen endometrium samples were collected at the Department of Gynecology, Erasmus Medical

Center, Rotterdam, The Netherlands. The samples were collected from premenopausal women who had undergone hysterectomies for diseases not involving the endometrium. Their ages ranged between 39 and 46 years (median of 43 years). Sections of 5 µm from each sample were counterstained and examined by the pathologist (JMK) to verify the presence of blood vessels (Figure 1). This study was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam, The Netherlands.

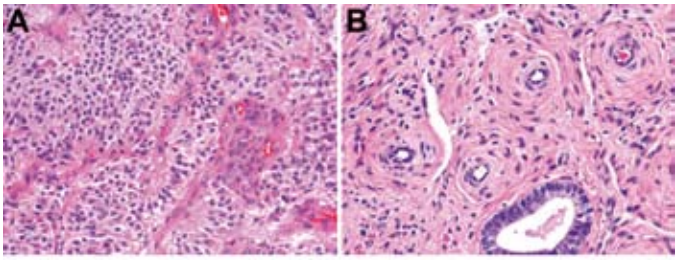

Figure 1
Proliferating blood vessels in A. glioblastoma and B. endometrium samples (H&E, x 400)

Laser capture microdissection

The previously described procedure was followed [16]. Briefly, cryosections of ten µm were made from each sample and mounted on polyethylene naphthalate (PEN) covered glass slides (P.A.L.M. Microlaser Technologies AG, Bernried, Germany). The slides were fixed in 70% ice-cold ethanol for a maximum of 2 hours. Before laser microdissection, the slides were stained with hematoxylin and dehydrated in series of ethanol solutions and left to air-dry for 5 minutes. The P.A.L.M. laser microdissector and pressure catapulting device, type P-MB, was used with PalmRobo version 2.2 software at 40x magnification. An area of 200,000 μm^2 was microdissected from each sample, resulting in ~2,000 cells/sample (estimated cell volume: 10x10x10 µm). Altogether, four groups of samples were laser microdissected: glioma blood vessels; glioma tissue surrounding the blood vessels; endometrium blood vessels and endometrium tissue surrounding the blood vessels. In addition, a corresponding area of the PEN membrane was laser microdissected and used as negative control. As an internal control, three full sections of a glioma sample were collected. The preparation and analysis of the internal controls was similar to that of the test samples.

Sample preparation

The procedure for samples preparation was as previously published [16]. For laser microdissection, 7µl of 0.1% RapiGest buffer (Waters, Milford, MA) was used to collect the laser

microdissected areas. Protein LoBind, 0.5-ml Eppendorf tubes (Eppendorf, Hamburg, Germany) containing the samples were stored at -80 ˚C until the time of preparation. After thawing the samples, 15 µl of RapiGest buffer were added to each tube to bring the final volume of each sample to 20 µl. The cells were disrupted by external sonification for 1 min at 70% amplitude at a maximum temperature of 25 °C (Branson Ultrasonics, Danbury, CT). For protein solubilization and denaturation, the samples were incubated at 37 °C and 100 °C for 5 and 15 min, respectively. To each sample, 1.5 µl of 100 ng/ µl gold grade trypsin (Promega, Madison, WI) in 3 mM Tris-HCl diluted 1:10 in 50 mM NH_4HCO_3 was added and incubated overnight at 37 °C for protein digestion. To inactivate trypsin and to degrade the RapiGest, 3 µl of 25% TFA were added and samples were incubated for 30 min at 37°C. Samples were centrifuged at maximum speed for 15 minutes at 4 °C and the supernatant was transferred to special glass vials to be measured in the Orbitrap. Samples were used for immediate measurements or stored for a maximum of 7 days at 4 °C. The internal control sample was diluted at 1:400 using distilled water in order to obtain a concentration comparable to that of the laser microdissected blood vessel samples. The internal control sample was prepared together with the other samples and stored at 4 °C for about 30 days until the end of all the measurements.

LTQ Orbitrap measurements

LC-MS measurements were carried out on a Ultimate 3000 nano LC system (Dionex, Germering, Germany) online coupled to a hybrid linear ion trap / Orbitrap MS (LTQ Orbitrap XL; Thermo Fisher Scientific, Bremen, Germany). The whole volumes of the digested samples (~20 µl) were loaded onto a C18 trap column (C18 PepMap, 300µm ID x 5mm, 5µm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands) and desalted for 10 minutes using a flow rate of 25 µl/min of 0.1% TFA. The trap column was switched online with the analytical column (PepMap C18, 75 µm ID x 5mm, 3 µm particle and 100 Å pore size; Dionex, Amsterdam, The Netherlands) and peptides were eluted with the following binary gradient: 0% - 25% solvent B in 120 min; 25% - 50% solvent B in 60 min; solvent A consists of 2% acetonitrile and 0.1% formic in water and solvent B consists of 80% acetonitrile and 0.08% formic acid in water. Column flow rate was set to 300 nl/min. For MS detection a data dependent acquisition method was used: high resolution survey scan from 400 – 1800 Th. was detected in the Orbitrap (value of target of automatic gain control AGC 10^6, resolution 30,000 at 400 m/z; locks mass was set to 445.120025 u (protonated $(Si(CH_3)_2O))_6$[1]). Based on this survey scan the five most intensive ions were consecutively isolated (AGC target set to 10^4 ions) and fragmented by collision activated dissociation (CAD) applying 35% normalized collision energy in the linear ion trap. After precursors were selected for MS/MS, they were excluded for further MS/MS spectra for 3 minutes. Samples were prepared and measured in a randomized way. For an internal control, we measured the same internal control sample once in every five measurements.

Mascot searching

From the raw data files of the Orbitrap mass spectrometer, MS/MS spectra were extracted by Mascot Deamon version 2.2.2 using the Xcalibur extract msn tool (version 2.07) into mgf files. All mgf files were analyzed using Mascot (Matrix Science, London, UK; 2.2). Mascot was set up to search the IPI.CHECK.v.3.12 database (version 3.12, 28229 entries) assuming trypsin digestion. The Mascot search engine was used with fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10 ppm. Oxidation of methionine was specified in Mascot as a variable modification. The Mascot server was set-up to display only peptide identifications with Mascot ion scores greater than 25.

Analysis of the LTQ Orbitrap measurements

The analysis of the Orbitrap measurements was done at two different levels. Firstly, at the level of the identified proteins, by using Scaffold software to compare the protein profiles of all four groups of samples. Secondly, at the level of the identified peptides, using Progenesis software to compare the peptide profiles of the two blood vessels groups.

Differentially expressed proteins using Scaffold

Scaffold software (Version, 2_05_01, Portland, OR, USA), [www.proteomesoftware.com], was used to summarize and filter MS/MS based peptides and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped.
Using these criteria, Scaffold generated a list of identified proteins including the number of sequenced peptides that were found in each sample. To analyze the data, we uploaded the list of identified proteins into Significance Analysis of Microarrays (SAM, Stanford University) version, 3.1 and performed a multi-class comparison with false rate discovery (FRD) of 5% to generate the differentially expressed proteins between the groups. SAM analysis calculated the relative frequency of occurrence of each protein for in the four groups of samples. Thus, we searched for those proteins which are significantly expressed in either one of the blood vessel groups as compared to the other three groups.

Differentially expressed peptides using Progenesis

Progenesis LC-MS Software package (Version, 2.5, Nonlinear Dynamics, UK) was used to align the measurements. According to the company's suggestions, a reference run was selected based on two criteria: 1. The position of the run in the middle of the measurement sequence. 2. A large file size: i.e. large number of data points. One of the glioma vessel samples was used as reference sample. After calculation of the number of vectors used to correlate the samples to the reference sample, the potential alignment is checked by a

quality control step consisting of two parts; the number of vectors and the length of the vectors. Firstly, the number of vectors found correlating with the reference run should be above 200. If the number is below 200, the length of the vectors is compared to that of the vectors in samples with high numbers of vectors. If the vectors in the alignment are more than twice as long as those in the other samples, exclusion from further analysis follows.
We aligned all blood vessel samples of glioma and endometrium. Because the control measurements were obtained from whole (not laser microdissected) tissue sections, we aligned all control samples in a separate run. Again, one sample was chosen as reference because of its adequate number of vectors and intermediate position in the order of processing. Following the alignment of the samples, the peptides were submitted to the Mascot search engine (Matrix Science, London, UK) using the UniProt (release 15.6) database, allowing 3 ppm peptide mass tolerance and one missed trypsin cleavage site.
To analyze the data, we used the data matrix generated after the first alignment for the two groups of vessels and log transformed the normalized abundance values. The list of peptides with identified proteins was uploaded into Partek® Genomics Suite™ version 6.09.0129 [http://www.partek.com/] and a principle component analysis (PCA) was performed. Subsequently, we performed an ANOVA analysis and found peptides which were differentially expressed between endometrium and GBM with a false discovery rate (FDR) lower than 0.05.

Pathway analysis

The resulting lists of differentially expressed proteins in glioma and endometrium blood vessels were uploaded into the Ingenuity Pathway Analysis system (IPA) version, 7.5 [www.ingenuity.com] as the starting point for the generation of biological networks. We used the final list of differentially expressed proteins generated by Progenesis and filtered by the Scaffold approach as a starting point to build networks. Subsequently, we used the differentially expressed proteins generated after analysis of the Scaffold data to build the networks.

Immunohistochemical validation

The proteomics results were confirmed by immunohistochemistry for some identified proteins (tenascin-C and calponin-1) using specific antibodies on paraffin-embedded sections of all the samples used for the proteomics analysis. To further investigate expressional variation between the two groups, five more samples of glioma, endometrium and normal brain were immunostained. Immunohistochemical staining was performed following the manufacturer's procedure (alkaline phosphatase technique) using mouse monoclonal antibody for tenascin-C at a 1:100 dilution (Abcam, Cambridge, UK) and mouse monoclonal antibody for calponin-1 antibody at a 1:200 dilution (Abcam, Cambridge, UK).

Results

LTQ Orbitrap measurements

The number of spectra for MS/MS generated from measuring the samples varied between 6,841 and 10,788 spectra, with a median of 8,403 spectra per sample. Based on the settings of the Orbitrap, a maximum of only five high abundant, non redundant peptides were sequenced in each spectra. The percentage of identifications in each spectrum varied between 0.03% and 0.3% with a median of 0.22 %. The controls yielded between 6,505 and 8,276 spectra with a median of 7,721 spectra per control sample. The percentage of identifications was between 0.18% and 0.26% with a median of 0.24%.

Differentially expressed proteins using Scaffold

Comparing the four groups of samples using Scaffold software generated a list of 694 identified proteins. After performing the SAM analysis with FDR of 5% a list of 152 differentially expressed proteins was generated. We categorized those 152 differentially expressed proteins based on their relative frequency of occurrence in the sample groups. We considered a protein to be differentially expressed in a particular sample group if its occurrence was at least twice the occurrence encountered in any of the other groups. There were 30 overexpressed proteins in the glioblastoma blood vessels (Table 1) and 12 overexpressed proteins in the endometrium blood vessels (Table 2).

Differentially expressed peptides using Progenesis

The alignment of both groups of endometrium and glioblastoma vessels by Progenesis was adequate to normalize the measured samples. Each sample shared more than 200 vectors in good position with the reference sample. The control samples shared also more than 200 vectors in good position to the reference sample. The data matrix obtained from Progenesis showed that overall 46,463 different masses (peptides) in all vessel samples were measured. In total, 31,614 peptides were sequenced and for 7,618 peptides the corresponding proteins could automatically be identified. The other peptides were linked with their accurate masses and sequences but there were no automatic protein identifications. The 7,618 peptides were linked to their corresponding protein identifications which resulted in 800 non-redundant identified proteins. The data matrix obtained by Progenesis contained on average 400 zero values in each sample, which had a significant influence on the distribution of the normalized abundance values. The distribution of the normalized abundance values without the zero values was log2-transformed (Figure 2). Progenesis considers the zero values as outliers and therefore, they were omitted from the analysis. Because of the random distribution of the zeros the analysis was not biased. After the transformations, the data showed a normal distribution allowing ANOVA testing in Partek to calculate the p-Values for further statistical comparisons between the two groups. The principle component analysis

Table 1

Differentially expressed proteins in glioma angiogenesis using Scaffold software

Protein	Accession #	GV contrast	EV contrast	GT contrast	ET contrast
40S ribosomal protein S16, GN=RPS16	P62249	1.582623447	-0.833402773	0.07652918	-0.833402773
Adenylyl cyclase-associated protein 1, GN=CAP1	Q01518	1.523673832	-0.951852184	-0.051660906	-0.514994652
Agrin, GN=AGRN***	O00468	2.016337036	-0.513013288	-0.658239144	-0.77926069
Band 3 anion transport protein, GN=SLC4A1	P02730	1.560701403	-0.444168284	-0.459356691	-0.611240758
Basement membrane-specific heparan sulfate proteoglycan core protein, GN=HSPG2***	P98160	2.268374171	-0.357697578	-1.154701171	-0.640505304
CD99 antigen, GN=CD99	P14209	1.073864505	-0.489275544	-0.323487963	-0.228752202
Chloride intracellular channel protein 1, GN=CLIC1	O00299	1.405522467	-0.441478211	-0.139241736	-0.810878346
Clathrin heavy chain 1, GN=CLTC	Q00610	0.671837816	-1.378684551	1.833800491	-1.310333806
Coactosin-like protein, GN=COTL1	Q14019	1.093286681	-0.56688939	0.036810999	-0.56688939
Collagen alpha-1(XVIII) chain, GN=COL18A1***	P39060	1.767677072	-0.381998011	-0.633258735	-0.689094452
Elongation factor 2, GN=EEF2	P13639	1.151635936	-0.621775271	-0.218727269	-0.289260669
Fermitin family homolog 3, GN=FERMT3	Q86UX7	1.275350321	-0.661292759	-0.309175835	-0.273964143
Fibronectin, GN=FN1	P02751	1.459146414	-0.430064206	-0.473000811	-0.508781315
Glutamate dehydrogenase 1, mitochondrial, GN=GLUD1	P00367	1.431931859	-0.742483186	-0.116515219	-0.561281932
Integrin alpha-V, GN=ITGAV	P06756	1.951762679	-0.891668229	-0.153114746	-0.891668229
Integrin-linked protein kinase, GN=ILK	Q13418	1.540645913	-0.373489918	-1.011535195	-0.05446728
Laminin subunit alpha-5, GN=LAMA5	O15230	1.897628696	-0.082805616	-0.931563178	-0.790103584
Laminin subunit beta-1, GN=LAMB1	P07942	1.434494228	-0.314628845	-0.53326923	-0.53326923
Laminin subunit beta-2, GN=LAMB2***	P55268	1.775016504	-0.758314013	-0.444264775	-0.528011239
Laminin subunit gamma-1, GN=LAMC1	P11047	2.328493269	-0.069554449	-1.460068605	-0.652863354
Nestin, GN=NES	P48681	1.464048594	-1.056230092	0.638737392	-1.110429633
Nidogen-1, GN=NID1	P14543	1.299567868	-0.153585293	-0.682004625	-0.395777487
Nidogen-2, GN=NID2	Q14112	1.281518532	-0.480905277	-0.490919049	-0.260602301
Periostin, GN=POSTN***	Q15063	1.061514938	-0.476036259	-0.099493109	-0.476036259
Plastin-3, GN=PLS3	P13797	1.982008532	-0.455861962	-0.726736462	-0.726736462
T-complex protein 1 subunit beta, GN=CCT2	P78371	1.439433434	-0.442260707	-0.037929735	-0.955450018

T-complex protein 1 subunit epsilon, GN=CCT5	P48643	1.430916612	-0.510290115	-0.373033074	-0.510290115
Tenascin, GN=TNC***	P24821	1.428421472	-0.679928621	-0.097825228	-0.6408851
Transforming growth factor-beta-induced protein ig-h3, GN=TGFBI***	Q15582	1.200152003	-0.40061651	-0.679011034	-0.052623355
von Willebrand factor A domain-containing protein 1, GN=VWA1	Q6PCB0	1.812515407	-0.72088681	-0.337037989	-0.72088681

GV= glioma blood vessels, GT= glioma surrounding tissue, EV= endometrium blood vessels, ET= endometrium surrounding tissue.

*** represent the overlap proteins between Scaffold and Progenesis analyses.

Table 2

Differentially expressed proteins in endometrium angiogenesis using Scaffold software

Protein	Accession #	GV contrast	EV contrast	GT contrast	ET contrast
Actin, aortic smooth muscle, GN=ACTA2	P62736	-0.241340252	1.241997826	-1.561872932	0.717402652
Cadherin-13, GN=CDH13***	P55290	-1.046111327	1.455838263	-0.721182808	0.383574153
Caveolin-1, GN=CAV1	Q03135	-0.361838834	1.532041021	-1.308778762	0.269454451
Complement factor B, GN=CFB	P00751	-0.71549225	1.240186568	-0.419177278	-0.063599311
Keratin, type II cytoskeletal 1b, GN=KRT77	Q7Z794	-0.590104111	1.27099347	-0.112214621	-0.557453276
Myoferlin, GN=FER1L3	Q9NZM1	-0.296696351	0.983782638	-0.354899942	-0.296696351
Myosin-Ic, GN=MYO1C	O00159	-1.156312207	2.394126596	-1.782860231	0.723331865
Poly(rC)-binding protein 1, GN=PCBP1	Q15365	-0.705312115	1.507784572	-0.893090016	0.17992656
Polymerase I and transcript release factor, GN=PTRF	Q6NZI2	-0.717892668	1.75115099	-1.1727165	0.256729828
Prolargin, GN=PRELP	P51888	-0.195674204	1.927966425	-1.611434624	0.040285866
Proliferation-associated protein 2G4, GN=PA2G4	Q9UQ80	-0.351567278	1.089858562	-0.351567278	-0.351567278
Protein kinase C delta-binding protein, GN=PRKCDBP	Q969G5	-0.671156984	1.392650741	-0.671156984	0.016778925

GV= glioma blood vessels, GT= glioma surrounding tissue, EV= endometrium blood vessels, ET= endometrium surrounding tissue.

*** represent the overlap proteins between Scaffold and Progenesis analyses.

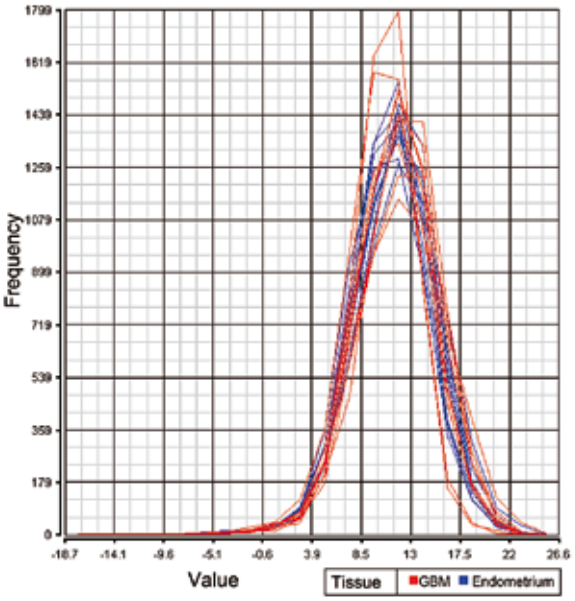

Figure 2

Histogram of the measured samples after log_2 normalization

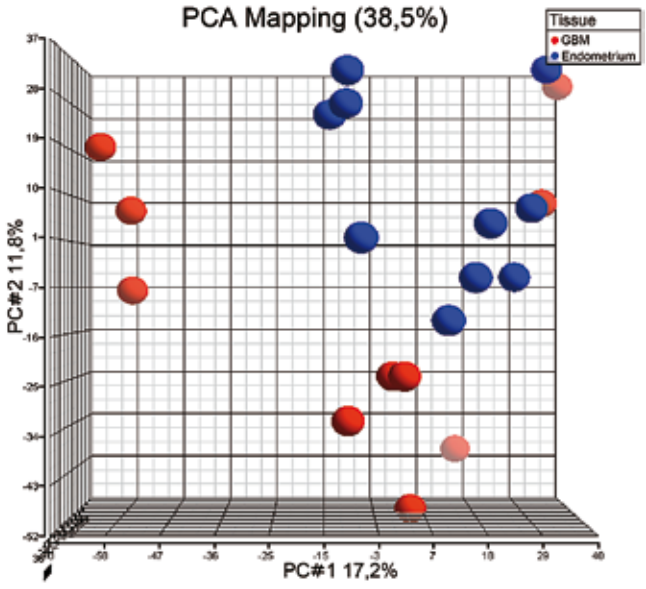

Figure 3

PCA of the protein expression patterns of the blood vessels in glioblastoma and endometrium

(PCA) yielded a rough separation of the glioma and endometrium samples (Figure 3). We concentrated our analysis on the 7,618 peptides with corresponding protein identifications. ANOVA analysis with an FDR of 0.05 resulted in 350 differentially expressed peptides. After removing the redundant protein identifications, a list of 134 differentially expressed peptides was obtained: 83 peptides with their corresponding proteins were overexpressed in glioma blood vessels while 51 were overexpressed in endometrium blood vessels.

Because the 134 differentially expressed peptides resulted from the comparison of the two blood vessels groups, we verified the expression of the corresponding proteins in all four groups included in this study. Therefore, we used Scaffold software (Version, 2_05_01, Portland, OR, USA). We used the same settings as before. We considered a protein to be exclusively expressed in one particular group if one sample of that group expressed at least two or more of its peptides, while none of the peptides of the protein was expressed by any sample of the other groups. Alternatively, a protein was considered to be exclusively expressed in one particular group if the total number of MS/MS peptides found in one group was more than three times the total number of these MS/MS peptides found in any of the other groups. This way, we confirmed the differentially expression of 12 proteins (out of 83 peptides) in the glioma blood vessels (Table 3) and 9 proteins (out of 51 peptides) in the endometrium blood vessels. (Table 4 and figure 4).

The data matrix obtained from the internal control samples showed an overall number of 27,426 measured peptides. In total, 10,826 peptides were sequenced and the corresponding proteins for 4,035 peptides could automatically be identified. The 4,035 peptides were linked to their corresponding protein identifications and resulted in 739 non-redundant identified proteins. The negative control sample did not yield any significant measurements.

Pathway Analysis

The list of 35 differentially expressed proteins from the glioma blood vessels (which were identified by Scaffold and Progenesis softwares) was uploaded into IPA and mapped against the database. The IPA could map all proteins in three different networks. The first matched network designated as "tissue development and cell-to-cell signaling" had a score of 50 and contained 20 of the identified proteins. Ten of the 20 proteins were associated with a function designated as "Cardiovascular System Development and function" and five proteins, namely: Collagen alpha-1(XVIII) chain, Laminin subunit alpha-5, Laminin subunit gamma-1, Fibronectin and Integrin alpha-V appeared to be related with angiogenesis. In addition, all 19 differentially expressed proteins identified in the endometrium blood vessels were uploaded into IPA and mapped against the database. They were mapped to four different networks. The first matched network called "Tissue development, embryonic development" contained 11 proteins. Nine proteins showed a direct relation with the

Table 3

Differentially expressed proteins in glioma blood vessels after validating the differentially expressed peptides generated by Progenesis software

Protein	Accession #	Number of peptides in each group			
		GV	GT	EV	ET
Basement membrane-specific heparan sulfate proteoglycan core protein***	P98160	101	5	32	4
Tenascin OS=Homo sapiens GN=TNC ***	P24821	89	29	2	0
Periostin***	Q15063	47	15	3	7
Laminin subunit beta-2***	P55268	32	2	0	0
Transforming growth factor-beta-induced protein ig-h3***	Q15582	28	0	3	1
Agrin***	O00468	23	0	0	0
Collagen alpha-1(XVIII) chain***	P39060	13	2	2	2
Leukocyte elastase	P08246	8	0	0	0
Adipocyte plasma membrane-associated protein	Q9HDC9	2	0	0	0
Aspartyl-tRNA synthetase, cytoplasmic	P14868	2	0	0	0
Cathepsin G	P08311	2	0	0	0
Laminin subunit alpha-4	Q16363	2	0	0	0

GV= glioma blood vessels, GT= glioma surrounding tissue, EV= endometrium blood vessels, ET= endometrium surrounding tissue.

*** represent the overlap proteins between Scaffold and Progenesis analyses.

Table 4

Differentially expressed proteins in endometrium blood vessels after validating the differentially expressed peptides generated by Progenesis software

Protein	Accession #	Number of peptides in each group			
		GV	GT	EV	ET
Collagen alpha-1(XII) chain OS=Homo sapiens GN=COL12A1	Q99715	10	2	57	3
Calponin-1 OS=Homo sapiens GN=CNN1	P51911	0	0	30	6
Polymerase I and transcript release factor OS=Homo sapiens***	Q6NZI2	0	0	28	2
Histone H1.0 OS=Homo sapiens GN=H1F0	P07305	0	0	28	6
EMILIN-1 OS=Homo sapiens GN=EMILIN1	Q9Y6C2	7	3	19	3
Tenascin-X OS=Homo sapiens GN=TNXB	P22105	0	0	17	3
Tropomyosin beta chain OS=Homo sapiens GN=TPM2	P07951	7	0	15	0
Cadherin-13 OS=Homo sapiens GN=CDH13 ***	P55290	0	0	10	2
Vigilin OS=Homo sapiens GN=HDLBP	Q00341	0	0	4	0

GV= glioma blood vessels, GT= glioma surrounding tissue, EV= endometrium blood vessels, ET= endometrium surrounding tissue.

*** represent the overlap proteins between Scaffold and Progenesis analyses .

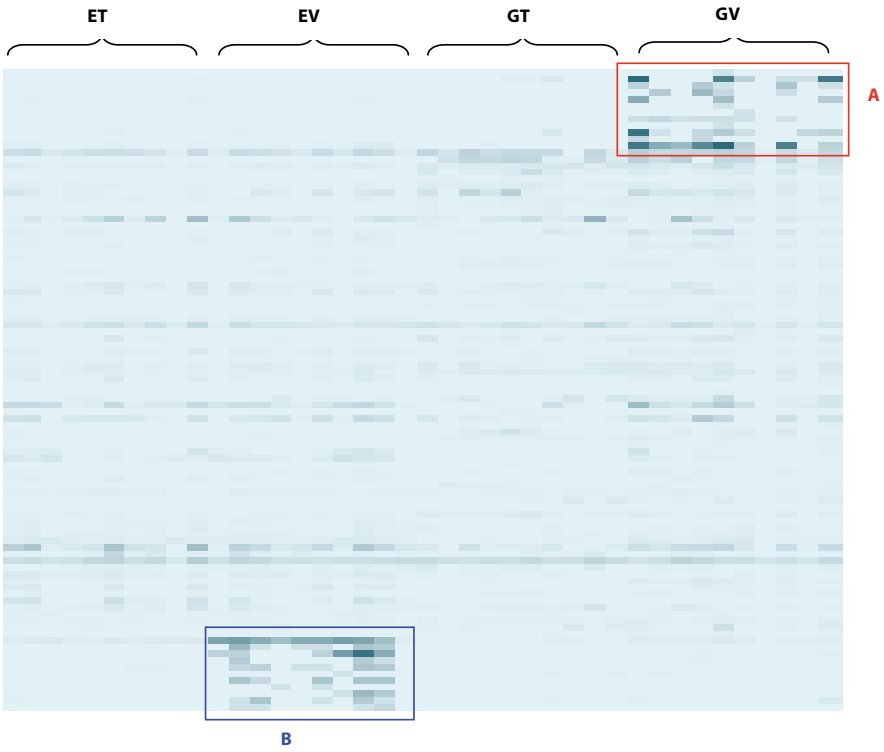

Figure 4

Heat map of the peptides belonging to the identified proteins by Progenesis in the respective tissue groups

Each row represents a different protein that corresponded to the differentially expressed peptides as calculated by the progenesis analysis. Each column represents one sample from the four respective groups. The intensity of the blue color represents the number of peptides that were measured in each sample. Light blue color = zero, and the darkest blue =100

A: the distribution of peptides for the proteins which were overexpressed in glioma angiogenesis.

B: the distribution of peptides for the proteins which were overexpressed in endometrium angiogenesis.

ET: endometrium Tissue, EV: endometrium blood vessels, GT: glioblastomatissue, GV: glioblastoma blood vessels

function called "Cardiovascular System Development and function" and eight proteins (Caveolin-1, Myosin-Ic, Protein kinase C delta-binding protein, Calponin-1 and Emilin-1) appeared to be related with proliferation of endothelial cells. At the level of molecular and cellular functions, the differentially expressed proteins that were identified in the glioma blood vessels had direct relation with cell-to-cell signaling, cellular movements and cell

morphology. In contrast, the differentially expressed proteins in the endometrium blood vessels had a relation with molecular transport and excretion of proteins.

Immunohistochemistry

The exclusive expression of tenascin-C in the glioma blood vessels, and the exclusive expression of calponin-1 in the endometrium blood vessels, was confirmed by immunohistochemistry (Figures 5 and 6).

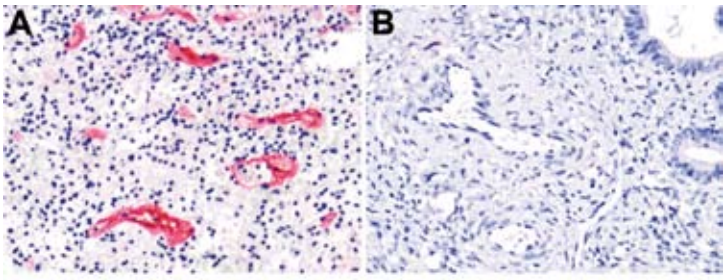

Figure 5
Expression of tenascin-C in blood vessels in glioma and endometrium

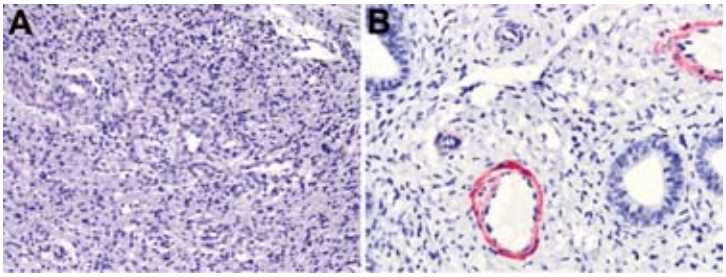

Figure 6
Expression of calponin-1 in glioma and endometrium blood vessels

Discussion

The analysis of complex protein mixtures is currently one of the most challenging subjects in the area of proteomics. The first step undertaken in the present study to reduce the complexity of the samples was by using laser microdissection to tissue structures of interest. The normal biological variation in protein expression between cells of identical lineage is

around 15% to 30% [29]. We expect an even higher variation in protein expression in situations of stress, or in tumors. The method of sample preparation is crucial to avoid false variations. Yet, a major source of variation lays in the structure and function of different tissue samples. The complexity of the samples was also reduced at the level of the measuring techniques. In several studies it has been shown that fractionation of biomaterials prior to measuring the peptide digest by mass spectrometers reduces the complexity of the test samples and increases the number of identified proteins [16, 26-28]. In the present study we used the LTQ Orbitrap to measure the protein profiles from 2,000 cells (~270 ng total protein, estimating that one cell has 137 ± 4 pg total proteins). The LTQ Orbitrap XL is an LC-MS combining three different and complementary fragmentation techniques with proven benefits of Orbitrap technology. These features allowed us to perform rapid, high resolution, automated separation and very sensitive, fast MS/MS sequencing. Because the measurements of the internal controls showed no significant differences, the LTQ Orbitrap is satisfactory as to stability and reproducibility of its measurements. In addition, these internal controls proved that the samples are stable after preparation and they can be stored at 4 ˚C up to 30 days until time of measurement.

We used Progenesis software to align the MS measurements obtained from the two vessel sample groups. The software was only recently made available and to the best of our knowledge, was used for the first time to align LC-MS measurements obtained from micro-dissected human tissue. In our hands, comparing glioma blood vessels with endometrium blood vessels resulted in a good alignment as determined by quality criteria such as the number of vectors, their lengths and their positions. We tried to align the four groups of samples, namely: glioma blood vessels, glioma surrounding tissue, endometrium blood vessels and endometrium surrounding tissue using Progenesis (data not shown). It was difficult to choose a reference sample with a good number of vectors in good positions as compared to tissue that originate from different organs, i.e. brains and endometriums. Because significant better results were obtained when two groups (instead of four) were aligned, we preferred to focus our analysis of the two sample groups of interest, viz., the blood vessels in glioma and endometrium. Aligning of the measurement using Progenesis yielded on average 400 zero values in each sample which significantly influenced the data analysis. The zeros may represent measurements below the threshold of detection, or alternatively, may be indicative of absence of a particular peptide. In any case, we considered the zero values as missing data. Because of the random distribution of the zero values, the data analysis was not biased by their omission. The comparison of the peptide spectra of the glioma blood vessels with the endometrium blood vessels yielded 46,463 different masses of which 70% (31,614 peptides) MS/MS were measured and their sequences were determined. Only 17% (7,618 peptides) were automatically identified. Direct searching of the database did not result in the identification of approximately 3% (23,996 peptides) of the peptides

with known sequences and accurate mass measurements. Improvement in identifications could be reached by generating specific databases for different tissue types. In order to generate such databases, large amounts of relevant tissues are needed for creating comprehensive databases. For 30% of the peptides measured (14,849 peptides) no sequences were obtained. Underlying these peptides, differentially expressed proteins may be present. Therefore, serious efforts should be made to include and sequence the maximum number of peptides measured.

The data obtained in this study was analyzed at the level of peptides and proteins. If Orbitrap measurements are analyzed at the level of proteins, only about 20% of the data are used. However, when comparisons are made at the level of peptides, far more differences between samples will be detected. Actually, 694 proteins were identified by introducing proteins to Scaffold, while 800 non-redundant proteins were identified if peptides were inserted to Progenesis. Using Progenesis allowed having access to the remaining data (about 80 % of the measured peptides). The number of sequenced peptides relating to proteins varied between the samples. The characteristics and abundances of the different proteins influence this number. High abundant proteins generate more measurable peptides, which may hamper the identification of peptides present in lower quantities. The setting used for the LTQ Orbitrap overcame this problem to some extent. Yet, very low abundant proteins are beyond the threshold of identification. In addition, high molecular weight proteins may generate more measurable peptides than proteins of lower mass weight. In Progenesis, the identification of a particular protein was based on the accurate mass measurement and the sequence of at least one peptide. Several hundreds of peptides from high abundant proteins as actin, collagen, glial fibrillary acidic protein (GFAP), vimentin were measured and sequenced. We noticed that some peptides belonging to the same protein may show differential expression in the opposite direction. In the present analysis we found that 15 out of 350 differentially expressed peptides were matched with proteins which were expressed in both groups. In order to generate a non-redundant list of peptides we decided to take the direction of the peptide with the lowest p-value, but confirmation at the protein level was necessary. To that aim Scaffold software was applied to filter out the misinterpreted proteins.

The differentially expressed proteins in glioma and endometrium angiogenesis were classified according to their function. The majority (59%) of the 39 proteins found in the glioma blood vessels were structural proteins which form the largest group of proteins in nature (Figure 7). Eight percent of the differentially expressed proteins appeared to be enzymes and another eight percent were integrins which have specific functions in angiogenesis. In addition, 6% were peptidases. The transporter proteins, receptors, proteases and peptidases are of specific interest because these molecules may become objects for the

design of anti-angiogenic therapies [30]. Classification of the 19 differentially expressed proteins of endometrium blood vessels showed that 44% were structural proteins and 11% were peptidases (Figure 8).

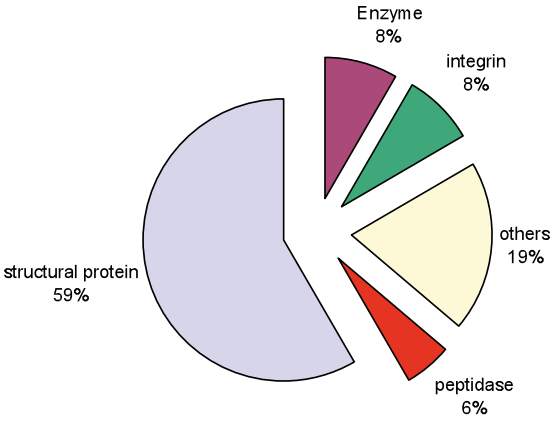

Figure 7
Classification of the 35 differentially expressed proteins in glioma angiogenesis

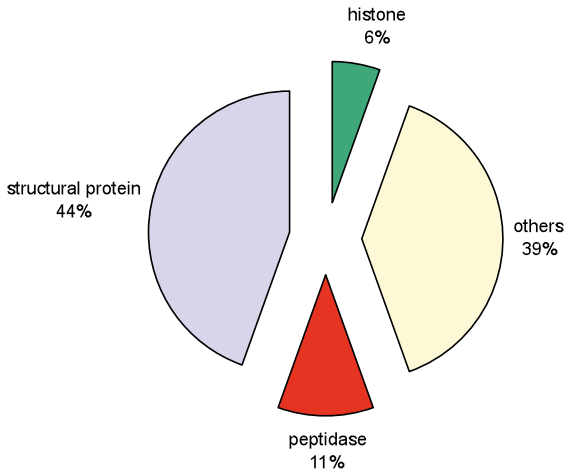

Figure 8
Classification of the 18 differentially expressed proteins in endometrium angiogenesis

Part of the differentially expressed proteins appeared to be associated with angiogenesis. For example, Tenascin-C which was found to be overexpressed in glioma blood vessels is an extracellular matrix protein which participates in various processes like normal fetal development and wound healing [31]. Its presence reportedly is strongly correlated with microvascular density (MVD) [31]. Additionally, tenascin-C regulates angiogenesis in tumor through the regulation of vascular endothelial growth factor expression (VEGF), particularly in glioblastoma [32]. VEGF is upregulated in glioblastoma under hypoxic conditions and mediated by hypoxia inducible factor (HIF-1) [33]. We validated the expression of tenascin-C by immunohistochemistry and confirmed its exclusiveness for glioma vasculature (Figure 5). Basement membrane-specific heparan sulfate proteoglycan core protein, also known as perlecan is another interesting protein which was upregulated in glioma blood vessels. It is an integral component of basement membranes that interacts with other basement membrane components such as laminin, prolargin and collagen type IV [34, 35]. In 2006 Kaji et al. showed that VEGF165 (a member of the VEGF family) increased the accumulation of basement membrane-specific heparan sulfate proteoglycan core protein in cultured endothelial cells human brain [36]. Yet another interesting finding is that of emilin1 which appeared to be overexpressed in endometrium. Emilin1 is a glycoprotein associated with the extracellular matrix of blood vessels [37]. Emilin1 binds to immature proTGF-β and prevents its maturation by protein convertases. TGF- β is a well known inducible factor for angiogenesis. Emilin regulates the extracellular TGF-β availability [38]. The relevance of its relative overexpression in endometrium as compared to glioma vasculature is not explained and whether this differential expression may assist in the development of anti-angiogenic therapy remains to be seen.

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Chapter | 7

Structural and transcriptional differences between the vasculature of pilocytic astrocytomas and diffusely infiltrating gliomas

Dana A.M. Mustafa; Marcel van der Weiden; Pim J. French;
Clemens M.F. Dirven; Peter .A.E. Sillevis Smitt; Andreas Kremer ; Johan M. Kros.

Submitted for Publication

Abstract

The formation of neovasculature in diffusely infiltrating gliomas is driven by various signaling pathways of which most prominently that of the hypoxia-induced VEGF/VEGFR. The identification of all pathways operative in glioma neoangiogenesis is important for the design of effective anti-angiogenic therapy. During progression of diffusely infiltrating glioma the tumor cellularity increases which causes hypoxia. In turn, hypoxia leads to apoptosis, hyperplasia of the microvasculature and necrosis. Paradoxically, in pilocytic astrocytoma there is notorious microvascular proliferation while proliferation parameters and cell density are low and apoptosis and necrosis are absent. So far, no data on the structural and immunophenotypical differences between the vasculature of both glioma subtypes have been published. We hypothesized that there may exist differences between the angiogenic triggers operative in high-grade diffuse astrocytomas (glioblastomas) and pilocytic astrocytomas. In the present study we compared the immunophenotypical characteristics of the microvasculature of pilocytic astrocytoma and glioblastoma using multiple markers for endothelial cells and pericytes (mural cells) using confocal microscopy. The intactness of the BBB was monitored by visualization of the expression of tight junction proteins. In addition, RNA expression profiles of both glioma subtypes were compared for expressional differences in angiogenesis-related pathways. We found that the newly formed blood vessels in pilocytic astrocytoma, including the glomeruloid vessels, have maintained a layered structure as compared to the haphazardly structured vessels in glioblastoma. In spite of this finding, the expression of tight junction proteins was diminished or absent in the pilocytic vasculature, just like the situation in the malignant gliomas. The RNA expression profiles showed, besides major differences in cell-cycle related gene expression, differences in the transcription of genes active in the VEGF/VEGFR pathway. Specifically, in the pilocytic astrocytomas upregulation of genes involved in capillary sprouting and development was present.

We conclude that there are essential structural and immunophenotypical differences between the blood vessels in pilocytic astrocytoma and diffuse glioma. Transcriptional differences point to differences in angiogenic triggers. Further detailing of these differences is necessary to understand the various mechanisms of neovascularization in gliomas and to define appropriate targets for anti-angiogenic therapy.

Introduction

Gliomas are among neoplasms with the highest degree of neovascularization. In diffusely infiltrating gliomas the vascular density increases over time and tumors with higher microvascular densities are more malignant [1-3]. Progressive proliferation of the vessel walls (called microvascular proliferation; MVP) will eventually result in the formation of glomeruloid vascular structures and ultimately develop into pseudo-sarcomatous proliferations. Angiogenesis is triggered and / or inhibited by molecules secreted by endothelial cells, tumor cells, connective tissue around tumor cells and blood cells [4]. In malignant (diffuse) glioma, tumor cells will co-opt brain blood vessels while increase in tumor cellularity causes local hypoxia. As a result, Ang-2 expression is upregulated by endothelial cells and in the absence of VEGF this leads to apoptosis, vascular collapse and more hypoxia. Part of the tumor cells will migrate from the hypoxic area and start to produce proangiogenic factors as VEGF, mediated by the transcriptional complex hypoxia inducible factor 1 (HIF-1) and Il-8 [5]. This will promote angiogenesis around necrotic foci which is the morphological hallmark of glioblastoma. It is speculated that particular genetic changes occurring in glial tumor cells like EGFR amplification and PTEN mutations may also have their influence on transformation of blood vessels by induction of VEGF [6].

In pilocytic astrocytomas MVP is also encountered and is responsible for contrast enhancement at radiological imaging [7]. In some cases the newly formed blood vessels may become so prominent that in obsolete literature the term "angioglioma" was used [8]. Remarkably, in pilocytic astrocytoma the presence of MVP is not associated with anaplasia or signs of increased malignancy. MVP is also encountered in pilomyxoid astrocytomas (to be regarded as a more aggressive pilocytic astrocytoma variant) but neither in this tumor the presence of MVP carries a prognostic dismal connotation [9]. The cell density in pilocytic astrocytoma is usually low and comparable to low-grade diffuse glioma in which MVP has not yet developed [10]. Other histological features associated with advanced tumor development like pseudopalisades and necrosis as encountered in glioblastomas are not present in pilocytic astrocytomas. Furthermore, there are no genetic aberrations like PTEN mutations or EGFR amplifications. Therefore, we wondered whether the angiogenic triggers and pathways involved in pilocytic astrocytoma overlap with those acting in malignant gliomas.

There are no specific data on angiogenic pathways or their relative contributions involved in the formation of the new and hypertrophied vessels in pilocytic astrocytoma. Moreover, no detailed description of the proliferated vessels in pilocytic astrocytoma is found in the literature. In a previous study we detailed the structure of high-grade glioma vessel walls in terms of cell types involved and found aberrant protein expression by these cells [11]. We also demonstrated diminished expression of the proteins occludin and the tight junction

protein 1 (ZO-1) as sign of the disruption of the BBB [12]. In the present study we compared the immunophenotypical characteristics of the microvasculature of pilocytic astrocytoma with that of glioblastoma using multiple markers for endothelial cells and pericytes (mural cells) using confocal microscopy. Since contrast enhancement on radiological scans of pilocytic astrocytoma is suggestive of leakiness due to disruption of the BBB in these tumors, we also included immunohistochemistry for occludin and cadherin. The state of hypoxia of the tissue was estimated by immunostaining for VEGF-A and HIF-1α. By using bio-informatics to gene expression profiles obtained from glioblastomas and pilocytic astrocytoma we investigated the involvement of hypoxia-related triggers in the development of MVP in these glioma subsets.

Material & Methods

Patients and tumor samples

Formalin-fixed paraffin-embedded samples of six pilocytic astrocytomas and five glioblastomas were taken from the files of the Department of Pathology, Erasmus MC, Rotterdam. For the application of confocal microscopy, the fresh-frozen samples of the same six pilocytic astrocytoma samples were used (Table 1). For comparison of RNA expression arrays, seven pilocytic astrocytomas were compared with six glioblastomas (Table 1). Glioma samples were collected from the Department of Pathology of the Erasmus MC tumor archive from patients as described before (Gravendeel et al., Cancer Res. 2009; accepted for publication).

Immunohistochemistry

Single staining procedures

From each paraffin embedded sample, adjacent slides of five µm sections were stained with the various antibodies. The antibodies and their specifications are summarized in Table 2. We followed the same procedure as published previously [11]. Immunohistochemical staining was performed following the manufacturer's instructions (alkaline phosphatase technique). Briefly, after deparaffinizing the sections in xylene for 15 minutes and rehydrated through graded alcohol and washed with water and with phosphate-buffer saline (PBS). The sections were incubated with the antibody for 30 minutes. After washing the sections with PBS, the corresponding secondary antibody was added and incubated 30 minutes at room temperature (RT). New Fuchsin Alkaline Phosphatase Substrate Solution (Dako, Denmark) was freshly prepared and the sections were incubated for about 30 minutes. Afterwards, the sections were washed with tap water, counterstained and cover-slipped with permanent mounting medium.

Table 1
Clinical data for the patient which had been used in this study

Diagnosis	Gender	Age	Localization	IHC / Expr
Pilocytic astrocytoma	f	1	Cerebellum	IHC
Pilocytic astrocytoma	m	5	Cerebellum	IHC
Pilocytic astrocytoma	f	5	Cerebellum	IHC
Pilocytic astrocytoma	m	51	Cerebellum	IHC
Pilocytic astrocytoma	f	36	Cerebellum / brain stem	IHC
Pilocytic astrocytoma	f	22	left Occipital	IHC
Pilocytic astrocytoma	f	12	Cerebellum	Expr.
Pilocytic astrocytoma	f	22	Cerebellum	Expr.
Pilocytic astrocytoma	f	24	Cerebellum	Expr.
Pilocytic astrocytoma	m	32	Cerebellum	Expr.
Pilocytic astrocytoma	m	34	Cerebellum	Expr.
Pilocytic astrocytoma	f	37	Cerebellum	Expr.
Pilocytic astrocytoma	m	16	Brain stem	Expr.
Glioblastoma	f	63	left frontal	IHC
Glioblastoma	f	54	left Fronto-Parietal	IHC
Glioblastoma	m	55	right Frontal	IHC
Glioblastoma	m	57	left Temporal	IHC
Glioblastoma	m	47	right Temporal-Parietal	IHC
Glioblastoma	m	46	right Fronto-Parietal	Expr.
Glioblastoma	f	61	right Frontal	Expr.
Glioblastoma	f	64	left Temporal	Expr.
Glioblastoma	m	67	right Temporal	Expr.
Glioblastoma	m	81	left Occipital	Expr.
Glioblastoma	m	61	left Temporal	Expr.

IHC = used for immunohistochemistry; Expr. = used for RNA expression arrays

Double staining procedures

Double immunolabelings for colligin 2 and various markers for endothelial cells and pericytes were carried out. We followed the same procedure as published previously [11]. Adjacent slides of five µm sections were made from six pilocytic astrocytoma samples and mounted onto non-coated microscope slides, fixed in acetone for 15 minutes and air dried. Sections were incubated with colligin 2 polyclonal antibody for 30 minutes, washed and incubated again with Cy3- conjugated goat-anti-rabbit for 30 minutes. After washing, sections were incubated with the second monoclonal antibody for 30 minutes followed by 30 minutes of labeling with biotin-horse-anti-mouse antibody. Detection was performed by FITC-conjugated-avidin. Nuclei were counterstained with DAPI in a vector sheet (1:1000)

and slides were covered. For all antibodies the staining were always performed single on each sample also, to control for the accuracy of the staining and specificity of the antibody. For each antibody, negative controls including the secondary antibodies only were obtained for both single and double stained slides.

Triple staining procedures

Triple immunolabelings were performed combining colligin 2 and CD31 antibodies with various markers either for activated endothelial cells using CD105 or for pericytes using SMA, NG2 and endosialin. The same six frozen biopsy samples of pilocytic astrocytoma were used for triple staining. Adjacent slides of 5 µm sections were mounted onto non-coated microscope slides, fixed in acetone for 15 minutes and then air-dried. Sections were incubated with colligin 2 polyclonal antibody for 30 minutes, washed and incubated again with Cy5- conjugated donkey anti-rabbit for 30 minutes. After washing, sections were incubated with the either one of CD105, SMA, NG2 or endosialin monoclonal antibody, washed and incubated with biotin- labeled-horse-anti-mouse antibody. Detection was performed by FITC-conjugated-avidin. After washing, sections were incubated with CD31 monoclonal antibody for 30 minutes followed by 30 minutes of labeling with Cy3-conjugated goat-anti-mouse-antibody. After washing, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in a vector sheet (1:1000) and slides were covered. For all antibodies the staining was always performed single on each sample also, to control for the accuracy of the staining and specificity of the antibodies used. For each antibody, negative controls including the secondary antibodies only were obtained for single-, double- and triple- stained slides. The specifications of the antibodies are summarized in Table 2.

Confocal laser scanning microscopy

Confocal images of double and triple stained sections were obtained using a confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc.) equipped with a Plan-Neofluar 40x/1.3 NA oil objective (Carl Zeiss MicroImaging, Inc.). A diode laser was used for excitation of DAPI at 405nm, an argon laser for FITC at 488nm, a HeNe-laser for Cy3 at 543nm and a HeNe-laser for Cy5 at 633nm. For DAPI an emission bandpassfilter of 420-480nm was used, for FITC a bandpassfilter of 500-530nm, for CY3 a bandpassfilter of 560-615nm and for Cy5 a longpassfilter of 650 nm. The signals were recorded sequentially (multi-track option) to avoid bleed-through of the signals and stored in separate channels.

RNA Expression profiles and pathway analysis

Expression levels were extracted from Affymetrix HU133 Plus 2.0 arrays and normalized with RMA using Partek® Genomics Suite™ [http://www.partek.com/]. A principle component analysis (PCA) was performed in Partek® and the two tumor groups were analyzed using

Table 2
Antibodies used in this study

Name	Dilution	Commercial source
monoclonal colligin 2	1:500	Stressgen, Michigan, USA
polyclonal colligin 2	1:100	MBL international, Woburn, Canada
CD31	1:40	Dako, Glostrup, Denmark
CD34	1:30	Dako, Glostrup, Denmark
CD105	1:2000	Dako, Glostrup, Denmark
NG2	1:100	ZYMED laboratories, California, USA
endosialin	1:500	Prof. Isacke, Institute of Cancer Research, London, UK
αSMA	1:40	Biogenex, California, USA
collagen I	1:100	Abcam, Cambridge, UK
collagen IV	1:25	Dako, Glostrup, Denmark
VEGF-A	1:200	Santa Cruz biotechnology, California, USA
HIF-1	1:100	BD bioscience, California, USA
Cy3-goat-anti-rabbit	1:100	BioLegend, California, USA
biotin-horse-anti-mouse	1:200	Vector, Peterborough, UK
FITC-conjugated-avidin	1:50	Jackson Immunoreasearch, Pennsylvania, USA
Cy5- conjugated donkey anti-rabbit	1:50	Jackson Immunoreasearch, Pennsylvania, USA
Cy3-goat-anti-mouse-antibody	1:100	Biolegend, San Diago, USA

Significance Analysis of Microarrays (SAM, Stanford University) as implemented in Biowisdom's OmniViz [http://www.biowisdom.com/content/omniviz]. Two-fold difference in expression between the two groups and a false discovery rate below 1% were used as cut-offs. The resulting list of differentially expressed transcripts was uploaded into Ingenuity IPA [Ingenuity Systems, Redwood City, CA, www.ingenuity.com] and BioBase ExPlain system [BIOBASE GmbH, Germany, www.biobase-international.com] as the starting points for the generation of biological networks. In both systems a p-Value is calculated determining the probability that each biological function and/or disease assigned to the data set of interest is due to chance alone. Selected genes were visualized in a molecular network using information contained in IPA and show the connectivity of the individual proteins. Additionally, differentially expressed transcripts and genes are classified either by proprietary Gene Ontology's (GO) or by Medical Subject Heading (MeSH) terms according to the categories of canonical pathways, therapeutic target, biomarker, and molecular mechanism. The significance of the association between the dataset and the categories is

measured by the ratio of the number of proteins from the dataset that map to the category divided by the total number of proteins that map to the canonical pathway.

Results

Immunohistochemistry for VEGF-A

Immonopositivity for VEGF-A in the glioblastoma specimens was concentrated around the areas of palissading necrosis (Figure 1A). In the pilocytic astrocytomas, all specimens contained areas of immunopositivity also (Figure 1B), but less cells were positive and no association with a ny particular histological feature was present. Roughly the same results were obtained by staining for HIF-1α.; in both pilocytic astrocytoma and in malignant glioma immunopositive cells were present.

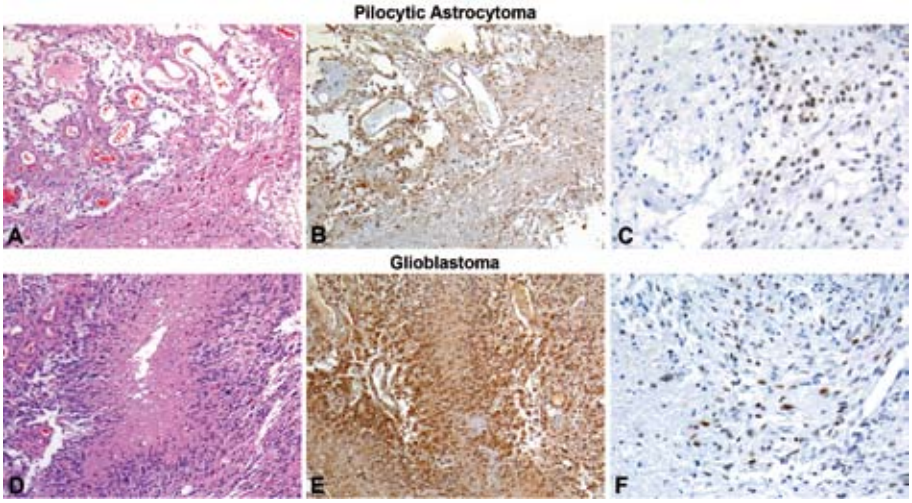


Figure 1
Expression of VEGF-A and HIF-1α in pilocytic astrocytoma and glioblastoma
A-: pilocytic astrocytoma (H&E; x250); **B:** pilocytic astrocytoma (VEGF-A; x250); **C:** pilocytic astrocytoma (HIF-1α; x400); **D:** glioblastoma (H&E; x250); **E:** glioblastoma (VEGF-A; x250); **F:** glioblastoma (HIF-1α; x400). The percentages of immunopositive cells differ between pilocytic astrocytoma and glioblastoma.

Confocal laser microscope scanning

The small, single-layered, normal looking vessels in both pilocytic astrocytoma and glioblastoma expressed the endothelial markers CD31 and CD34 and the extracellular

proteins collagen types I and IV. In addition, the cells showed abnormal expression of CD105, endosialin, NG2, colligin 2 and αSMA (Table 3; Figure 2).

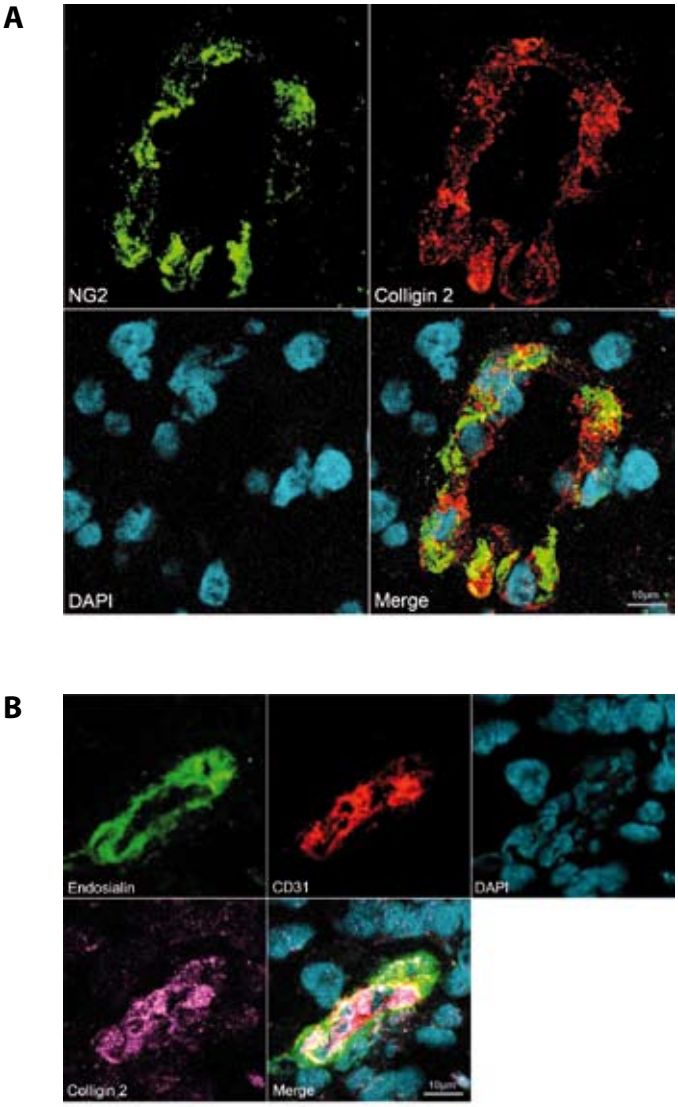
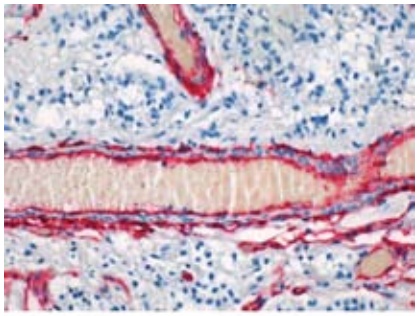


Figure 2
Expression of the various markers in small vessels in pilocytic astrocytoma
A: Expression of NG2 and colligin 2 in the endothelium of small vessels in pilocytic astrocytoma.
B: Expression of endosialin, CD31 and colligin 2 in the endothelium in small vessels of pilocytic astrocytoma.

Similar results were obtained in hypertrophied vessels in both pilocytic astrocytoma and glioblastoma. However, the distribution of these markers within the inner, middle and outer layers of these vessels differs between the two glioma subtypes (Table 3). In the hypertrophied vessels in pilocytic astrocytoma, CD34 expression is not confined to the inner (endothelial) layer, but also present in the outer cell layers (Figure 3A). In pilocytic astrocytoma CD105 is expressed in all layers, while it is absent from the middle layer in glioblastoma (Figure 3B). In contrast to glioblastoma, the expression of αSMA in pilocytic astrocytoma is restricted to the middle layer (Figure 3C). In addition, endosialin is ubiquitously expressed in pilocytic astocytoma but missing from the middle layer in

A



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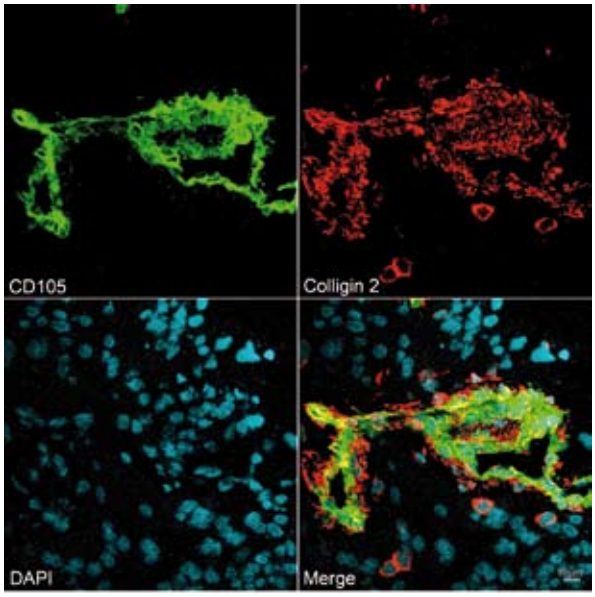

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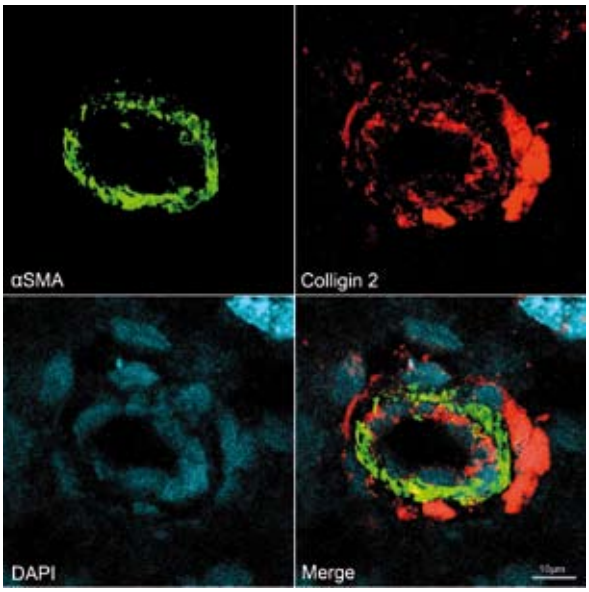


Figure 3
Expression of the various markers in hypertrophic blood vessels in pilocytic astrocytoma
A: The expression of CD34 is not restricted to the endothelial layer, its also present in the outer cell layer of the hypertrophied blood vessels (x 250).
B: CD105 expression is noticed in all layers of the hypertrophied vessels.
C: Expression of αSMA in pilocytic astrocytoma is restricted to the middle layer.

glioblastoma. NG2 is only expressed in the middle layer of pilocytic vasculature while in glioblastoma, NG2 is found in all, except the inner, layer.

In contrast to the glomeruloid vessels in glioblastoma, in the pilocytic astrocytomas these vessels are luminized and keep a well-defined structure of an inner endothelial layer expressing CD31, CD34, CD105 and endosialin (Figure 4A). The outer layer also shows expression of endosialin. The expression of αSMA and NG2 is restricted to this layer in the pilocytic astrocytomas (Figure 4B) (Table 3).

Another distinguishing feature between the vasculature of glioblastoma and pilocytic astrocytoma is the absence of thrombotic and re-canalized vessels in the latter. Remarkably, no differences in the expression patterns of colligin 2, collagen types I and IV were found between the pilocytic and glioblastoma vessels (Table 3). Colligin 2 is present in all vessel subtypes and in all layers of the blood vessels in pilocytic astrocytoma. In general, collagen I and IV are expressed in the extracellular matrix of all vessels subtypes (Figure 5).

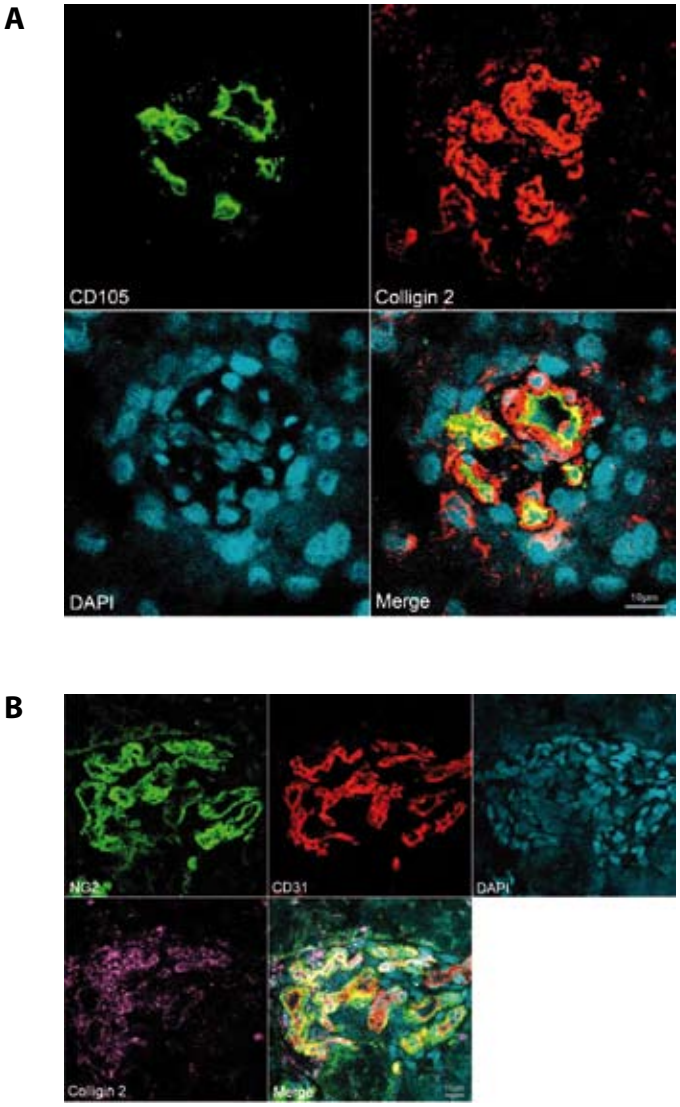


Figure 4

Expression of the various markers in glomeruloid blood vessels in pilocytic astrocytoma

A: Expression of CD105 is restricted to the endothelial layers of the glomeruloid vessels.

B: The expression of NG2 is seen in the outer layer only.

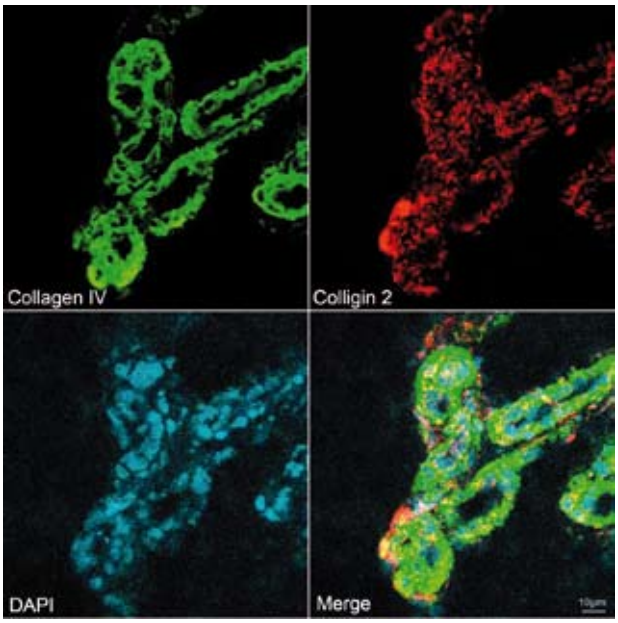


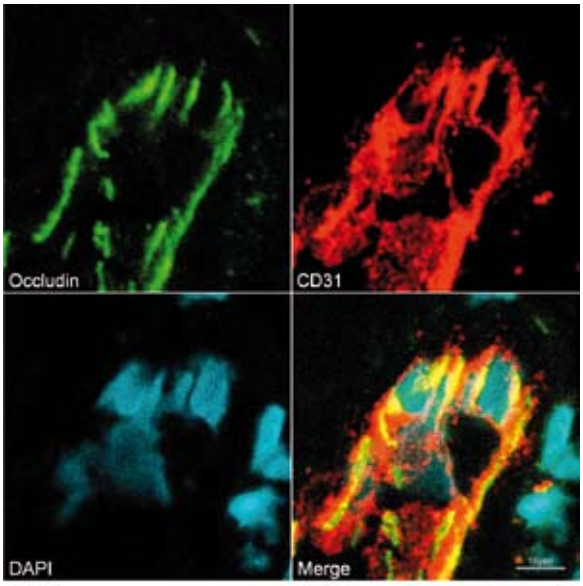
Figure 5
Expression of collagen I in glomeruloid blood vessels in pilocytic astrocytoma

Table 3
Immunostaining of blood vessels in pilocytic astrocytoma

Blood vessel structure	Layers	CD31	CD34	CD105	αSMA	endosialin	NG2	colligin 2	collagen IV
small, normal-looking vessels	single	+	+	+	+	+	+	+	+
Hypertrophied vessels	inner	+	+	+	-	+	-	+	-
	middle	-	-	+ (*)	+	+ (*)	+	+	+
	outer	-	+ (*)	+	+	+	- (*)	+	+
Glomeruloid vessels	inner	+	+	+	- (*)	+	- (*)	+	-
	middle	-	-	- (*)	+	+	+	+	+

(*) different from staining results in similar vessel types in diffuse glioma [11].

A



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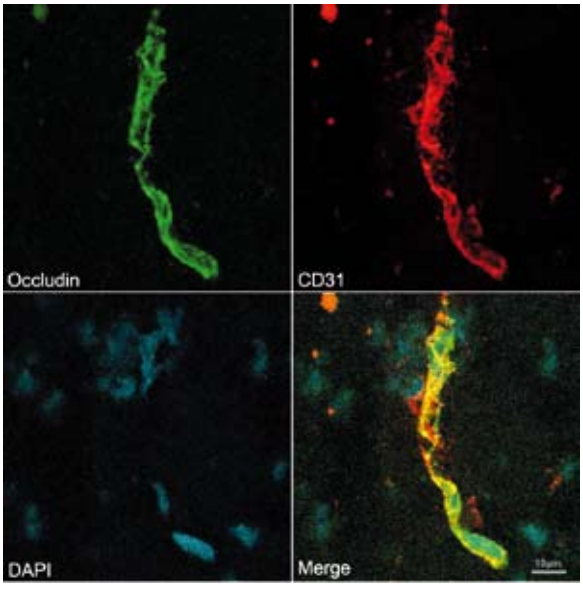

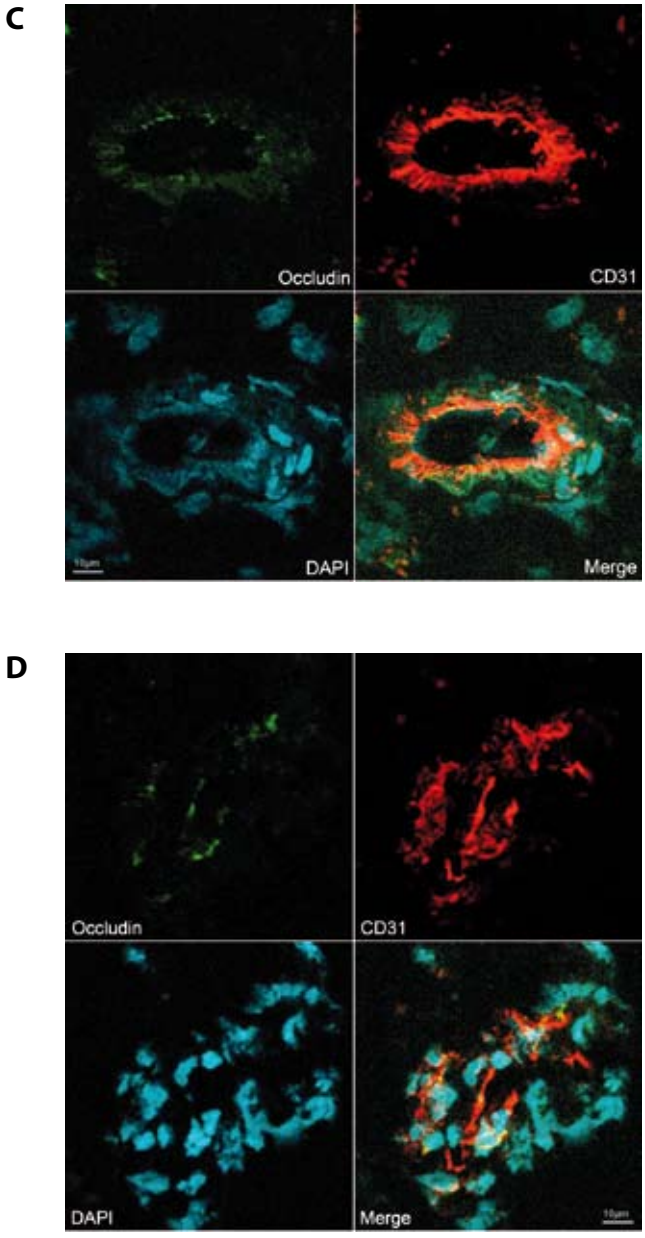

Figure 6

Expression of tight junction protein occludin in normal and pilocytic astrocytoma

A: Expression pattern of occludin in blood vessels in normal brain.

B: Intact expression of occludin in small blood vessels in pilocytic astrocytoma.

C: Reduced expression of occludin in hypertrophied blood in pilocytic astrocytoma.

D: Reduced to absent expression of occludin in hypertrophied blood in pilocytic astrocytoma.

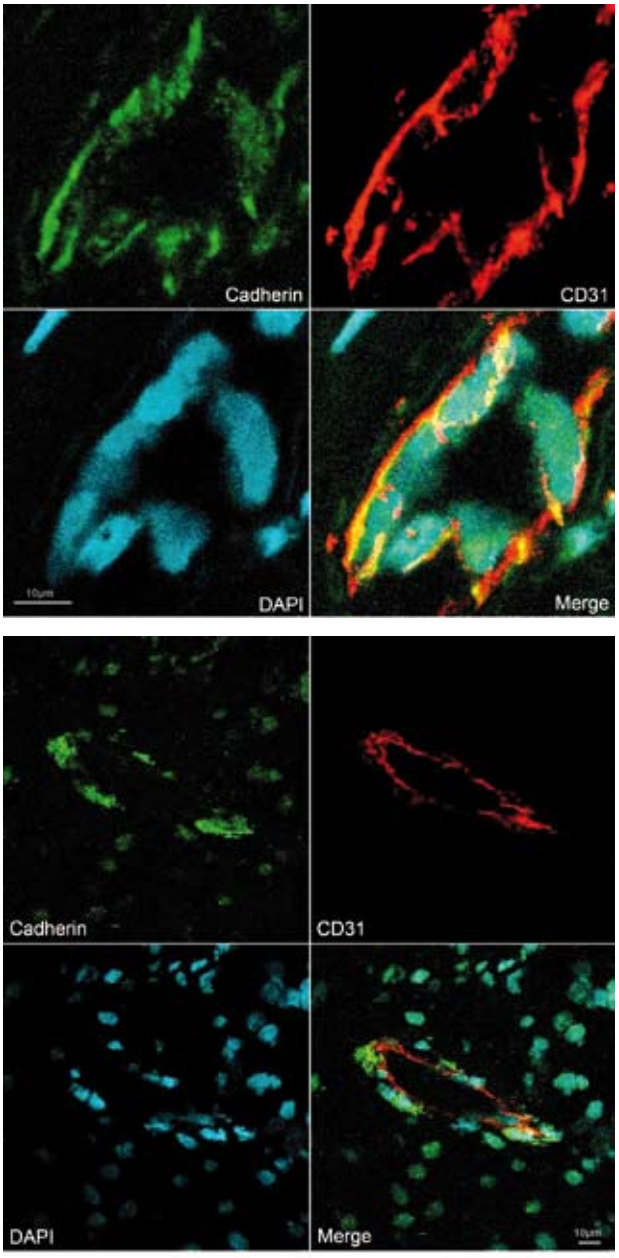

Figure 7

Expression of tight junction protein cadherin in normal and pilocytic astrocytoma

A: Intact expression of cadherin in blood vessels of normal brain samples.

B: Intact expression of cadherin in small blood vessels of pilocytic astrocytoma.

In normal brain samples, all blood vessels showed expression for the tight junction proteins occludin and cadherin (Fig 6A and 7A). While the expression of both proteins in small blood vessels of pilocytic astrocytoma is similar to that in normal brains (Fig 6B and 7B), the expression in the hypertrophied and glomeruloid vessels is strongly reduced or absent (Figures 6C and 7C).

RNA profiles and pathway analysis

The PCA analysis in Partek® showed a clear separation of the pilocytic astrocytomas from the glioblastomas (Figure 8). To identify differentially expressed transcripts, we performed a SAM analysis on the two groups and obtained a list of 2,081 differentially expressed transcripts after applying selection criteria of a minimum of two-fold differences in expression and an FDR of <1%.

This list of transcripts was uploaded into both IPA and ExPlain systems for functional analysis in order to identify biological functions and/or diseases that were most significant to the

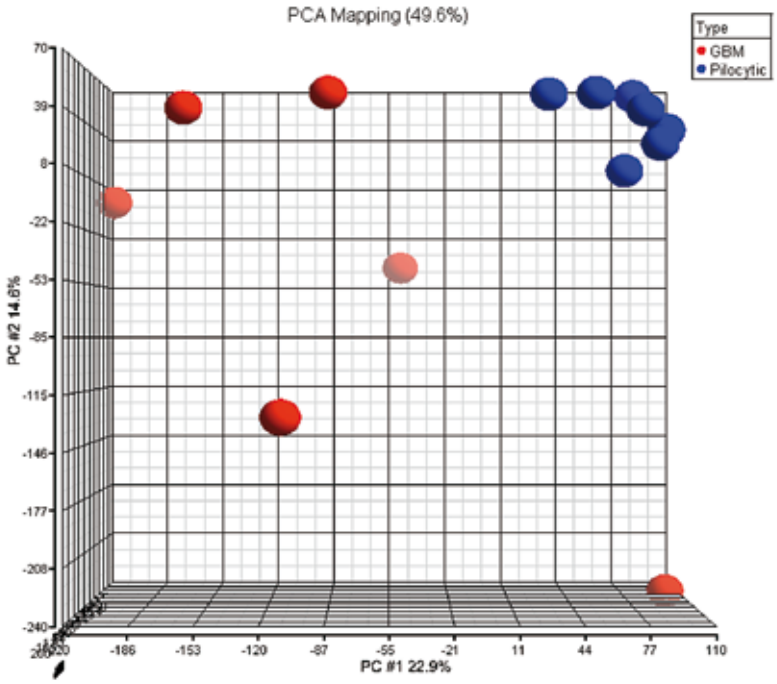

Figure 8

Principle component analysis (PCA) plot

The samples of pilocytic astrocytoma (blue dots) and the glioblastoma (red dots) are separated. The pilocytic astrocytomas show strong clustering while the glioblastomas are scattered.

dataset. In IPA, 595 network eligible genes could be identified while 689 eligible genes were identified in Explain. Differentially expressed genes are classified according to the categories of canonical pathways, therapeutic target, biomarker, and molecular mechanism.

Both systems indicated networks related to cell-to-cell signaling and cell-cycle regulation appeared to be highly significant within the data set. Subsequently, we investigated the presence of angiogenesis-related genes in the list of differential expressed genes based on gene ontology (GO) categories. Several networks were identified and the number of genes that were involved in each of the networks varied. It is important to note that a given gene can be associated with more than one category. For example, in ExPlain, 134 genes were related to "cell-cycle" (randomly expected: 60); 19 genes were related to "blood vessel formation" (randomly expected: 12) and 16 genes were related to "vascular endothelial growth factor receptor activity" (randomly expected: 6).

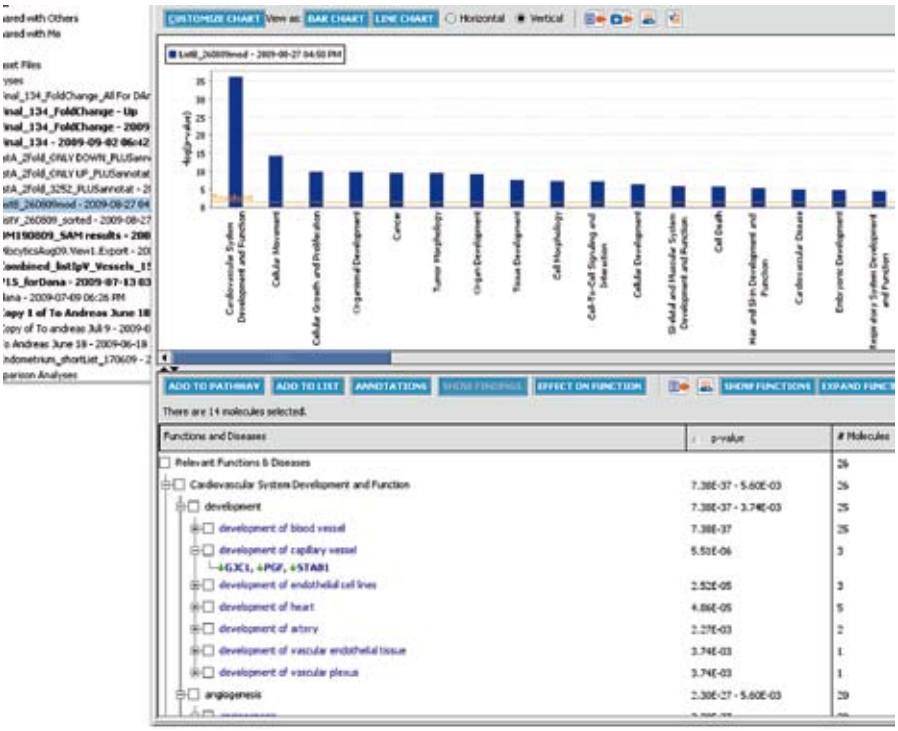

Figure 9
Ingenuity IPA result
Blood vessel development related genes were mapped to the network of "cardiovascular system development and function". The subgroups "development of capillary vessels" contains three genes that were overexpressed in pilocytic astrocytoma. The green arrows represent the overexpression of the genes in pilocytic astrocytoma.

The two systems use different ontologies which may cause slight differences in identification and classification results. Therefore, we investigated the details of the sub-lists and networks in both systems. For example, the list of genes related to "blood vessel development" identified in ExPlain showed an association to "cardiovascular system development and function" in IPA (Figure 9). The results appeared to be coherent. Among the subgroup of blood vessel development, three genes (*GJC1, PGF, STAB*) were upregulated in pilocytic astrocytoma, belonging to "development of capillary vessels". Further, five genes (*COL18A1, KLF5, MMP2, NRP1, PGF*) were upregulated in pilocytic astrocytoma belonged to "vascularization". For "sprouting of endothelial cells" two genes (*COL18A1, EPHB4*) were upregulated in pilocytic astrocytoma, while for "Sprouting of capillary vessels" one gene (*NRP1*) was upregulated in glioblastoma (Figure 10).

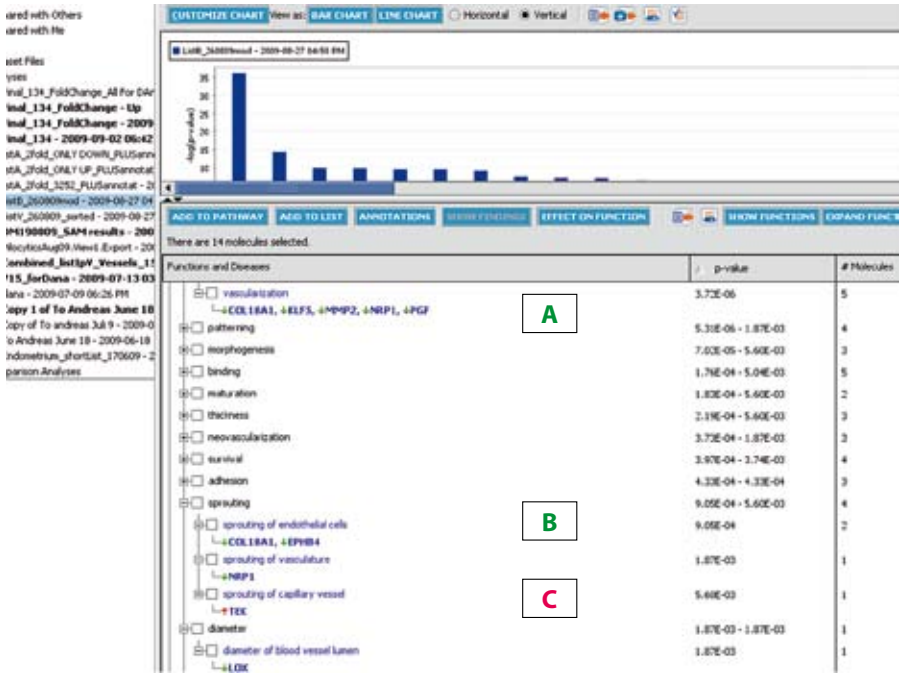

Figure 10
Ingenuity IPA networks
A: The subgroup "vascularization" contains five genes which are all overexpressed in pilocytic astrocytoma as compared to glioblastoma. **B**: The subgroup "sprouting of endothelial cells" contains 3 genes found overexpressed in pilocytic astrocytoma. **C:** The subgroup "sprouting of capillary vessels" contains a gene that is overexpressed in glioblastoma.
The green arrows indicate overexpression in pilocytic astrocytoma; the red arrows overexpression in glioblastoma.

Discussion

In the present study we investigated whether there are structural and immunophenotypical differences between the hypertrophied vessels of pilocytic astrocytomas and high-grade diffuse gliomas. In addition, based on transcriptional profiles we compared the involved angiogenic pathways. In the blood vessels of both glioma subtypes aberrant expression of proteins / cell markers was found. The aberrant expression was not restricted to morphologically abnormal vessels, but was also found in normal-looking vasculature, indicative of changes in protein expression patterns preceding structural changes of the vessels. This finding corroborates the results of an *in vitro* study in which morphologically similar endothelial cells taken from the vasculature of low-and high-grade gliomas differed in protein expression patterns [13]. Much of the literature on tumor neovascularization suffers from lack of definition of the cells taking part in the newly formed blood vessels. In a previous study on the vascularization of glioblastoma, we delineated the cellular constituents of the blood vessels according to their position in the vessel wall and their immunophenotyping [11]. It appeared that all distinct cellular subtypes take part in the proliferated blood vessels of both glioma subtypes. Lumen-lining (CD31+, CD34+) endothelial cells express CD105 and colligin 2, which is not expressed by the endothelium of normal brain microvasculature. In addition, pericytes (abluminal situated cells) express NG2 and endosialin, proteins which are not found in the vessel walls of normal brain either. There are, however, subtle differences in the composition of the layers of the vessels in which CD34, CD105, SMA, endosialin and NG2 are expressed. In the glomeruloid vessels in pilocytic astrocytomas, αSMA is never expressed by endothelial cells. Further, the putative pericyte marker NG2 is expressed with more restriction in the pilocytic astrocytomas. In contrast, endosialin is found ubiquitously in the multilayered vessels [11]. Overall, the layering of the hypertrophied vessels in pilocytic astrocytoma is more regular. The layers of the hypertrophied vessels have not lost organization as is the case in the malignant gliomas [11]. In the few descriptions of the pilocytic vasculature it is mentioned that endothelial cells have not proliferated, but no specific lineage markers were used [14, 15] and the present results do not confirm this statement. The present results show that, unlike the situation in glioblastoma, the lumina of the glomeruloid vessels in pilocytic astrocytoma are patent and their walls are structured in an orderly fashion. In all vessel types depositions of collagen types I and IV were present (data not shown). The results corroborate textbook descriptions of the structure of MVP in pilocytic astrocytoma which mention that the vessels are loose, dilated and teleangiectatic, unlike the glomeruloid MVP in malignant gliomas. The formation of glomeruloid vessels could be a reflection of either an accelerated form of angiogenesis, or an abortive type of proliferation [16]. In xenografts the level of Ang1 expression is correlated with the formation of glomeruloid vascular structures, while Ang1 inhibition by blocking its cognate receptor Tie2 had the opposite effect [17]. We found that the RNA

expression levels of Ang1 showed a slight, though not significant, overexpression in the pilocytic astrocytomas as compared to the glioblastomas. The structural differences of the glomeruloid vessels in the glioma subsets may reflect the influence of the differentially expressed genes involved in vascular development. We found such differences, but are unable to pin-point which genes or pathways could be responsible for the structural differences. Despite the structural differences, we found signs of BBB breakdown in the proliferated vessels of both glioma subtypes.

The expression of proteoglycans like endosialin and NG2 in the neovasculature of diffuse glioma has been described previously [18]. While in the experimental tumors endosialin was only seen in pericytes, we found the expression more ubiquitously in the vessels of the human gliomas [11]. Endosialin (initially called tumour endothelial marker 1 (TEM1)) was introduced as a marker for tumor-associated endothelial cells [19, 20] but appeared to be expressed by cells surrounding the endothelial cells. However, in the vasculature of experimental tumors the expression of endosialin is not restricted to pericytes [21-23]. In mouse development, the expression is restricted to endothelium and fibroblast-like cells [23, 24]. The expression in fibroblast-like cells is prominent around buds of developing endothelia as seen in developing kidney and lung [24]. In mouse knockouts of endosialin the development of tumor xenografts is hampered, mainly because of maldevelopment of vasculature [24]. These data point to a role of endosialin in the interplay between the tumor cells, endothelium and surrounding mesenchymal cells. Perhaps the more extensive expression of endosialin in the vessels of pilocytic astrocytoma corresponds to overexpression of other proteins involved in blood vessel development and the more organized structure of the vessel walls - but such an association is entirely speculative at this point. NG2 is a transmembrane proteoglycan serving as a promoter of angiogenesis and is reportedly expressed in pericytic cells or stromal cells around newly formed blood vessels [25]. In the vasculature of diffusely infiltrating glioma NG2 is expressed in all but the endothelial cell layer. Immunohistochemical studies of developing human brain demonstrated that NG2-positive migrating pericytes guide the earliest stages of vessel growth [26]. We found expression of NG2 in all types of glioblastoma blood vessels in our previous study and also - though more restricted - in the pilocytic vasculature shown in the present study. The differences in expression of SMA, endosialin and NG2 may well reflect differences in proliferation or recruiting the cellular constituents of the hypertrophied blood vessels. Again, the association with differences in involvement of certain angiogenic molecules needs further exploration.

As we anticipated, the strongest discriminating networks of genes between pilocytic astrocytoma and glioblastoma appeared to be those involved in cell cycle regulation [27]. In addition, networks involved in cell-cell signaling were identified as prominent

discriminators. These results confirm the scarce data in the literature on gene expression in pilocytic astrocytoma. In a study in which pilocytic astrocytomas and diffusely infiltrating gliomas of low malignancy grade were compared for differentially expressed pathways, immune system-related genes; genes involved in cell adhesion, migration, and also angiogenesis-related genes, were identified [27]. Unsupervised analyses of gene expression in glioma subsets revealed tumor lineage [27, 28] and tumor grade [29] as major classifiers. Within astrocytic gliomas of various malignancy grades, angiogenic activity of VEGF was correlated with that of IGFBP2 and both molecules appeared to be overexpressed around palisading necrosis, while their activity significantly differed between tumors of different malignancy grades [29]. Because it is hard to conceive that there is hypoxia in pilocytic astrocytomas we anticipated differences in activation of pathways of angiogenesis and structural differences of the blood vessels reflecting co-option versus hastily formed, chaotically structured vessels. However, our presumption that the hypoxia-induced activity of VEGF / VEGFR pathways would discriminate between the glioma subsets was not supported by the present data. The results of immunohistochemistry for VEGF-A and HIF-1α (considered as indicators of hypoxia) showed a considerable number of hypoxic cells in the pilocytic astrocytoma samples (Figure 1), corroborating the finding that the VEGF / VEGFR pathway is involved in diffuse - and pilocytic astrocytoma. The pathway analysis revealed eight genes specifically overexpressed in pilocytic astrocytoma, namely (*GJC1, PGF, STAB, COL18A1, KLF5, MMP2, NRP1, EPHB4*). Some of these proteins are components of the identified pathways indeed. The relation of specific expression of these genes in the pilocytic vasculature remains unsolved; we are unable to directly link these proteins to the structural differences between the vasculature of pilocytic astrocytoma and glioblastoma, respectively.

In conclusion, cell types with similar immunophenotypical characteristics contribute to the formation of new vessels in pilocytic astrocytoma and glioblastoma, but there are differences in their positioning. In the hypertrophied vessels of pilocytic astrocytomas an organized, well-layered structure is conserved. Although differences in the activity of genes involved in the hypoxia-induced VEGF /VEGFR pathway were not revealed, differences in expression profiles of genes involved in development and sprouting of blood vessels were identified. Future studies are necessary to reveal whether these differences reflect the involvement of particular genes and how all this relates to the structural differences of the vasculature of the glioma subsets.

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Summary and concluding remarks

Identification of glioma neovascularisation-related proteins by using MALDI-FTMS and nano-LC fractionation to microdissected tumor vessels

By using matrix-assisted laser desorption \ ionization Fourier transform mass spectrometry (MALDI-FTMS) to laser microdissected blood vessels taken from glial neoplasms and normal brain for comparison, specific glioma angiogenesis-related proteins were sought. The results of this investigation are reported in **Chapter 3**. In order to enrich the samples used for the analysis fractionation by nano-LC was applied. The analysis resulted in the identification of four proteins which were exclusively present in the glioma vasculature: fibronectin, colligin 2, fibrinogen β-chain precursor and acidic calponin 3. By using specific antibodies to tissue sections of the glioma samples, colligin 2 and fibronectin were validated. Immunohistochemistry to a set of additional sections including various types of tumors and tissues in which reactive angiogenesis is present, revealed that the expression of colligin 2 and fibronectin is indicative of active angiogenesis under both neoplastic and reactive conditions.

For this study the vasculature of glioma was compared with that of normal brain tissue. Since the normal brain vasculature is to be considered as resting vasculature without angiogenic activity, the proteins identified are representative of active angiogenesis, more than specific for angiogenesis under neoplastic conditions. Not surprisingly, positive immunostaining for colligin 2 and fibronectin was not restricted to the glioma vasculature (and vasculature of other tumors), but also present in situations of reactive angiogenesis within and outside of the brain (cerebral contusion, ischemic infarction; dural membrane; inflammation) and physiological angiogenesis (placenta and endometrium). Further proteomics comparisons of glioma blood vessels with physiological angiogenesis are reported in Chapter 7.

Specific expression sites of colligin 2 in glioma blood vessels

Elaborating on the finding of colligin 2 which was specifically expressed in glioma neovasculature, in **Chapter 4** we related the expression to the phenotypically different blood vessels in glioma. There are normal looking small vessels, vessels with hyperplasia of their walls and vessels with extensive glomeruloid-like changes in glioma. Because no univocal immunophenotypical description of the various types (stages) of glioma blood vessels exist in the literature we first characterized the cell types encountered in the vessels walls using markers for endothelial, pericytic and smooth muscle differentiation and described their position relative to each other. The expression of colligin 2 was plotted against this lineage

scaffold. We found expression of colligin 2 in all blood vessel subtypes in cells with endothelial -, and also pericytic lineage. Interestingly, some GFAP-positive cells within the blood vessels as well as scattered (GFAP-negative) cells around the blood vessels also expressed colligin 2. These cells point to a more ubiquitous involvement of cellular subsets in neoangiogenesis in glioma. It remains to be elucidated what the precise nature and origin of these cells are. We also found overlap of the expression of colligin 2 with that of collagens types 1 and 4, consistent with colligin 2 being a chaperon for these collagens. The results underline that without exception, all cellular components of the vessel walls are involved in the expression of colligin 2. Moreover, even the normal looking capillaries show expression, representing an early stage of neo-angiogenesis. This means that expression of glioma angiogenesis-related proteins precede morphological changes in the vessels. It remains to be explored how extensive these expressional changes are throughout brains with gliomas.

Overexpression of colligin 2 in glioma vasculature is associated with overexpression of heat shock factor 2

So far, colligin 2 (heat shock factor 47 (HSP47; *SERPINH1*) appeared to be overexpressed in glioma vasculature while absent from normal brain vessels. In **Chapter 5** we investigated the expression of candidate regulators of this HSP47 expression viz., HSF1, HSF2 and HSF4 in low- and high grade glioma and used endometrium samples as controls representing physiological angiogenesis. For simultaneous monitoring the blood vessel density and neovascularization, expression of CD31 (*PECAM1*) and NG2 (*CSPG4*) was measured. We found that together with overexpression of colligin 2, *HSF2* is overexpressed in glioma; more precisely, the overexpression was significant in low-grade glioma, not in glioblastoma. There was only minor overexpression of *HSF1* while *HSF4* was underexpressed in GBM. While colligin 2 expression levels were elevated, none of the HSFs were overexpressed in endometrium as compared to the normal brain samples. The data suggest specific regulation of the expression of colligin 2 by HSF2 and HSF4 in glioma neovascularization.

The data point to specificity in regulation, rather than specificity of the upregulated protein itself. The data show that in both during glioma angiogenesis and during the physiological angiogenesis in endometrium, the overexpression of colligin 2 is regulated at the level of mRNA rather than at the protein level. However, the expression of the heat shock factors differs for the angiogenesis in these contexts. The angiogenesis in the endometria is certainly triggered by hormonal influences while angiogenesis in glioma seems mostly hypoxia-driven. It seems that both situations elicit different stress responses at the level of the expressional status of the heat shock factors. Future studies should include the heat

shock factor status in other tumors than gliomas, and also in other types of physiological neovascularization like angiogenesis in wound healing. Analysis of the involvement of the various heat shock factors in these different situations in which angiogenesis takes place will reveal the specificity in their up- or downregulation. If specificity for neoplastic angiogenesis at the level of the regulation of the heat shock response exists, intervention at this level should be considered for anti-angiogenic therapy.

Angiogenesis proteom: a comparison between physiological angiogenesis and angiogenesis in glioma

In **Chapter 6** the blood vessels of glioblastoma were compared with blood vessels involved in active angiogenesis under physiological, not neoplastic, circumstances. To this aim, we laser microdissected the blood vessels and surrounding tissue of samples of glioblastomas and endometrium in proliferation. The samples were introduced in the LTQ Orbitrap mass spectrometer. The resulting peptide spectra from the blood vessels from glioblastoma and endometrium were analyzed by alignment and comparison at peptide level. In addition, the resulting protein lists were compared to the proteins identified in both groups of blood vessels and those identified in the surrounding tissue. In the blood vessels in glioblastoma 39, and in those of endometrium, 13 differentially expressed proteins were identified. Part of the shortlist of proteins was validated by immunohistochemistry. The 39 proteins found in the glioblastoma vessels could be related to three pathways, among which two were associated with vascular development and angiogenesis. The 13 proteins found specifically in endometrium blood vessels were mapped to four networks among which one involved in cardiovascular development and another associated with the proliferation of endothelial cells.

Structural and transcriptional differences between the vasculature of pilocytic astrocytomas and diffusely infiltrating gliomas

Because pilocytic astrocytomas are slowly progressing gliomas with low proliferation indices and low cell density without necrosis but nevertheless contain florid microvascular proliferation, we assumed that pathways involved in the neoangiogenesis in this glioma subtype may differ essentially from that in the diffusely infiltrating anaplastic gliomas. In **Chapter 7** we compared the microvasculature of diffusely infiltrating gliomas with that of the pilocytic astrocytomas at the structural and cellular level and sought differences possibly reflecting different angiogenic triggers. Hypoxia was monitored by immuno-

histochemistry for VEGF-A and HIF-1α. In addition, we compared both glioma subtypes for transcriptional differences reflecting differences in the pathways involved. We found that the newly formed blood vessels in pilocytic astrocytoma, in contrast to the vessels in high grade glioma, maintained an organized structure. Particularly, the glomeruloid blood vessels in the pilocytic astrocytomas had still open lumina. Nevertheless, disruption of the BBB was present in pilocytic astrocytoma just as well. The transcriptional profiles showed, besides major differences in cell-cycle related gene expression, differences in the expression of genes active in the VEGF/VEGFR pathway. Specifically, there was upregulation of genes involved in capillary sprouting and development in the pilocytic astrocytomas as compared to the glioblastomas.

We hypothesized that angiogenesis in pilocytic astrocytoma could serve as a model for hypoxia-independent glioma angiogenesis. However, the data of RNA microarrays did not directly confirm this. Rather, the pathway analyses illustrate the prominent involvement of VEGF in the angiogenesis in the pilocytic astrocytomas. Nevertheless, the differences of transcriptions may well lead to the discovery of additional pathways involved in glioma angiogenesis. Such alternative pathways could gain importance when anti-agiogenic therapies would lame the VEGF pathway. Consequently, targeting all involved pathways for anti-angiogenic therapy would be necessary for effective and successful anti-angiogenic therapy. Comparisons of the activation of such alternative pathways in glioma biopsies taken after the administration of the anti-VEGF drugs are necessary to check for activation of alternative pathways.

Samenvatting

Gliale tumoren zijn rijk gevasculariseerd. De bloedvaten in gliomen ondergaan een continue verandering en breiden zich steeds verder uit. Het aantal vaten neemt toe en de wanden van de vaten tonen sterke verdikking. Daarbij stapelen endotheelcellen en cellen die wel als pericyten, murale cellen of gladde spiercellen worden aangeduid, zich op. Met het verstrijken van de tijd transformeren capillairen tot dikwandige vaten met onregelmatig opgebouwde wanden, die uiteindelijk glomeruloide vormsels worden die nauwelijks nog een lumen hebben. Voor het bestrijden van een glioom worden niet alleen middelen ingezet die zijn gericht tegen de tumorcellen zelf, maar ook middelen die vaatnieuwvorming tegengaan. De gedachte is dat het vernielen van de vaten, of afremmen van hun groei, schadelijk is voor de tumorcellen die dan immers verstoken blijven van bloed met zuurstof en nutrienten. De anti-angiogene middelen zijn vooral gericht tegen vascular endothelial growth factor (VEGF) en haar receptoren, een cascade van interacterende moleculen die wordt aangezwengeld door hypoxie. In de praktijk is echter gebleken, dat anti-angiogene therapie niet zo succesvol is als gehoopt werd. In het eerste deel van de Inleiding (**Hoofdstuk 1**) wordt een kort overzicht van de huidige kennis over angiogenese in gliomen gepresenteerd.

In dit proefschrift worden experimenten beschreven waarin werd gezocht naar eiwitten die specifiek in glioombloedvaten tot expressie komen. Deze eiwitten zouden als nieuwe targets kunnen gaan dienen voor anti-angiogene therapie in gliale tumoren. De methodieken waarmee de eiwitten werden gezocht worden als “proteomics” aangeduid. “Proteomics” betekent het inventariseren van alle eiwit expressie van een bepaalde cel, een bepaalde celpopulatie, een bepaald weefsel, of een heel organisme. Er werd massa spectrometrie op gemicrodisseceerde weefselfragmenten toegepast. De massaspectrometrie werd uitgevoerd met behulp van matrix-assisted laser desorption / ionization – time of flight (MALDI-tof) en Fourier transformer mass spectrometry (FTMS). De weefselmonsters werden door middel van microdissectie verrijkt. De analyses werden uitgevoerd met fragmentatietechnieken als liquid chromatography electrospray (LCE) en high performance liquid chromatography (HPLC) om maximale expositie van de te detecteren eiwitfragmenten te verkrijgen. In het tweede deel van de Inleiding (**Hoofdstuk 2**) wordt op deze technieken nader ingegaan.

In **Hoofdstuk 3** wordt met behulp van MALDI-FTMS en nano-LC fractionering in gemicrodisseceerde bloedvaten van gliomen naar eiwitten gezocht die niet voorkomen in bloedvaten van normaal hersenweefsel. De eiwitten fibronectine, colligin 2 (heat shock eiwit 47), fibrinogen β-chain precursor en zure calponine type 3 bleken specifiek in de vaatwanden van de gliomen voor te komen. Met behulp van immunohistochemie kon de specifieke aanwezigheid van fibronectine en colligine 2 in de glioomvaatwanden worden gevalideerd. Het eiwit colligine 2 bleek ook in vaten van andere tumortypen voor te komen.

Tevens bleek colligine 2 niet alleen in tumorvaten, maar in alle vaten waarin actieve angiogenese voorkomt, aanwezig te zijn.
Gliale bloedvaten zien er onderling verschillend uit. Er bestaan capillaire vaten die morfologisch niet te onderscheiden zijn van die welke in normaal hersenweefsel voorkomen, en er zijn vaten waarvan de wanden sterk verdikt zijn, soms zodanig dat er een soort weefselkluwens zijn ontstaan die nauwelijks of geen lumen meer hebben. Een gedetailleerde karakterisering van de gliale tumorvaten, ofwel het beschrijven in hoeverre de vaatwanden bestaan uit cellen met endotheliale- pericytaire – of gladde spiercel eigenschappen is nodig om precies te kunnen beschrijven in welke cellen de eiwitten die onderwerp van dit proefschrift zijn, tot expressie komen.

In **Hoofdstuk 4** werden glioomvaten getypeerd naar de expressie van celtype merkers voor endotheel, pericyten en gladde spiercellen. De verdikte glioomvaatwanden bleken verschillen te kunnen tonen in hun wandopbouw. Vervolgens werd de expressie van colligine 2 bekeken in de verschillende celtypen in de vaatwanden. Alle celtypen bleken colligine 2 tot expressie te kunnen brengen. In sporadische cellen buiten de vaatwanden werd ook colligine 2 aangetoond. De expressie van colligine 2 in de vaatwanden ging steeds gepaard met die van de collageen types 1 en 4, hetgeen goed past bij het gegeven dat de expressie van colligine 2 geassocieerd is met de afzetting van deze collageentypes. Bemerkenswaardig was dat er ook sporadische cellen met een gliaal merkerprofiel in de vaatwanden aanwezig waren, en dat ook die cellen colligine 2 tot expressie konden brengen.

In **Hoofdstuk 5** wordt de regulatie van de expressie van colligine 2 bestudeerd. Colligine 2 (heat shock protein 47; *SERPINH1*) is een heat shock proteine. Heat shock proteinen hebben verschillende functies (assisteren bij vorming van 3-dimensionale structuur van eiwitten; afbreken van eiwitten) en komen tot expressie bij verschillende vormen van "stress". Daarbij spelen heat shock transcriptiefactoren (HSF) een rol. In humane weefsels komen drie van de vier HSF voor, namelijk de subtypen 1, 2 en 4. Deze HSFs komen onder verschillende omstandigheden in verschillende mate tot expressie. Met behulp van real-time PCR reacties werd de expressie van de drie HSFs in relatie tot die van colligine 2 nagegaan. In gliomen bleek met name HSF2 verhoogd tot expressie te komen, terwijl HSF1 nauwelijks verhoogd aanwezig is. Daarbij bleek ook dat deze overexpressie vooral aanwezig is in laaggradige gliomen, en afneemt met de maligniteitsgraad. Interessant is de gelijktijdige afname van transcriptie van HSF4. Zowel HSF2 als HSF1 waren niet verhoogd aanwezig in endometriumweefsel dat als controle voor fysiologische angiogenese in de experimenten werd betrokken.
In Hoofdstuk 3 werd naar verschileiwitten gezocht tussen de bloedvaten in gliomen en die in normale hersenen. In **Hoofdstuk 6** werden boedvaten van glioblastomen vergeleken

met bloedvaten die deelnemen aan actieve angiogenese. Daarvoor werden de vaten in endometrium in proliferatiefase gebruikt. De bloedvaten alsmede omliggend weefsel werden gemicrodisseceerd en gemeten met behulp van geavanceerde massa-spectrometrie, i.c., de LTQ Orbitrap massaspectrometer. De peptidenspectra uit de twee bloedvatgroepen werden vergeleken en er werden verschileiwitten geidentificeerd. Alle vier testgroepen (vaten en omliggend weefsel) werden onderling vergeleken en zo kon de lijst verschileiwitten tussen de vaten worden gereduceerd. Uiteindelijk werden 39 eiwitten in de glioom bloedvaten, en 13 eiwitten in de endometriumvaten geidentificeerd. De specifieke aanwezigheid van een deel van deze eiwitten kon vervolgens met behulp van immunohistochemie worden bevestigd. De eiwitten in de glioblastoom bloedvaten bleken deel uit te maken van drie verschillende moleculaire netwerken, waarvan twee geassocieerd bleken met vasculaire ontwikkeling en angiogenese. De eiwitten uit de endometriumvaten bleken deel uit te maken van vier verschillende netwerken, waarvan twee gerelateerd zijn met angiogenese.

De meeste gliomen behoren tot de categorie van “diffuus infiltrerende” gliomen. De individuele tumorcellen van deze gliomen migreren tussen de normale gliale en neurale cellen door. Daarbij treedt een transformatie van het tumorweefsel op: de cellen worden steeds meer anaplastich en genetisch instabieler, gepaard gaande met het ontstaan van steeds meer verdikte tumorvaten en necrotische gebieden. Een minder vaak vookomende gliale tumorcategorie is die van de circumscripte gliomen, waarvan het pilocytaire astrocytoom een voorname representant is. Dit glioomtype is veel minder infiltratief en ook treedt de progressie naar een genetisch instabiel, histologisch hooggradig glioom niet, of minder snel, op. Toch bevinden zich in de pilocytaire astrocytomen, ondanks de lage celrijkdom en afwezigheid van necrose, wel sterk geprolifereerde vaten met verdikte wanden. In **Hoofdstuk 7** werden deze vaten immunophenotypisch vergeleken met die in de diffuus infiltrerende, hooggradige gliomen (glioblastomen). De vraag was in hoeverre de tumorvaten in de pilocytaire astrocytomen lijken op die van glioblastomen, aannemend dat er minder hypoxie in de pilocytaire tumoren aanwezig zou zijn. Afgezien van morfologische verschillen werden ook RNA expressie data vergeleken met behulp van bio-informatica analyses, waarbij we ons concentreerden op de moleculaire cascades betrokken bij angiogenese. Het bleek dat er wel degelijk hypoxie in de pilocytaire astrocytomen aanwezig is, want immunohistochemisch kon pleksgewijze nucleaire expressie van VEGF A en HIF-1α worden aangetoond. Dit kwam overeen met de bevinding dat de hypoxie-gestuurde VEGF / VEGFR respons in beide tumortypen actief bleek te zijn. Er waren echter ook verschillen: de vaten in de pilocytaire astrocytomen toonden een geordende wandstruktuur, waarbij de celtypen steeds geordend bleven. Mogelijk hangt dit samen met de bevinding dat in dit glioomsubtype, en niet in de diffuus infiltrerende gliomen, een aantal genen tot expressie komen die vooral te maken hebben met vertakking

en ontwikkeling van vaten. Het belang van het opsporen van zulke genen en paden van genexpressie is dat deze ook betrokken zouden moeten worden in het ontwikkelen van anti-angiogene therapieën.

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List of Publications

Mustafa D, Kros JM, Luider T: **Biomarker discovery in glioma by combined approach of laser microdissection and advanced mass spectrometry.** *Predictive Diagnostics and Personalized Treatment: Dream or Reality* 2009, 471-476.

Mustafa D, van der Weiden M, Zheng P, Nigg A, Luider TM, Kros JM: **Expression Sites of Colligin 2 in Glioma Blood Vessels**. *Brain Pathol* 2008.

Mustafa D, Kros JM, Luider T: **Combining laser capture microdissection and proteomics techniques**. *Methods Mol Biol* 2008, **428**:159-178.

Titulaer MK, Mustafa DA, Siccama I, Konijnenburg M, Burgers PC, Andeweg AC, Smitt PA, Kros JM, Luider TM: **A software application for comparing large numbers of high resolution MALDI-FTICR MS spectra demonstrated by searching candidate biomarkers for glioma blood vessel formation**. *BMC Bioinformatics* 2008, **9**:133.

Mustafa DA, Burgers PC, Dekker LJ, Charif H, Titulaer MK, Smitt PA, Luider TM, Kros JM: **Identification of glioma neovascularization-related proteins by using MALDI-FTMS and nano-LC fractionation to microdissected tumor vessels**. *Mol Cell Proteomics* 2007, **6**(7):1147-1157.

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Al-Alami JR, Tayeh MK, Mustafa D, Abu-Rubaiha ZA, Majeed HA, Al-Khateeb MS, El-Shanti HI: **Familial Mediterranean fever mutation frequencies and carrier rates among a mixed Arabic population**. *Saudi Med J* 2003, **24**(10):1055-1059.

PhD Portfolio Summary

Summary of PhD training and teaching

Name PhD student: Dana A.M. Mustafa **Erasmus MC Department:** Pathology **Research School:** Erasmus Postgraduate School Molecular Medicine (MolMed).	**PhD period:** 15th June 2005 to 10th November 2009 **Promotor(s):** Prof.dr. Johan M. Kros **Supervisor:** Prof.dr. Johan M. Kros

1. PhD training

	Year	Workload	
		Hours	**ECTs**
General courses			
- Biomedical English Writing and Communication	2006	120h	4 ECTs
- Introduction to clinical Research	2009	15h	0.54 ECTs
Specific courses			
- Biomedical Research Techniques Course	2005	40h	1.43 ECTs
- Molecular Medicine Course	2006	32h	1.14 ECTs
- Spotfire Course	2006	16h	0.57 ECTs
- Course Ensemble	2007	16h	0.57 ECTs
- Bioinformatics Analysis Course	2008	16h	0.57 ECTs
- Course Molecular Diagnostics III	2008	16h	0.57 ECTs
Seminars and workshops			
- Writing Grand Proposal workshop	2008	8h	0.29 ECTs
- Educational Days in the SNO	2008	28h	1 ECTs
- Witting Grand Proposal in the AACR course	2009	5h	0.18 ECTs
Presentations			
- Identification of Glioma neovasculature- Specific Proteins by Proteomics techniques, INC, San Francisco, USA	2006	40h	1.43 ECTs
- Identification of glioma neovascularisation-related proteins by using MALDI-FTMS and nano-LC fractionation to microdissected tumor vessels, ECP, Istanbul, Turkey	2007	40h	1.43 ECTs
- Colligin 2 in Glioma Angiogenesis, Landelijke Werkgroep Neuro-Oncologie, Groningen, The Netherlands	2008	40h	1.43 ECTs
Poster Presentations			
- Identification Of Glioma Neovascularisation-Related Proteins By Using MALDI-FTMS and Nano-LC Fractionation To Microdissected Tumor Vessels, NPC, Utrecht, The Netherlands	2007	40h	1.43 ECTs
- Differential Expression of Colligin 2 In Neovasculature Of Glioma Subtypes, ECN, Athens, Greece	2008	40h	1.43 ECTs
- New Targets In Anti -Angiogenesis: The Identification Of Colligin 2 In The Microvasculature Of Gliomas, SNO, Las	2008	40h	1.43 ECTs

Vegas, USA - Identification Of Colligin 2 In The Microvasculature Of Gliomas; A study At The Proteomics Level, AACR, Denver, USA	2009	40h	1.43 ECTs
(Inter)national conferences - The European Association of Neuro-Oncology - 5th Asia Pacific International Academy Pathology Congress	2006 2007		
Winner of the travel award of the XVI International congress of Neuropathology 2006			