Identification of Glioma Neovascularization-related Proteins by Using MALDI-FTMS and Nano-LC Fractionation to Microdissected Tumor Vessels*  

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The identification of angiogenesis-related proteins is important for the development of new antiangiogenic therapies, and such proteins are potential new biomarkers for gliomas. The aim of this study was to identify proteins that are exclusively present in glioma neovasculature and not in the vasculature of normal brain. We combined advanced proteomics 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 MALDI-FTMS. Subsequently, the samples were fractionated by nano-LC prior to MALDI-TOF/TOF. This combined approach enabled us to identify four proteins that appeared to be exclusively expressed in the glioma blood vessels. Two of these proteins, fibronectin and collagen 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 neoangiogenesis 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.  

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Gliomas are the most common primary brain tumors; the incidence in the United States is about ~25,000 new cases per year (1). The diagnosis of these tumors and the decisions regarding therapy is based almost exclusively on the tissue histopathology (2, 3). Diffuse gliomas are highly infiltrative and heterogeneous. Gliomas are among the neoplasms with the highest degree of vascularization (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 a few useful biomarkers or therapeutic targets have been identified so far (7). In a previous study, we identified glioma-related proteins by using two-dimensional PAGE followed by MALDI-TOF-MS analysis. By using specific antibodies raised to low molecular weight caldesmon on tissue sections of glioma it was shown that this protein was exclusively expressed in the neovasculature (8) and that it is a potential serum marker for glioma (7). Rapid and major developments in proteomics technology and methodology over the last decade have opened a new stage in the identification of proteins (9). MALDI-TOF-MS recently became available as a flexible tool in the search for disease markers (10). Moreover, the recently introduced technique of MALDI-FTMS provides a powerful technique for accurate peptide 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 prefractionation 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 proteomics analysis.

The aim of this study was to identify proteins that are specifically expressed in glioma vasculature but not in the normal blood vessels of the brain. To this end, microdissected hypertrophied 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 expressed peptides was achieved by combining nano-LC fractionation of samples with...
Protein Identification in Glioma Neovascularization

**TABLE I**

Clinical data

| Sex | Age | 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  | f   | 32  | Bi F                        |
| G10 | f   | 30  | Ri F                        |

**Normal brain samples**

| Sex | Age | Tumor location               |
|-----|-----|------------------------------|
| 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 wk | Intraterine infection      |

**MATERIALS AND METHODS**

**Sampling**

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

**Laser Capture Microdissection**

Cryosections of 8 μm were made from each sample and mounted on polyethylene naphthalate (PEN)1-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 s in hematoxylin, 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 device, type P-MB, was used with PalmRobo version 2.2 software at 40× magnification. Estimating that a cell has a volume of 10×10×10 μm, we microdissected an area of about 190,000 μm² of blood vessels and another area of the same size of the surrounding tumor tissue from each sample, resulting in ~1500 cells/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 analyzed in the same way as the other samples. This negative control experiment was performed three times.

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). 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 min. 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 min at 70% amplitude at a maximum temperature of 25 °C (Branson Ultrasonics, Danbury, CT). The samples were incubated at 37 and 100 °C for 5 and 15 min, respectively, for protein solubilization and denaturation. To each sample, 1.5 μl of 100 ng/μl gold grade trypsin (Promega, Madison, WI) in 3 ml Tris-HCl diluted 1:10 in 50 mM NH₄HCO₃ 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 min at 37 °C. Samples were dried in a SpeedVac (Thermo Savant, Holbrook, NY) and reconstituted in 5 μl of 50% ACN, 0.5% TFA in 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. Each sample (0.5 μl) was mixed on the spot with 1 μl of a
2,5-dihydroxybenzoic acid matrix solution (10 mg/ml in 0.1% TFA in 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-tesla magnet (Bruker Daltonics). For each measurement, 450 scans of 10 shots each were accumulated with 60% laser power. Mass spectra were acquired in the mass range of 800–4000 Da. FTMS spectra were processed with a Gaussian filter and two zero fillings.

**MALDI-FTMS External and Internal Calibration**—A standard peptide calibration mixture (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 version 3.4, build 169 software (Bruker Daltonics) was performed. Ubiquitous 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; all accession numbers cited are from the Swiss-Prot database) present in the samples were compared with the calculated masses for MH+ (1165.58475, 1234.67896, 1365.63930, 1381.64814, 1390.68085, 1707.77211, 1797.01161, and 2096.04673 Da).

**Data Analysis**—Monoisotopic peaks with signal-to-noise ratio >3 were annotated with the Sophisticated Numerical Annotation Procedure (SNAP) algorithm using the prerelease version of the DataAnalysis software package (version 3.4, build 169). 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 (18). 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 in at least five 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. The masses of the differentially expressed peptides were submitted to the MASCOT search engine (Matrix Science, London, UK) using the Swiss-Prot (release 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).

**Sample Preparation for Nano-LC**

Sample G8 was selected for fractionation (Table I). One, four, and eight 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. RapiGest buffer (20 μl; Waters) was added to the frozen sections followed by 1 min of sonication, 5 min at 37 °C, and finally 15 min at 100 °C. For each section 10 μl of 100 ng/μl gold grade trypsin (Promega) in 3 mM Tris-HCl was added and samples were incubated overnight at 37 °C. Finally 50 μl HCl was added. For comparison, eight sections from normal brain sample N5 were prepared in exactly the same way.

In addition, an area of about 900,000 μm² of blood vessels from each of the glioma samples and the normal control samples was 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. RapiGest buffer (20 μl) 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 PepMap column (75-μm inner diameter × 150 mm, 3 μm; Dionex, Sunnyvale, CA). The sample (5 μl) was loaded onto the trap column (300-μm inner diameter × 5 mm, 5 μm; Dionex). Fractionation was performed for 130 min with a gradient of buffer A (100% H2O, 0.05% TFA) and buffer B (80% ACN, 20% H2O, 0.04% TFA): 0–15 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) containing α-cyano-4-hydroxycinnamic acid matrix using a robotic system (Probot Micro Fraction Collector, Dionex). To each fraction, 1 μl of water was added. Finally we used a 10 mM (NH)4H2PO4 in 0.1% TFA, water solution to wash the prespotted plate for 5 s to remove salts. The plates were subsequently measured by automated MALDI-TOF/TOF (Ultraflex, Bruker Daltonik GmbH, Leipzig, Germany) using WARP-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 WARP-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 (release 40.21) database allowing 150-ppm parent mass tolerance, 0.5-dalton fragment tolerance, and one missed trypsin cleavage site. In addition, identification was confirmed by exact mass measurements on the MALDI-FTMS instrument, adding 1 μl of 2,5-dihydroxybenzoic acid solution to the fractionated spot and allowing it to dry.

**Backward Database Searching**

By in silico digestion of the identified proteins, theoretical peptides were generated that were sought for in the monoisotopic peaks of the MALDI-FTMS data. 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 paraffin sections of the samples. We first confirmed our results using the 10 glioma samples and the 10 normal brain samples that 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 neoangiogenesis takes place, and tissues with notorious neoangiogenesis were also tested for the presence of these proteins (Table II).

Immunohistochemical staining was performed following the manufacturer’s procedure (alkaline phosphatase technique) using rabbit polyclonal antibody for fibronectin at a 1:1000 dilution (DakoCytomation, Glostrup, Denmark) and mouse monoclonal antibody for colligin 2 at a 1:500 dilution (Stressgene, Victoria, British Columbia, Canada). Paraffin sections (5 μm) were mounted onto poly-lysine-coated microslides, deparaffinized in xylene for 15 min, rehydrated through graded alcohol, and then washed with water. The sections were washed with PBS and incubated with the antibody for 30 min. After washing the sections with PBS, the corresponding antigen was
added and incubated for 30 min at room temperature. New fuchsin alkaline phosphatase substrate solution was freshly prepared, and the sections were incubated for about 30 min. Afterward the sections were washed with tap water, counterstained, and coverslipped with permanent mounting medium.

RESULTS

**FTMS Measurements**—The MALDI-FTMS measurements of the microdissected samples yielded ~700–1100 monoisotopic 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 mixture. 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 autodigestion 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 III). All 16 peptides were expressed in the glioma vessel group only. The MASCOT database search resulted in matching of four of the 16 peptides to fibronectin precursor protein (P02751). 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 up to 30 daltons to the masses of the 16 peptides that 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.

**TABLE III**

| Peptide measured masses (m/z) | p value | Number of samples in which these peptides were found |
|-----------------------------|---------|-----------------------------------------------------|
| 1926.04820*                 | 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                                              |
| 1275.55361*                 | 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                                              |
| 1848.85488                  | 0.0136  | 5 0 0 0                                              |
| 1912.04220                  | 0.0136  | 5 0 0 0                                              |

* Peptides resulted in protein identification.
the Spotfire program (Fig. 2). A cluster of eight glioma vessel samples is observed. From the two samples that 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 that 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.

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, including 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 IV). The capacity of the nano-LC 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.

The data obtained from MALDI-TOF/TOF after the fractionation procedure were compared with the MALDI-FTMS data, searching specifically for the 16 differentially expressed peptides. Nine of 16 peptides matched within 200 ppm. To obtain a higher mass accuracy for the peptides, the corresponding spots of these nine peptides were remeasured in the MALDI-FTMS instrument. The exact mass of five of nine peptides matched within 3 ppm (external calibration) with the masses originally obtained by FTMS. 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 β chain precursor (P02675), one peptide matched to colligin 2 (P50454), and one peptide matched 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 β 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 V). The same search for the in silico digestion of fibrinogen yielded nine additional peptides of which three were exclusively found in the glioma vessel group and the others were found in one sample of the normal vessels (Table VI). Searching for the theoretical peptides of colligin 2 and acidic calponin 3 did not result in the finding of any extra peptide.

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

In Fig. 5 the results of additional immunostaining of various gliomas, carcinomas, vascular malformations, and tissues

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**Table IV**

| No. of sections and sample type | 1 section, glioma | 4 sections, glioma | 8 sections, glioma | 8 sections, normal brain | 15,000 microdissected cells, glioma | 15,000 microdissected cells, 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                                |

**Table V**

| Number of samples in which these peptides were found | Glioma vessels | Glioma surrounding tissue | Normal brain vessels | Normal brain surrounding tissue |
|------------------------------------------------------|---------------|---------------------------|----------------------|--------------------------------|
| Da                                                   | 8             | 0                         | 0                    | 0                              |
| 2470.3207a                                            | 6             | 0                         | 0                    | 0                              |
| 1593.8172a                                            | 5             | 0                         | 0                    | 0                              |
| 1807.90584a                                           | 5             | 0                         | 0                    | 0                              |
| 1629.8232a                                            | 4             | 1                         | 0                    | 0                              |
| 2692.3750b                                            | 4             | 0                         | 0                    | 0                              |
| 1349.68509b                                           | 3             | 0                         | 0                    | 0                              |
| 1401.6682b                                            | 3             | 0                         | 0                    | 0                              |
| 2524.36562b                                           | 3             | 0                         | 0                    | 0                              |
| 3042.59234b                                           | 3             | 0                         | 0                    | 0                              |

*Peptides matching the criteria used in this study.

b Peptides derived from in silico digestion.

**Table VI**

| Number of samples in which these peptides were present | Glioma vessels | Glioma surrounding tissue | Normal brain vessels | Normal brain surrounding tissue |
|--------------------------------------------------------|---------------|---------------------------|----------------------|--------------------------------|
| Da                                                     | 5             | 0                         | 0                    | 0                              |
| 1239.51764                                             | 5             | 0                         | 0                    | 0                              |
| 2385.15768                                             | 4             | 0                         | 0                    | 0                              |
| 1275.55961                                             | 4             | 1                         | 0                    | 0                              |
| 1544.69498                                             | 3             | 1                         | 0                    | 0                              |
| 1668.71478                                             | 2             | 1                         | 0                    | 0                              |
| 886.38736                                              | 2             | 1                         | 0                    | 0                              |
| 1951.00371                                             | 2             | 1                         | 0                    | 0                              |
and reactive conditions in which neoangiogenesis takes place are shown. 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 (arteriovenous malformation (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 (Fig. 5 D).

**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 three-dimensional structures of a 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). 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 resolution, which in the present study generated relatively complex spectra consisting of
700–1100 monoisotopic peaks per spectrum. Yet another advantage is the very high sensitivity and reproducibility of FTMS (11), which is higher than that of any other mass spectrometric techniques currently available. In addition, FTMS provides an excellent signal-to-noise ratio because the source of noise in MALDI-FTMS is of physical origin and is not a chemical-based noise as that generated in 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 microdissected 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 abundance proteins is achieved by using a higher number of cells than can be

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. H&E, hematoxylin and eosin.

Fig. 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 the case of active blood vessel formation in tumors and in reactive and normal tissues, staining is present. The AVM (D) and the cavernous hemangioma (E)
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 peptides identified previously 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 a few 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 increasing the confidence in the identified proteins (Table VII). 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 extradomain B splice variant was not specifically identified, but neither was its presence excluded.

Two of the four proteins identified by the proteomics 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 (Table V). 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 demonstrates that colligin 2 and fibronectin both are expressed in the context of neoangiogenesis. The expression was not specific for glioma neovascularization but also was 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 neoangiogenesis have been identified in gliomas, such as vascular endothelial growth factor (26) and platelet-derived growth factor (27). Relations have been discovered between some cytokines such as transforming growth factor-β and tumor blood vessels (28). Furthermore, endogenous expression of angiogenesis inhibitor factors, e.g. angiostatin, endostatin, and thrombospondin-1 and -2, by glioma tumor vessels also have been reported (29). Some of these proteins have been used to monitor therapy effects (7). Despite the gradual unraveling of the roles of these regulatory proteins in the process of tumor neovascularization, 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 antiangiogenic therapies. Such discoveries may well increase our understanding of the formation of neovascularity in glioma.

In the present study, we identified fibronectin, fibrinogen, colligin 2, and acidic calponin 3 as proteins that are expressed in the glioma vasculature. Fibronectin is a high molecular weight, multifunctional matrix protein that binds to other extracellular matrix proteins such as collagen, fibrin, and heparin. Several studies addressed the relation between fibronectin and tumors, including breast cancer, melanoma (30, 31), and gliomas. Overexpression of fibronectin in glioblastoma as detected by immunohistochemistry was reported previously (32). The expression of fibronectin by glioma blood vessels suggests 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 with glioblastoma, fibronectin was found to be differentially expressed; the glioblastomas expressed fibronectin, whereas the pilocytic astrocytomas did not (34). However, we did not find a difference in the expression of fibronectin between these two tumor types. Because hypertrophied microvasculature is a hallmark of both glial tumor types, despite their different World Health Organization grades, this finding

| Identified proteins (accession number) | Specific peptide masses by FTMS (prefractionation) (m/z) | Calculated mass (MH+) | Δppm | Score | Sequence coverage | Sequence obtained after nano-LC fractionation and MALDI-TOF/TOF measurements | No. of extra peptides identified after nano-LC fractionation |
|----------------------------------------|----------------------------------------------------------|------------------------|-------|-------|-------------------|-----------------------------------------------------------------|-------------------------------------------------|
| Fibrinogen β chain (P02675)            | 1535.72354 2257.07971                                    | 1535.72366 2257.08046  | 0.13 0.35 | 52 48            | 13 6                                                          | AHYGGFTVONEANK GGETSEMYLIQPDSSVKPYR              | 5 1                                               |
| Colligin 2 (P50454)                    | 1659.80041 1659.80126                                     | 1659.80081 1659.80128  | 0.54 0.35 | 39 39            | 6 3                                                          | LYGPSSVSFADDVFR YDHOAEEDLR                       | 5 3                                               |
| Acidic calponin (Q15417)               | 1275.55961                                            | 1275.56000                                    | 0.31                           | 39 39            | 6 3                                                          | YDHOAEEDLR                                      | 5 3                                               |

**TABLE VII**

Differentially expressed proteins identified by nano-LC fractionation

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did not surprise us.

Colligin 2, also called heat shock protein-47, is a collagenbinding 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 neoangiogenesis 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 stimulates motility of the activated endothelial cells involved in this process. Furthermore, 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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