Toward understanding recurrent meningioma: the potential role of lysosomal cysteine proteases and their inhibitors

Clinical article

TAMARA T. LAH, PH.D.,1,2 ISABELLE NANNI, PH.D.,3 MIHA TRINKAUS, M.D., PH.D.,1 PHILIPPE METELLUS, M.D.,4 CHRISTOPHE DUSSELT, M.D.,5 LEÓ DE RIDDER, M.D., PH.D.,6 UROŠ RAJČEVIĆ, D.V.M., PH.D.,1 ANDREJ BLEJEC, PH.D.,1

AND PIERRE-MARIE MARTIN, M.D., PH.D.3

1Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Ljubljana; 2Department of Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia; 3Laboratoire de transfert d’OncoLogie Biologique, Faculté de Médecine de Marseille–Secteur Nord; 4Service de Neurochirurgie, Hôpital de la Timone; 5Laboratoire de Cancérologie Expérimentale, INSERM, Faculté de Médecine Marseille–Secteur Nord, Marseille, France; and 6Laboratory of Histology, University of Ghent, Belgium

Object. The first aim of this study was to diagnose more aggressive and potentially recurrent meningiomas using an in vitro embryonic chick heart invasiveness assay in which lysosomal enzyme cathepsin B was used as the invasiveness marker. The second aim was to confirm if cathepsin B and/or cathepsin L and their endogenous inhibitors were also prognostic parameters in the clinical study of 119 patients with meningioma.

Methods. Primary meningioma cultured spheroids were “confronted” with embryonic chick heart spheroids in vitro, and cathepsin B was used as molecular marker to immunolabel the invasive tumor cells. In vitro invasion assays of the malignant meningioma cells were used to assess the invasive potential related to the cysteine cathepsins. As to the second aim, the possible association of cathepsin B along with selected molecular markers, cathepsin L, and endogenous cysteine protease inhibitors (stefins A and B and cystatin C) with meningioma malignancy was determined using enzyme-linked immunosorbent assays in tumor homogenates. Univariate and multivariate analyses were used to compare these parameters with established biological markers of meningioma recurrence in 119 patients with meningiomas.

Results. The more invasive tumors, which characteristically overgrew the normal tissue, were identified even within a group of histologically benign meningiomas. More intensive staining of cathepsin B in these tumors was not only found at the tumor front, but also in the invading pseudopodia of a single migrating tumor cells. Matrigel invasion of malignant meningioma cells was significantly altered by modulating cathepsin B activity and by stefin B silencing. In the clinical samples of meningioma, the levels of cathepsins B and L, stefin B, and cystatin C were highest in the tumors of higher histological grades, whereas stefin A and progesterone receptor were the only markers that were significantly increased and decreased, respectively, in WHO Grade III lesions. With respect to the prognosis of relapse, cathepsin L (p = 0.035), stefin B (p = 0.007), cystatin C (p = 0.008), and progesterone receptor (p = 0.049) levels were significant, whereas cathepsin B was not a prognosticator. As expected, WHO grade, age, and Simpson grade (complete tumor resection) were prognostic, with Simpson grade only relevant in the short term (up to 90 months) but not in longer-term follow-up. Of note, the impact of all these parameters was lost in multivariate analysis, due to overwhelming prognostic impact of stefin B (p = 0.039).

Conclusions. The data indicate that the cysteine cathepsins and their inhibitors are involved in a process related to early meningioma recurrence, regardless of their histological classification. Of note, the known tumor invasiveness marker cathepsin B, measured in whole-tumor homogenates, was not prognostic, in contrast to its endogenous inhibitor stefin B, which was highly significant and the only independent prognostic factor to predict meningioma relapse in multivariate analysis and reported herein for the first time. Stef in B inhibition of local invasion was confirmed by in vitro invasion assay, although its other functions cannot be excluded. (DOI: 10.3171/2009.7.JNS081729)

Key Words • cysteine protease • cathepsin • cystatin • invasion • meningioma • prognosis of recurrence • stefin

Numerous histological markers—such as cell atypia, high cellularity, increased mitotic rate, necrosis, prominent nucleoli, and nuclear polymorphism—can be used to classify the tumors into benign, atypical, and anaplas-
Cathepsins and steins in recurrent meningioma

tic or malignant types. It has been postulated that tumor invasion in brain tissue is uncommon, but if it occurs these tumors are malignant and have a high rate of recurrence. A critical step in the progression and recurrence of these tumors might therefore be the invasion and infiltration into adjacent brain tissue, a more common feature of atypical (WHO Grade II) and anaplastic (WHO Grade III) meningiomas. However, it was hypothesized that even benign meningiomas differ in their invasiveness and may relapse faster than predicted by histological parameters. Therefore, benign meningiomas that invade the brain may have a similar clinical outcome to atypical meningiomas. The notion led de Ridder and colleagues to propose in vitro confrontation assays as the most reliable diagnostic and prognostic tools for determining meningioma aggressiveness and recurrence and were expanded into several variations.

We therefore hypothesize that if the key issue in the prognosis of meningioma relapse is invasion of the cells at the tumor edges, the biological markers—such as lysosomal cathepsin B, which is linked to the invasive character of putative aggressive tumor cells—may help us to diagnose potentially recurrent meningiomas. These markers, when translated to clinical practice, may identify the patients with earlier relapse and who would need more aggressive postoperative treatments.

Proteases are involved in the invasion of a variety of tumors and include lysosomal cysteine proteases such as cathepsins B and L. Many authors have reported a close association of cathepsins B and L with cancer progression, suggested as prognostic markers in various types of tumors, as reviewed recently by Lah et al. Their activity is regulated by a set of endogenous cysteine protease inhibitors, particularly by intracellular steins A and B and secreted cystatins C and M. The balance between these inhibitors and the cysteine cathepsins seems to be highly relevant and may possibly serve as biomarkers for tumor progression, as well as having an impact on prognosis. On the one hand, the altered balance with enhanced expression of cystatins would diminish the tumor-associated proteolytic activity, responsible for tumor spread, indicating more favorable prognosis. However, there is also evidence that higher levels of steins A and B and cystatin C in tumor tissues correlates with poor prognosis.

On the other hand, these cystatins may also have other, yet unknown functions not related to proteolysis, as similarly found for tissue inhibitors of metalloproteases and plasminogen activator inhibitors, both elevated in various types of tumors, which have less favorable prognosis.

The cysteine peptidase system has been shown to play a role in the invasion and other processes associated with tumor progression, such as apoptosis, proliferation, and angiogenesis. Collectively, literature data indicate that cathepsin B is highly relevant for increased invasion of tumor cells, whereas cathepsin L may be more important for cell proliferation, cell senescence, and apoptosis and the role of steins and cystatins in these processes is less clear. Previously, we found that both cathepsin B and cathepsin L were increased in the tumor cells of more aggressive meningiomas and were potentially also prognostic for meningioma relapse. Here, we aim to compare the concentrations of the 2 enzymes and their inhibitors in whole-tumor homogenates to evaluate their clinical application in prognosis. As cathepsins are also highly expressed in the cells of activated tumor microenvironment—such as macrophages and fibroblasts—and are involved in the process of tumor progression, either by suppressing or facilitating its malignancy, total cathepsins and inhibitor levels within the entire tumor mass may be more relevant for its potential of early relapse than the tumor-associated levels of these biomarkers alone. Furthermore, protease may also play a dual role—either as tumor facilitating or tumor protecting—as reviewed recently, adding to the difficulty of understanding their impact on tumor prognosis.

We addressed 2 primary questions in this study. First, we endeavored to determine whether the invasion biomarker cathepsin B is also expressed at the invasive edges of invading meningioma in 2 types of in vitro models, as was similarly observed in GBM invasion. Second, we sought to reveal if, similar to GBM, cathepsin B is also prognostic when measured in whole-tissue homogenates. Third, our goal was to demonstrate the prognostic importance of other potential biomarkers, cathepsin L and the cathepsin inhibitors, steins A and B and cystatin C. The prognostic impact of other potential biomarkers, cathepsin L and cysteinie cathepsins inhibitors, was also determined and compared with the known molecular marker progesterone receptor and to histopathological and clinical markers, such as WHO grade, age, and the extent of tumor resection, in uni- and multivariate analyses.

**Methods**

**Chick Heart Spheroid Invasion Assay**

The invasion of meningial tumor–derived cells in vitro was evaluated using a modified 3D model introduced by Mareel and coworkers in 1979. In the first step, a tumor biopsy specimen was transferred into a Petri dish containing modified Eagle medium enriched with 2 mM glutamine and 10% calf serum. The tissue was cut into 2 × 2 × 2–mm fragments and transferred into plastic dish, covered by 2 ml of the aforementioned medium. During incubation at 37°C, the tumor fragments adhered to the bottom of the flask and the outgrowing cells formed a monolayer of primary tumor-derived cell culture. After confluence, the cells were scraped from the bottom of the flask. These flaps were transferred into 15-ml Erlenmeyer flasks filled with 2 ml of the same growth medium. In the next step, the flaps were incubated at 37°C for 48 hours under constant gyratory shaking at 0.05 G, when 0.2–0.3–mm spheroids, which were not necrotic, formed. In the third step, freshly cut 0.5-mm-diameter heart fragments from 9-day-old chick embryos were incubated at 37°C on a gyratory shaker for 3 days, rendering them spheroids. In the final step, the tumor-derived aggregates were confronted with the chick spheroids attaching to each other when placed in contact on a semisolid agar medium for about 6 hours. After adhering, the confronta-
tion mixture was transferred into 3 ml of the aforementioned medium and rotated at 0.05 G at 37°C for 1–8 days under a constant flow of 5% CO\textsubscript{2}/air and finally fixed in Bouin-Holland fixative.

**In Vitro Matrigel Invasion Assay**

The in vitro invasion assay was performed in Matrigel-coated modified Boyden chambers. The upper surface of the chambers (12-well polycarbonate filter inserts with 12-μm pores, Sigma) were coated with Matrigel (50 μg/well, Becton Dickinson) in serum-free medium. The filters were dried overnight and reconstituted with 0.2 ml of medium 1 hour prior to cell seeding. We have used IOMM Lee malignant meningioma cells,\textsuperscript{21} siStefB IOMM cells (see below), and negative control non-siIOMM cells. Treated cells were detached using 0.4% EDTA/0.1% bovine serum albumin/phosphate-buffered saline and seeded in triplicate on the upper chamber at a density of 100,000 cells/well in 0.5 ml of medium, and 1.5 ml of medium was added to the lower chamber. After 36 hours, the MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; Sigma), at 0.5-mg/ml final concentration, was added to both chambers. Three hours later the formazan crystals were collected separately from the upper and lower chambers, pelleted by centrifugation, and dissolved in 1 ml of dimethyl sulfoxide. The optical density of each well was measured at 570 nm (reference filter 690 nm) with a GENios spectrofluorometer (Tecan). Three individual wells were prepared for each treatment, and the experiment was repeated 3 times. Invasion was calculated as the ratio of the number of cells in the lower compartment to the sum of cells in both compartments. In parallel to the stefin B silencing experiment, cathepsin B in IOMM Lee malignant meningioma cells was inhibited using Ca074Me inhibitor (Calbiochem) in a final concentration of 0.5 μM. The invasion assay and MMT test were performed as stated above. The significance of the differences among these assays was tested using a paired t-test.

**Stefin B Silencing**

The IOMM Lee malignant meningioma cells\textsuperscript{21} were seeded 100,000 cells/well (6-well plate) a day before a knockdown experiment. At 50–80% confluence, the cells were transfected with Hs\_CSTB-4 siRNA vector (HP GenomeWide siRNA) or nonsilencing duplex RNA as a negative control using RNAiFect transfection reagent (all from Qiagen).

---

**Table 1: Summary of patient and tumor characteristics**

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of Cases</th>
<th>Nonrecurrent Tumors</th>
<th>Recurrent Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of patients</td>
<td>119</td>
<td>102</td>
<td>17</td>
</tr>
<tr>
<td>WHO Grade I (benign)</td>
<td>57</td>
<td>52</td>
<td>5</td>
</tr>
<tr>
<td>WHO Grade II (atypical)</td>
<td>33</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>WHO Grade III (anaplastic)</td>
<td>7</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>not known</td>
<td>22</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>age (yrs)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>59.3</td>
<td>58.6</td>
<td>6.25</td>
</tr>
<tr>
<td>range</td>
<td>21–88</td>
<td>21–88</td>
<td>25–88</td>
</tr>
<tr>
<td>resection†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>complete (SG &lt;2)</td>
<td>87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>incomplete (SG &gt;2)</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Patient classification was performed according to WHO grade. The relapse was determined within the period from 0 to 156 months (mean 13 years). Mean survival rates are given for each stage. Abbreviations: PgR = progesterone receptor; SG = Simpson grade.
† Data obtained in 118 patients.
‡ Significant difference in nonrecurrent versus recurrent tumors.
Immunohistochemical Staining of Cathepsin B

Intense cathepsin B staining in meningioma sections was achieved with the biotinylated tyramide method. This differs from the classic biotin method in that biotinylated tyramide is loaded to the secondary antibody-bound horseradish peroxidase after the first incubation step, intensifying the reaction with horseradish peroxidase. We have used the TSA Biotin System NEL 700 kit (NEN Life Science Products), according to the manufacturer’s protocol. Antigen retrieval was achieved by heating in a microwave for 15 minutes in 0.01 M citrate buffer. The first incubation, for 10 minutes with 0.2% H$_2$O$_2$, was followed by addition of blocking solution and then primary mouse antibodies against human cathepsin B (MOAb 3E1) used in 1:100 dilution. After extensive washing with Tris-HCl, pH 7.5, the sections were treated with streptavidin horseradish peroxidase diluted 1:200. The sections were again washed 4 times for 5 minutes and incubated for 5 minutes in freshly prepared 1:50 diluted biotinylated tyramide. The sections were then washed, and streptavidin horseradish peroxidase was again applied for 30 minutes, washed in TNT (Tris, NaCl, and Tween), and transferred to Tris buffer (pH 7.5) followed by a 5-minute incubation in the dark with diaminobenzidine solution (Sigma). Sections were contrast stained with diluted hematoxylin (1:4 in bi-distilled water) for 15 seconds and then washed for 10 minutes with tap water. Finally, the sections were dehydrated and inserted in Canada balsam and dried.

Patients and Clinical Materials

Included in the study were 119 patients who underwent surgery at the Hôpital de la Timone in Marseille, France, between 1989 and 2003. All the tumor tissue samples were collected and conserved in the oncological laboratory at the Hôpital Nord, Marseille. The study was approved by the relevant institutional board, and written informed consent was obtained from the patients. The efficacy of the removal after resection was evaluated. Patient characteristics are listed in Table 1. Follow-up ranged from a few months to a maximum of 13 years. During this period, 102 tumors did not recur, 12 recurred once, and 5 relapsed twice. Tumor samples, taken during the operation, were frozen in liquid nitrogen before preparing the cytosols by homogenization of frozen tissue in 0.01 M Tris buffer, pH 7.4, containing 1.5 mM EDTA, 0.01 M Na$_2$MoO$_4$, 0.5 mM dithiothreitol, and 1% glycerol. Homogenates were centrifuged at 40,000 g for 1 hour to separate the supernatant (cytosol) fraction, which was kept at −70°C. Cytosol extract protein concentrations were determined using the bicinchoninic acid assay (Peirce).

Enzyme-Linked Immunosorbent Assays

The ELISA kits were used to determine cathepsin B, cathepsin L, stefins A and B, and cystatin C. Polyclonal mouse and monoclonal rabbit and sheep polyclonal antibodies were used as catching antibodies, according to the producer’s instructions. Tumor cytosols were thawed only once and diluted in 3 different concentrations (50–100 times). For cathepsin L and cystatin C, the cytosols were diluted 5–10-fold to ~ 0.2 mg/ml total protein concentration. To determine stefin B, the cytosols were diluted from 50–200-fold to protein concentrations between 0.015 and 0.02 mg/ml; for stefin A, samples were diluted ~ 2-fold. Recombinant human proteins were used...
as standards, and the assays were carried out according to the manufacturer’s protocols. The antibodies were highly selective for human cathepsins and stefins. All forms of the cathepsins, either native or bound to the cystatins, were recognized by the antibodies.

The concentration of progesterone receptors was determined using a progesterone receptor-EIA commercial kit from Abbott Laboratories, according to the manufacturer’s instructions, and expressed as ng/mg protein.

Statistical Analysis

The comparison of parameters as a function of histological grade was done using ANOVA. Survival was analyzed by the Kaplan-Meier method using the log-rank comparison test; the Cox multivariate analysis method was used. Disease-free survival was considered to be the time of primary tumor removal (intervention) to tumor recurrence or the date of the last information about the patient’s status. Cut-off values for continuous variables were calculated using isotonic regression analysis. Comparison between randomly selected samples (training set) and the validation set gave the cut-off value, which was used for the entire sample population. The cut-off values were tested on 2 independent, randomly selected sets of patients and the closest cut-off point was confirmed in the total population. In all analyses, statistical significance was taken as $p < 0.05$.

Results

Cathepsin B Localization in Meningioma and its Localization in Confrontation Assays

Cathepsin B in the meningioma tissue, grown as primary culture in spheroids, was labeled immunohistochemically using the enhanced labeling with biotinylated tyramide. The positive reaction of cathepsin B is observed as a granular ring around the nuclei, indicating its lysosomal localization. The labeling was markedly stronger in tumor spheroids in vitro than in previously reported biopsy specimens or the tumors, probably due to the different fixation procedures; the latter were fixed in 4% formaldehyde, which causes tissue shrinking, whereas the Bouin-Holland fixative used for the tumor spheroids provides better preservation of cell morphology and structure. In the confrontations with the chick heart spheroids, 2 types of confrontation patterns were observed in the group of meningiomas diagnosed as benign transitional types. The immunohistochemical staining of spheroid sections was usually done on the 1st, 4th, and 8th days. Of the noninvasive pattern, after the tumor spheroid attached to the normal chicken tissue spheroid during the 1st day, there was no massive invasion observed over several days of incubation (Fig. 1A). After 8 days, the myocytes completely encircled the tumor spheroid; however, single tumor cells loaded with cathepsin B can also be seen in the region of chick tissue (Fig. 1C). The antibody used was specific for human cathepsin B and did not interact with chick cathepsin B, as no single cell reactivity was observed in the chick tissues after 1 day of incubation.

In contrast, the invasive type is characterized by a process in which tumor-derived cells infiltrate the chick heart (Fig. 2). This process is time dependent, and progressive replacement of the chick heart tissue by the infiltrating cells is observed. There is no clear-cut border between the confronting pairs. Invading tumor-derived cells progressively overgrew the chick heart spheroid. The process is shown on Days 2 (Fig. 2A), 4 (Fig. 2B),
and 8 (Fig. 2C) of confrontation. The tumor overgrew the entire chick tissue, and cathepsin B labeling appeared to intensify at the cells of the invasive front, which have extended pseudopodia loaded with cathepsin B and which surrounded the chick myocytes.

In the 10 benign meningiomas of different histological subtypes, noninvasive (Fig. 1) and invasive (Fig. 2) patterns were seen. The patterns were not related to the histological subtype.

In Vitro Invasion of Cathepsin-Inhibited and Stefin B–Silenced Malignant Meningioma

To assess the potential impact of cathepsin B activity in the process of meningioma invasion, we blocked cathepsin B using a specific inhibitor, Ca074Me, in an in vitro invasion assay in Matrigel-coated, modified Boyden chambers; we used IOMM Lee malignant meningioma cells. We found that cathepsin B inhibition significantly (p < 0.04) decreased cell invasion compared with negative controls (Fig. 3 left). Moreover, the potential impact of stefin B in the process of meningioma invasion was measured in a stefin B–silencing experiment in which we used siStefin B RNA IOMM cells. This procedure significantly (80–90%) reduced mRNA levels of stefin B expression (data not shown). Figure 3 right shows that Stefin B RNA silencing highly significantly (p < 0.008) increased the invasion of malignant meningioma cells compared with the cells transfected with nonsilencing duplex RNA.

Cathepsin B in Meningioma Tissue: Correlation With Other Candidate Markers of Meningioma Progression and WHO Grade

Of 119 patients in the study, 17 had a tumor relapse (Table 1). The meningiomas were classified according to WHO grades as follows13,26: 57 were benign, 33 atypical, and 7 malignant. No WHO grade data were available for 22 patients. Patient age ranged from 21 to 88 years (mean ~ 60 years). A Simpson grade was determined, but for survival analysis only total and partial resections were taken into consideration. All patient data are available upon request and as a supplement. The levels of molecular parameters are listed in Table 1 for all the primary tumors, 102 samples of nonrecurrent tumors, and 17 samples of recurrent tumors. No significant differences were found between the primary and the recurrent tumors, except significantly lower levels of progesterone receptor in the recurrent tumors.

With respect to distribution of these molecular parameters according to WHO tumor grade, 97 patients were included for correlation of the antigens cathepsins B and L, stefins A and B, cystatin C, and progesterone receptor with WHO grade (Fig. 4). The mean levels of cathepsin B were 175 ng/mg in benign, 164 ng/mg in atypical, and 180 ng/mg protein in malignant meningiomas, and these values did not differ significantly (Fig. 4A). Cathepsin L protein was expressed at higher levels in atypical (mean 58 ng/mg) and malignant (mean 60.5 ng/mg) meningiomas than in benign (mean 56 ng/mg) meningiomas, but the differences were only close to being significantly different (p = 0.06) (Fig. 4B). Stefin A levels were significantly lower in the benign (mean 12.3 ng/mg) than in atypical (mean 16.8 ng/mg) and malignant (16.9 ng/mg) meningiomas, the differences being highly significant (p = 0.006) (Fig. 4C). The mean levels of stefin B, which ranged from 291 to 305 ng/mg in benign to malignant meningioma, and the levels of cystatin C, which ranged from 32 to 36 ng/mg protein, were also not significantly different according to WHO grades. The levels of progesterone receptor, known to be a good prognostic factor for meningioma,26 were significantly lower in the cases of malignant meningioma (49 µg/mg protein) than in the benign (170 µg/mg protein) and the atypical (140 µg/mg protein) meningiomas, and these differences were statistically significant (p < 0.05) (Fig. 4D).

Correlation With Prognosis for Disease-Free Survival: Univariate Analysis

Survival analysis included 100 nonrecurrent and 8 recurrent meningiomas. Patients were followed from 1989 to 2004. The shortest follow-up period was 1 month and the longest 13.3 years (mean 2.1 years). The variables tak-
into consideration with respect to survival were WHO grade, Simpson grade, age, and levels of progesterone receptor, cathepsin B, cathepsin L, stefin A, stefin B, and cystatin C. All patient data are available upon request. Univariate analysis was performed for the continuous variables for which statistically significant (p < 0.05) correlation was found in the period of relapse-free survival, and the optimum cut-off was determined using isotonic regression analysis. The results of the survival analysis are shown in Table 2 and the survival curves are shown in Fig. 5. As expected, patients with the highest grades (WHO Grades II and III) had significantly (p = 0.027) shorter survival times than patients with lower grades (Fig. 5A). However, differences between the atypical and malignant meningiomas were not statistically significant. For progesterone receptor, the cut-off level was taken as 10 µg/mg protein, and patients with higher levels had longer survival at borderline significance (p = 0.049; Fig. 5B). For cathepsin L, the patients with values > 60 ng/mg (2.14 pmol/mg) exhibited a significantly (p = 0.035) higher probability of relapse (that is, a worse prognosis) (Fig. 5C). Furthermore, patients with levels of the cysteine protease inhibitor cystatin C that were > 10.8 ng/mg (0.83 pmol/mg) in the primary meningioma had a significantly (p = 0.008) better prognosis (that is, longer disease-free survival) (Fig. 5D). In contrast, patients in whom levels of stefin B, another cysteine protease inhibitor, were > 500 ng/mg (45.5 pmol/mg) had a significantly (p = 0.007) poorer prognosis (Fig. 5E). Of note, for stefin A—the only factor that positively correlated with WHO grade—no correlation with survival was observed at any cut-off point, although the 9.5-ng/mg (0.86 pmol/mg) level was close to statistical significance (p = 0.059) (Fig. 5F).

We also stratified the tumors into cases of complete resection (Simpson grade < 2 in 87 cases [73%]) and cases of incomplete resection (Simpson grade > 2) resection (19 cases [15%]); for the remaining tumors, no data were available (Table 1). Statistically significant differences were observed between the survival rates in the 2 groups of patients (p = 0.045) in univariate analysis, but only when the disease-free survival was followed up to 90 months (Table 2). When longer follow-up (up to 150 months) was taken into consideration, the extent of resection was not relevant for relapse. Finally, the age of the patients had an impact in univariate analysis (p = 0.028) but not in multivariate analysis.
Multivariate Cox Regression Analysis

Survival analysis data are summarized in Table 2. The variables included in the Cox³ regression analysis were as follows: cathepsin B, cathepsin L, stefins A and B, cystatin C, and progesterone receptor levels. When these variables were collectively compared with the WHO grade, they were not statistically relevant for prognosis (p = 0.109). Single-variable comparison in multivariate Cox analysis, however, revealed that only stefin B was a statistically significant prognostic marker (p = 0.039), whereas progesterone receptor was only close to borderline significance for recurrence in multivariate analysis. Abbreviations: FU = follow-up; NS = not significant.

Discussion

A meningioma is a solid, well-circumscribed tumor that can attach to and often invade the dura mater, and even the bone, causing hyperostosis.¹³ Although such tumors have not been reported to have poor prognosis, because complete tumor removal can often not be achieved, this can affect the prognosis.²⁸ In this study we also observed significantly lower disease-free survival rate in patients in whom incomplete tumor resection was achieved, although we found that this surgical result may be responsible only for early, but not for late, recurrences (Table 2). Although the invasion of meningioma into normal brain tissue is rarely observed, it can occur in all histological grades, as well as in benign meningioma.²⁸ Nakasu and coworkers³¹ classified meningioma invasion into brain tissue in vivo into 4 types, with the invasive type exhibiting poor demarcation between the brain and neoplasm and no encapsulation of the tumor. They concluded that this type of benign meningioma is most prone to recurrence. Invasiveness may therefore be considered a hallmark of
malignancy, which, along with high tumor proliferation, differentiates malignant from benign tumors. Because detailed histological examination is not always possible, we revisited the in vitro biological tissue assays proposed by Mareel and coworkers and de Ridder and colleagues, and used the in vitro chick heart confrontation assay to diagnose the invasive meningioma. Cysteine cathepsin B, which is considered an invasive marker in GBM and other tumors, labeled the tumor cells and demonstrated that those cells leaving the bulk of meningioma spheroid are strongly positive for cathepsin B. The enzyme is expressed in high concentrations not only at the invasion front of the tumors, but also in the pseudopodia of solitary invading cells. This also demonstrates that meningiomas, similar to GBM, invade the host tissue both frontally and as single “guerilla cells” infiltrating the host tissue. In these cells, the enzyme was expressed in high concentrations in the pseudopodia of solitary invading cells. Unfortunately, the levels of cystatins were too low to be detected by immunohistochemistry. Therefore, in an in vitro invasion assay using Boyden chambers, we additionally confirmed the role of both cathepsin B and stefin B in the invasion, showing that altering the level of active cathepsin B by synthetic inhibitor or the endogenous inhibitor stefin B alters the invasion of malignant meningioma cells. However, neither of these antagonistic molecules was significantly elevated in the whole malignant meningioma tissue, indicating that their focal localization at the tumor edge is more crucial for the invasion process.

Reports in the literature confirm the complex interplay between proteases and their inhibitors in cancer progression and proteolytic cascade, initiated by cathepsins (cathepsin B); this includes activation of urokinase plasminogen activators and plasmin, along with several MMPs, in the invasion of extracellular matrix. The 2 secretory gelatinases, MMP2 and MMP9, have mostly been studied in meningioma, and the results of in vivo study of MMP2 and mMP9 could not confirm previous reports that these MMPs correlated with histological subtypes, as found in the in vitro cell cultures. In contrast, MMP9 was downregulated in WHO Grade III lesions, similar to tissue inhibitors of metalloprotease, the endogenous inhibitor of MMPs. Nevertheless, the authors concluded that the balance between the enzyme and the inhibitor, still in favor of the MMPs, may be more relevant for the impact on invasive behavior of meningioma, which has been reported to correlate with recurrence of intracranial meningioma. Recent reports on the RNA interference–mediated simultaneous downregulation of cathepsin B and MMP9 in malignant meningioma cell lines, however, clearly led to not only decreased invasion but also to tumor growth and angiogenesis, underscoring that these enzymes are relevant for various processes in these tumor.

We have previously demonstrated higher labeling of cathepsin B in border benign and atypical meningiomas in a series of 88 meningiomas and suggested that this marker may be useful to distinguish the clear benign tumors from the histomorphologically benign but invasive meningiomas. We confirmed these findings in a further immunohistochemical study; however, in the total tumor extracts, differences in the expressions of this biomarker at the mRNA level were not found. Similarly, in the present study we observed no significant differences in cathepsin B protein by ELISA among the tumor extracts of benign, atypical, and malignant meningioma. Taken together, these data suggest that meningioma invasiveness may be caused by only a small fraction of the tumor cells, located at the tumor edges and expressing higher levels and activities of cathepsin B; these higher cathepsin B levels and activity can be detected by submitting tumor specimens to immunohistochemical analysis, but there is no significant overall increase in the whole cancer tissue, comprising the tumor and stromal cells. Furthermore, the invasive tumor cells infiltrating the surrounding tissue may not be included in the tumor samples that are obtained for analysis. In contrast to cathepsin B, a trend to higher expression of cathepsin L and stefin A was observed in high-grade meningiomas, as was the well-known downregulation of progesterone receptor. However, no correlation of overall cystatin C and stefin B levels with WHO grade was made.

With respect to prognosis, a univariate analysis revealed that cathepsin L, stefin B, and cystatin C were significant prognostic factors for early recurrence of meningioma, as were high WHO grade and low progesterone receptor levels. These findings indicate that cysteine-dependent proteolysis is associated with malignant progression of meningioma, independent of other parameters contributing to WHO grade, the latter being known as good indicator of early recurrence. High cathepsin L levels have also been found to be significant for poor prognosis in breast carcinoma but not for glioma. This protease may play an important role in processes, such as cell proliferation, other than invasion, and it has been suggested it may also be related to apoptosis and tumor resistance to therapy, although its role remains to be revealed. We may conclude that the recurrence of meningioma may not only be dependent on the subpopulation of the invasive cells and on complete tumor resection, but also on the tumor cell subpopulation, which acquires higher resistance to chemotherapy.

In a multivariate analysis, only low stefin B levels remained significant parameter for a better prognosis (that is, later relapse). In contrast to this, higher levels of cysteine protease inhibitors, stefin A, stefin B, and cystatin C have been shown to correlate with better prognosis in various types of cancer, although the opposite findings have also been reported. High stefin B levels have been shown to indicate better prognosis in breast carcinoma, but the opposite was found in head and neck cancer. Enhanced expression of cystatins in cancer progression, a trend noted in malignant meningioma, can be explained as a response of the tumor cells and/or the tumor microenvironment cells to impaired tumor-associated proteolytic activity. Alternatively, increased intracellular stefin B levels in the tumor cells protect them from the harmful effect of the increased enzymes, whose goal is to damage the tumor microenvironment. This has similarly been suggested for PI-1 and for stefin A in other tumors. For example, intense stefin A staining
Cathepsins and stefins in recurrent meningioma

has been seen in tumor cells of aggressive breast carcinoma, and we found this inhibitor highly prognostic for disease-free survival of cancer patients. In contrast, in this study, meningioma-associated stefin A also correlated with disease progression but had no prognostic impact on disease-free survival.

One limitation of our study may be that it included < 10% of recurrent tumors, which may result in a statistical distortion, and we would suggest that all biomarkers should be evaluated in a larger patient population. In this study, however, WHO grade, tumor resection, and progesterone receptor status were found to be significant prognosticators, as has been reported in many other studies, underscoring the justification for our study design.

Conclusions

This study suggests that immunolabeling of the invasive marker cathepsin B is useful for the immunohistochemical assessment of invasive tumor cells and it is also useful when in vitro confrontation assays are used as biological assays to diagnose more aggressive and recurrent meningioma. However, cathepsin B was not found to be a prognostic parameter when determined in whole-tumor tissue extracts by ELISA, possibly due to the fact that cathepsin B at the invasive edges of the tumor represents only a marginal fraction of the total amount of cathepsin B in the entire tumor mass. Cystatin C and stefin B, potentially associated with meningioma invasion and aggressiveness, were prognostic in the univariate analysis, but only stefin B proved to be an independent prognostic factor for meningioma recurrence in the multivariate analysis, overwhelming even the classic histopathological parameters. Our results on one hand emphasize the important role of cysteine cathepsins in meningioma aggressiveness and, on the other, suggest the need for designing larger clinical studies to test the impact of stefin B on meningioma recurrences for clinical application. This is of particular importance because of the long relapse-free intervals experienced by patients with these tumors, a period during which patients at risk can be thoroughly and more aggressively treated.

Disclosure

This work was supported by the Slovenian Research Agency Research Program (grant no. 0105-507 to Dr. Lah), the Integrated Project CANCER DEGRADOME (EU 6FP LSH-2002-2.2; no. 503297), and by FNR Luxembourg (grant AFR PDR-08-077 to Dr. Rajčević). Dr. Lah is also a member of the EORTC–PathoBiology Group.

Acknowledgments

We thank Prof. Dr. Janko Kos (Faculty of Pharmacy, University of Ljubljana, Slovenia) for providing us with selective antibodies for cathepsin B and ELISA assays for cathepsins, stefins, and cystatin C. We also thank Dr. Bojan Sedmak for technical help in graphics design, and Prof. Dr. Roger Pain for carefully reviewing the manuscript and for his substantial professional and grammatical improvements of this work. Drs. David Gillespie and Randy Jensen (Departments of Neurosurgery, Radiation Oncology, and Oncological Sciences, University of Utah) are acknowledged for kindly providing the IOMM Lee cell line.

References


Manuscript submitted December 26, 2008. Accepted July 15, 2009. Please include this information when citing this paper: published online September 11, 2009; DOI: 10.3171/2009.7.JNS081729. Portions of this paper represent the doctoral thesis of Dr. Trinkaus, presented at his defense in 2006. Other portions were included in an oral presentation by Dr. Lah at the International Proteolysis Society Conference in Patras, Greece, October 2007. Address correspondence to: Tamara T. Lah, Ph.D., Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia. email: tamara.lah@nib.si.