Immunodetection of Endogenous Opioid Peptides in Human Brain Tumors and Associated Cyst Fluids

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ABSTRACT

The antitumorigenic effects of endogenous opioid peptides and their presence in extracerebral tumors are well documented. In this study, methionine-enkephalin (met-enkephalin) was measured by radioimmunoassay in 108 glial and nonglial brain tumors and in 44 associated cyst fluids. By immunohistochemistry, the distribution of the peptide and its precursor, preproenkephalin A, was also analyzed. Met-enkephalin and preproenkephalin were detected in the cytoplasm and cell processes of all tumors. Moreover, for neuroectodermal tumors (i.e., gliomas, gangliogliomas, and dysembryoplastic neuroependelial tumors), a strong inverse correlation (P < 0.0001) was observed between the met-enkephalin levels and the degree of malignancy (242.9, 148.3, 55.3, and 30.3 pg/mg protein for grade 1, 2, 3, and 4, respectively). When compared to normal tissue, this differential expression mainly resulted from a decrease in the opioid peptide content in high-grade neuroectodermal tumors. Meningiomas and cerebral metastases displayed low met-enkephalin levels, similar to those of grade 4 neuroectodermal tumors. Large amounts of met-enkephalin were found in all cyst fluids. These data suggest that the endogenous opioid system is an integral component of brain tumors and that met-enkephalin may represent a useful malignancy marker in neuroectodermal tumors.

INTRODUCTION

Human brain tumors represent about 9% of all solid tumors. Despite progress in medical and surgical treatments, the prognosis of most cerebral tumors remains invariably poor, and better understanding of neuro-oncogenesis is needed to define new therapeutic approaches.

Endogenous opioid peptides have been implicated in a wide variety of functions, including the regulation of neuronal and glial proliferation in the developing nervous system (1–5). Moreover, the antitumoral properties of opioids are now well documented, and opioid agonists have been reported to exert a growth-inhibitory effect on several tumor types (6–13). This control of tumorigenesis can be blocked by opioid antagonists (6, 8, 10) and seems to be mediated by a specific receptor, the zeta (ζ) receptor (14).

Met-enkephalin, the proteolytic end product of preproenkephalin A, is the most potent opioid compound associated with growth modulation (1, 3, 8, 10). This pentapeptide, and other closely related molecules, have been detected in a wide range of human and animal neoplasms (15–23). However, the presence of endogenous opioid peptides in human brain tumors has not yet been investigated. In the present study, we looked for met-enkephalin and preproenkephalin immunoreactivity in a series of glial and nonglial human cerebral tumors and in several cyst fluids associated with these tumors. We also analyzed the relationship between the level of met-enkephalin expression and the degree of tumor malignancy evaluated by histopathological criteria.

MATERIALS AND METHODS

Tumors. One hundred eight brain tumors were harvested at the time of surgery, immediately frozen in isopentane chilled in liquid nitrogen, and kept at −80°C. For one recurrent glioma, primitive and recurrent tumor tissue were obtained. Forty-four cyst fluids withdrawn during stereotactic biopsy or tumor resection were also stored at −80°C. In 9 cases, both cyst fluid and corresponding tumor tissue were collected. Normal white matter taken from 5 patients who had undergone surgical cortectomy for cryptogenic epilepsy and from 1 monkey served as controls. Cyst fluids were compared with normal serum and cerebrospinal fluids.

Histological classification was based on the World Health Organization system (24) and gliomas were graded according to the Kernohan system (25). The distribution of the tumors is summarized in Tables 1 and 2.

Tissue Extraction. After removal of necrotic and hemorrhagic tissue, samples (weighing 50–250 mg) were cut from tumor specimens with a cryotome, boiled in 1 ml of 1 M acetic acid for 15 min, and homogenized by sonication for 45 s at 0°C. The homogenates were centrifuged at 2000 × g for 30 min at 4°C, and the supernatants were divided into aliquots for radioimmunoassay and protein determination (26).

Radioimmunoassay. For radioimmunoassay, tumor extracts were lyophilized, redissolved in 0.9 ml of BSA-phosphate buffer, and centrifuged again. Cyst fluids, sera, and cerebrospinal fluids were briefly centrifuged and directly assayed. Met-enkephalin measurements were performed in duplicate with an 125I reagent kit from Incstar Corp. (Stillwater, MN).

Immunohistochemistry. Tumor specimens were cut (20 μm) on a cryotome, and the sections were collected on poly-L-lysine-coated slides. Tissues were fixed in 4% paraformaldehyde at 4°C for 10 min and rinsed in PBS. Endogenous peroxidase activity was blocked by immersion in 0.3% hydrogen peroxide. Some sections were counterstained with Giemsa. Incubations were done in high-grade neuroectodermal tumors. Meningiomas and cerebral metastases displayed low met-enkephalin levels, similar to those of grade 4 neuroectodermal tumors. Large amounts of met-enkephalin were found in all cyst fluids. These data suggest that the endogenous opioid system is an integral component of brain tumors and that met-enkephalin may represent a useful malignancy marker in neuroectodermal tumors.
For NETs, the levels of met-enkephalin were strongly inversely correlated with grade (analysis of variance, \( P < 0.0001 \)), with the lowest concentrations recorded in the most malignant tumors. The same relationship was found when considering only gliomas or only astrocytomas. Furthermore, values in grade 3 and 4 gliomas were consistently lower than in normal white matter obtained from patients who had undergone surgery for cryptogenic epilepsy and in monkey normal brain tissue (Student’s \( t \) test, \( P < 0.0001 \)). Grade 1 NETs contained more met-enkephalin than normal white matter, but the difference was not significant. There was no significant correlation with histological type of NETs, even if met-enkephalin levels were consistently lower than in normal white matter obtained from patients with histological type of NETs, even if met-enkephalin levels were consistently lower than in normal white matter obtained from patients.

Amounts of met-enkephalin detected in meningiomas and cerebral metastases were relatively small and similar to those observed in grade 4 gliomas (Table 3). Variability in met-enkephalin content between tumors was high in the low-grade NET group (range, 6.9–783.9 pg/mg protein). On the other hand, the high-grade glioma group, the meningioma group, and the metastasis group were more homogeneous (range, 6.7–215.3, 13.2–65.7, and 10.8–62.2 pg/mg protein, respectively). There were also consistent variations within tumors, as suggested by measurements performed in central and peripheral fragments of three NETs. In the 2 low-grade NETs (one grade 1 astrocytoma and one grade 1 dysembryoplastic neuroepithelial tumor), met-enkephalin concentrations in peripheral regions (paucicellular infiltration) were much lower than in the tumor core (75.2 and 327.3 pg/mg protein, respectively). On the contrary, in the high-grade glioma (grade 3 oligodendroglioma), values were higher in the peripheral part of the tumor than in the central region (217.4 versus 29.7 pg/mg of protein).

### Table 1 Distribution of the tumors examined for met-enkephalin immunoreactivity

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroectodermal tumors, grade 1</td>
<td>17</td>
</tr>
<tr>
<td>DNE* tumors</td>
<td>5</td>
</tr>
<tr>
<td>Astrocytomas</td>
<td>6</td>
</tr>
<tr>
<td>Gangliogliomas</td>
<td>6</td>
</tr>
<tr>
<td>Neuroectodermal tumors, grade 2</td>
<td>18</td>
</tr>
<tr>
<td>DNE tumors</td>
<td>2</td>
</tr>
<tr>
<td>Astrocytomas</td>
<td>8</td>
</tr>
<tr>
<td>Oligodendrogliomas</td>
<td>7</td>
</tr>
<tr>
<td>Gangliogliomas</td>
<td>1</td>
</tr>
<tr>
<td>Neuroectodermal tumors, grade 3</td>
<td>21</td>
</tr>
<tr>
<td>Astrocytomas</td>
<td>19</td>
</tr>
<tr>
<td>Oligodendrogliomas</td>
<td>1</td>
</tr>
<tr>
<td>Ependymomas</td>
<td>1</td>
</tr>
<tr>
<td>Neuroectodermal tumors, grade 4</td>
<td>18</td>
</tr>
<tr>
<td>Glioblastomas</td>
<td>17</td>
</tr>
<tr>
<td>Gliosarcomas</td>
<td>1</td>
</tr>
<tr>
<td>Radiation necrosis</td>
<td>5</td>
</tr>
<tr>
<td>Meningiomas</td>
<td>16</td>
</tr>
<tr>
<td>Meningotheliomatous</td>
<td>9</td>
</tr>
<tr>
<td>Fibrous</td>
<td>1</td>
</tr>
<tr>
<td>Transitional</td>
<td>3</td>
</tr>
<tr>
<td>Angiomatos</td>
<td>3</td>
</tr>
<tr>
<td>Cerebral metastases</td>
<td>10</td>
</tr>
<tr>
<td>Epidermoid carcinoma (lung)</td>
<td>2</td>
</tr>
<tr>
<td>Adenocarcinoma (lung)</td>
<td>1</td>
</tr>
<tr>
<td>Carcinoma (unknown)</td>
<td>1</td>
</tr>
<tr>
<td>Adenocarcinoma (lung)</td>
<td>3</td>
</tr>
<tr>
<td>Adenocarcinoma (colon)</td>
<td>1</td>
</tr>
<tr>
<td>Adenocarcinoma (unknown)</td>
<td>1</td>
</tr>
<tr>
<td>Melanoma</td>
<td>1</td>
</tr>
<tr>
<td>Cavernous angiomas</td>
<td>2</td>
</tr>
<tr>
<td>Lymphomas</td>
<td>1</td>
</tr>
</tbody>
</table>

* DNE, dysembryonic neuroepithelial.

### Table 2 Distribution of the tumors associated with cyst fluids

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroectodermal tumors, grades 1, 2</td>
<td>13</td>
</tr>
<tr>
<td>Astrocytomas</td>
<td>9</td>
</tr>
<tr>
<td>Oligoastrocytomas</td>
<td>1</td>
</tr>
<tr>
<td>Oligodendrogliomas</td>
<td>2</td>
</tr>
<tr>
<td>Gangliogliomas</td>
<td>1</td>
</tr>
<tr>
<td>Neuroectodermal tumors, grade 3</td>
<td>17</td>
</tr>
<tr>
<td>Astrocytomas</td>
<td>17</td>
</tr>
<tr>
<td>Neuroectodermal tumors, grade 4</td>
<td>4</td>
</tr>
<tr>
<td>Glioblastomas</td>
<td>4</td>
</tr>
<tr>
<td>Meningiomas</td>
<td>2</td>
</tr>
<tr>
<td>Meningotheliomatous</td>
<td>1</td>
</tr>
<tr>
<td>Angiomatos</td>
<td>1</td>
</tr>
<tr>
<td>Cerebral metastases</td>
<td>4</td>
</tr>
<tr>
<td>Epidermoid carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Melanoma</td>
<td>1</td>
</tr>
<tr>
<td>Hemangioblastomas</td>
<td>3</td>
</tr>
<tr>
<td>Cranioopharyngiomas</td>
<td>1</td>
</tr>
</tbody>
</table>

### Table 3 Met-enkephalin immunoreactivity in human brain tumors

<table>
<thead>
<tr>
<th>Tumors</th>
<th>( n )</th>
<th>Tissue (pg/g)</th>
<th>Protein (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroectodermal tumors, grade 1</td>
<td>17</td>
<td>9726 ± 2250a</td>
<td>242.9 ± 45.6a</td>
</tr>
<tr>
<td>Neuroectodermal tumors, grade 2</td>
<td>18</td>
<td>4772 ± 1243</td>
<td>148.3 ± 48.2</td>
</tr>
<tr>
<td>Neuroectodermal tumors, grade 3</td>
<td>20</td>
<td>2564 ± 351e</td>
<td>55.3 ± 18.9e</td>
</tr>
<tr>
<td>Neuroectodermal tumors, grade 4</td>
<td>18</td>
<td>1835 ± 252d</td>
<td>30.3 ± 4.6d</td>
</tr>
<tr>
<td>Radiation necrosis</td>
<td>5</td>
<td>7895 ± 3895</td>
<td>128.9 ± 56.2</td>
</tr>
<tr>
<td>Meningiomas</td>
<td>15</td>
<td>1570 ± 166d</td>
<td>29.5 ± 3.7d</td>
</tr>
<tr>
<td>Cerebral metastases</td>
<td>10</td>
<td>2170 ± 270e</td>
<td>31.3 ± 5.0e</td>
</tr>
<tr>
<td>Cavernous angiomas</td>
<td>2</td>
<td>5209 ± 3407</td>
<td>74.9 ± 43.4</td>
</tr>
<tr>
<td>Lymphomas</td>
<td>1</td>
<td>2279 ± 0</td>
<td>24.9 ± 0.0</td>
</tr>
<tr>
<td>Normal white matter</td>
<td>5</td>
<td>4522 ± 430</td>
<td>198.8 ± 43.3</td>
</tr>
<tr>
<td>Monkey white matter</td>
<td>1</td>
<td>4193 ± 0</td>
<td>150.8 ± 0.0</td>
</tr>
</tbody>
</table>

* Significant difference from neuroectodermal tumors grade 3 (\( P < 0.002 \)), grade 4 (\( P < 0.0004 \)), meningiomas (\( P < 0.0004 \)), and metastases (\( P < 0.005 \)) with Scheffe F test.

** Immunohistochemistry. Eighteen tumors were examined for immunohistochemistry. All of them demonstrated immunostaining for met-enkephalin and its precursor, preproenkephalin A. In low-grade NETs, preproenkephalin immunoreactivity was mostly localized to cell processes and displayed a fibrillar pattern similar to that of glial fibrillary acidic protein labeling (Fig. 1). Some neurofilament-positive fibers were also stained with preproenkephalin antibodies. Met-enkephalin reactivity had the same cellular distribution as that of preproenkephalin (Fig. 1) but was fainter and was also found in the extracellular spaces. Only few cell bodies were immunoreactive for preproenkephalin and met-enkephalin. In high-grade gliomas, the labeled cell processes were sparser, but perikarya of tumor cells, including monstrous giant cells, often exhibited immunoreactivity for both antibodies (Fig. 2). Several grade 4 gliomas displayed very heterogeneous immunostaining; some tumor regions were devoid of any labeling.
In cerebral metastases, preproenkephalin and met-enkephalin immunoreactivity was detected in the cytoplasm of most neoplastic cells (Fig. 2). Meningiomas were stained diffusely with met-enkephalin antibodies but showed no reactivity for preproenkephalin. No labeling of cell nuclei was observed.

Cyst Fluids. All cyst fluids exhibited sizable levels of met-enkephalin (Table 4). When expressed as a function of protein content, levels measured in cyst fluids associated with low-grade NETs were slightly higher than those associated with high-grade glioma and metastasis and 1.4-fold higher than in serum. On the contrary, menin-
gioma, haemangioblastoma, and craniopharyngioma cyst fluids contained much smaller amounts of met-enkephalin than serum. However, there was no statistically significant difference between the various tumor groups, nor between tumor groups and serum.

**DISCUSSION**

**Brain Tumors.** The presence of met-enkephalin and other related peptides in benign or malignant tumors of all histological origins is now well documented (15–22), except for brain tumors which have been of little interest. Preproenkephalin mRNA has also been detected in several extracerebral neoplasms (20, 22, 27, 28). This study shows the presence of met-enkephalin and its precursor, preproenkephalin A, in a wide range of glial and nonglial brain tumors. Identification of the two peptides in the cytoplasm and processes of tumor cells by immunohistochemistry suggests an effective synthesis of opioids by these cells. Detection of preproenkephalin mRNA in the same tumor samples using polymerase chain reaction analysis reinforces this hypothesis. The fact that met-enkephalin immunostaining is also observed in the extracellular spaces is consistent with several studies indicating either the secretion of the peptide itself or an extracellular processing of the precursor following secretion (29, 30). Since opioid receptors and the putative \( \xi \) receptor, specific for met-enkephalin, have been discovered in brain tumors (15, 31, 32), one could speculate that met-enkephalin acts as an autocrine-regulating factor.

Moreover, the present study demonstrates for the first time an inverse relationship between the level of met-enkephalin expression and the degree of malignancy within a tumor type, i.e., neuroectodermal tumors. When compared with normal tissue, this differential expression mainly results from a decrease in met-enkephalin production in high-grade tumors. Furthermore, the peptide is probably overexpressed in some low-grade NETs that can contain up to 4 times more met-enkephalin than normal white matter. The large variations in opioid content between the tumor core and the peripheral paucicellular regions could be interpreted in the same way.

The mechanisms underlying the relationship between met-enkephalin production and malignancy progression remain to be elucidated. Several investigations have demonstrated that opioid peptides, and especially met-enkephalin, exert a growth inhibitory effect *in vivo* or *in vitro* on various tumor types, including brain tumors. On the other hand, we demonstrated for the first time a strong correlation between endogenous opioid peptides expression in neuroectodermal tumors and the degree of tumor malignancy. In this regard, met-enkephalin may be considered as a useful tumor marker; in particular, the lowering of its production in low-grade neuroectodermal tumors may indicate an evolution to anaplasia. Further studies are needed to precise this point. Since opioid peptides have antitumorigenic effects against several neoplasms, it will be of interest to investigate this action in brain tumors using an animal model of glioma.

**Cyst Fluids.** Since the pathogenesis of brain tumor cysts remains unclear, it is difficult to determine the origin of the met-enkephalin they contain. Recent investigations (42) have shown that more than 90% of cyst fluid proteins derive from blood plasma. It is therefore very likely that cyst fluid met-enkephalin has in large part the same origin. However, when related to protein content, the met-enkephalin levels are higher in cysts associated with NETs and metastases than in serum. This may suggest that the peptide is also secreted by these tumors into the cyst fluid.

**Conclusion.** As suspected by some authors (15), the endogenous opioid system seems to be an integral component of most tumor types, including brain tumors. On the other hand, we demonstrated for the first time a strong correlation between endogenous opioid peptides expression in neuroectodermal tumors and the degree of tumor malignancy. In this regard, met-enkephalin may be considered as a useful tumor marker; in particular, the lowering of its production in low-grade neuroectodermal tumors may indicate an evolution to anaplasia. Further studies are needed to precise this point. Since opioid peptides have antitumorigenic effects against several neoplasms, it will be of interest to investigate this action in brain tumors using an animal model of glioma.

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**REFERENCES**


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