Differential Expression of sst\textsubscript{1}, sst\textsubscript{2A}, and sst\textsubscript{3} Somatostatin Receptor Proteins in Low-Grade and High-Grade Astrocytomas

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Abstract. We have previously reported that sst\textsubscript{2A} somatostatin receptors are frequently overexpressed in human meningiomas. Initial clinical observations suggest that somatostatin analogues may also be of value for imaging and treatment of other human intracranial tumors, including astrocytomas. However, contradictory results have been reported regarding the expression of somatostatin receptors in low-grade and high-grade astrocytomas. Therefore, we determined the precise pattern of somatostatin receptor protein expression in 8 diffuse astrocytoma (DA), 10 anaplastic astrocytomas (AA), and 32 glioblastoma multiforme (GBM) using immunohistochemistry and Western blot analysis. sst\textsubscript{1} and sst\textsubscript{2A} somatostatin receptors were not present in DA and only occasionally detected in AA. In GBM, sst\textsubscript{1} was present in 66\%, and sst\textsubscript{2A} was found in 44\% of the tumors. sst\textsubscript{2} receptors were present in 38\% of DA, 40\% of AA, and 84\% of GBM. Thus, loss of differentiation was significantly associated with increased expression of sst\textsubscript{1}, sst\textsubscript{2A}, and sst, somatostatin receptors. In contrast, sst\textsubscript{1} and sst\textsubscript{2} receptors were found in 80\% and 25\% of all cases, respectively, in a manner independent of histological grade. No significant correlation was found between somatostatin receptor expression and the proliferation rate of the tumors as determined by MIB-1 immunostaining. Furthermore, the presence or absence of the 5 somatostatin receptor subtypes did not significantly influence survival time in 14 GBM patients.

Key Words: Astrocytoma; Immunohistochemistry; Somatostatin receptor.

INTRODUCTION

It is well known that somatostatin receptors can be found in many human tumors (1). This is the molecular basis for the clinical application of long-acting somatostatin analogues for tumor imaging and treatment. Five subtypes of somatostatin receptors, designated sst\textsubscript{1}–\textsubscript{5}, have been identified (2). Four (sst\textsubscript{1}, sst\textsubscript{3}–\textsubscript{5}) of the somatostatin receptor genes do not have introns, whereas sst\textsubscript{2} is alternatively spliced into sst\textsubscript{2A} and sst\textsubscript{2B}. Although the distribution of the sst\textsubscript{2B} receptor has been studied in detail in mouse and rat tissues, little is known about the expression and function of the human sst\textsubscript{2B} (3–5). All 5 receptors bind natural somatostatin with high affinity, but differ in their binding characteristics to various long-acting somatostatin analogues (6). While sst\textsubscript{2}, sst\textsubscript{3}, and sst\textsubscript{5} exhibit high affinity for the synthetic somatostatin analogues octreotide and lanreotide, sst\textsubscript{1} and sst\textsubscript{2A} do not bind these compounds.

It is now generally accepted that meningiomas frequently overexpress sst\textsubscript{2A} receptors (7–9). Based on these findings, [\textsuperscript{111}In-DTPA-D-Phe\textsuperscript{1}-Tyr\textsuperscript{3}]octreotide has been successfully applied for in vivo imaging of meningiomas.

Besides meningioma, glioblastoma multiforme (GBM) is among the most frequent brain tumors, accounting for approximately 12\% to 15\% of all intracranial neoplasms (10). GBM preferentially affects adults with a peak incidence between 45 and 70 years, and despite multimodal treatment strategies combining surgery, radiation therapy, and adjuvant chemotherapy, GBM still yields a poor prognosis with a median postoperative survival time not exceeding 12 months (11, 12). Recent clinical observations suggest that local injection of [\textsuperscript{90}Y-DOTA\textsuperscript{0}-D-Phe\textsuperscript{1}-Tyr\textsuperscript{3}]octreotide may be a promising modality for treatment of astrocytic tumors, including GBM (13). However, earlier studies reported contradictory findings with regard to the expression of somatostatin receptors in low-grade and high-grade astrocytomas. While several studies detected somatostatin receptor mRNA and binding sites (14, 15), a recent report failed to detect immunoreactive sst\textsubscript{2A} receptors in 1 GBM sample (16).

Because the distribution of individual somatostatin receptor proteins in astrocytomas is not well understood, we investigated the immunoexpression of all 5 somatostatin receptor subtypes in a large collective of low-grade and high-grade astrocytomas using immunohistochemistry and Western blot analysis. The results were correlated with the proliferation rate of the tumors and the survival time of 14 GBM patients.

MATERIALS AND METHODS

Paraffin-embedded biopsy samples from 8 patients (4 males, 4 females) with diffuse astrocytomas (DA), 10 patients (4 males, 6 females) with anaplastic astrocytoma (AA), and 32 patients (16 males, 16 females) with GBM were retrieved from the neuropathology files of the Otto-von-Guericke University,
Magdeburg, Germany. The mean age at diagnosis was 32.8 ± 5.1 years (DA), 65.3 ± 3.5 years (AA), and 60.7 ± 2.8 years (GBM), respectively.

All tumors were histologically assessed and graded using standard hematoxylin and eosin (H&E) sections by 2 neuropathologists (C.M. and K.D.) on the basis of the World Health Organization 4-tiered grading scale (17). The astrocytic origin of the tumors was confirmed by positive immunoreaction for the glial fibrillary acidic protein (GFAP; Sigma, Deisenhofen, Germany; dilution 1:1,500). Oligodendroglial features were not present in this series. In addition, to determine the proliferation activity of each tumor, a labeling index (LI [%]) was calculated after immunostaining for MIB-1 (monoclonal, clone Ki-S5; dilution 1:50, DAKO, Hamburg, Germany) by determining the number of immunopositive nuclei among 100 tumor cells per high power field (×400) in a total of 10 high power fields. For this purpose, tumor areas with the highest density of immunopositive nuclei were chosen. Only a distinct nuclear immunoreaction was judged as positive for MIB-1.

Survival data were available for 14 GBM patients. The clinical data of these patients are summarized in Table 2. All of these patients received standardized neuro-oncological treatment starting 2 to 6 weeks postoperatively. Radiotherapy was applied by a 6M-photon linear accelerator with focal doses of 55 to 60 Gy. Chemotherapy included 90 mg/m² 1-(4-amin-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) on day 1 and 60 mg/m² teniposide (VM26) on days 1 to 3 of each cycle. Patients received 4 to 10 cycles in 6-week intervals. The clinical progression or regression and side effects determined the number of treatment cycles each individual patient received.

**Immunohistochemistry**

Seven-μm sections were cut and floated onto positively charged slides for immunohistochemical staining. Sections were dewaxed 3 times in xylol and rehydrated in a graded series of ethanol. After rinsing in TPBS (10 mM Tris, 10 mM phosphate buffer, 137 mM NaCl, 0.05% thimerosal, pH 7.4), sections were incubated in methanol containing 0.3% H₂O₂ for 30 min at room temperature. Sections were transferred into TPBS and subsequently microwaved in 10 mM citric acid (pH 6.0) for 20 min at 600 W. Specimens were then allowed to cool to room temperature, washed in TPBS and preincubated in TPBS containing 3% normal goat serum for 1 h at room temperature. Sections were then incubated with affinity-purified anti-sst, (4819), anti-sst₁, (6291), anti-sst₂, (4823), anti-sst₃, (4802), and anti-sst₄, (6006) antibodies at a concentration of 1 μg/ml in TPBS containing 1% normal goat serum overnight at 4°C. These polyclonal rabbit antisera were generated against the carboxy-terminal tails of the human somatostatin receptors and have been characterized extensively (9, 18), and all slides were evaluated by the same investigator. Briefly, the presence or absence of staining and depth of color was noted, as well as the number of cells showing a positive reaction and whether the staining was localized to the plasma membrane.

**Assessment of Staining Patterns**

Immunohistochemical staining patterns were assessed as previously described (9, 18), and all slides were evaluated by the same investigator. Briefly, tissue was lysed in homogenization buffer (5 mM EDTA, 3 mM EGTA, 250 mM sucrose, and 10 mM Tris-HCl [pH 7.6] containing 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). The homogenate was spun at 500 × g for 5 min at 4°C to remove unbroken cells and nuclei. Membranes were then pelleted at 20,000 × g for 30 min at 4°C. Membranes were dissolved in lysis buffer (150 mM NaCl, 5 mM EDTA, 3 mM EGTA, and 20 mM HEPES [pH 7.4]) containing 4 mg/ml dodecyl-β-maltoside and proteinase inhibitors (as described above) and incubated with 150 μl of wheat germ lectin-agarose beads for 90 min at 4°C. Beads were washed 5 times in lysis buffer and adsorbed glycoproteins were eluted with SDS-sample buffer for 60 min at 37°C. The protein content was determined using the BCA method according to the manufacturer’s instructions (Pierce, Rockford, IL), and aliquots of each sample containing equal amounts of protein were subjected to 8% SDS-PAGE and immunoblotted onto nitrocellulose. Blots were incubated with affinity-purified anti-sst₁, (4819), anti-sst₂, (6291), anti-sst₃, (4823), anti-sst₄, (4802), and anti-sst₅, (6006) antibodies at a concentration of 1 μg/ml overnight at 4°C. Blots were developed using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. For absorption controls, antisera were preincubated with 10 μg/ml of their cognate peptide for 2 h at room temperature.

**Western Blot Analysis**

In a separate study, 16 freshly isolated GBM samples (average wet weight 300 mg), 3 AA samples (average wet weight 30 mg), and 3 DA samples (average wet weight 30 mg) were collected and stored at −70°C until analysis. Membranes were prepared, and glycoproteins were partially purified using wheat germ lectin-agarose (Pharmacia, Freiburg, Germany) essentially as described previously (9, 19). Briefly, tissue was lysed in homogenization buffer (5 mM EDTA, 3 mM EGTA, 250 mM sucrose, and 10 mM Tris-HCl [pH 7.6] containing 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). The homogenate was spun at 500 × g for 5 min at 4°C to remove unbroken cells and nuclei. Membranes were then pelleted at 20,000 × g for 30 min at 4°C. Membranes were dissolved in lysis buffer (150 mM NaCl, 5 mM EDTA, 3 mM EGTA, and 20 mM HEPES [pH 7.4]) containing 4 mg/ml dodecyl-β-maltoside and proteinase inhibitors (as described above) and incubated with 150 μl of wheat germ lectin-agarose beads for 90 min at 4°C. Beads were washed 5 times in lysis buffer and adsorbed glycoproteins were eluted with SDS-sample buffer for 60 min at 37°C. The protein content was determined using the BCA method according to the manufacturer’s instructions (Pierce, Rockford, IL), and aliquots of each sample containing equal amounts of protein were subjected to 8% SDS-PAGE and immunoblotted onto nitrocellulose. Blots were incubated with affinity-purified anti-sst₁, (4819), anti-sst₂, (6291), anti-sst₃, (4823), anti-sst₄, (4802), and anti-sst₅, (6006) antibodies at a concentration of 1 μg/ml overnight at 4°C. Blots were developed using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. For absorption controls, antisera were preincubated with 10 μg/ml of their cognate peptide for 2 h at room temperature.

**Statistical Evaluation**

Patient follow-up was defined as the interval from initial diagnosis of the GBM to the patient’s death or to the last official
contact. Time dependent survival probabilities were estimated by the Kaplan-Meier method. An association between sst staining (positive vs negative) and survival time was assessed by the log rank test. Differences between tumor grade (DA = WHO grade II; AA = WHO grade III; GBM = WHO grade IV [17]) and somatostatin receptor expression were assessed with the Chi-square test. An association between the presence of somatostatin receptor immunoexpression and proliferation of somatostatin receptor protein expression in a panel of immunoreactive somatostatin receptors.

**RESULTS**

The immunohistochemical distribution of the 5 somatostatin receptor subtypes in our series of 8 DA, 10 AA, and 32 GBM is summarized in Table 1. In most cases, immunoreactivity for somatostatin receptors was predominantly confined to the plasma membrane of the tumor cells (representative cases are shown in Fig. 1). In addition, immunostaining of the cytoplasm was also observed in a subset of cases. No specific staining of tumor cell nuclei was observed. Occasionally, tumor blood vessels in AA and GBM showed a moderate staining of endothelial cells for sst3a, sst4, and/or sst5, whereas sst was not observed in tumor blood vessels. Strong immunoreactivity was observed in tumor cells surrounding foci of necrosis in the GBM samples. In cases containing areas of non-tumoral cerebral cortex, plasma membrane and cytoplasmic immunoexpression of all somatostatin receptor subtypes except for sst4 were seen in cortical neurons, but were nearly absent in astrocytes and oligodendrocytes, as well as in blood vessel endothelial cells. In Western blots, immunoreactive bands migrating at the appropriate molecular weight of each somatostatin receptor were detected in membrane extracts prepared from freshly isolated GBM tumor samples (Fig. 2). However, presumably due to very small sample size no immunoreactive bands were detectable in AA and DA samples (data not shown). Immunostaining for each antibody was completely abolished by preabsorption with 10 μg/ml immunizing peptides (Fig. 1A–E, insets; Fig. 2).

**DISCUSSION**

In the present study, we determined the precise pattern of somatostatin receptor protein expression in a panel of 50 randomly selected low-grade and high-grade astrocytomas. We provide unequivocal evidence for the presence of immunoreactive somatostatin receptors on the tumor cells using immunohistochemistry and Western blot analysis. We also showed that the immunoreexpression of somatostatin receptor subtypes significantly differed between low-grade and high-grade astrocytomas, indicating that loss of differentiation is associated with increased expression of sst1, sst3a, and sst somatostatin receptors. These findings are of particular relevance given the recent clinical finding that local injection of [15SOMATOSTATIN RECEPTORS IN ASTROCYTOMAS

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Distribution of Immunoexpression in the 5 Somatostatin Receptor Subtypes in Low-Grade and High-Grade Astrocytomas</th>
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<tbody>
<tr>
<td></td>
<td>DA (n = 8)</td>
</tr>
<tr>
<td>sst1</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>sst2a</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>sst3</td>
<td>3 (38%)</td>
</tr>
<tr>
<td>sst4</td>
<td>7 (88%)</td>
</tr>
<tr>
<td>sst5</td>
<td>2 (25%)</td>
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Percentage of positive cases is indicated in parentheses. Abbreviations: sst, somatostatin receptor; DA, diffuse astrocytoma WHO grade II; AA, anaplastic astrocytoma WHO grade III; GBM, glioblastoma multiforme WHO grade IV.
Fig. 2. Western blot analysis of somatostatin receptor immunoreactivity in GBM. Membrane preparations from 5 surgically removed individual glioblastoma multiforme samples were separated on 8% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were then incubated with either anti-sst1 (4819), anti-sst2A (6291), anti-sst3 (4823), anti-sst4 (4802), or anti-sst5 (6006) antibodies in the absence (−) or presence (+) of peptide antigen (10 μg/ml). Blots were developed using enhanced chemiluminescence. Ordinate, migration of protein molecular weight markers (M, × 10^2).
somatostatin receptor mRNA was successfully detected in later studies by RT-PCR and in situ hybridization in both GBM and malignant glioma cell lines (14, 15, 21, 22). Until now, no carefully controlled immunohistochemical studies examining the somatostatin receptor status in astrocytic tumors has been performed. In a previous investigation, sst$_{2A}$ receptors were detected in a single GBM sample examined, but the presence of other receptor subtypes was not determined (14). Another study detected somatostatin-binding sites but failed to detect sst$_{2A}$ or sst$_{3}$ somatostatin receptors in a small number of GBM samples (16). Based on the simultaneous demonstration of synaptophysin immunoreactivity in these GBM samples, the authors from the latter study supposed that somatostatin binding sites in these tumor samples may arise solely from “contaminating” residual brain tissue. In contrast, the present study revealed in a large collective of GBM specimens that somatostatin receptors were expressed in a significant fraction. Furthermore, the immunoreexpression was clearly localized on the tumor cells. While we also detected expression of sst$_{3}$ in non-tumoral cortical tissue, it was significantly less intense than in tumor areas. Northern blot analysis has recently demonstrated that mRNA transcripts of sst$_{3}$ are detectable in both normal brain tissue and gliomas, but with 7- to 14-fold higher levels of sst$_{3}$ within the tumor samples (14). Although the presence of preexisting non-tumoral tissue in our samples cannot be excluded, it seems rather unlikely that it represents the sole source of somatostatin receptor immunoreactivity in our study. Finally, in line with previous immunohistochemical studies (14, 16), expression of somatostatin receptors was also found within glomeroid vascular proliferations, which is regarded as a histopathological hallmark of GBM (17). Our study revealed a particular frequent expression of sst$_{3}$ (66%), sst$_{3}$ (84%), and sst$_{4}$ (72%) receptor subtypes in GBM. Together with the detection of sst$_{3}$ in approximately half of the samples in our series, the presence of sst$_{2A}$ and/or sst$_{3}$ could provide a plausible explanation for the clinical utility of $[^{90}$Y-DOTA$^6$-D-Phe$^1$-Tyr$] $octreotide in GBM (13). In this first clinical trial including 3 GBM patients, 1 patient without autoradiographically detectable sst$_{2A}$ receptor showed a stabilization of the disease with an overall survival of 15 months, whereas another patient with strong expression of sst$_{2A}$ demonstrated a progressive disease with only a 6-month survival time. This raises the question about the prognostic significance of somatostatin receptor expression in GBM. In the present study, we were unable to identify any specific expression pattern of somatostatin receptor subtypes to be associated with a favorable prognosis. The sst$_{2A}$ subtype, which has been focused as a prognostic marker in the clinical trial mentioned above (13), was present in only a third of the patients in our series. Therefore, although according to the present data GBM seems to contain a sufficient number of somatostatin receptors, they are supposed to be less important for the prognosis of highly dedifferentiated gliomas such as GBM. This is further supported by our observation that the proliferation rate of the tumors as determined by MIB-1 immunostaining, which has been shown to be a valuable prognostic marker in GBM (23), seems not to be related to the expression of specific somatostatin receptors.

The observation of a frequent expression of somatostatin receptors in low-grade but not in high-grade astrocytomas in previous reports has been interpreted to reflect the loss of differentiation seen in high-grade gliomas (7, 15, 20). In the present study we show that a change in the receptor distribution, rather than a complete loss of receptors, seems to be characteristic for the process of dedifferentiation that occurs from low-grade to high-grade astrocytomas. This view is supported by a report demonstrating all 5 somatostatin receptor subtypes by RT-PCR in low-grade as well as high-grade astrocytomas (14). However, to further elucidate this hypothesis, the investigation of tumor samples that show malignant progression from astrocytoma WHO grade II to AA and GBM could be of important value.

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REFERENCES


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