Estramustine-Binding Protein and Specific Binding of the Anti-Mitotic Compound Estramustine in Astrocytoma

A. Tommy Bergenheim, Per Björk, Jonas Bergh, Eva von Schoultz, Helena Svedberg, and Roger Henriksson

Departments of Oncology (A. T. B., E. v. S., R. H.) and Neurosurgery (A. T. B.), Umeå University Hospital, S-901 85, Umeå; Department of Immunology, Pharmacia Oncology Immunology, S-223 63 Lund (P. B., H. S.); and Department of Oncology, Akademiska Sjukhuset, S-751 85 Uppsala (J. B.), Sweden

ABSTRACT

Estramustine-binding protein (EMBP) is a Mr 46,000 heterodimeric protein originally isolated from prostate tissue. It has a demonstrated high affinity for, and selective binding of, estramustine, which is a derivative of 17β-estradiol and nonnitrogen mustard with antimitotic activity. In this study, we have analysed the expression of an EMBP-like protein in astrocytoma specimens. Immunohistochemistry revealed a pronounced reactivity for EMBP in astrocytoma grades III-IV as well as in metastatic prostatic adenocarcinoma used as positive control. In astrocytoma grades I-II, the expression was weak. The EMBP-like protein was quantified by radioimmunooassay in astrocytoma tumor tissue with higher concentrations in malignant astrocytoma, grades III-IV, compared to grades I-II tumors. Western immunoblotting of immunopurified EMBP-like protein under nonreducing conditions revealed an immunoreactivity corresponding to Mr, 138,000 and 200,000, indicating a different structure of EMBP in astrocytoma compared to prostate tissue. Specific binding and the presence of saturable binding sites for 3H-labeled estramustine were demonstrated in astrocytoma tissues expressing EMBP-like protein. Scatchard plot analysis showed a Kd at ~30 nM, which suggests a binding affinity for estramustine in the same range as previously reported for EMBP in the prostate. Moreover, the number of estramustine binding sites/g tumor as calculated from the Scatchard plots was well correlated with the EMBP levels determined in the radioimmunoassay. In conclusion, an EMBP-like protein is expressed in astrocytoma. This protein may be responsible for the specific binding of estramustine in the tumor tissue. Whether this specific binding of estramustine is of importance for the cytotoxic effect in glioma cells remains to be evaluated.

INTRODUCTION

The treatment of primary malignant supratentorial brain tumors is disappointing. In malignant astrocytoma, the extent of surgery and postoperative irradiation have been shown to increase survival (1, 2), and adjuvant chemotherapy may improve survival in selected patients, but only marginally (3, 4).

EMB (Estracyt; Emcyt), a combination of 17β-estradiol phosphate and nonnitrogen mustard used in the treatment of prostatic carcinoma, has recently gained interest in the management of malignant glioma. The metabolites of EMB have been shown to exert specific antiproliferative effects in glioma cells in vitro (5–7). The main mechanism of action seems to involve the microtubule system (8–12) with arrest of glioma cells in G2/M of the cell cycle (5, 6). Other targets have also been suggested to be of importance, such as membrane-coupled cytotoxic effect in glioma cells remains to be evaluated.

The treatment of primary malignant supratentorial brain tumors is disappointing. In malignant astrocytoma, the extent of surgery and postoperative irradiation have been shown to increase survival (1, 2), and adjuvant chemotherapy may improve survival in selected patients, but only marginally (3, 4).

Received 4/18/94; accepted 7/20/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This investigation was supported by grants from The Swedish Cancer Society and The Lion Cancer Foundation, Umeå; Swedish Society for Medical Research, Karolinska Institutet, Stockholm; Umeå University; and Lundbergs Foundation for Medical Research, Gothenburg, Sweden.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: EMB, estramustine phosphate; EMBP, estramustine-binding protein; Eam, estramustine; Eom, estromustine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; mAb, monoclonal antibody; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate.

4974

4974

4974
sodium phosphate, 10 mM EDTA, and 10 mM NaCl containing 0.2% w/v BSA (pH 7.4) using an Ultra-Turrax homogenizer (4 × 5 s with 20-s cooling intervals). The homogenate was centrifuged for 20 min at 2000 × g (4°C), and the supernatant was collected for determination of EMBP. TheRIA was carried out as described previously (29) using a polyclonal rabbit antiserum raised against purified rat EMBP at a dilution of 1:5000 and with purified rat EMBP as tracer and standard. The chloramine-T technique (30) was used for radioiodination with specific activity ranging from 50 to 100 μCi/μg protein. A standard curve ranging from 0.1 to 20 pmol/ml of purified rat EMBP was established. One hundred μl of the sample, the tracer, and the antiserum were incubated at room temperature for 2 h. For separation of free and bound antigen, sheep anti-rabbit-IgG-coated particles were added, and the test tubes were slowly rotated overnight. Thereafter, the samples were centrifuged at 3000 × g for 3 min, washed three times in saline, and counted in a gamma counter. The concentration of EMBP-like protein in astrocytoma was obtained from the rat-EMBP standard curve and was expressed as pmol/g tumor tissue or pmol/mg protein, assuming equal amounts of bound antibody per antigen molecule in rat EMBP and in astrocytoma EMBP-like protein. Protein was determined by the method of Lowry et al. (31).

Partial Purification of EMBP. An immunoabsorbent was prepared to enrich cross-reactive components in a sample of astrocytoma grade III (no. 1) for analysis in a Western immunoblot. Briefly, 2.5 mg of the anti-EMBP mAb was coupled to an N-hydroxysuccinimide-activated HITrap resin (1 ml; Pharmacia Biotech, Sollentuna, Sweden) in 0.2 M sodium bicarbonate-0.5 M sodium chloride at pH 8.3, according to the instructions given by the manufacturer. Blocking of unreacted sites was performed by sequential washings in 0.5 M ethanolamine (pH 8.3) and 0.1 M sodium acetate (pH 4.0), where both buffers contained 0.5 M sodium chloride. The coupling yield was about 97% and with minimal antibody leakage from the column. The antigen-binding capacity was estimated to about 0.1 mg, as determined by loading of rat EMBP to the column.

The astrocytoma sample was poured through PD-10 columns (Pharmacia Biotech) and equilibrated in PBS; the excluded volume was filtered on 0.2 μm filters. The sample was loaded onto the immunoabsorbent by recirculation overnight and washed until baseline absorbance at 280 nm was reached; finally, retained protein was eluted with 0.1 M glycine-HCl buffer (pH 2.5) and with 0.1 M sodium carbonate buffer (pH 11.5), interrupted by washing in PBS in between. Eluted fractions were immediately neutralized to near physiological pH, the buffer was changed to PBS, and finally the samples were concentrated and frozen until further use.

Western Blot Analysis. Gel electrophoresis in the presence of SDS was run with denatured samples on 8–25% gradient gels (PhastGel Gradient 8–25) using the PhastSystem (Pharmacia Biotech) according to the instructions given by the manufacturer. Transfer to 0.45 μm nitrocellulose was carried out using the PhastTransfer kit (Pharmacia Biotech). The membranes were blocked for 30 min in 3% w/v BSA in Tris-buffered saline (TBS; pH 7.5), washed three times for 5 min in TBS containing 0.05% v/v Tween-20 (TTBS) and incubated overnight with rabbit polyclonal anti-EMBP serum, diluted 1:2000 in TTBS containing 1% v/v nonimmune goat serum. After washing three times for 5 min with TTBS, incubation for 30 min was performed with goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Bio-Rad, Richmond, CA) diluted 1:4000 in TTBS. Finally, immunodetection was made using the enhanced chemiluminescence system from Amersham. In the negative, as well as the negative serum controls, excluding nonspecific immunoreactivity (data not shown). In metastatic prostatic carcinoma, a variation in the proportion of cells positively stained was also observed, as well as a variation in the staining intensity. As in astrocytoma, the staining was located in the cytoplasm of the tumor cells, leaving the stroma unstained (Fig. 1D).

RIA. A comparison between RIA analysis and immunohistochemical detection of the EMBP-like protein in the same astrocytoma tumors is presented in Table 2. The RIA values suggested an increase in EMBP levels with higher malignancy grade of the tumor. The

**RESULTS**

Immunohistochemistry. The results from immunohistochemical staining for EMBP-like protein is presented in Table 1. In all positive astrocytoma cells, the immunostaining was encompassed within the cytoplasm. Occasionally, the cells stained were randomly scattered in the tumor tissue, and the staining intensity varied considerably between different cells in the same tumor. In low grade astrocytomas (grades I-II), there was generally a weak staining of the tumor cells (Fig. 1A). High grade astrocytomas (grades III-IV) showed a higher staining intensity also within the cytoplasm of the cells and usually with a higher proportion of cells stained compared to low grade astrocytomas (grades I-II; one-way analysis of variance; P < 0.001 and P < 0.05, respectively; Table 1; Fig. 1, A and B). In many tumors, there was a moderate positive reaction in the majority of cells with a strongly stained subpopulation scattered in the tumor (Fig. 1C). In astrocytomas with areas of necrosis, there was usually a high staining intensity in the necrotic area.

All positive controls were positively stained and served as reference for evaluation of staining intensity. The negative controls were negative, as well as the negative serum controls, excluding nonspecific immunoreactivity (data not shown). In metastatic prostatic carcinoma, a variation in the proportion of cells positively stained was also observed, as well as a variation in the staining intensity. As in astrocytoma, the staining was located in the cytoplasm of the tumor cells, leaving the stroma unstained (Fig. 1D).

**Determination of EaM Binding Characteristics.** Three astrocytoma grade III specimens (nos. 1, 2, and 3) previously investigated for the expression of EMBP-like protein using RIA were selected for binding analysis.

Extracts from the astrocytoma specimens were prepared from minced frozen tissue by homogenization for five times 10 s in 5–7 volumes of 0.25 M sucrose, 50 mM Tris, 10 mM KCl, and 1.5 mM MgCl₂ (pH 7.4) containing 8 mM 3-[(3-chomaidopropyl)dimethylamino]-1-propanesulfonate and protease inhibitors (EDTA, phenylmethylsulfonyl fluoride, pepstatin, and leupeptin). The extracts were obtained by collecting the supernatant after high-speed centrifugation of the homogenates for 1 h at 105,000 × g.

In order to avoid underestimation of the EaM binding sites, endogenous ligand(s) that might occupy the binding sites were removed by acetone precipitation, mainly according to the method described by Ichii (32). Briefly, the tumor extracts (1 ml) were added drop-wise to 9 volumes of ice-cold acetone in the "cold room." The precipitated protein was recovered by centrifugation for 30 min at 10,000 × g. The supernatant was discarded, and the precipitate was washed with 10 volumes of ice-cold diethyl ether and centrifuged as above. The supernatant was discarded, and the remaining ether was evaporated under a stream of nitrogen gas. Finally, the precipitate was dissolved in 2 ml TEN buffer (50 mM Tris, 1 mM EDTA, and 10 mM NaCl, pH 7.5) and clarified by centrifugation under a stream of nitrogen gas. Finally, the precipitate was dissolved in 2 ml TEN buffer (50 mM Tris, 1 mM EDTA, and 10 mM NaCl, pH 7.5) and clarified by centrifugation as above. The delipidated samples were immediately frozen at −30°C pending further use.

Aliquots of the delipidated extracts were incubated in duplicates with serially diluted [³H]EaM at a final concentration ranging from 1 to 544 nm. Briefly, 10 μl of the radioligand dissolved in ethanol were added to 150-μl sample and incubated for 30 min at 30°C. The incubation was stopped by placing the samples in an ice-bath for 5 min. The protein-bound radioactivity was assessed by filtration of the incubate (100 μl) through Sephadex G-25 medium (Pharmacia Biotech) packed in 2-ml plastic syringes. Twenty-five μl were transferred directly into counting vials for determination of total radioactivity. The apparent dissociation constant, Kᵢ, and the number of available binding sites, Bₘᵦ, were calculated according to Scatchard (33).

**Table 1 Immunohistochemical staining for estramustine binding protein in astrocytoma and metastatic prostatic carcinoma**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No.</th>
<th>Cells in %</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytoma I</td>
<td>2</td>
<td>75 (75–75)</td>
<td>1.0 (1–1)</td>
</tr>
<tr>
<td>Astrocytoma II</td>
<td>16</td>
<td>52 (10–100)</td>
<td>1.3 (1–2)</td>
</tr>
<tr>
<td>Astrocytoma I + II</td>
<td>18</td>
<td>54 (10–100)</td>
<td>1.3 (1–2)</td>
</tr>
<tr>
<td>Astrocytoma III</td>
<td>27</td>
<td>67 (25–90)</td>
<td>2.0 (1–3)</td>
</tr>
<tr>
<td>Astrocytoma IV</td>
<td>6</td>
<td>76 (65–95)</td>
<td>2.2 (2–3)</td>
</tr>
<tr>
<td>Astrocytoma III + IV</td>
<td>33</td>
<td>68 (25–95)</td>
<td>2.0 (1–3)</td>
</tr>
<tr>
<td>Prostatic carcinoma</td>
<td>6</td>
<td>64 (25–80)</td>
<td>1.7 (1–3)</td>
</tr>
</tbody>
</table>
concentration of EMBP-like protein was, as an average, 2.3 (0.4–7.0) pmol/g tumor tissue or 0.06 (0.01–0.12) pmol/mg protein.

Western Blot Analysis. The EMBP-like protein in astrocytoma no. 1 was enriched by immunoaffinity chromatography on an anti-EMBP immunoabsorbent. The adsorbed protein was eluted both at acidic and alkaline pH (Fig. 2). The crude astrocytoma extract as well as the fractions eluted at pH 2.5 and 11.5, respectively, were analysed by immunoblotting after SDS gel electrophoreses under denaturing conditions (Fig. 3). Purified rat EMBP showed the typical two bands corresponding to subunits S1 (Mr 19,300) and S2 (Mr 22,100). In addition, there was a weak specific band at Mr 35,700, which is often

Table 2  Immunohistochemical staining for EMBP and concentrations of EMBP in astrocytoma tumors expressed in binding sites/g tumor tissue and binding sites/mg protein as detected by radioimmunoassay

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cells (%)</th>
<th>Intensity</th>
<th>pmol/g tumor tissue</th>
<th>pmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytoma II</td>
<td>2 55 (50-60)</td>
<td>2.0 (2–2)</td>
<td>0.9 (0.8–1.1)</td>
<td>0.028 (0.026–0.030)</td>
</tr>
<tr>
<td>Astrocytoma III</td>
<td>8 68 (50-85)</td>
<td>1.8 (1–3)</td>
<td>2.4 (0.4–7.0)</td>
<td>0.047 (0.010–0.116)</td>
</tr>
<tr>
<td>Astrocytoma IV</td>
<td>2 65 (65-85)</td>
<td>2.0 (2–3)</td>
<td>2.9 (2.5–3.1)</td>
<td>0.117 (0.110–0.124)</td>
</tr>
<tr>
<td>All astrocytoma</td>
<td>12 64 (50-85)</td>
<td>2.1 (2–3)</td>
<td>2.3 (0.4–7.0)</td>
<td>0.056 (0.010–0.124)</td>
</tr>
</tbody>
</table>

a Means and ranges are given.

Fig. 1. Microphotographs demonstrating immunoreactivity for the EMBP-like protein in homogeneous staining of an astrocytoma grade III specimen from patient no. 1 (B); heterogeneous staining in a specimen of astrocytoma grade III (C); and staining of metastatic prostatic carcinoma (positive control) with invasive growth adjacent to the vertebral bone (D); bar, 50 μm.

EMBP/ESTRAMUSTINE IN ASTROCYTOMA
tigated in order to assess the binding affinity and the concentration of saturable EaM sites. The Scatchard plot analysis for binding of EaM to the astrocytoma specimen no. 1 is shown in Fig. 4. In all three samples, a high degree of nonspecific or low-affinity binding was obtained, which had to be subtracted in order to calculate the binding characteristics for the high-affinity sites. The results from the in vitro binding experiments are summarized in Table 3. An apparent dissociation constant, $K_d$, of approximately 30 nM was obtained for the high-affinity sites. The number of EaM binding sites were also calculated and expressed as pmol bound EaM per g tumor tissue. These values were comparable to the RIA levels of the EMBP-like protein, which suggest that EaM is bound to this protein with a stoichiometric relation of 1:1. This finding demonstrates that the two methods used for determination of EMBP-like protein (RIA and in vitro binding assay) are well correlated.

The astrocytoma specimen no. 1 was also subject to immunohistochemical analysis (Fig. 1B), demonstrating a staining intensity for EMBP of 2+ and with 80% of the cells displaying immunoreactivity.

**DISCUSSION**

Using immunohistochemistry and different immunochemical and ligand-based techniques, the present study demonstrates the existence of an EMBP-like protein in astrocytoma tumors with similar epitopic

---

**Fig. 2.** Enrichment of EMBP-like protein from a human astrocytoma extract (patient no. 1) by chromatography on immobilized mouse anti-rat EMBP mAb. Arrows, the start of elution at low pH (pH 2.5) and high pH (pH 11.5), respectively.

**Fig. 3.** Partial characterization of the immunoabsorbent fractions (Fig. 2, patient no. 1) by Western blot analysis after SDS electrophoresis in 8–25% gradient gels under nonreducing conditions. Samples were purified rat prostate EMBP (rat-EMBP), crude astrocytoma extract (starting material), and immunoabsorbent fractions after elution at pH 2.5 and pH 11.5. Negative controls (neg.) were incubated in the absence of rabbit anti-rat EMBP. Dashes, the positions of subunits S1 and S2 of the rat prostate EMBP as well as the two main specific bands visualized in the astrocytoma samples. The molecular masses were calculated from Rainbow markers 12,300–200,000 (Amersham, Solna, Sweden) run in the same gels (data not shown).

seen when rat EMBP is overloaded in gels and when the more sensitive enhanced chemiluminescence technique is used for development. In the astrocytoma extract before (starting material) and after immunoaffinity chromatography (pH 2.5 and 11.5, respectively), specific staining was observed as two bands with a molecular mass of 138 and 200 kilodaltons, respectively. Other bands most probably represent nonspecific staining or cross-reactivity with preimmune serum since they were also visualized in the negative controls.

**In Vitro Binding of EaM.** The binding of EaM to extracts from three astrocytoma specimens with high EMBP expression was inves-
EMBP-like protein in human astrocytoma tissue compared to what concentrations in serum and tumor tissue (23). This is in accordance with the data of Heyns and Bossyns (48) that prostate EMBP carries only one binding site for EaM. It is of great interest that an uptake of EaM has been demonstrated in the clinical situation (23). In one of the patients (no. 1 in this paper), the uptake and accumulation of EaM after a single oral dose of 280 mg EMP was 13 ng/g tissue 12 h after drug administration, while at the same time no EaM was detectable in serum. Thus, a very high concentration gradient in the tumor versus serum was obtained in this patient (23). It is also of interest to recall the observation that the concentrations of EaM and EoM in glioma (49).

Comparison of binding site levels determined in the ligand binding assay and in the RIA.

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>$K_d$ (nM)</th>
<th>Regression coefficient</th>
<th>$B_{max}$ (pmol/g tumor)</th>
<th>RIA levels (pmol/g tumor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>-0.90</td>
<td>6.7</td>
<td>7.0</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>-0.65</td>
<td>4.6</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>-0.83</td>
<td>3.8</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*EMBP levels estimated from the number of available binding sites at saturation, $B_{max}$, obtained in the Scatchard plot.

The degree of specific binding was low due to analysis of a diluted tumor extract, resulting in a low regression coefficient.

EMBP-like protein may play an important role for the cytotoxicity of EMP treatments in astrocytoma. The skillful technical assistance of Annika Holmberg, Emma Lundkvist, Kristin Persson, and Ulrika Andersson is acknowledged.

Acknowledgments

The skillful technical assistance of Annika Holmberg, Emma Lundkvist, Kristin Persson, and Ulrika Andersson is acknowledged.

References


Estramustine-Binding Protein and Specific Binding of the Anti-Mitotic Compound Estramustine in Astrocytoma

A. Tommy Bergenheim, Per Björk, Jonas Bergh, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/18/4974

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.