Increased Expression of Diazepam Binding Inhibitor in Human Brain Tumors

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Abstract

Benzodiazepines, which are in extensive clinical use, can regulate neoplastic growth via benzodiazepine receptors. We have studied the expression of the diazepam binding inhibitor (DBI) polypeptide, a putative endogenous ligand for benzodiazepine receptors, in normal and pathological human brain. In normal brain, DBI immunoreactivity (IR) and mRNA were detected in all brain areas, with the highest levels in the cerebellum, amygdala, and hippocampus. In light and electron microscope immunohistochemistry, DBI-IR was only detected in glial and ependymal cells. In brain tumors, such as astrocytomas, glioblastomas, and medulloblastomas, a much higher content of DBI-IR and -mRNA was found than in normal tissues. The highest level of DBI expression was found in the most anaplastic tumors. DBI-IR was virtually undetectable in meningiomas and pituitary adenomas. The high expression of DBI in brain tumors might play a role in the neoplastic growth of glial cells via the mitochondrial benzodiazepine receptor, or it may be involved in the regulation of the high energy consumption of these tumors via acyl-CoA metabolism.

Introduction

DBI is a M, 10,000 polypeptide that was originally isolated from rat brain by its ability to displace [3H]diazepam from GABA_A receptors on neuronal membranes (1). Since then, it has also been isolated from bovine (2), frog (3) and human brains (4) and from many peripheral organs (5–8). Furthermore, an identical M, 10,000 polypeptide that is capable of binding and inducing synthesis of fatty acid esters of the acetyl-CoA has been isolated from bovine liver (9) and named acetyl-CoA-binding protein.

In humans, increased DBI concentration has been observed in the cerebrospinal fluid in chronic hepatic encephalopathy and in depressed and paranoid schizophrenic patients (10–12). The presence of DBI-like immunoreactivity has been shown both in normal human brain (13, 14) and in some cerebral tumors (15). In the rat and monkey central nervous system, DBI-IR has been localized in many glial, ependymal (16–18), and some neuronal cells (19, 20).

However, the exact localization of DBI-IR in normal and neoplastic human brain is not known.

DBI is involved in the regulation of multiple biological processes such as neuronal Cl− influx (21), steroidogenesis (7, 22), acyl-CoA metabolism (6, 23), and glucose-mediated insulin secretion (5). It has been suggested that some of these biological events are mediated either via the central GABA_A or peripheral benzodiazepine receptors (reviewed in Refs. 24 and 25). In the brain, there are two pharmacologically distinct types of high affinity benzodiazepine receptors. The central benzodiazepine receptor is prevalently present in neurons and is associated with GABA_A receptors, which are ligand-gated Cl− channels (26). GABA_A receptors have also been found in some glial cell lines and gliomas (27), but the physiological role of these receptors in neoplastic tissues remains unclear. The second type of benzodiazepine binding site, the PBR, is widely distributed in peripheral organs (28). It is also found in normal brain glial cells, in human glia cell lines, and in neoplastic brain tumors (15, 29, 30). PBR is associated with a protein complex preferentially found in the outer membranes of mitochondria, and on this basis, it is also called mitochondrial benzodiazepine receptor (31). Recent studies indicate that PBRs are involved in a number of intracellular functions such as cell proliferation, steroidogenesis, immune response, and mitochondrial respiration (reviewed in Refs. 25, 32, and 33). The stimulation of these receptors by PBR ligands exert either proliferative or antiproliferative effect, depending upon the ligand concentration (reviewed in Ref. 33). However, no universal mechanism of action has been found for these receptors.

Although PBR and its putative endogenous ligand, DBI, are present in pathological human brain, and a significant correlation between high PBR binding and the malignancy of gliomas in humans has been found (29, 34), it is not known if DBI expression is related to the malignancy of brain tumors. Another observation that encouraged us to investigate the localization and expression of DBI in different human brain tumors was the recent finding that in Drosophila, DBI is highly expressed in cell types using fatty acids as a primary energy source (35). The fact that brain tumors have a high level of fatty acid and energy consumption (reviewed in Ref. 36) is also relevant in this regard.

Results

In normal brain, DBI-IR was only observed in glial and ependymal cells. No staining was seen in neurons (Fig. 1). The highest DBI-IR cell density was found in cerebellum and amygdala (Table 1). In cerebellum, the majority of the cells were Bergmann glial cells around the Purkinje neurons (Fig. 1). In other brain areas, the immunopositive cells were identified by morphological and electron microscopic criteria as podoplasmic astrocytes (Fig. 1).

In low grade (grade I-II) astrocytomas, DBI-IR was identified in some of the astrocyte-like cells in the tumors. In glioblastomas, medulloblastomas, and high-grade astro-
cytomas (grade III), both the number and the percentage of DBI-immunoreactive cells was significantly higher (Fig. 2; Table 2). In particular, large multinuclear anaplastic cells showed intense immunoreactivity. RNAse protection assays of RNA from normal brain tissues and different brain tumors also demonstrated that DBI mRNA is found both in normal and pathological brain. In normal brain, the highest DBI mRNA content was detected in cerebellum. In brain tumors, a significantly higher level of mRNA expression was observed in high-grade astrocytomas compared to normal cortex (Fig. 3). A small number of tumors were also analyzed for progesterone content. This analysis indicated a tendency for malignant tumors to have a higher progesterone content (Table 3).

**Table 1**  Density of DBI-immunoreactive cells in different areas of the normal human brain

<table>
<thead>
<tr>
<th>Brain region</th>
<th>DBI-IR* cell density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum, cortex</td>
<td>112 ± 13</td>
</tr>
<tr>
<td>Amygdala</td>
<td>97 ± 12</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>Thalamus (dorsomed nuc)</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>Striatum</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Neurohypophysis</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Adenohypophysis</td>
<td>1 ± 0.1</td>
</tr>
</tbody>
</table>

* IR, immunoreactive.

**Discussion**

This study is the first immunohistochemical demonstration of DBI-IR in human brain. Our observations of the regional distribution of DBI-IR and mRNA within the brain are fully consistent with the previously reported distribution of DBI-like immunoreactivity in human brain, with the highest concentration in cerebellum (13). Earlier reports on the immunohistochemical localization of DBI in neurons and/or glial cells give different results from species to species, and in some cases, even within same species. In rat brain, some studies show DBI both in glial and neuronal cells (19, 20), while others have found it only in glial cells (16, 36). In the frog, DBI has been reported only in glial cells (3), and in fish brain, only in neurons (37). In the human brain, we showed with light and electron microscopy that DBI was only localized in glial and ependymal cells. This is in agreement with a previous study by Slobodiansky et al. (18), who found that, in the monkey, DBI-IR was only localized in glial cells.

Our study also demonstrates the presence of DBI in human gliomas, thus confirming the finding of Ferrarese et al. (15). They reported a lower DBI content in gliomas compared to normal brain tissue. Our study, on the contrary, demonstrates clearly a higher number and higher percentage of DBI
immunoreactive cells and mRNA in malignant cerebral gliomas compared to normal brain. This discrepancy may be due to two main reasons: (a) we used antiserum raised against human-DBI antigen, whereas Ferrarese et al. (15) had antiserum raised against rat-DBI antigen. The specificity of our antiserum has been characterized previously (14), and it was confirmed here by preabsorption controls; and (b) the determinations by Ferrarese et al. (15) were performed by RIA measurements from biopsied brain tissue homogenates, which may also contain nonmalignant brain areas. This is indicated by the wide variation in DBI levels. Our material was obtained from open operations, and only clearly defined malignant tissue was accepted for analysis.

The role of DBI in glial cells and in gliomas is unknown. Originally, it was suggested that DBI modulates the function of GABA<sub>δ</sub>-benzodiazepine receptors (1). Although some gliomas contain these receptor complexes (15, 27), it is possible that DBI is not an endogenous ligand for the central GABA/benzodiazepine receptors, as originally suggested. This is indicated by the presence of DBI in peripheral tissues (38, 39), yeast (40), and plants (41).

Several authors have suggested that DBI may act as an endogenous ligand for the peripheral benzodiazepine receptors (7, 16, 17, 24, 42). In normal brain, PBRs have a low expression, but in brain tumors, the expression is rather high (15, 29, 30). Our observation of high DBI expression in anaplastic astrocytomas indicate a possible connection between DBI and PBRs in glial tumors. While PBRs regulate cell proliferation and/or steroid synthesis (reviewed in Refs. 25 and 33), it is possible that, in brain tumors, DBI modulates cellular growth either by acting directly on these receptors or indirectly via neurosteroids. Neurosteroids, which are pregnenolone derivatives (43), can be produced by glial cells, and their synthesis can be regulated with PBR

Fig. 2. DBI-like immunoreactivity in different brain tumors. A, normal cortex; B, astrocytoma grade II; C, astrocytoma grade III; D, glioblastoma; E, a higher magnification of glioblastoma; F, meningioma. Bar, 100 µm, except in (E) and (F), 50 µm.
The cell density is calculated as absolute number of immunoreactive cells/1.6 mm² and the percentage from all cells (hematoxylin counterstained) in the same area. Values are expressed as mean ± SD. **, P < 0.001; *, P < 0.05, when tumors were compared to normal cortex. Numbers of samples are in parentheses.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell density</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Normal cortex (4)</td>
<td>45 ± 6</td>
<td>22.0 ± 2.9</td>
</tr>
<tr>
<td>Astrocytoma, grades 1 and 2 (4)</td>
<td>57 ± 6</td>
<td>44.0 ± 12.2</td>
</tr>
<tr>
<td>Glioblastoma, grade 3 (5)</td>
<td>124 ± 11**</td>
<td>93.3 ± 14.1**</td>
</tr>
<tr>
<td>Glioblastoma multiforme (5)</td>
<td>136 ± 12**</td>
<td>96.2 ± 16.9**</td>
</tr>
<tr>
<td>Oligodendroglioma (3)</td>
<td>44 ± 2</td>
<td>28.7 ± 1.9</td>
</tr>
<tr>
<td>Ependymoma (2)</td>
<td>67 ± 6</td>
<td>95.1 ± 11.1</td>
</tr>
<tr>
<td>Medulloblastoma (2)</td>
<td>135 ± 25</td>
<td>96.6 ± 18.8*</td>
</tr>
<tr>
<td>Ganglioglioma (1)</td>
<td>71</td>
<td>NA*</td>
</tr>
<tr>
<td>Meningioma (4)</td>
<td>0.5 ± 0.1</td>
<td>NA</td>
</tr>
<tr>
<td>Lung CA metastases (3)</td>
<td>1 ± 0.1</td>
<td>NA</td>
</tr>
<tr>
<td>Pituitary adenoma (1)</td>
<td>12</td>
<td>NA</td>
</tr>
</tbody>
</table>

* NA, not applicable; CA, cancer.

Table 3 Progesterone content in different brain tumors

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Progesterone concentration (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytoma grade 1 (2)</td>
<td>14.6 ± 2.3</td>
</tr>
<tr>
<td>Astrocytoma grade 3 (2)</td>
<td>45.4 ± 14.0</td>
</tr>
<tr>
<td>Oligodendroglioma (1)</td>
<td>19.9</td>
</tr>
</tbody>
</table>

Protein levels of DBI were measured from two different samples in each specimen. The values represent the mean ± SD.

Materials and Methods

Materials. Pathological tissues were collected at the Tampere University Hospital from neurosurgical operations on 26 primary brain tumors and four metastatic brain tumors (11 males and 16 females; mean age, 42 years; range, 14–69). None of the patients had received benzodiazepine therapy during the previous 7 days, except in connection with preoperative medication. The tumors were classified by a neuropathologist according to the WHO nomenclature (47). The astrocytomas (n = 14) were divided into: (a) grade 1 pilocytic astrocytomas and grade 2 astrocytomas (n = 4); (b) grade 3 anaplastic astrocytomas (n = 5); and (c) grade 4 glioblastomas (n = 5). Normal postmortem brain tissue was obtained from four patients who had died without a neurological or psychiatric disease (two males and two females; mean age, 57 years; range, 38–65; mean autopsy delay, 6 h; range, 2–12 h). Regions of each brain were dissected as described earlier (47) and immersion fixed as the tumor samples. The Tampere University Hospital Ethical Committee has previously approved the clinical investigation protocol for these studies (48).

Immunohistochemistry. After dissection, the tissues were cut into small pieces of similar size and immersed overnight in 4% paraformaldehyde/PBS (pH 7.4) at 4°C. The fixed tissue samples were washed for several hours in PBS at 4°C. The tissues were cut either on a vibrating microtome (Vibratome) or embedded in polyester wax and cut according to the method of Kusakabe et al. (49). Prior to staining, the sections were incubated with normal goat serum (5% for...
10 min in PBS), and staining was carried out by incubating the sections with a rabbit polyclonal antiserum against human-DBI (1:4), dilution 1:4000 in 1% normal goat serum-PBS for 18 h at 4°C. The immunoreaction was visualized either for fluorescence by incubating the sections with FITC-conjugated goat anti-rabbit IgG or for ABC immunohistochemistry with biotin-conjugated goat anti-rabbit IgG. ABC and diaminobenzidine reagents were used as described by the ABC-elite kit manufacturer (Vector, Burlingame, MO). Some sections were counterstained with the routine hematoxylin counterstaining procedure. Immunohistochemical specificity was verified by incubating the sections with preimmune serum instead of primary antiserum and by preabsorbing the human DBI antiserum with the corresponding DBI peptide (1, 5, and 10 μm). No specific reaction was observed after these incubations.

Semiquantitative analysis of staining intensity, i.e., the number of DBI positive cells/area, and cell density was performed with an image analyzer. The immunoreactive and counterstained microscopic images were grabbed with an MTF 2 CCD-72E camera (Dage, Inc., Michigan City, IN) and further processed with a DSP-200 MTL image processor (Dage) and quantitated with image analyzing software (Microscale, Digilab Ltd., Herts, England). The mean gray value of cells stained with preimmune serum was used as background value, and on the antiserum-stained sections, cells exceeding this value were considered as immunopositive cells. Cell density was calculated from each specimen from three different 1.6-mm² areas in four consecutive sections (20-μm distance); the density is expressed as the mean of all of these areas. Statistical analysis were performed using Students’s two-tailed t test.

**RNA Isolation and RNase Protection Assays.** RNAs from normal and pathological brain tissues were prepared as described in Chirgwin (50). Human DBI was cloned by reverse transcriptase-assisted PCR from jejunum total RNA using oligonucleotide primers designed on the basis of previously published DBI cDNA sequence (51). For RNase protection assays, Xhol/SmaI fragment from DBI cDNA was subcloned into pBluescript SK vector (in which the Xhol site was from the 5’ primer and Smal was located at position 192). The cRNA probe was synthesized using T7 RNA polymerase (Promega) and [α-32P]CTP (Amersham) on linearized plasmid templates covering DBI cDNA sequence from nucleotide 48 to 192. RNase protection assays were carried out with an RPA II RNase protection assay kit (Ambion). Protected RNA fragments were analyzed by electrophoresis on denaturating polyacrylamide gel.

**Progesterone Assay.** Progesterone content was measured by a specific RIA progesterone assay kit [Spectra Progesterone (125); Diagnostica, Farmos, Finland]. For these measurements, the brain homogenates were centrifuged at 1500 x g and stored at –20°C. Before assaying, the samples were extracted with dichloromethane, the organic phases were dried under an N₂ stream and dissolved in assay buffer (1:20 of original volume), and 0.1 ml of the concentrate was used for assays.

**Electron Microscopy.** A conventional pre-embedding immunohistochemical electron microscopy method was used. Briefly, the samples were fixed as above, but 0.1% of glutaraldehyde was added to the solution. Immunohistochemistry was performed as above. Then the samples were postfixed with ice-cold 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h, dehydrated in ethanol, and flat embedded with Epon 812 (LADD Research Industries, Inc., Burlington, VT). After polymerization, the sample was cut into ultrathin sections with ULTRATOME III (LKB, Wallac, Turku, Finland), mounted on precoated grids, and investigated under a JEOL electron microscope (JEM-1200 Ex).

**Acknowledgments**

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**References**

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