Expression of Two Types of Receptor for Insulin-like Growth Factors in Human Malignant Glioma

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ABSTRACT

Two types of receptors for insulin-like growth factors (IGFs) were characterized in glioma cell lines established from different human brain tumors of glial origin (astrocytoma grades III and IV) by competitive binding assay, affinity labelling, and protein phosphorylation. Type I IGF receptor is a heterodimer composed of α-subunits (M, 130,000), which bind IGF I and II with equal affinity, and of β-subunits (M, 98,000), which show tyrosine kinase activity and autophosphorylation stimulated by IGF I and II with equal potency. The type II IGF binding site is a monomer (M, 250,000) which binds IGF II with 10 times higher affinity than IGF I. The cellular concentration of type II IGF binding site is about 2- to 5-fold higher than the amount of type I IGF receptor. The characteristics of the two types of IGF receptors in human glioma cell lines are similar to those described recently in fetal rat astrocytes. In contrast the type I IGF receptor in glioma cells is different from that studied previously in normal adult brain regarding the equal affinity for IGF I and II, and the higher molecular size of the α-subunit (130,000 versus 115,000). It is suggested that glioma cells may represent a fetal cell type in tumor development of adult human brain. A role of IGF in malignant glioma has not yet been determined, but the presence of IGF receptors is a prerequisite for cellular actions of IGF.

INTRODUCTION

IGF I and II belong to the insulin family of peptides and act as growth promoters on several mammalian cells (1, 2). IGF I is identical with somatomedin C, which stimulates skeletal growth under the control of pituitary growth hormone, and IGF II acts as a growth factor in the fetus (3, 4). Both IGF I and II are present in mammalian brain. IGF I is produced by fetal rat brain explants (5, 6) and IGF II and its putative precursors have been identified in extracts of adult human brain (7). Expression of IGF I and II mRNAs has been found in fetal and adult brain of rats and humans (8–11). Furthermore, two types of IGF binding sites have been identified in human and rat brain, which interact with IGF with high affinity (Kd = 1–3 nmol/liter) (12, 13). The type I IGF receptor in brain is a tyrosine-specific protein kinase (14, 15) and belongs to the family of receptors for growth factors and hormone with protein–tyrosine kinase activity (14, 15). The type II IGF binding site has no intrinsic kinase activity and a role in cellular uptake of IGF II has been suggested (16). In mammalian brain growth-promoting actions of IGFs have been demonstrated in cultured rat astrocytes (11, 17).

The discovery of IGF I and II, and the two types of binding sites in mammalian brain raises the question whether IGFs could play a role in development of malignant brain tumors like gliomas. Previously, production of PDGF by a human clonal glioma cell line has been reported (18), and enhanced expression and rearrangement of the gene for EGF receptor in human brain tumors of glial origin have been described (19). PDGF is homologous to the v-sis oncogene product, and the EGF receptor to the v-erb B oncogene product (20). The type I IGF receptor and insulin receptor have a strong sequence homology, and their tyrosine kinase domains show a partial identity with the v-ros oncogene product (21).

The aim of the present study is to characterize IGF receptors on cultured human glioma cell lines with respect to protein structure and tyrosine kinase activity in search for possible structural and functional alterations, which may be of importance for development of brain tumors of glial origin. Our data show that glioma cells express two types of IGF binding sites, the properties of which are similar to those in fetal rat astrocytes, but different from IGF receptors in adult brain.

MATERIALS AND METHODS

Isotopes. (A14–125I) Monoiodoinsulin was a gift from NOVO Research Institute, Copenhagen, Denmark. (B26–125I) Monoiodoinsulin was purchased from the Radiochemical Centre, Amersham, UK. [125I]-IGF I and [125I]IGF II were prepared by the chloramine T method (13).

Peptides. Purified IGF I and II (22) were kindly donated by R. E. Humbel, Institute of Biochemistry, University of Zurich, Switzerland. At concentrations above 10 nmol/liter a partially purified preparation of IGF I and II (about 30% purity) was used. In this mixture there were roughly equal amounts of IGF I and II. Insulin was purchased from NOVO Research Institute.

Antiserum. Monoclonal antibody (mouse) to human IGF I receptor, oIR-3 (23) was a gift from S. Jacobs, Welcome Labs., Triangle Park, NC. Antiserum (human) to human insulin receptor, Bo (24) was a gift from C. R. Kahn, Joslin Research Laboratory, Boston, MA. Antiserum to mouse immunoglobulins was from DAKO, Copenhagen, Denmark.

Chemicals. The materials for SDS-PAGE were from Bio-Rad, Pan sorbin (staphylococcus protein A) was from Calbiochem, wheat germ agglutinin agarose and peptide poly(Glu, Tyr 4) from Sigma.

Cell Cultures. Six human glioma cell lines U-178 MG, U-251 MG, U-343 MG, U-343 MG Cl 26, U-489 MG, and U-563 MG were established from human malignant gliomas classified histologically as grade III and IV astrocytomas. Their morphology showed great variation ranging from astrocytic to spindle cells (25). The cells were routinely grown in 25-cm² flasks (NUNC) in RPMI 1640 medium supplemented with 10% newborn calf serum (GIBCO) and antibiotics (100 units of penicillin and 50 mg streptomycin per ml). Cultures were maintained at 37°C in humidified air containing 5% CO2. Cultures were subcultivated twice a week at 1:2 split ratio using 0.2 mg of EDTA and 2.5 mg trypsin per ml (GIBCO) in PBS as detaching agents.

Competitive IGF Binding Assay. Cells were cultured in multidish ×24 (NUNC or Falcon) to confluence and washed ×2 in PBS containing 2 mM CaCl₂ and incubated at 20°C with [125I]IGF I, [125I]IGF II or [125I]-insulin (100 pm) in 250 µl KRH buffer (pH = 7.4) with addition of bovine serum albumin (10 mg/ml) and bacitracin (1 mg/ml). After 2 h steady state binding was achieved and the reaction was stopped by washing 3 times with cold PBS. The cells were solubilized in 500 µl NaOH (0.2 M) and counted in a gamma counter. Nonspecific binding
of 125I-labeled peptide was determined by incubation in the presence of partially purified IGF I + II or insulin (1 μM). No degradation of 125I-labeled peptides occurred during the 2-h incubation period when controlled by precipitation with trichloroacetic acid (10% w/v). Bound radioactivity was expressed as cpm/100 μg cell protein in per cent of free radioactivity (cpm/250 μl) (11).

Affinity Labeling. Confluent cells in 25-cm² flasks were washed and incubated in KRH-buffer added albumin and bacitracin with [125I]IGF I, [125I]IGF II, or [125I]insulin (10 nM) in the absence or presence of unlabeled IGF 1 + II or insulin (1 μM) for 2 h at 20°C. After washing with cold PBS 3 times, the cells were incubated with KRH-buffer (without albumin and bacitracin) at 4°C. Cross-linking was initiated by addition of disuccimidyldi sulfosuccinate (0.1 mM) dissolved in dimethyl sulfoxide (2% v/v) and stopped after 15 min by addition of Tris (10 mM, pH 7.4) and EDTA (1 mM). Labeled proteins were analyzed by SDS-PAGE (11, 13).

Protein Phosphorylation. Confluent cells in 150-cm² flasks were solubilized in NaCl (150 mM) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (50 mM) buffer (pH 7.6) with bacitracin (1.8 mg/ml), trysiol (100 KIU/ml), phenylmethylsulfonyl fluoride (0.17 mg/ml), and Triton X-100 (1% v/v). Glycoproteins were purified by wheat germ agglutinin-agarose chromatography and incubated in absence or presence of IGF I or II (10 nM) for 30 min, at 20°C. Receptor autophosphorylation was studied by incubation with [γ-32P]ATP (15 μM/liter) for 15 min at 20°C after addition of MgCl₂ (8 mM/liter) and MnCl₂ (4 mM/liter). The reaction was stopped by addition of EDTA (20 mM/liter) and NaF (50 mM/liter). The phosphorylated receptors were immunoprecipitated by antibodies to receptor and analyzed by SDS-PAGE (13, 26). Tyrosine kinase activity of receptors was assayed by phosphorylation of synthetic substrate poly(Glu Tyr; 4:1) (27).

RESULTS

Specificity of IGF Receptor Binding. The specificity of binding of 125I-labeled IGF I, IGF II, or insulin in one human glioma cell line U-489 MG is shown in Fig. 1. [125I]IGF I binding was inhibited by IGF I and II with the same affinity (apparent Kᵦ value of 1 nM/liter) and insulin was bound with an affinity of 1% relative to the IGFs. [125I]IGF II binding was inhibited by IGF II with an apparent Kᵦ value of 1 nM/liter, IGF I being only 10% as potent and insulin showing no inhibitory effect (relative affinity less than 0.1%). [125I]Insulin was bound with an apparent affinity of 1 nM/liter and the relative affinities of IGF I and II were about 1 and 5%.

The peptide specificity of IGF I and II binding was determined in the other five glioma cell lines: U-178 MG, U-251 MG, U-343 MG, U-343 MG Cl 2;6, and U-563 MG (data not shown). The specificity patterns were similar to that shown for glioma cell line U-489 MG in Fig. 1 suggesting that the two types of IGF receptor are identical in different glioma cell lines. Scatchard plots of the IGF I and II binding data in glioma cell line U-489 MG were curvilinear with upward concavity (Fig. 2). This may be interpreted that the two types of IGF receptor are heterogeneous and/or influenced by negatively cooperative interactions. The slopes of the Scatchard plots of IGF I and II binding in the six glioma cell lines were approximately similar (data not shown). This suggests that the two types of IGF receptor bind their respective ligands with the same affinity in different cell lines.

The total cellular concentrations of IGF receptors were estimated from the Scatchard plots as represented in Table 1. In the six glioma cell lines the total amount of IGF II binding sites was 2- to 5-fold higher than the amount of IGF I binding sites. Insulin receptors were only detectable at very low levels in the six glioma cell lines and their concentration was approximately 10-fold lower than the concentration of IGF II binding sites (Fig. 2 and Table 1). For comparison previous data from our laboratory on IGF and insulin receptor concentrations in fetal and adult rat brain have been included in Table 1. The relative amounts of type I and type II IGF receptors in glioma cells resemble that in fetal rat astrocytes, but is different from that in adult rat brain cortex.

Affinity Labeling of Receptors. Chemical cross-linking of 125I-labeled IGF I or II to glioma cell line U-343 MG and analysis on SDS-PAGE under reducing conditions showed two strongly labeled proteins with molecular weights of 130,000 and 250,000 (Fig. 3). The labeling of both proteins by [125I]IGF I was completely inhibited by IGF (I + II) in a concentration of 1 μmol/liter and partly by insulin 1 μmol/liter (Fig. 3, A-C). Based on the peptide specificity described in Fig. 1, it is likely that these two bands represent monomers and cross-linked dimers of the type I IGF receptor α-subunit (M, 130,000).

Longer exposure of the autoradiogram showed a weak but specific labeling of three proteins with molecular weights of 18,000, 44,000, and 65,000, which may correspond to IGF carrier proteins (28, 29).

The M, 250,000 complex cross-linked with [125I]IGF II was completely inhibited by IGF (I + II) but also significantly reduced by insulin 1 μmol/liter (Fig. 3, D-F). This finding may be interpreted that IGF II binds to a cross-linked dimer of the type I IGF receptor α-subunit (M, 130,000), which is inhibited by insulin, and to a monomer of the type II IGF binding site (M, 250,000), which does not interact with insulin. The M, 130,000 protein labeled with [125I]IGF II was completely inhibited by IGF (I + II) and insulin suggesting that it corresponds to the type I receptor α-subunit. A faint staining of an M, 65,000 IGF carrier protein was also observed on the autoradiogram after longer exposure (data not shown).

Affinity labeling of glioma cell line U-343 MG with [125I]-

Fig. 1. Specificity of IGF I (left), IGF II (middle), and insulin (right) binding in human glioma cells. Glioma cell line U-489 MG was incubated with [125I]IGF I, [125I]IGF II, or [125I]insulin (100 pmol/liter) in presence of increasing concentrations of IGF (C), IGF II (D), or insulin (Δ). After 120 min at 20°C cell-bound radioactivity was measured and expressed as cpm/100 μg cell protein in percentage of free radioactivity (cpm/250 μl).
Table 1 Concentrations of type I IGF, type II IGF, and insulin receptors in human glioma cells, fetal rat astrocytes, and adult rat brain cortex membranes estimated by Scatchard analysis

<table>
<thead>
<tr>
<th>Glioma cell line or tissue</th>
<th>Receptor concentration (pmol/g protein)</th>
<th>Type I IGF</th>
<th>Type II IGF</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-178 MG</td>
<td>60</td>
<td>300</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>U-251 MG</td>
<td>80</td>
<td>275</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>U-343 MG</td>
<td>50</td>
<td>300</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>U-343 MG Cl 2:6</td>
<td>75</td>
<td>250</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>U-489 MG</td>
<td>120</td>
<td>250</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>U-563 MG</td>
<td>70</td>
<td>200</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Fetal rat astrocytes</td>
<td>100</td>
<td>500</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>Adult rat brain cortex</td>
<td>120</td>
<td>40</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* Data from Ballotti et al. (11).
* Data from Gammeltoft et al. (13).

Fig. 2. Scatchard plots of the IGF I, IGF II, and insulin binding data on Fig. 1.

Table 2 Molecular weights of IGF receptors in human glioma cells, fetal rat astrocytes, and adult rat brain cortex membranes estimated on SDS-PAGE under reducing conditions

<table>
<thead>
<tr>
<th>Glioma cell line or tissue</th>
<th>Molecular weight of receptor (M&lt;sub&gt;r&lt;/sub&gt;, 10&lt;sup&gt;3&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I IGF (α-subunit)</td>
<td></td>
</tr>
<tr>
<td>U-178 MG</td>
<td>130</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>130</td>
</tr>
<tr>
<td>U-343 MG</td>
<td>130</td>
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<tr>
<td>U-343 MG Cl 2:6</td>
<td>130</td>
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<tr>
<td>U-489 MG</td>
<td>130</td>
</tr>
<tr>
<td>U-563 MG</td>
<td>130</td>
</tr>
<tr>
<td>Fetal rat astrocytes</td>
<td>130</td>
</tr>
<tr>
<td>Adult rat brain cortex</td>
<td>115</td>
</tr>
</tbody>
</table>

* Data from Ballotti et al. (11).
* Data from Gammeltoft et al. (13).

insulin (Fig. 3, G–J) showed two proteins of M<sub>r</sub> 130,000 and 250,000, which were completely reduced by insulin and almost completely by IGF (I + II) in concentrations of 1 μmol/liter. These bands may represent monomers and cross-linked dimers of the insulin receptor α-subunit. No labeled proteins of lower molecular weight were seen on the autoradiogram with insulin thus supporting that the M<sub>r</sub> 18,000–65,000 complexes seen with IGF I and II are IGF-carrier proteins (28, 29). The cross-linked complexes with IGF I or II in the six glioma cell lines showed the same molecular sizes of the two major bands: M<sub>r</sub> 130,000 and 250,000 (Table 2). Previous data from our laboratory on molecular weight estimates of IGF receptors in fetal and adult rat brain have been included in Table 2 for comparison. The size of type I IGF receptor α-subunit in glioma cells is similar to that in fetal rat astrocytes, but significantly larger than that in adult rat brain cortex.

Receptor Tyrosine Kinase Activity. Autophosphorylation of type I IGF receptor and insulin receptor β-subunits was demonstrated by immunoprecipitation of partially purified receptors from glioma cell line U-563 MG with specific antibodies to type I IGF and to insulin receptors, respectively. As shown in Fig. 4 the labeling of IGF I or insulin receptor β-subunits with a molecular weight of 98,000 was increased approximately.
2-fold by IGF I or insulin when estimated by visual examination. In all six glioma cell lines IGF-sensitive phosphorylation of type I receptor β-subunits and insulin-sensitive phosphorylation of insulin receptor β-subunits could be demonstrated (data not shown).

Tyrosine kinase activity of partially purified receptors from glioma cell line U-343 MG was assayed with the synthetic substrate poly(Glu, Tyr 4:1) (Fig. 5). The phosphorylation was increased by IGF I, IGF II, or insulin with almost identical dose-response curves, although the maximal effect with insulin was smaller than those obtained with IGF I or II. Half-maximal stimulation of tyrosine kinase activity was seen with IGF I, IGF II, or insulin at a concentration of about 0.1 nmol/liter. Based on the fact that IGF and insulin cross-react with type I IF and insulin receptors only with 1% affinity (compare with Fig. 1), these results may be interpreted that IGF I and II stimulate the type I IGF receptor kinase, whereas insulin activates the insulin receptor kinase. Similar results were obtained with solubilized receptor preparations from the other glioma cell lines (data not shown).

DISCUSSION

Two types of IGF receptor have been characterized for the first time in human glioma cell lines by their binding specificity, subunit structure, and intrinsic protein kinase activity. The characteristics of IGF receptors in six different glioma cell lines are similar, and resemble those described recently in cultured fetal rat astrocytes (11) regarding the subunit composition, molecular size, binding affinity, and cellular concentration. In contrast, significant differences are observed between IGF receptors expressed on human glioma cells, and those characterized previously in normal adult brain of humans and rats (13). The size of type I IGF receptor α-subunits in glioma cells (Mr 130,000) is significantly higher than that observed previously on adult brain cortical membranes (Mr 115,000) (13, 30). We also found that the insulin receptor α-subunit in glioma cells (Mr 130,000) is larger than that found in normal adult brain (Mr 115,000) (26, 31), in agreement with a recent study by others (32). The smaller molecular size of IGF I and insulin receptors in adult brain may be due to absence of sialic acid in the N-linked glycosylation of membrane proteins (30, 31), a different processing of proreceptor proteins or alternative splicing of mRNAs.

Another difference between IGF binding sites in glioma cells and normal adult brain tissue is the equal binding affinity of IGF I and II for the type I IGF receptor in glioma cells, compared with adult brain membranes where the affinity of IGF I is about 2-fold higher than that of IGF II (13). In glioma type I IGF receptor kinase is activated by IGF I and II with equal potency, suggesting that both IGFs may act as potent agonists in stimulation of glioma cell proliferation. Furthermore, glioma cells express significantly higher amounts of type II than type I binding sites, whereas in several regions of normal adult rat brain the concentrations of the type I IGF receptor are higher than those of type II binding sites (13). The high levels of IGF II binding in glioma cells may be due to higher content of type II IGF binding sites as well as IGF-carrier proteins with high affinity for IGF II, which are present in the human central nervous system (33). The stronger labeling of IGF receptors compared with IGF carrier proteins in our cross-linking experiments suggest, however, that IGF receptors constitute the major binding components in glioma cells.

Finally, insulin receptors are present in very low amounts in human glioma cells compared to adult brain (13). It has been reported that the concentrations of insulin receptors increase, and those of IGF receptors decrease during development of the central nervous system of human fetus (12) and of chick embryo (34) as well as during differentiation of cultured myoblasts into myotubes (35). In cultured developing rat astrocytes insulin receptor levels are very low (11). Thus, it may be concluded that glioma cells resemble developing astrocytes regarding the biochemical characteristics of IGF and insulin receptors. On the basis of these similarities it is tempting to speculate that human glioma cells may represent a fetal cell type in tumor development of adult brain.

The role of IGFs in malignant tumor development and growth is not well understood. Recent observations of increased expression of IGF II in Wilms’ tumor, phaeochromocytoma and embryonal tumors (36, 37), and previous demonstrations of growth-promoting effects of IGFs in rat chondrosarcoma and human embryonal carcinoma cells (38, 39) suggest that IGFs may be involved in growth stimulation of various tumors. The mechanisms of brain tumorigenesis are not known, but other growth factors like PDGF have been implied as brain tumor promoters after findings of synthesis of a PDGF analogue by human glioma cell lines (18). An autocrine mechanism of action by PDGF on tumor cell growth has been suggested (20). Preliminary findings of expression of the IGF II gene and release of IGF II-like immunoreactivity from glioma cell lines may suggest that IGF II could act as an autocrine growth factor in human glioma.*

Increased expression and rearrangement of EGF receptors in primary human glioblastomas have been reported, suggesting that EGF and TGFα and their receptor could be involved in stimulation of brain tumor growth (19). The type I IGF receptor on glioma cells show fetal characteristics regarding its structure, binding properties, and cellular concentration. In fetal astrocytes IGF I stimulates cell division via activation of its receptor tyrosine kinase (11). Thus, it is possible that IGFs act as growth promoters in human glioma by interaction with a fetal type I

Fig. 5. Tyrosine kinase activity of partially purified receptors from glioma cells. Purified glycoproteins from glioma cell line U-343 MG were incubated with the indicated concentrations of IGF I (□), IGF II (○), or insulin (△) followed by addition of synthetic peptide poly(Glu Tyr, 4:1), and phosphorylation with [γ-32P]ATP. The phosphorylated peptide was precipitated on filter paper with trichloroacetic acid (20% w/v), and the radioactivity measured by Cerenkov counting.

* S. Gammeltoft and G. Haselbacher, manuscript in preparation.
IGF receptor. In vitro, however, we were unable to demonstrate stimulatory effects of IGF I and II on thymidine incorporation, glucose uptake, and amino acid accumulation of cultured glioma cells. A possible explanation of this failure may be that growth and metabolism of glioma cells are maximally stimulated by other autocrine growth factors.

In conclusion, glioma cell lines originating from six different human brain tumors classified as astrocytoma grades III and IV, express two types of IGF binding sites. The cellular level, subunit structure, and peptide specificity of IGF receptors in malignant glioma cells are similar to those in developing astrocytes, but different from those in normal adult brain. This may indicate that glioma cells represent a fetal cell type. A role of IGFs as growth promotores in human gliomas has not yet been shown, but the presence of cellular receptors is a prerequisite for regulatory actions of IGF I and II on brain tumors.

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