Expression and Growth Dependency of the Insulin-Like Growth Factor I Receptor in Craniopharyngioma Cells: A Novel Therapeutic Approach

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Abstract
Craniopharyngioma is a rare benign intracranial epithelial tumor that, however, often recurs and sometimes kills the affected patients, one-third of which are children. In many cases, the patients acquire growth hormone deficiency and postoperatively need substitution. Generally, growth hormone promotes local release of insulin-like growth factor I (IGF-I), which in turn activates the IGF-I receptor (IGF-IR) if present. Together, these circumstances raise the question whether IGF-IR may be involved in craniopharyngioma growth. To address this issue, we analyzed phenotypically well-characterized primary low-passage craniopharyngioma cell lines from nine different patients for IGF-IR expression and IGF-I dependency. Two of the cell lines showed no/very low expression of the receptor and was independent on IGF-I, whereas five cell lines exhibited a strong expression and was clearly contingent on IGF-I. The two remaining cell lines had low receptor expression and IGF-I dependency. Upon treatment with an IGF-IR inhibitor, cells with high IGF-IR expression responded promptly with decreased Akt phosphorylation followed by growth arrest. These responses were not seen in cells with no/very low receptor expression. Growth of cell lines with low IGF-IR expression was only slightly affected by IGF-IR inhibition. Taken together, our data suggest that IGF-IR may be involved in the growth of a subset of craniopharyngiomas and points to the possibility of the involvement of IGF-IR inhibitors as a treatment modality to obtain complete tumor-free conditions before growth hormone substitution.

Craniopharyngiomas are benign epithelial neoplasms most often found in the intra/suprasellar region. They represent one of the most difficult benign intracranial lesions to treat, because of their close relation to the surrounding structures and high tendency to recur. They are classified into two histopathologic subtypes: adamantinomatous type and squamous papillary type (1). The adamantinomatous types are considered to be derived from an enamel anlage in Rathke’s pouch, and squamous papillary types are assumed to originate in the metaplastic squamous cell nests of the adenohypophysis (1).

The incidence of craniopharyngioma is about 1.3 per million, with one-third occurring in children under age 15 (2). Little is known about the natural course of the disease but continuing tumor growth after partial tumor removal is reported to be 63% to 90% (3–10), with only 20% to 42% 10-year survival rates (6, 7, 11, 12). The main treatment options, microsurgery and radiation, render a majority of the patients growth hormone–deficient (5, 13–15). The use of growth hormone to promote growth in children with craniopharyngioma has been in practice for more than two decades and is now a part of the standard treatment for this patient group (16, 17). The fact that growth hormone is mitogenic (18–21) either directly or indirectly via the insulin-like growth factor I (IGF-I) has raised the question of a possible role for growth hormone in inducing the recurrence of craniopharyngiomas in children (8, 21, 22). The overall opinion based on the few clinical studies addressing this relationship is still that growth hormone treatment does not increase the recurrence rates (8, 21–23). However, those studies have some weaknesses, and thus far, no experimental studies have been done to confirm this statement. In theory, one can expect growth hormone to stimulate growth of craniopharyngioma cells. The craniopharyngioma cells are histologically similar to basal epidermal cells (24), and it is well known that the main stimulatory effects of growth hormone on the growth of normal epithelial cells are exerted through the IGF-I system (25–28).

The specific aim of our study is to evaluate the expression status and possible dependency of IGF-I receptor (IGF-IR) in promoting the growth of craniopharyngioma cells.
Materials and Methods

Compounds and antibodies. Picropodophyllin (99.97% purity) was prepared as described (29). A monoclonal antibody against phosphotyrosine (PY99), polyclonal antibodies to the β-subunit of IGF-IR (C-20), β-subunit IGF-IR (H-60), pAkt1 (Se257) and Akt1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell cultures. Primary cultures of human craniopharyngioma cells were isolated and prepared from tumor samples in a similar manner as for keratinocytes according to the methods described (30). Briefly, after surgical removal, a portion of the tumors was immediately put in DMEM with 10% FCS and stored at 4°C. A fresh tumor sample was cut down to small pieces and washed in PBS + penicillin/streptomycin solution. The tumor cells were dispersed by treatment with trypsin and then cultured on mitomycin-pretreated 3T3 cells in DMEM/Ham’s F12 medium (3:1; Life Technologies, Gaithersburg, MD) containing insulin (10 µg/mL), cholera toxin (2 × 10−8 mol/L), hydrocortisone (0.4 µg/mL), choler toxin (2 × 10−10 mol/L), antibiotics (penicillin 50 µg/mL and streptomycin 50 units/mL) and 10% FCS (Life Technologies; referred to as complete keratinocyte medium without epidermal growth factor). After 2 days of culture, epidermal growth factor (10 ng/mL, Sigma, St. Louis, MO) was added to the culture medium. The obtained cultures were used for experiments.

Immunocytochemical stainings. Immunostainings of tumor tissues and cell cultures for cytokeratin-7 and IGF-IR were done using the standard avidin-biotin complex technique as previously described (31). We classified the results of IGF-IR stainings as negative (−) when <10%, weakly positive (+) when 10% to 30%, intermediate positive (+++) when 30% to 60%, and strongly positive (++++) when >60% of the epithelial cells were positive.

Assay of cell growth. Measurement of de novo DNA replication was done by assessment of [3H]thymidine incorporation, mainly as described by Kratz et al. (32). Craniopharyngioma cells were seeded in 96-well plates (Corning Life Sciences, Schipol-Rijk, Netherlands, 3 × 103 cells per well) and were allowed to grow for 24 hours before 12 hours of serum starvation. Cells were then incubated for 24 hours in DMEM with IGF-I, T3, and growth hormone at indicated concentrations. Controls were incubated with DMEM only. To all the media, [3H]thymidine (Amersham Biosciences, Umeå, Sweden, 5 Ci/mmol) was added to a final concentration of 0.5 µCi/L (0.5 µCi/mL). After 24 hours, the cells were rinsed twice with PBS, lysed with 0.1% Triton X-100 in distilled water, and harvested with a Scatron cell harvester. Radioactivity was measured in a scintillation counter (Beckman, Fullerton, CA). Results are expressed as means ± SD of percentages of increase in quadruplicate cultures.

Cell proliferation determinations were also done using the Cell Proliferation Kit II (Roche Diagnostics, Indianapolis, IN), which is based on colorimetric change of the yellow 2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxanilide inner salt in orange formazan dye by the respiratory chain of viable cells (33). All standards and experiments were done in triplicate.

Expression and phosphorylation of IGF-IR. Exponentially growing cells were serum-depleted for 20 hours, after which they were treated with desired concentrations of picropodophyllin for 60 minutes. After this treatment, IGF-I (100 ng/mL; control cells with 0 ng/mL) was added

Table 1. Clinical information on patients from which cell lines were derived

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Growth hormone – deficiency before operation</th>
<th>Operation (y, m)</th>
<th>Tumor resection</th>
<th>Cell line</th>
<th>Growth hormone after operation</th>
<th>Recurrence (y, m)</th>
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<tr>
<td>32</td>
<td>no</td>
<td>2000, 01</td>
<td>subtotal</td>
<td>C1</td>
<td>no</td>
<td>2002, 06</td>
</tr>
<tr>
<td>8</td>
<td>unknown</td>
<td>2000, 04</td>
<td>radical</td>
<td>C2</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>45</td>
<td>unknown</td>
<td>2000, 05</td>
<td>subtotal</td>
<td>C3</td>
<td>yes</td>
<td>2001, 03</td>
</tr>
<tr>
<td>11</td>
<td>yes</td>
<td>2000, 09</td>
<td>subtotal</td>
<td>C4</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>9</td>
<td>yes</td>
<td>2000, 09</td>
<td>subtotal</td>
<td>C5</td>
<td>yes</td>
<td>2004, 06</td>
</tr>
<tr>
<td>35</td>
<td>yes</td>
<td>2000, 12</td>
<td>radical</td>
<td>C6</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>14</td>
<td>yes</td>
<td>2001, 03</td>
<td>radical</td>
<td>C7</td>
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<td>no</td>
</tr>
<tr>
<td>8</td>
<td>probably</td>
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<td>radical</td>
<td>C8</td>
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<td>2002, 11</td>
</tr>
<tr>
<td>6</td>
<td>unknown</td>
<td>2001, 07</td>
<td>radical</td>
<td>C9</td>
<td>no</td>
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</table>

Table 2. Immunohistochemical analysis of craniopharyngioma cell lines and corresponding tumor specimens

<table>
<thead>
<tr>
<th>Cell line/tumor sample</th>
<th>Cytokeratin 7 in cell lines</th>
<th>Cytokeratin 7 immunoreactivity in tumors</th>
<th>IGF-IR grade immunoreactivity in cell lines</th>
<th>IGF-IR grade immunoreactivity in tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Pos.</td>
<td>ND</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>C2</td>
<td>Pos.</td>
<td>Pos.</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C3</td>
<td>Pos.</td>
<td>Pos.</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>C4</td>
<td>Pos.</td>
<td>Pos.</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>C5</td>
<td>Pos.</td>
<td>ND</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>C6</td>
<td>Pos.</td>
<td>ND</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>C7</td>
<td>Pos.</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>C8</td>
<td>Pos.</td>
<td>Pos.</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C9</td>
<td>Pos.</td>
<td>ND</td>
<td>+++</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE: −, negative; +, weakly positive; ++, intermediately positive; ++++, strongly positive; ND, not determined; Pos., positive without quantification.
to the medium and the cells were incubated for another 5 minutes. Cells were then harvested for assay of IGF-IR phosphorylation using immunoprecipitation and Western blotting as described (29, 34). The membranes were probed with PY99 and after stripping with an IGF-IR β-subunit antibody (loading control). Determination of pAkt/Akt was done directly by Western blotting using specific antibodies.

**Results**

**Establishment and characterization of primary craniopharyngioma cell lines.** Primary cell lines obtained from nine tumor specimens from nine different craniopharyngioma patients were included in the study. Clinical information for each of the cases is listed in Table 1. All nine tumors exhibited adamantinomatous histology. From each tumor specimen, a primary craniopharyngioma cell line was prepared according to established procedures to selectively raise epithelial cells (30) (see Materials and Methods). It was proven that all investigated cells of each cell line expressed cytokeratin-7 (Table 2), which is a well-documented immunomarker for craniopharyngioma cells irrespective of histological subtype (35–37). Based on the selective cell culture procedures employed, together with the epithelial cytomorphology and the cytokeratin-7 phenotype, it is evident that primary cell lines represent homogenous cultures of craniopharyngioma cells.

Table 2 also shows the level of immunoreactivity of the nine different cell lines (denoted C1-C9) to IGF-IR, as assayed by immunostaining. Five of the craniopharyngioma cell lines (C1, C3-5 and C9), exhibited strong immunoreactivity, whereas C2 and C8 were negative. For three of the cell lines (C2, C3, and C8), we had access to paraffin-embedded materials from the corresponding tumor specimens. In agreement with the cell lines, immunohistochemical stainings with antibodies to IGF-IR show that the C2 and C8 tumors were IGF-IR-negative, whereas C3 was positive (Table 2). Figure 1A shows the histology of the tumors in cases 2 and 3. Both samples are predominated by an epithelial component. Several cysts are shown in case 2. Figure 1B shows microphotographs of the cytokeratin-7 expression (localized in the cytoplasm) of the two cell lines, C2 and C3. Figure 1C shows immunoreactivity to IGF-IR in case 2 (negative) and case 3 (positive) tumors. The positive epithelial cells in case 2 show brown staining in the cytoplasm/cell membrane components. This staining is not shown in the epithelial cells in case 2. For further details, see legend of Fig. 1.

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![Fig. 1. Histology and IGF-IR expression of craniopharyngioma cases. A, histology of two craniopharyngioma tumor specimens (C2 and C3) stained routinely with H&E (×200 magnification). Epithelial tumor tissue is indicated by E. Cysts appearing in case 2 are indicated by (C). B, cytokeratin-7 immunostainings of cell lines C2 and C3 (×200 magnification). Arrows, some positive cells (brown staining). C, IGF-IR immunostainings of two craniopharyngioma tumors specimens (C2 and C3; ×200 magnification). The epithelial component of case 3 shows IGF-IR-positive cells (brown staining).](image-url)
Growth dependence of IGF-I. All nine cell lines, at indicated passages (passages 2-6), were investigated for growth-stimulatory effects of IGF-I in comparison with T3, and growth hormone as well as T3 and IGF-I in combination. Serum-depleted cells were treated with these compounds for 24 hours, whereupon [³H]thymidine uptake was determined (Fig. 2). As can be seen, IGF-I was the most effective stimulant and caused alone significant growth response in four of the cell lines (C1, C3, C4, and C9) and together with T3 in five cell lines (C1, C3, C4, C5, and C9).

As assessed by Western blotting, strong IGF-IR expression was found in five of the cell lines (C1, C3-5, and C9; Fig. 3). The four other cell lines showed low (C6 and C7) or no/very low (C2 and C8) IGF-IR signals. These data are well consistent with the growth stimulatory effects of IGF-I (cf. Fig. 2). Four of the cell lines with high IGF-IR expression were responsive to IGF-I. Also, the data fit with the IGF-IR immunostaining data obtained from the corresponding immunocytostainings as well as the three available immunohistostainings (cf. Table 2).

Phosphorylation of IGF-IR and effects of inhibition. Two IGF-IR-positive (C1 and C9) and two cell lines with no/very low IGF-IR expression (C2 and C8) were selected for analysis of IGF-I induced phosphorylation of IGF-IR and Akt (Ser⁴⁷³). Figure 4A shows that IGF-I stimulates IGF-IR phosphorylation in the C1 and C9 cells. In contrast, no such effects were seen in C2 and C8. It is also shown that treatment with picropodophyllin, administered at concentrations of 0.5 or 2.5 μmol/L, ablates the IGF-I-stimulated phosphorylation of IGF-IR. Picropodophyllin is a cyclolignan compound that inhibits tyrosine phosphorylation of IGF-IR selectively (29, 38).

The phosphatidylinositol-3 kinase/Akt (protein kinase B) branch constitutes a major pathway mediating IGF-IR-dependent intracellular signaling for cell proliferation and survival (39). After IGF-I-induced phosphorylation of the receptor phosphoinositide-3-kinase becomes activated and phosphor-

Fig. 3. Expression of IGF-IR in craniopharyngioma cells. C1-C9 lines were analyzed for the expression of the β-subunit of IGF-IR using Western blotting (top). Actin was used as loading control. The signals were quantified by densitometry and the IGF-IR expression calculated for each cell line as (absorbance of IGF-IR) / (absorbance of actin) × 100. Bottom, means and SDs of IGF-IR expression of four determinations.
Effect of IGF-IR inhibition on growth of craniopharyngioma cells. All nine craniopharyngioma cell lines were assayed for effect of IGF-IR inhibition on proliferation. Cells were treated with picropodophyllin at different concentrations (50-2,500 nmol/L) for 48 hours and assayed by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt for cell growth. As shown in Fig. 5A, proliferation of C1, C3 to C5, and C9 was markedly reduced with IC_{50} values of \leq 0.5 \mu mol/L. The maximal inhibitory effect (80%) after 48 hours of treatment was seen at the highest concentration. To achieve a complete inhibition of cell growth, the picropodophyllin treatment had to be continued for another 24 to 48 hours. This delayed response of picropodophyllin could be due to autocrine IGF-I/II expression. Growth of C5 and C6 cells were slightly reduced but C2 and C8 were hardly affected at all. Table 1 and Fig. 5B summarize the proliferation data and relate them to expression levels of IGF-IR for each cell line (cf. Fig. 2). As shown, responsiveness to IGF-IR inhibition correlates well with IGF-IR expression. For closer details, see legend of Table 3.

Discussion

One of the major issues regarding treatment of craniopharyngioma patients, is not only the ability to accomplish tumor-free survival but at what price in terms of quality of life. Morbidity and mortality in this patient group results from injury to the critical structures surrounding the tumor, inflicted by the tumor itself and the treatment. Recent series reports 81% to 91% 10-year recurrence-free survival rates after subtotal tumor removal followed by radiotherapy, with 46% to 58% of the survivors living a normal independent life (40, 41). Still, a substantial part of the patients are carrying considerable morbidity. Ten years after diagnosis, most craniopharyngioma patients can be expected to have panhypopituitarism, and many to have serious visual disturbances.
morbid obesity, memory disturbances, lack of libido, etc. Much of this morbidity is caused by the treatment and the impact differs between surgery and radiation. Although the morbidity from surgery does arise directly after treatment, the effect of radiation can unfold much later. Studies analyzing the late effects of radiation in craniopharyngioma patients are few. One such study reports radiation-related complications in 58% of the children and 46% of the adults (42). Of the adult patients who died without tumor growth, 41% of the deaths were related to complications from radiation treatment.

Because the clinical course of a craniopharyngioma is known to vary greatly and there are historical reports on subgroups of patients living for many decades with mild symptoms and a slowly progressing disease (43, 44), we need to find methods predicting the long-term outcome at the time of diagnosis to enable individual treatment strategy. No such methods are known today.

This is the first study to show how craniopharyngioma cells can be cultured systematically to produce cell lines. Not only can this method be used as an experimental tool for studying the versatile biological behavior of craniopharyngiomas but it can also be helpful for the patients from whom the cultures are retrieved. One could establish an in vitro environment for each craniopharyngioma patient, which takes into account the sensitivity of the tumor cells to different treatment modalities.

By such means, one can hopefully get one step closer in estimating future prognosis of patients with a newly diagnosed craniopharyngioma, and make an individualized treatment strategy, based on tumor biology, possible.

In this study, we show for the first time that craniopharyngioma cells express IGF-R both in cell culture and in available paraffin-embedded material and that expression varies between patients. Craniopharyngioma cell cultures from five out of nine patients showed high expression of the IGF-IR and that IGF-I alone promoted growth in four of them. The fifth cell line (C5) with high IGF-IR expression was not responsive to IGF-1 alone, but in combination with T3, this cell line was growth-stimulated. T3 alone had no mitogenic effect. Inhibition of phosphorylation of IGF-IR resulted in marked reduction in proliferation of all five cell lines (also C5) with high expression. This suggests that IGF-IR is also critical for C5 growth, even though this cell line requires permissive factors (e.g., T3) in addition to IGF-I for proliferation.

According to our present results, one could expect that patients harboring viable craniopharyngiomas with high expression of IGF-IR may have higher incidence of tumor recurrence during growth hormone therapy because most of the growth stimulatory effect of growth hormone is mediated via the IGF-I/IGF-IR pathway in vivo (18, 20). A reported study showing that growth hormone therapy might increase the likelihood of tumor recurrence support this notion (22, 45). On the other hand, three other clinical studies addressing this issue are not in concordance with this assumption (8, 21, 23). However, all these three studies are associated with some limitations. Most important, they do not report adequate control groups for the treatments given before or during the growth hormone therapy, nor do they note the growth pace of the tumor before starting growth hormone therapy (8, 21, 23).

To exemplify the impact of these variables, patients having no signs of tumor remnants and patents receiving radiotherapy before or during the study should be excluded or analyzed separately because the recurrence rates in those groups of patients are much lower (40, 41, 46–50) compared with patients harboring viable tumor after surgery (8). Also, because the growth potential of craniopharyngiomas differs between individuals, one has to note the growth pace of the tumor in each patient both before and after start of growth hormone treatment. It is difficult to evaluate the possible effects of growth hormone therapy on craniopharyngiomas that are growing rapidly because they are likely to continue to grow irrespective of growth hormone treatment or not.

To summarize, our data show that craniopharyngioma cells with high IGF-IR expression are IGF-I-dependent and that attenuation of IGF-IR activity blocks their growth. These observations suggest a functional impact of IGF-IR in a subset of craniopharyngiomas. Further studies on larger collections of cases are needed to evaluate the expression level and distribution of IGF-IR and its correlation to clinical variables.

Our study also points to the possibility of using IGF-IR inhibition as a treatment complement to radiotherapy in the future and in this way reduce the long-term complications for craniopharyngioma patients.

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Table 3. Relationship between IGF-IR expression and growth inhibition

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IGF-R expression level*</th>
<th>Picropodophyllin IC&lt;sub&gt;25&lt;/sub&gt; †</th>
<th>Picropodophyllin IC&lt;sub&gt;50&lt;/sub&gt; †</th>
<th>Level of picropodophyllin response †</th>
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<tbody>
<tr>
<td>C1</td>
<td>88</td>
<td>&lt;50</td>
<td>≤500</td>
<td>high</td>
</tr>
<tr>
<td>C2</td>
<td>10</td>
<td>≥2,500</td>
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<td>no</td>
</tr>
<tr>
<td>C3</td>
<td>113</td>
<td>&lt;50</td>
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<td>high</td>
</tr>
<tr>
<td>C4</td>
<td>106</td>
<td>≤50</td>
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<td>96</td>
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<tr>
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<tr>
<td>C8</td>
<td>11</td>
<td>≥2,500</td>
<td>≥2,500</td>
<td>no</td>
</tr>
<tr>
<td>C9</td>
<td>96</td>
<td>&lt;50</td>
<td>≤500</td>
<td>high</td>
</tr>
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</table>

*Relative values (obtained from Fig. 3).
†IC<sub>25</sub> and IC<sub>50</sub> (nmol/L picropodophyllin resulting in 25% and 50% inhibition of cell proliferation, respectively). Data obtained from Fig. 5A.
‡Picropodophyllin responses defined as follows: no, IC<sub>25</sub> > 2,500 nmol/L; low, IC<sub>25</sub> < 2,500. But IC<sub>50</sub> > 2,500 nmol/L; high, IC<sub>50</sub> ≤ 500 nmol/L. Data obtained from Fig. 5A.
Human Cancer Biology

References

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