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-1, 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.
30 minutes at 37°C. The tumor cells were dispersed by treatment with trypsin and subsequently cut down to small pieces and washed in PBS + penicillin/streptomycin (50 μg/mL), transferrin (5 μg/mL), T3 (2 × 10−10 mol/L), hydrocortisone (0.4 μg/mL), cholera toxin (2 × 10−10 mol/L), antibiotics (penicillin 50 μg/mL and streptomycin 50 units/mL) and 10% FCS. Each tumor specimen was cut down to small pieces and washed in PBS + penicillin/streptomycin solution. The tumor cells were dispersed by treatment with trypsin for 30 minutes at 37°C and 5% CO2. The growing cells were then cultured on mitomycin-pretreated 3T3 cells in DMEM/Ham’s F12 culture medium. The obtained cultures were used for experiments.

We classified the results of IGF-IR stainings as negative (−) when <10%, weakly positive (+) when 10% to 30%, moderately positive (+++) when 30% to 60%, and strongly positive (++++) when >60% of the epithelial cells were positive.

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 (Ser473) 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. Each tumor specimen was cut down to small pieces and washed in PBS + penicillin/streptomycin solution. The tumor cells were dispersed by treatment with trypsin for 30 minutes at 37°C and 5% CO2. The growing cells were then cultured on mitomycin-pretreated 3T3 cells in DMEM/Ham’s F12 medium (3:1; Life Technologies, Gaithersburg, MD) containing insulin (5 μg/mL), transferrin (5 μg/mL), T3 (2 × 10−9 mol/L), hydrocortisone (0.4 μg/mL), cholera 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 μg/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). A monoclonal antibody against phospho-h20), pAkt1 (Ser473) and Akt1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

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

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Growth hormone – deficiency after operation</th>
<th>Operation (y, m)</th>
<th>Tumor resection</th>
<th>Cell line</th>
<th>Growth hormone – deficiency after operation (y, m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>no</td>
<td>2000, 01</td>
<td>subtotal</td>
<td>C1</td>
<td>no</td>
</tr>
<tr>
<td>8</td>
<td>unknown</td>
<td>2000, 04</td>
<td>radical</td>
<td>C2</td>
<td>yes</td>
</tr>
<tr>
<td>45</td>
<td>unknown</td>
<td>2000, 05</td>
<td>subtotal</td>
<td>C3</td>
<td>yes</td>
</tr>
<tr>
<td>11</td>
<td>yes</td>
<td>2000, 09</td>
<td>subtotal</td>
<td>C4</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>
</tr>
<tr>
<td>35</td>
<td>yes</td>
<td>2000, 12</td>
<td>radical</td>
<td>C6</td>
<td>no</td>
</tr>
<tr>
<td>14</td>
<td>yes</td>
<td>2001, 03</td>
<td>radical</td>
<td>C7</td>
<td>yes</td>
</tr>
<tr>
<td>8</td>
<td>probably</td>
<td>2001, 04</td>
<td>radical</td>
<td>C8</td>
<td>yes</td>
</tr>
<tr>
<td>6</td>
<td>unknown</td>
<td>2001, 07</td>
<td>radical</td>
<td>C9</td>
<td>no</td>
</tr>
</tbody>
</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>ND</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>–</td>
</tr>
<tr>
<td>C8</td>
<td>Pos.</td>
<td>ND</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.
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 $[^{3}H]$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 immunocyto-stainings 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-I 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-IIR phosphorylation in the C1 and C9 cells. In contrast, no such effects were seen in C2 and C8. IGF-IIR phosphorylation is a cyclolignan compound that inhibits tyrosine phosphorylation of IGF-IIR selectively (29, 38).

The phosphatidylinositol-3 kinase/Akt (protein kinase B) branch constitutes a major pathway mediating IGF-IIR-dependent intracellular signaling for cell proliferation and survival (39). After IGF-I-induced phosphorylation of the receptor phosphoinositol-3-kinase becomes activated and phosphorylates Akt at Ser473 (39). This is also shown to occur in craniopharyngioma cell lines with high expression of IGF-IIR (C1 and C9; Fig. 4B). In the C2 cell line, being without IGF-IIR activity (see Fig. 4A), the ligand did not induce Akt phosphorylation. Surprisingly, in serum-depleted C8 cells (also IGF-IIR negative), there was a high basal Akt phosphorylation, which was neither increased by IGF-I nor inhibited by picropodophyllin (Fig. 3B). This means that Akt is constitutively activated and serum growth factor-independent in this cell line. The underlying mechanism for this is unknown and not relevant to this study, but could be due to PTEN (phosphatase and tensin homologue gene) mutation.
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 assay for cell growth. As shown in Fig. 5A, proliferation of C1, C3 to C5, and C9 was markedly reduced with IC₅₀ values of 0.5-2.5 nmol/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.
retrieved. One could establish an in vitro can also be helpful for the patients from whom the cultures are the versatile biological behavior of craniopharyngiomas but it can this method be used as an experimental tool for studying can be cultured systematically to produce cell lines. Not only methods are known today.

To summarize, our data show 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-I 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.
References


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