Insulin-like Growth Factor I Receptor Activity in Human Medulloblastomas¹

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ABSTRACT
Medulloblastomas represent about 25% of all pediatric intracranial neoplasms. These highly malignant tumors arise from the cerebellum affecting mainly children between ages 5 and 15. Although the etiology of medulloblastomas has not yet been elucidated, several reports suggest that insulin-like growth factor I (IGF-I) may contribute to the development of these tumors. Results of this study show that the majority of cases examined were characterized by the abundant presence of the receptor for IGF-I (IGF-IR) protein (16 of 20 cases), and its major signaling molecule, insulin receptor substrate 1 (IRS-1; 15 of 20). Protein levels for IGF-IR and IRS-1, determined by Western blot and immunohistochemistry, were significantly higher in medulloblastoma biopsies than in control cerebellar tissue. By immunohistochemistry, 10 of 17 biopsies examined were also positive for the anti-pY1316 antibody staining that specifically recognizes the phosphorylated (active) form of the IGF-IR. These findings correlate with the fact that phosphorylated forms of the downstream-signaling molecules Erk-1, Erk-2, and Akt/protein kinase B were found in medulloblastoma biopsies but not in control cerebellar tissue. Importantly, there is a strong inverse correlation between biopsies that are positive for anti-pY1316 and for anti-Trk-C immunoactivity. These observations direct our attention to the IGF-IR system as a potential therapeutic target in medulloblastomas and suggest a possibility of using the anti-pY1316 antibody as a potential prognostic marker for medulloblastomas.

INTRODUCTION
The IGF-IR¹ is a multifunctional membrane-associated tyrosine kinase that sends a mitogenic signal (1, 2), protects normal and tumor cells from apoptosis (3, 4), may induce cell differentiation (5), and plays an important role in the maintenance of the transformed phenotype by different viral and cellular oncogenes (6, 7). After ligand binding (to the α-subunit), the cytoplasmic portion of the receptor (the β-subunit) becomes phosphorylated, creating specific binding sites for direct IGF-IR substrates. Signaling molecules that bind activated IGF-IR include the family of IRS-1 proteins (8). She proteins (8), p85 subunit of PI3-K (9), GRB-10 (10), 14-3-3 (11), SH-containing phosphotyrosine phosphatase (12), and COOH-terminal Src kinase (13). Among multiple IGF-IR substrates, IRS-1 and PI3-K have been reported to play a role in IGF-IR-mediated cell proliferation and cell protection from apoptosis by recruiting downstream phosphorylation events such as the activation of the Ras/MAPK pathway and PI-3 kinase-mediated phosphorylation of Akt/ PKB (14–16).

In the nervous system IGF-IR and its ligand(s) are present during embryonal and early postnatal development of the brain, decreasing substantially during adolescence and adult life (17, 18). In cerebellar cortex the IGF-IR is found in cells of the granular layer, where IGF-I is mitogenic and protects them from low potassium induced apoptosis (19, 20). Tumors of the nervous system secrete both IGF-I and IGF-II and often over-express IGF-IR (18). The presence of an active IGF system has been established in glioblastomas (21–23), neuroblastomas (24), astrocytomas and meningiomas (25). Despite data that point to the IGF system involvement in the cerebellum and in embryonic tumors of the peripheral nervous system (neuroblastomas), IGF-I and IGF-IR have been investigated minimally in medulloblastomas, the tumors that arise from the cerebellum and that are the most common embryonic tumors of the central nervous system. These few studies have demonstrated the presence of IGF-I binding sites in one medulloblastoma specimen (26), as well as the presence of IGF-IR protein and IGF-I mRNA in human medulloblastoma cell lines (27), and have shown over-expression of IGF-2 in medulloblastomas and rhabdomyosarcomas, which develop in mice with deletions of the PTCH gene (28). Finally, activation of the IGF-IR system has been demonstrated in human medulloblastoma cell lines, and in mouse medulloblastoma cells obtained from cerebellar tumors, which develop in transgenic mice carrying JC virus T-antigen and the JC virus archetype promoter (29).

It has been previously established that besides IGF-I, there are other factors that control growth and development of medul-

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The abbreviations used are: IGF-I, insulin-like growth factor I; IGF-IR, receptor for IGF-I; IRS-1, insulin receptor substrate 1; Akt/PKB, Akt/protein kinase B; MAP, mitogen-activated protein; PI3-K, phosphatidylinositol 3'-kinase; GRB-10, growth factor receptor-bound protein-10; MAPK, MAP kinase; GFAp, glial fibrillary acidic protein kinase; DAB, 3,3'-diaminobenzidine; Erk, extracellular signal-regulated protein kinase; Trk, tyrosine receptor kinase.
loblastomas. For instance, neurotrophins and their cognate receptors, Trk-A, Trk-B, and Trk-C, were shown to mediate proliferation, differentiation, and apoptosis of neuronal progenitor cells (30, 31). Importantly, Trk-C has been repeatedly shown to induce apoptosis in medulloblastoma cell lines (31, 32), and its elevated expression has been associated with favorable clinical outcome in medulloblastoma patients (32, 33).

To further explore the involvement of the IGF-IR system in medulloblastomas, we have examined 17 paraffin-embedded and 3 frozen cerebellar biopsies from patients with this tumor. Several parameters of the IGF-IR system were examined in this study: (a) the protein levels for IGF-IR, IRS-1, and PI3-K; (b) the cellular localization of IGF-IR and IRS-1; and (c) the phosphorylation status of the IGF-IR and its downstream signaling molecules, including Erk-1, Erk-2, and Akt/PKB. In addition, we made an attempt to correlate activation of the IGF-IR system with Trk-C expression in multiple biopsies isolated from medulloblastoma patients.

MATERIALS AND METHODS

Clinical Samples and Cells. A total of 17 human medulloblastoma samples were obtained from the pathology archives of Medical College of Pennsylvania-Hahnemann University, Philadelphia, PA. Formalin-fixed, paraffin-embedded surgical resections were histologically classified according to WHO classification of tumors of the nervous system (34). Three frozen medulloblastoma biopsies (p138, p494, and p609) were obtained from The Cooperative Human Tissue Network (CHTN). Finally, samples of frozen human cerebellum (N906, N989, and N1061) were kindly provided by Drs. Mary Herman and Joel E. Kleinman from the Neuropathology Section-Clinical Brain Disorder Branch, National Institute of Mental Health, Bethesda, MD.

Previously characterized R600 mouse embryo fibroblasts were used in Western blot analyses as a positive control to monitor the presence and activity of different components of the IGF-IR signaling pathways (2). Briefly, R600 cells were derived from R− cells (35), which originated from mouse embryos with a targeted disruption of the IGF-IR gene (36). After stable transfection with the pMRIGFIR12 plasmid, which contains the hygromycin B phosphotransferase gene of Escherichia coli, and the human IGF-IR cDNA under control of the rat (−2350 + 1640) IGF-IR promoter (2), clones with different numbers of the IGF-IR were selected (35). By Scatchard analysis, clone R600 was shown to express $3 \times 10^{5}$ IGF-IR molecules per cell (35). To determine the presence of the phosphorylated forms of Akt/PKB and MAPKs, quiescent R600 cells were stimulated with IGF-I (50 ng/ml), and total proteins were extracted 15 min after stimulation.

Western Blots. To determine total IGF-IR, IRS-1, and PI3-K protein levels, monolayer cultures of R600 cells or small fragments of cerebellar tissues were lysed on ice with 400 μl of lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, and 10 μg/ml aprotinin]. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), and 50 μg of total proteins were separated on a 4–15% gradient SDS-PAGE (Bio-Rad) and transferred into nitrocellulose membranes. Blots were blocked with 5% nonfat dry milk in TBST [10 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20] and were probed with rabbit anti-IGF-IRβ antibody (Santa Cruz Inc., Santa Cruz, CA), rabbit anti-IRS-1 antibody (Upstate Biotechnology Inc., Lake Placid, NY), rabbit anti-PI3-K (p85) antibody (Upstate Biotechnology), and antirabbit horseradish peroxidase-conjugated secondary antibody (Calbiochem, San Diego, CA). Blots were developed with ECL detection reagents (Amersham, Arlington, IL). Phosphorylated forms of Akt/PKB were detected with the PhosphoPlus Akt((Ser473) antibody kit (New England BioLabs, Beverly, MA), and phosphorylated Erk-1 and Erk-2 were detected by PhosphoPlus Phospho-p44/42 MAPK (Thr202/Tyr204) antibody kit (New England BioLabs, Beverly, MA). To determine equal loading conditions, corresponding blots were “striped” and reprobed with anti-Grb-2 antibody (Transduction Laboratories, Lexington, KY). Finally, to evaluate IGF-IR tyrosine phosphorylation, frozen tissues were lysed on ice with 400 μl of lysis buffer, and protein concentration was determined by a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). A 500 μg of total protein extract was used for immunoprecipitation the anti-εIGF-IR antibody (Ab-1, Calbiochem) in the presence of agarose conjugated mouse IgG (Sigma). The immunoprecipitated proteins were resolved on 4–15% gradient polyacrylamide gels containing SDS and electrophoresed onto nitrocellulose filters. Membranes were blocked with 5% BSA in TBST buffer overnight at 4°C. Blots were probed with the mouse anti-phosphotyrosine horseradish peroxidase-conjugated antibody (PY20; Transduction Laboratories) and developed with ECL reagents (Amersham).

Immunohistochemistry. Formalin-fixed, paraffin-embedded samples were sectioned at 4-μm thickness and stained with H&E for routine examination and the histological classification of the tumors. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex system, according to the manufacturer’s instructions (Vectastain Elite ABC Peroxidase Kit; Vector Laboratories). Briefly, sections were deparaffinized in xylene and rehydrated through descending alcohols to water. For nonenzymatic antigen retrieval, sections were heated in 0.01 M sodium citrate buffer (pH 6.0) to 95°C for 40 min and allowed to cool for 20 min at room temperature. To quench endogenous peroxidase, slides were then rinsed with PBS and incubated in methanol/3% H₂O₂ for 20 min. Sections were then washed with PBS and blocked in PBS/0.1% BSA containing 5% normal horse or goat serum for 2 h at room temperature, and incubated overnight at room temperature with primary antibodies. The primary antibodies used were rabbit anti-pY1316 IGF-IR (1:500 dilution; Ref. 36); rabbit anti-IGF-IR (1:500 dilution; Santa Cruz Inc.); rabbit anti-IRS-1 (1:100 dilution; Upstate Biotechnology Inc.); mouse anti-GFAP (1:100 dilution, clone b2F; DAKO); mouse antineurofilaments antibody (1:500 dilution, clone SMⅠ-312; Sternberger Monoclonals); and mouse anti-synaptophysin antibody (1:500 dilution, clone SY38; Roche Molecular, Indianapolis, IN). Finally, rabbit anti-Trk-C antibody (sc-117, 1:500 dilution; Santa Cruz Inc.), which specifically recognizes the COOH terminus of the Trk-C and does not cross-react with Trk-A and Trk-B, was used as well.
Secondary antibodies were biotin-conjugated horse anti-mouse and goat antirabbit IgGs (Vector). After secondary antibodies, avidin-biotin peroxidase complexes (Vector), counterstained with hematoxylin, and mounted with Permount.

RESULTS

Localization and Activity of the IGF-IR in Biopsies from Patients with Medulloblastoma. A recently developed and characterized antibody against the phosphorylated (active) form of the IGF-IR was used in these immunohistochemical experiments (37). The antibody specifically recognizes phosphotyrosine 1316 (pY1316) of the β subunit of IGF-IR, and does not cross-react with the corresponding moiety of the activated insulin receptor (37). To determine the status of IGF-IR activity in human medulloblastomas, paraffin-embedded cerebellar sections taken from 17 biopsies of patients diagnosed with medulloblastoma were stained with the anti-pY1316 antibody. Ten of 17 medulloblastomas were positive when tested with the anti-pY1316 antibody, indicating the presence of the phosphorilated (active) form of the IGF-IR in these samples.

Table 1 Clinical and immunohistochemical analysis of human medulloblastomas

<table>
<thead>
<tr>
<th>No.</th>
<th>Diagnosis</th>
<th>Gender/Age</th>
<th>Location</th>
<th>GFAP</th>
<th>SY-38</th>
<th>NF</th>
<th>IRS-1</th>
<th>IGF-IR Total</th>
<th>IGF-IR Phosp</th>
<th>Trk-C</th>
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<tr>
<td>1</td>
<td>Desmoplastic medulloblastoma</td>
<td>Male/6 yr</td>
<td>Left hemisphere</td>
<td>–</td>
<td>+</td>
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<td>++</td>
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<td>Vermis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
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<td>Male/7 yr</td>
<td>Right hemisphere</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
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<td>Female/3 yr</td>
<td>Vermis</td>
<td>–</td>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Female/17 yr</td>
<td>Right hemisphere</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>Female/16 mo</td>
<td>Posterior fossa</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
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<td>Posterior fossa</td>
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<td>+</td>
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<tr>
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<td>Male/5 yr</td>
<td>Posterior fossa</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>12</td>
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<td>Male/7 yr</td>
<td>Vermis</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>Male/9 yr</td>
<td>Vermis</td>
<td>–</td>
<td>++</td>
<td>–</td>
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<tr>
<td>14</td>
<td>Desmoplastic medulloblastoma</td>
<td>Female/8 yr</td>
<td>Posterior fossa</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>15</td>
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<td>Male/5 yr</td>
<td>Posterior fossa</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>16</td>
<td>Medulloblastoma (neuroblastic)</td>
<td>Male/12 yr</td>
<td>Left hemisphere</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>17</td>
<td>Medulloblastoma (neuroblastic)</td>
<td>Male/3 yr</td>
<td>Posterior fossa</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
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* Focal positivity.
* Sy-38, Synaptophysin.

GFAP immunostaining; and, finally, (e) presence or absence of the Trk-C in the neoplastic cells. In general, results of these multiple comparisons did not show a clear association with the anti-pY1316 antibody staining. The only exception was found with Trk-C-positive human medulloblastoma samples, in which a strong inverse correlation with anti-pY1316 immunostaining was detected. As illustrated in Table 1, all of the strongly positive Trk-C samples (biopsies 1, 5, 10, 11, and 12) were negative for anti-pY1316 antibody staining. In agreement with this finding, all of the strongly positive pY1316 samples (biopsies 2, 3, 15, and 16) were negative for the anti-Trk-C antibody staining. There are two cases however, biopsies 6 and 8, in which a weak anti-pY1316 immunostaining was accompanied by the presence of a weak Trk-C protein detection.
uniformly stained. Sections of normal human cerebellum were also positively for the total IGF-IR (Fig. 1F) and were completely negative for anti-pY1316 antibody staining (Fig. 1G). Lack of the staining in parallel cultures that were left without IGF-I stimulation confirmed again specific recognition of the phosphorylated form of the IGF-IR by this antibody (Fig. 1H).

Fig. 2 shows representative immunohistochemical staining with anti-pY1316 (A and C) and anti-Trk-C (B and D) antibodies. Biopsy 2, which has a strong anti-pY1316 immunoreactivity, is completely negative for anti-Trk-C antibody staining (Fig. 2; compare C and D). Conversely, biopsy 1, strongly positive for the presence of Trk-C (see Table 1) is negative for anti-pY1316 antibody staining (Fig. 2; compare A and B). Although this inverse correlation between phosphorylation of the IGF-IR and the Trk-C expression is not absolute, compare, for instance, biopsies 6 and 8 (Table 1), an opposite immunostaining for these two receptor proteins was detected in the majority cases examined in this study (15 of total 17 cases).

Protein Levels for IGF-IR, IRS-1, and PI3-K in Medulloblastoma Biopsies. To analyze protein levels for IGF-IR, IRS-1, and PI3-K in medulloblastoma biopsies, Western blot analysis was performed on frozen biopsy samples from three patients with classic medulloblastoma (p138, p609, and p494). Thick frozen sections of human normal cerebellum collected from autopsies of an 11-week-old (N906), a 26-week-old (N1061), and a 36-year-old (N989) were used as reference samples. Fig. 3A (top) demonstrates elevated levels of the IGF-IR protein in three medulloblastoma frozen biopsies. By densitometry, the IGF-IR from biopsies showed, on average, a 4-fold increase in comparison with three samples isolated from human normal cerebellum. Additionally, IGF-IR protein levels in medulloblastoma biopsies were compared with IGF-IR protein levels detected in R600 fibroblasts, previously shown by Scatchard analysis to express $3 \times 10^4$ IGF-IR molecules/cell (35). Again densitometry allowed us to estimate the quantity of IGF-IR protein in normal and neoplastic cerebellum. These semiquantitative determinations showed that the population of cells from the biopsies p138, p609, and p494 expressed an average of $1.3 \times 10^3$; $2.6 \times 10^3$; and $2.1 \times 10^4$ IGF-IR/cell, respectively. In comparison, cells from normal human cerebellum, N906 and N1061, expressed an average of $0.6 \times 10^4$ and $0.8 \times 10^4$ IGF-IR/cell, respectively. It is noteworthy that IGF-IR protein level in cerebellar tissue from the adult (N989) is significantly lower (0.14 $\times 10^4$ IGF-IR/cell) than the IGF-IR level detected in two cerebellar autopsies from the newborns (N906 and N1061). Importantly, the IGF-IR protein, abundantly present in all of the medulloblastoma biopsies, was tyrosine phosphorylated as indicated (Fig. 3B) by immunoprecipitating the IGF-IR and developing corresponding blot with anti-phosphotyrosine antibody (pY). Because of restricted sensitivity of the Western blot in comparison with immunohistochemistry, detection of the phosphorylated band was possible when the IGF-IR protein was immunoprecipitated from 500 µg of total proteins. Control samples from normal cerebellar tissue did not show any traces of the IGF-IR phosphorylation (Fig. 3B). These results confirmed our immunohistochemical evaluation of the IGF-IR ty-
Rosine phosphorylation depicted in Fig. 1, by using a different antibody and different biopsy samples (Fig. 3B).

Western blot for PI3-K is shown in the Fig. 3A, middle panel. In comparison with IGF-IR detected in the same set of protein samples, PI3-K has a different pattern of expression. It appears to be abundant in both normal cerebellar tissue and medulloblastoma biopsies. One could even argue that in three medulloblastoma biopsies examined, PI3-K protein levels were slightly lower than in control cerebellar tissues. Interestingly, an additional slightly lower band was detected in all three of the samples from control cerebellum. Although we did not attempt to investigate the nature of this lower band in this study, it may reflect the presence of hypophosphorylated p85 in normal cerebellar tissue, and its disappearance in actively growing medulloblastomas.

Fig. 3A, bottom panel, illustrates IRS-1 protein levels determined in the samples previously analyzed for the IGF-IR and PI3-K. The IRS-1 protein was detected in all of the medulloblastoma samples within seconds after exposing ECL-treated blots to photographic material. In contrast, control samples obtained from normal cerebellar tissues did not show any detectable traces of IRS-1 signal at these short exposure times. Overexposure of the film for 15 min resulted in visualization of weak bands at a Mr range of 160,000 in all of the control samples (Fig. 3C). This may reflect a low level of IRS-1 expression in normal human cerebellum. Because reactivation of the IRS-1 expression in combination with an apparent IGF-IR activation in medulloblastomas may potentially affect aggressiveness of the tumor cells, it was critical to confirm these findings by using different medulloblastoma samples and a different methodological approach. In this respect, Fig. 4 illustrates IRS-1 immunostaining of paraffin-embedded biopsies obtained from two additional patients with a diagnosed classic medulloblastoma. As expected, the IRS-1 staining appears to be cytoplasmic because DAB-positive granules (light brown) are visible in perinuclear regions of the cells. Control sections, treated with secondary antibody in the presence of an irrelevant antibody, were negative (data not shown). In comparison, a faint anti-IRS-1 immunostaining of the granular layer (arrows) and Purkinje cells (arrowheads) was observed in normal cerebellum (Fig. 4A), whereas the medulloblastoma samples were characterized by much stronger cytoplasmic staining (Fig. 4, B and C). This result supports our previous data from the Western blot (Fig. 3A), confirming apparent up-regulation of the IRS-1 protein in medulloblastomas.

Activity of the Downstream Signaling Pathways. Activation of two downstream signaling pathways, Ras/MAPK and the PI3-K-mediated phosphorylation of Akt/PKB, is thought to facilitate IGF-IR-mediated cell proliferation and cell protection from apoptotic death (5, 27, 38, 39). We first compared levels of Erk1 and Erk2 phosphorylation between medulloblastoma biopsies and normal cerebellar tissue (Fig. 5, top two panels). IGF-1-stimulated R600 fibroblasts were used as a positive control for Erk1/Erk2 phosphorylation (2). Two medulloblastoma biopsies (p138 and p494) were characterized by Erk1/Erk2 phosphorylation on threonine 202 and tyrosine 204, an event that triggers MAPK activity (40). In contrast, the third biopsy (p609), and all of the control samples (N906, N989, and N1061) were negative for the anti-phospho-Erk-1 antibody staining.

The Western blot depicted in Fig. 5, third panel, is the same one shown in the top two panels after reprobing it with the...
levels independently from MAPKs and Akt, the same blot was additionally reprobed with the anti-Grb-2 antibody (41). This confirms that, despite of the presence of total Erks, p85, and Akt in all of the samples examined, only actively growing medulloblastoma cells are characterized by the presence of phosphorylated forms of the signaling molecules, which are likely activated by the IGF-IR signaling system in these tumors.

DISCUSSION

Despite the data that point to the involvement of the IGF system in the cerebellum (19, 20, 27) and in embryonic tumors in the peripheral nervous system, neuroblastomas (24), the IGF-I, and the IGF-IR have been investigated minimally in medulloblastomas, which arise from the cerebellum and are the most common embryonic tumors of the central nervous system. These studies include detection of the IGF-I-binding sites in one medulloblastoma specimen (26), and the demonstration of the presence of IGF-IR protein by Western blot and the presence of IGF-1 transcripts by RT-PCR (27). We have recently demonstrated the up-regulation of the IGF-IR system in human and mouse medulloblastoma cell lines, as well as the phosphorylation of the IGF-IR in several medulloblastoma biopsies (29). These preliminary studies, together with recent reports by Patti et al. (27) emphasize the potential involvement of the IGF-IR system in the development and progression of medulloblastomas. To further analyze the IGF-IR system in medulloblastomas, we have examined 17 paraffin-embedded and 3 frozen biopsies from patients with medulloblastomas.

In agreement with previous results obtained from medulloblastoma cell lines (27, 29), both IGF-IR and its major substrate IRS-1 were significantly up-regulated in medulloblastoma biopsies. Importantly, the phosphorylated (active) form of the IGF-IR was detected in 59% of all medulloblastoma cases examined. Because not all medulloblastoma samples demonstrated positive staining with the anti-pY1316 antibody, it was relevant to ask whether the presence of the phosphorylated form of the IGF-IR correlates with some of the parameters commonly used for the clinical characterization of medulloblastomas. We were initially able to demonstrate that all of the pY1316-positive samples were also positive for the IGF-IR and IRS-1 immunostaining. Importantly, all IGF-IR-negative samples were also negative for anti-pY1316 antibody staining, further confirming the specificity of the anti-pY1316 antibody. In the next set of comparisons, we expected to find phosphorylated IGF-IR in more aggressive classic and/or neuroblastic medulloblastomas rather than in the less aggressive desmoplastic medulloblastomas. However, the data shown in Table 1 indicate the absence of such a correlation. This was somewhat surprising because activation of the IGF-IR mediates both cell proliferation (1, 2) and cell motility (42–44), parameters that could potentially increase invasiveness of the tumors. On the other hand, elevated IRS-1 expression, although favorable for the IGF-I-dependent primary tumor growth (45), has been conversely related to metastatic potential of breast cancer (46), and prostate cancer (43, 44) cell lines. In this respect, overexpression of IRS-1 in the metastatic prostate cancer cell line, LNCaP, markedly attenuated cell motility and increased cell aggregation and adhesion to extracellular matrix proteins (43, 44). Therefore, it is possible
that, in medulloblastomas, activation of the IGF-IR, together with strong up-regulation of the IRS-1, is associated with primary tumor growth rather than with tumor invasiveness (ability to escape from the primary site into the subarachnoid space and infiltration of medulloblastoma cells into healthy cerebellar tissues). Other comparisons made with respect to anti-pY1316 antibody staining included: gender and age of the patient; location of the tumor; presence of cellular markers such as neurofilaments and synaptophysin, and absence of the GFAP. These multiple comparisons did not show any significant association with the IGF-IR activity in medulloblastoma samples. In contrast, a strong inverse correlation was noticed between the presence of the phosphorylated IGF-IR and Trk-C expression (last two columns in Table 1). Elevated Trk-C levels were already proposed as a molecular marker associated with good prognosis in medulloblastomas (32, 33). Results in this study (Table 1 and Fig. 2) suggest that the presence of the activated form of the IGF-IR may also carry some diagnostic values; however, more studies are required to correlate low levels of the Trk-C expression, activation of the IGF-IR phosphorylation, and the clinical outcome in medulloblastoma patients.

Fig. 1, A and B, show two different examples of medulloblastoma biopsies. In these samples, there are groups of neoplastic cells strongly positive for the anti-pY1316 antibody staining. Cytoplasmic location of the staining may suggest that the activated (Y1316-positive) receptor was internalized at the time the biopsy was collected. Sections from the same samples were also probed with the antibody that recognizes total IGF-IR (Fig. 1, D and E). Cells within these sections were uniformly stained, suggesting the presence of the IGF-IR protein in the majority of neoplastic cells in the affected region of the cerebellum. On the other hand, the phosphorylated form of the IGF-IR was detected only in certain areas within the tumors and only some of the cells showed strong anti-pY1316 reactivity (Fig. 1, A and B). This irregular pattern of IGF-IR activation may suggest that, only in certain discrete regions within the cerebellar tumor IGF-IR, ligand(s) are secreted and activate IGF-IR-dependent cellular processes that may possibly contribute to the malignant growth within the neoplasm.

The results presented in Fig. 3 show apparent increases in the IGF-IR and IRS-1 protein levels in medulloblastoma samples. These up-regulations were evaluated in biopsy samples obtained at surgery, in relation to control samples isolated from frozen autopsies of normal cerebellar tissue. The biopsy samples were frozen within minutes after their surgical excision, whereas the normal cerebellar tissues were obtained between 13.5 to 36 h postmortem. Therefore, it is possible that the proteins isolated from the control sections are not well preserved. Microscopic examination of the sections, and the abundant presence of the PI3-K band (M, 85,000), Grb-2, Erk1, and Erk2, detected in all of the protein samples, including normal cerebellar tissues (Figs. 3 and 5), partially excludes this possibility. Additionally, we
have shown, in our previous work, blots containing proteins from the postmortem cerebellum in which both cytoplasmic and membrane-associated proteins were well preserved (29).

Ligand-activated IGF-IR leads to the recruitment and the activation of the Ras/MAPK pathway via the IRS-1 and/or Shc proteins (16, 47). In parallel, PI3-K can also be activated via a direct interaction with the β-subunit of the IGF-IR or by an interaction with tyrosine-phosphorylated IRS-1 (16). Once PI3-K is activated, it mediates phosphorylation, and activates Akt/PKB, an event repeatedly shown in many different systems to protect cells from apoptosis (39). Fig. 5, top, illustrates the phosphorylation status of Erk-1 and Erk-2. Although both IGF-IR and IRS-1 were strongly up-regulated in all three of the medulloblastoma samples examined (Fig. 3A), biopsy p609 did not exhibit a detectable level of phosphorylated Erk-1 and Erk-2 (Fig. 5, top panel). Interestingly, when the same blot was probed with the antibody that recognizes the phosphorylated Akt/ PKB, biopsy p609 was characterized by the strongest Akt/ PKB phosphorylation (Fig. 5, middle panel). The explanation for this peculiar phosphorylation pattern may come from several recent reports showing the inhibition of the Ras/MAPK pathway in cells with constitutively active Akt/ PKB (48). Although the question as to whether medulloblastoma cells are able to proliferate without activating Ras/MAPK when Akt/ PKB is constitutively active remains to be addressed, our present results indicate activation of different signal transduction pathways that may originate from the auto and/or paracrine IGF-IR system in medulloblastomas.

In summary, we have detected the IGF-IR and its major signal transduction substrates, IRS-1 and PI3-K, in the majority of paraffin-embedded and frozen sections from patients with medulloblastoma. Protein levels and the intensity of immunostaining for IGF-IR and IRS-1 show strong up-regulation in medulloblastoma biopsies when compared with control, age-appropriate, normal cerebellar tissue. Importantly, 10 of 17 paraffin-embedded biopsies from patients diagnosed with medulloblastoma demonstrated the presence of the phosphorylated form of IGF-IR (pY1136) and subsequent phosphorylation of downstream IGF-IR signaling molecules, Erk-1, Erk-2, and Akt/ PKB. Finally, an inverse pattern for anti-pY1316 and anti-Trk-C immunostaining was demonstrated in 15 of 17 medulloblastoma cases examined. These results strongly suggest the involvement of the IGF-IR system in the development of medulloblastomas and direct our attention to the IGF-IR molecule as a potential therapeutic target in these cerebellar tumors.

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