Efficacy of Transferrin Receptor-targeted Immunotoxins in Brain Tumor Cell Lines and Pediatric Brain Tumors

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ABSTRACT

The efficacy and cytotoxic properties of immunotoxin conjugates directed against the transferrin receptor were examined in cell lines and operative specimens from pediatric brain tumors. Dose-response relationships were assessed for immunotoxin-mediated inhibition of protein synthesis for two immunotoxins, 454A12-rRA and anti-thr-CRM 107. Three target medulloblastoma cell lines (DAOY, D283MED, and D341MED), a glioblastoma (U373), and a neuroblastoma (SH-SYSY) cell line exhibited similar sensitivity to both immunotoxins with IC50 in the 10^-10^-10 M range. The time course of protein synthesis inhibition by the immunotoxins in DAOY cells showed that inhibition by anti-thr-CRM 107 was rapid and apparent by 6 h of incubation. In contrast, a response to 454A12-rRA was not observed until 16 h. Cell viability was decreased 30-40% by 24 h after removing 454A12-rRA (1 x 10^-9 M) and was maximally decreased 70-80% after 3 days. The efficacy of the immunotoxins on a variety of fresh specimens of pediatric brain tumors was also examined. The more aggressive and malignant tumor types such as glioblastoma multiforme and medulloblastoma had low IC50 values (10^-12 M), indicating that these tumors were extremely sensitive to transferrin receptor-targeted immunotoxins. In general, protein synthesis in slow-growing and benign tumors was not as greatly affected by immunotoxins. Immunoblots showed expression of transferrin receptors on the cell lines and tumors which correlated with in vitro sensitivity to immunotoxin. The results demonstrate that two immunotoxins targeted to the transferrin receptor are efficacious in killing brain tumor cell lines and primary tumor cultures at very low concentrations and that highly malignant tumors are especially sensitive to this cytotoxic response.

INTRODUCTION

Central nervous system tumors such as astrocytomas and medulloblastomas are exceeded only by leukemia as the most common form of childhood malignancy; however, they are the leading cause of cancer-related death. These types of tumors often disseminate through cerebrospinal fluid pathways, resulting in leptomeningeal neoplasia. Particularly in the pediatric population, the most common therapy for these tumors, radiation therapy, has unacceptable long-term sequelae. Chemotherapy has also been tried but has minimally altered the survival of pediatric brain tumor patients. Thus, research efforts have focused on developing more specific forms of therapy. ITs are covalently linked conjugates of enzymatically active toxins that are linked with monoclonal antibodies against cell surface antigens (for reviews, see Refs. 1–5) or linked with cell binding proteins such as growth factors, the receptors of which are abundantly expressed on tumor cells. The concept of ITs is to combine the potency of the toxin with the specificity of an antibody against a cell surface antigen or a receptor ligand. The directed toxin enters the cytosol of the target cell and inactivates protein synthesis machinery in a catalytic manner (6).

ITs containing the ricin A chain or CRM 107 are effective in killing tumor cells in vitro and in cultured cell lines (7–14). The transferrin receptor has been utilized as a target for ligand toxin or monoclonal anti-receptor antibody-toxin conjugates (7, 8, 15–20). Transferrin receptors are overexpressed in some cancers, including CNS malignancies, and thus may be a reasonable target for IT therapy. It has been postulated that the expression of transferrin receptors increases during cell proliferation to fulfill the need of the tumor cell for iron.

In vivo studies and clinical trials using ITs to date indicate that they may play an important role in therapy for compartmentalized tumors in the CNS (21–24). This study compares the efficacy of transferrin receptor-targeted ITs in three recently isolated medulloblastoma cell lines (DAOY, D283MED, D341MED) (25), a glioblastoma cell line (U373), and a neuroblastoma (SH-SYSY) cell line in vitro. In addition, we examine the cytotoxic activity of ITs toward primary tumor cell cultures derived from pediatric brain tumor surgical specimens.

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium, Eagle’s minimal essential medium, Hanks’ basal salt solution, penicillin/streptomycin/Fungizone, L-glutamine, bovine serum albumin, 4-chloro-1-naphthol, and iron-stained transferrin were from Sigma Chemical Co. (St. Louis, MO). Tissue culture supplies were from Costar Corp. (Cambridge, MA). 14C]Leucine (specific activity, 314 mCi/mmol) was from Du Pont New England Nuclear (Boston, MA). Monoclonal anti-transferrin receptor antibody was from Amersham (Arlington Heights, IL). Anti-mouse IgG horseradish peroxidase and molecular weight standards were purchased from Bio-Rad (Richmond, CA).

Tumor Cell Lines and Surgical Specimens. RT-4, HL-60, U373, DAOY, D283 MED, and D341 MED were all obtained from the American Type Culture Collection and cultured in the medium suggested by the supplier with 10% FBS in a humidified atmosphere of 5% CO2 at 37°C. The SH-SYSY neuroblastoma cell line was a gift from Dr. Margaret E. Gnegy (Department of Pharmacology, University of Michigan), and cells were cultured as previously described (26). Brain tumor specimens and normal control brain tissue were obtained in the operating room and sterilely placed in RPMI 1640 containing L-glutamine, penicillin/streptomycin, Fungizone, and 10% FBS for transport to the laboratory. The tumor tissue was mechanically dissociated with a razor blade, stirred at room temperature for 1 h, and purified on a Hypaque (Sigma) gradient.

Immunotoxins. 45A12-rRA (Cetus Corp., Emeryville, CA), 45A12 MAh, CRM 107, anti-thr-CRM 107 (Inland Laboratories, Austin, TX), and transferrin-CRM 107 were generously provided by Dr. Richard J. Youle (Surgical Neurology Branch, National Institutes of Neurological Disorders and Stroke). Ricin A chain was purchased from Vector Laboratories (Burlingame, CA). ITs were diluted to appropriate concentrations in phosphate-buffered saline plus 0.1% bovine serum albumin.

In Vitro Cytotoxicity Assay. Inhibition of protein synthesis was used to assay the cytotoxic effect of ITs and was performed according to the method of Johnson et al. (9). In brief, cells were plated in leucine-free RPMI 1640 at a density of 2 x 10^4 cells/well for cell lines and 1 x 10^5 cells/well for primary tumor cells. Monolayer cultures were allowed to reattach overnight before adding ITs. Both suspension and monolayer cultures were washed once with Hanks’ solution and placed in 100 µl of leucine-free RPMI 1640 containing penicillin/streptomycin and Fungizone. ITs were added at the indicated concentrations in a final volume of 11 µl and incubated for 16 h at 37°C. The cells...
were then pulsed with 0.1 μCi of [14C]leucine in 20 μl for 2 h for cell lines or 6 h for primary tumor cultures and harvested onto glass fiber filters with a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA). The filters were dried and counted in a beta scintillation counter. All cytotoxicity assays with the cell lines were performed 2-3 times in sextuplet, and those using the tumor specimens were performed once in triplicate. The results are expressed as a percentage of [14C]leucine incorporation in vehicle-treated control cultures.

**Cell Survival Assay.** Cells were plated as described for cytotoxicity bioassays. After incubation with 1 × 10^-8 M 454A12-rRA for 16 h, the cells were washed free of IT and serially diluted in sextuplet into Eagle’s minimum essential medium with 10% FBS, penicillin/streptomycin, and L-glutamine. At various times up to 7 days, the cells were counted with a hemocytometer in a 1:1 dilution with trypsin blue. Each value represents the mean ± SE of 6 wells counted.

**Immunoblot.** Cell lines and frozen tumor specimens were homogenized on ice in a buffer containing 40 mM Tris-HCl (pH 8), 3 mM MgCl2, 0.32 mM sucrose, 1 mM leupeptin, 1 mM pepstatin, and 1 mM phenylmethylsulfonyl fluoride using a Teflon-glass homogenizer. The homogenates were centrifuged at 500 X g to remove debris. The homogenates (30 μg protein) were diluted in sample buffer and separated on a reducing sodium dodecyl sulfate-polyacrylamide gel (10% acrylamide). The proteins were electrophoretically transferred to Immobilon-P membranes. Blots were incubated in 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl with 0.1% Tween 20 and 1% (w/v) bovine serum albumin (blocking buffer) for 1 h at 4°C. The monoclonal anti-transferrin receptor antibody (Amersham) was diluted by 1:200 in blocking buffer and incubated with the blots overnight at 4°C. After incubation with goat anti-mouse IgG horseradish peroxidase for 8 h at 4°C, the immunoreactive products were visualized using 4-chloro-l-naphthol as the substrate. Protein was quantified by the method of Bradford (28), using bovine serum albumin as a standard.

**RESULTS**

**IT Cytotoxic Activity against Medulloblastoma, Glioblastoma, and Neuroblastoma Cells.** *In vitro* inhibition of protein synthesis was used to assay the cytotoxic activity of two transferrin receptor-directed ITs, 454A12-rRA and anti-tfnR-CRM 107. Fig. 1 (A and B) shows representative dose-response curves for IT inhibition of protein synthesis in human medulloblastoma cell lines DAOY, D283MED, and D341MED. The dose-response curve for the DAOY cells treated with 454A12-rRA was steep, with a maximal inhibition of protein synthesis observed at 10^-6 M and an IC50 of 7.3 × 10^-10 M. The curve for D283MED cells was more shallow with an IC50 of 1 × 10^-9 M. D341MED cells were very sensitive to 454A12-rRA, with an IC50 of 8.5 × 10^-11 M. In contrast, all three medulloblastoma cell lines responded similarly to treatment with anti-tfnR-CRM 107 and displayed a steep dose-response curve, with IC50s of 3-6 × 10^-9 M. Fig. 1 (C and D) shows the dose-response curves for both ITs in U373 human glioblastoma and SH-SY5Y human neuroblastoma cells. The U373 cells were susceptible to 454A12-rRA, with an IC50 of 1.7 × 10^-10 M. The level of protein synthesis in the SH-SY5Y cells was affected to a greater extent than in the U373 cells by 454A12-rRA, with an IC50 of 3.1 × 10^-11 M. Interestingly, even with 10^-6 M IT, the extent of protein synthesis inhibition appeared to saturate at 80%. For anti-tfnR-CRM 107, both the U373 and SH-SY5Y cells showed a similar dose-response curve, with IC50s of 2-3 × 10^-10 M. The anti-tfnR-CRM 107 inhibited protein synthesis by 100% in both cell lines.
Table 1 summarizes the 50% inhibition of protein synthesis values for various cell lines. Bladder cancer cells (RT-4) and HL-60 promyelocytic leukemia cells are susceptible to transferrin receptor-targeted toxins. In general, the IC_{50} values for all of the cell lines tested range from 10^{-9} to 10^{-11} M.

Specificity of IT Cytotoxicity. The effect of unconjugated toxin on protein synthesis in DAOY cells was examined to show the specificity of IT cytotoxicity. Fig. 2 shows that unconjugated RA chain is 1000 times less potent at inhibiting protein synthesis than when it is conjugated with the anti-transferrin receptor Mab 454A12. The inhibition of protein synthesis mediated by 454A12-rRA was blocked by the addition of 1 × 10^{-9} M 454A12 Mab alone, resulting in a dose-response curve shifted to the right that resembles the response observed with RA chain alone. The effect of unconjugated CRM 107 on protein synthesis in a primary cell culture derived from a medulloblastoma surgical specimen is shown in Fig. 3. Compared with anti-tfnR-CRM 107, the CRM 107 is less potent, with an IC_{50} of approximately 7 × 10^{-9} M. Thus, the ITs are 2–3 log units more potent than the unconjugated toxin.

Time Course of IT Inhibition of Protein Synthesis. The kinetics of 454A12-rRA and anti-tfn-CRM 107 inhibition of protein synthesis in DAOY cells were investigated. A response to 454A12-rRA is not observed until 16 h (IC_{50} = 5.8 × 10^{-9} M) of incubation (Fig. 4A), with a maximal inhibition of protein synthesis occurring at 24 h. In contrast, the cytotoxic activity of anti-tfn-CRM 107 is apparent by 6 h of incubation (Fig. 4B). The inhibition of protein synthesis continues to increase at 16, 20, and 24 h. The IC_{50} for anti-tfn-CRM 107 cell cytotoxicity were 8 × 10^{-10} M after 6 h, 7.2 × 10^{-11} M after 16 h, and 2.5 × 10^{-11} M after 20 and 24 h.

Cell Survival after Treatment with IT. Since inhibition of protein synthesis does not necessarily indicate cell death, the effects of IT on DNA synthesis and cell survival were assessed. As measured by [3H]thymidine assay, IT 454A12-rRA inhibited the rate of DNA synthesis in DAOY and U373 cells to an extent similar to that observed for protein synthesis (data not shown). The number of cells excluding trypan blue were counted after treatment of the cells with 454A12-rRA and anti-tfnR-CRM 107 inhibition of protein synthesis continues to increase at 16, 20, and 24 h. The IC_{50} for anti-tfnR-CRM 107 cell cytotoxicity were 8 × 10^{-10} M after 6 h, 7.2 × 10^{-11} M after 16 h, and 2.5 × 10^{-11} M after 20 and 24 h.

Table 1 Cytotoxic activity of transferrin receptor-targeted immunotoxins for various cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} (m)</th>
<th>454A12-rRA</th>
<th>Transferrin-CRM107</th>
<th>Anti-tfnR-CRM107</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-4 (bladder cancer)</td>
<td>1 × 10^{-9}</td>
<td>4 × 10^{-10}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL-60 (promyelocytic leukemia)</td>
<td>1.1 × 10^{-11}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK-N-SH (neuroblastoma)</td>
<td>3.1 × 10^{-11}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH-SY5Y (neuroblastoma)</td>
<td>3.2 × 10^{-11}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U373 (glioblastoma)</td>
<td>1.7 × 10^{-10}</td>
<td>1.6 × 10^{-11}</td>
<td>2.3 × 10^{-10}</td>
<td></td>
</tr>
<tr>
<td>DAOY (medulloblastoma)</td>
<td>7.3 × 10^{-10}</td>
<td>5.1 × 10^{-10}</td>
<td>3.2 × 10^{-9}</td>
<td></td>
</tr>
<tr>
<td>D283 MED (medulloblastoma)</td>
<td>1 × 10^{-9}</td>
<td></td>
<td>5.7 × 10^{-9}</td>
<td></td>
</tr>
<tr>
<td>D341 MED (medulloblastoma)</td>
<td>8.5 × 10^{-11}</td>
<td></td>
<td>4.8 × 10^{-7}</td>
<td></td>
</tr>
</tbody>
</table>

70% decrease in cell viability; however, the U373 cells appeared to recover, since they displayed only a 27% decrease in viability.

Cytotoxic Activity of ITs against Cultured Cells from Pediatric Tumor Specimens. Since the ITs were effective in killing brain tumor cell lines, we examined the efficacy of the ITs in cells cultured from pediatric brain tumor surgical specimens. A variety of tumor types were assayed, ranging from benign tumors such as choroid plexus papilloma to highly invasive and malignant tumors such as medulloblastoma and glioblastoma multiforme (Table 3). Several of the tumor types, such as craniopharyngioma, a recurrent giant cell astrocytoma, and choroid plexus papilloma, were not sensitive to the ITs. The more malignant tumors, such as medulloblastoma and glioblastoma multiforme, were susceptible to the killing activity of ITs, with IC_{50} in the 10^{-12} M range. Interestingly, two low-grade gliomas, pilocytic astrocytomas, responded to ITs and yet are thought to be slow-growing tumors and are not invasive.

Expression of Transferrin Receptors on Tumor Cell Lines and Pediatric Brain Tumor Specimens. The expression of transferrin receptors was examined to determine the relationship with IT sensitivity. Cell homogenates from DAOY, D283MED, D341MED, U373, and SH-SY5Y were prepared, and the proteins were electrophoretically separated under reducing conditions and transferred for Western blot analysis with monoclonal anti-transferrin receptor antibodies. This approach measures both internal and external tfnRs, whereas IT
sensitivity is dependent only on the surface expression of receptors. Fig. 5A shows the 90,000 molecular weight immunoreactive protein present in all of the cell lines examined. The intensity of the staining did not appear to differ extensively between cell lines. In contrast, the homogenates from pediatric brain tumor specimens showed obvious differences in staining intensity of the 90,000 molecular weight immunoreactive product using Western blot analysis (Fig. 5B). The M, 90,000 band was much more prominent in specimen numbers 6 (medulloblastoma), 8 (glioblastoma), 10 (anaplastic ependymoma), 16 (medulloblastoma), and 18 (medulloblastoma), which are all malignant tumors. No immunoreactive products were observed in specimens 4 (choroid plexus papilloma), 5 (craniopharyngioma), and 7 (metastatic congenital mesoblastic nephroma).
IMMUNOTOXIN EFFICACY IN PEDIATRIC BRAIN TUMORS

DISCUSSION

In this study, we have shown that ITs have potent cytotoxic activity against brain tumor cell lines, including medulloblastoma and glioblastoma, and cultures obtained from various pediatric brain tumors. Medulloblastomas are poorly differentiated and are among the most common CNS neoplasms of childhood. Other intracranial tumors common in childhood include astrocytomas and ependymomas. Medulloblastomas and ependymomas are often difficult to treat due to their ability to spread through the cerebrospinal fluid pathways. Recent advances in surgical techniques and radiation therapy have improved survival in children with these tumors. Chemotherapy regimens have also been developed to augment radiation therapy (29-31). There are unacceptable long-term sequelae to radiation therapy and chemotherapy. The unacceptable side effects include a decrease in intellectual function, hypothalamic and pituitary dysfunction, a decrease in stature and development of leukomalacia, and radiation necrosis of the brain.

ITs have properties different from those of conventional chemotherapy agents which render them useful as a complement to or in combination with chemotherapy. ITs have a different mechanism of action, affecting protein synthesis and killing nondividing cells. Most chemotherapeutic drugs, which disrupt DNA synthesis and cell division, require actively proliferating cells to be effective. In addition, those cancer cells expressing resistance to conventional therapies may be susceptible to ITs. The cytotoxicity of ITs is not affected by conditions of hypoxia, an important factor in the resistance observed with radiation and chemotherapy. In addition, because of the high enzymatic activity of the toxins ricin and diphtheria toxin, the entry of one or very few molecules into the cytosol can kill a cell (32).

Ricin and diphtheria toxin consist of two functionally distinct polypeptides linked by a disulfide bond (33). The A chain carries the enzymatic activity, whereas the B chain binds to receptors at the cell surface and contains the internalization functions of the toxin. Ricin toxin acts directly on the ribosomes, where it inactivates the large 60S ribosomal subunit (34). Diphtheria toxin inactivates elongation factor 2, an enzyme required for the translocation of the growing peptide chains from the A site to the P site of the ribosome (35). CRM 107 is an enzyme required for the translocation of the growing peptide chains from the A site to the P site of the ribosome (35). CRM 107 is identical to diphtheria toxin except for a single point mutation in the B chain, which inactivates intrinsic toxin binding (36). CRM 107 killing at a faster rate than 454A12-rRA. This result has been described previously by others in other cell lines (38) and may suggest a greater utility for the CRM 107 conjugate over the ricin A chain conjugate. The two Mabs directed against the human transferrin receptor, 454A12 and anti-tfnR, do not compete with transferrin for binding to the receptor and are thought to be directed against different domains on the same receptor.

Tumor heterogeneity remains a significant obstacle with regard to implications for the therapeutic sensitivity of a tumor to ITs and traditional chemotherapy, as well as metastatic potential (39-42). An ideal target antigen for IT therapy would be expressed only on the tumor cells. However, since this level of specificity may not be attainable, a more realistic approach is to seek antigens expressed at high levels on target cells and low levels on normal cells. The expression of transferrin receptors is related to cellular iron requirements and is thought to represent the proliferative potential of the cell. In normal brain, the most prominent immunostaining for transferrin receptors was observed in the capillary endothelial cells (43). In addition, Recht et al. (20) have shown that immunostaining for transferrin receptor is much more abundant in high-grade astrocytic tumors and glioblastomas than normal brain and that the staining pattern and intensity roughly correlated with increasing tumor grade. The use of transferrin receptors as a target for toxins, first proposed by Trowbridge and Domingo (7), has been shown to be an effective means of killing tumor cells. Transferrin receptors are effectively internalized upon binding of antibody or ligand and are linked with the rate of DNA synthesis (44). It has been suggested that IT therapy targeted to the transferrin receptor in glioblastomas and medulloblastomas would not be affected by antigenic heterogeneity, antigenic modulation, or genetic loss since the receptor is up-regulated by higher iron requirements by the rapidly dividing cells (24). We have examined the effect of the simple addition of iron-loaded transferrin to cultures of DAOY and U373 cells and found a 50% increase in the rate of DNA synthesis.2

A number of requirements must be fulfilled for an immunoconjugate to be of therapeutic value in vivo. The conjugate must remain intact in the circulation for a sufficient period of time, it must be able to penetrate through capillary walls and into tumors, and it must be specific for tumor tissue over normal tissue. In the treatment of brain tumors and other "compartmentalized" tumors, these issues may not be so critical, since conjugates are administered locally in a compartmentalized space, where high concentrations can be reached without great risk to the systemic circulation. ITs may play an important role in the treatment of residual disease, which commonly results in tumor


5 L. A. Martell and K. M. Muraszko, unpublished observation.
recurrence. In the case of tumors which spread through cerebrospinal fluid pathways, ITs may provide a means of controlling dissemination and improving morbidity as well as mortality. Thus, the results suggest that ITs are potent and efficacious in killing aggressive tumors common in childhood and that these agents should be examined in an in vivo model of neoplastic disease.

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