The effect of transforming growth factor-\(\beta_2\)-specific phosphorothioate-anti-sense oligodeoxynucleotides in reversing cellular immunosuppression in malignant glioma

PIOTR JACHIMCZAK, M.D., ULRICH BOGDAN, M.D., JÖRG SCHNEIDER, PH.D., CHRISTIAN BEHL, PH.D., JÜRGEN MEINENSBERGER, M.D., RAINER APPEL, PH.D., RÜDIGER DORRIES, PH.D., KARL-HERMANN SCHLINGENSIEPEN, M.D., and WOLFGANG BRYSCH, M.D.

Department of Neurology and Neurosurgery, University of Würzburg, and Institute of Virology and Immunobiology, Würzburg, and Department of Neurobiology, Max-Planck Institute of Biophysical Chemistry, Göttingen, Germany

This in vitro study was aimed at restitution of transforming growth factor (TGF)-\(\beta\)-mediated suppression of T-lymphocyte activation within malignant gliomas. In early-passage tumor cell cultures of two glioblastomas (HTZ-153 and HTZ-209) and one malignant astrocytoma classified as World Health Organization Grade III (HTZ-243), autologous peripheral blood mononuclear cells were activated by interleukin-1 \(\alpha\) and interleukin-2 in vitro (lymphokine-activated killer cells) and tested for cytotoxic and proliferative activity. In expression studies (Western blot and Northern hybridization) of all three tumors, TGF-\(\beta\) could be detected at the protein and messenger ribonucleic acid (mRNA) levels. A polyclonal anti-TGF-\(\beta\) neutralizing antibody did not enhance lymphocyte proliferation upon stimulation with tumor targets (\(^3\)H-thymidine incorporation) and slightly stimulated lymphocyte cytotoxicity against autologous target cells. Preincubation of target cells for 12 hours with TGF-\(\beta\)-specific phosphorothioate-anti-sense oligodeoxynucleotides (S-ODNs) did, however, enhance lymphocyte proliferation up to 2.5-fold and autologous tumor cytotoxicity up to 60%, compared to controls not treated with S-ODN's. Incubation of tumor cells with TGF-\(\beta\)-specific S-ODN's resulted in decreased TGF-\(\beta\)-specific immunoreactivity in cultured glioma cells. In reduced TGF-\(\beta\): protein concentration (Western blot), and in a change in the expression pattern of TGF-\(\beta\): mRNA's. These observations may have implications for in vivo and in vitro activation of a cellular immune response against autologous malignant glioma cells.

KEY WORDS • transforming growth factor-\(\beta\) • cellular immunology • cytokine • malignant glioma • phosphorothioate-anti-sense oligodeoxynucleotide • interleukin-2 • lymphokine-activated killer cell

Depression of immune responsiveness has been well documented in patients with malignant gliomas; these patients express a variety of immunological deficiencies including cutaneousergy, depressed antibody production, and diminished numbers of circulating T cells.\(^5\)\(^,\)\(^6\) More recent studies indicate that these impairments may result from malfunctions in physiological pathways required for normal T-cell activation and from quantitative and qualitative defects in T-cell subsets.\(^1\)\(^,\)\(^2\)

Prostaglandin E\(_2\) (PGE\(_2\)) has been recognized as an immunosuppressive substance produced by glioma cells.\(^1\)\(^,\)\(^2\) The suppressive effect of PGE\(_2\) is mediated in part by inhibition of interleukin (IL)-2 production and transferrin receptor expression.\(^7\) The transforming growth factor (TGF)-\(\beta\) family, the second known immunosuppressive factor produced by malignant gliomas,\(^6\)\(^,\)\(^13\)\(^,\)\(^14\)\(^,\)\(^8\) is characterized by a wide range of immunoregulatory properties including depression of T cell-mediated tumor cytotoxicity in vitro,\(^20\)\(^,\)\(^29\) and in vivo,\(^20\)\(^,\)\(^29\) inhibition of IL-2- or IL-1-dependent T-cell proliferation,\(^2\) lymphokine-activated killer (LAK) and natural killer (NK) cell activation,\(^3\) generation of cytotocytic macrophages,\(^2\) and B-cell function.\(^2\) Moreover, TGF-\(\beta\) also inhibits constitutive and induced expression of the IL-1 and IL-2 receptor,\(^2\) human lymphocyte antibody (HLA)-DR antigen, Fc receptor,\(^1\)\(^,\)\(^3\)\(^,\)\(^4\) and transferrin receptor.\(^2\) The IL-2- and IL-4-dependent S-
Reversal of cellular immunosuppression in gliomas

phase progression is inhibited,\(^2\) and synthesis of IL-2, IL-6, interferon (IFN)-\(\gamma\), and tumor necrosis factor (TNF)-\(\alpha\) is suppressed.\(^2\)

In human gliomas in particular, TGF-\(\beta\), which was initially isolated from glioblastoma cell culture supernatants and described as a glioblastoma-derived T-cell suppressor factor,\(^3\) is associated with immunosuppression.\(^5\) It is known from neuropathological studies that patients with tumor-infiltrating lymphocytes (TIL's) may have slightly prolonged survival times;\(^27\) however, in most patients TIL's have no lasting effect upon the clinical prognosis, which may be due to endogenous production of TGF-\(\beta\).\(^2,20,23\)

In this study, we investigated whether modified anti-sense oligodeoxynucleotides (phosphorothioate-anti-sense oligodeoxynucleotides (S-ODN's)) may reverse TGF-\(\beta\)-mediated suppressive effects upon the proliferative and cytotoxic functions of autologous lymphocytes in glioma patients.

Materials and Methods

Characterization of Tumor Cells (Autologous Target Cells)

Tumor cells from three patients with high-grade malignant gliomas (HTZ-153 and HTZ-209 glioblastomas, and HTZ-243 World Health Organization Grade III astrocytoma) and their respective autologous lymphocytes were studied. Standard tumor cell cultures were established in Dulbecco’s minimum essential medium (MEM), containing 20% fetal calf serum, 1 mM L-glutamine, MEM vitamin solution, and nonessential amino acids, as described previously.\(^1\) Other target cells included K562, an NK cell-sensitive erythroleukemia cell line.\(^6\) Tumor cell cultures were characterized by immunocytochemistry employing the peroxidase-antiperoxidase method, as described previously, in tissue culture slides with the mono- or polyclonal antibodies to glial fibrillary acidic protein (GFAP), cytokeratin, neurofilament, desmin, vimentin, neuron-specific enolase, HLA-DrQ, W6/32 (Class I antigen), \(\beta\)-microglobulin, fibronectin, laminin, Ki 67, and anti-TGF-\(\beta\).\(^4\) The TGF-\(\beta\)-specific immunocytochemistry was performed after incubation of glioma culture slides with TGF-\(\beta\)-specific S-ODN’s (final concentration 1 \(\mu\)M) and non-sense S-ODN-treated controls (final concentration 1 \(\mu\)M) for 48 hours.

Characterization of Lymphocytes (Effector Cells)

Peripheral blood mononuclear cells (PBMC’s) from the three glioma patients were isolated from heparinized venous blood at the time of surgery employing gradient centrifugation as described previously, and cryopreserved in liquid nitrogen under standard conditions. Lymphocytes were cultured in RPMI 1640 with 10% human pooled AB-serum and 2 mM L-glutamine. Native and activated (see below) PBMC’s were characterized by immunocytochemistry employing alkaline phosphatase and monoclonal anti-alkaline phosphatase complexes (APAAP method), as described previously,\(^6\) with monoclonal antibodies to the following antigens: CD3, CD4, CD8, CD16, CD25, and HLA-DR.

Generation of LAK Cells

As the proliferative and cytotoxic response of PBMC’s from glioma patients is suppressed, cells (2 \(\times\) \(10^8\) cells/ml) were preactivated in vitro for 6 days with IL-1\(\alpha\) (10 U/ml) and IL-2 (100 U/ml) in 48-well flat-bottom tissue culture plates.†

Proliferation Assay

In mixed lymphocyte tumor cell (MLTC) cultures, lethally irradiated (60 Gy, \(\rho\) Co-source) tumor cells (15 \(\times\) \(10^8\)) served as stimulators and were cocultivated with preactivated mononuclear cells (LAK cells, 25 \(\times\) \(10^8\), see above) for 6 days in 96-well flat-bottom tissue culture plates.‡ In MLTC culture experiments, the same culture medium conditions were employed as during preactivation. In anti-sense experiments, TGF-\(\beta\)-specific S-ODN’s and non-sense oligodeoxynucleotides (see below) were added to the cultures 12 hours before MLTC assay. Anti-TGF-\(\beta\) neutralizing anti-

---

*Fetal calf serum supplied by Seromed, Berlin, Germany; amino acids obtained from Gibco, Paisley, Scotland.
†K562 cell line supplied by American Type Culture Collection, Rockville, Maryland.
‡Labeck tissue culture slides obtained from Miles Laboratories, Naperil, Illinois; Ki 67 supplied by Dakopatts, Glostrup, Denmark; anti-TGF-\(\beta\) supplied by R & D Systems, Inc, Minneapolis, Minnesota.
§Ficoll-Hypaque centrifugation system obtained from Pharmacia, Uppsala, Sweden.

---

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HLA</td>
<td>human lymphocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LAK</td>
<td>lymphokine-activated killer</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MLTC</td>
<td>mixed lymphocyte tumor cell</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PAP</td>
<td>peroxidase-antiperoxidase</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>S-ODN</td>
<td>phosphorothioate anti-sense oligodeoxynucleotide</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIL</td>
<td>tumor-infiltrating lymphocyte</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
</tbody>
</table>

---

* Alkaline phosphatase and anti-alkaline phosphatase complexes supplied by Dakopatts GmbH, Hamburg, Germany; monoclonal antibodies obtained from Becton Dickinson, Mountain View, California.
* Interleukin-1\(\alpha\) supplied by R & D Systems, Inc., Minneapolis, Minnesota; IL-2 obtained from Bectest AG, Frankfurt, Germany; culture plates manufactured by Costar, Cambridge, Massachusetts.
* Tissue culture plates manufactured by Nunc, Copenhagen, Denmark.

J. Neurosurg. / Volume 78 / June, 1993
bodies§ were added to the culture 2 hours before MLTC assay.

**Cytotoxicity Assay**

Cytotoxicity experiments were performed with a modified microcytotoxicity assay as described previously.¹² Briefly, target cells (1.5 × 10⁴) were seeded into 96-well flat-bottom tissue culture plates; 12 hours later, TGF-β₂-specific S-ODN’s and non-sense oligodeoxyribonucleotides (anti-sense controls) were added to the culture. Anti-TGF-β neutralizing antibodies and normal rabbit serum][ were added to the culture 22 hours after plating. Various target-effector ratios (1:1, 1:5, and 1:10) of preactivated effector cells (LAK cells) were irradiated (30 Gy), and added to respective targets 24 hours after plating for 3 days under standard culture conditions (RPMI 1640 culture medium containing 10% pooled AB-serum and 2 μM L-glutamine). No cytokines were added to the culture during cytotoxicity experiments. An incubation period of 3 days was selected as statistical evaluation of data turned out to be optimal at that time. Killing of target cells was demonstrated by incorporation of trypan blue dye (data not presented). Target cell proliferation in LAK cell-treated targets versus anti-sense, non-sense, or untreated (no LAK cells) controls (glioma targets) was assessed with a standard ³H-thymidine incorporation assay (6³H-thymidine, 1 μCi/well, specific activity 27 Ci/mmol). Liquid scintillation counting of ³H-thymidine incorporation was performed after 18 hours of incubation as previously described.⁴ The specific cytotoxicity was calculated as:

\[
\text{specific cytotoxicity} = \frac{\text{cpm}_{\text{treated}} - \text{cpm}_{\text{probe}}}{\text{cpm}_{\text{control}}} \times 100.
\]

**Northern and Western Blot Analysis**

Cytosplasmic ribonucleic acid (RNA) was prepared by lysing glioma cells treated with TGF-β₂-specific S-ODN’s (final concentration 1 μM) for 48 hours and untreated controls in buffer containing 0.5% NP-40 as described previously.¹³ For Northern hybridization, aliquots of denatured RNA (20 μg) were separated by electrophoresis on 1% agarose-formaldehyde gel. The quality and quantity of immobilized RNA was verified by methylene blue staining of the membranes* after transfer.¹⁵ Blots were hybridized overnight with specific TGF-β₁, or TGF-β₂-synthetic oligonucleotide probes (40 oligomer) 5’ labeled with (γ⁻³²P)-adenosine triphosphate employing T4 polynucleotide kinase, and exposed to x-ray film.†

For Western blot analysis, TGF-β₂-S-ODN-treated (48 hours, final concentration 1 μM) and untreated glioma cells were grown in medium containing 10% fetal calf serum, then washed and further cultured in defined serum-free medium for 24 hours. The cells were lysed employing a lysis buffer containing NP-40 as described previously.¹⁶ Total cellular protein (30 μg) was loaded onto each lane of a 12% polyacrylamide/sodium dodecyl sulfate gel.¹⁷ Fractionated proteins were then electrophoresed to a nitrocellulose membrane for 20 minutes at 0.8 mA/sq cm as described previously.¹⁸ Filters were probed with a polyclonal antibody to TGF-β₂ employing standard techniques, with pure TGF-β₂ (50 ng) serving as the control.‡

**Phosphorothioate-Modified Anti-Sense Oligodeoxyribonucleotides**

The TGF-β₂-specific anti-sense oligodeoxyribonucleotides (anti-sense direction of TGF-β₂ mRNA primer sequence: oligonucleotide sequence: CAGCACACAG-TAGT) and a randomized non-sense sequence with the same GC content as the specific S-ODN’s (non-sense oligonucleotide sequence: GTCCCTATACGAAACG) were synthesized on an Applied Biosystems Model 380 B deoxyribonucleic acid (DNA) synthesizer, as described previously.¹⁹ The S-ODN’s were removed from the solid support with 33% ammonia. Oligonucleotides still bearing the 5'-trityl-protecting group were purified by reverse-phase high-performance liquid chromatography using a C8-column chromatograph,§ 0.1 M tetraethylammonium amide (pH 7) and acetonitrile as solvents, and a gradient 3% to 35% acetonitrile over 30 minutes, linear. The trityl-bearing fraction of oligonucleotides, corresponding to the full-length product, was detritylated in 80% acetic acid/ethyl alcohol for 20 minutes, and concentrated. The concentrated product was washed with diethyl ether, desalted on a Sephadex G25 column,† ethanol-precipitated twice, and finally diluted in 0.1 M Tris/HCl (pH 7.6). The S-ODN’s were judged from polyacrylamide gel electrophoresis to be more than 85% full-length material.

**Results**

**Characterization of Tumor Cells**

All glioma cell cultures expressed GFAP, TGF-β₂, vimentin, and HLA-Class I antigens, as well as β₁-microglobulin, fibronectin, and KI 67. Inconsistent expression was found with desmin HLA-Class II antigen (positive for the HTZ-209 cells) and neuron-specific enolase (positive for the HTZ-209 and HTZ-243 cells). No expression was found for cytokeratin, laminin, or neurofilaments, indicating the glial origin of these tumor cells.

**Western and Northern Blot Analysis**

Western blot analysis of tumor cell lysates revealed that HTZ-153, HTZ-209, and HTZ-243 cells produced TGF-β₂ protein (Fig. 1). Northern blot analysis of cytoplasmic RNA’s from all three tumors revealed evidence of TGF-β₂ mRNA expression....

---

§ Anti-TGF-β neutralizing antibodies supplied by R & D Systems, Inc., Minneapolis, Minnesota.
* Hybond-N membranes supplied by Amersham/Buchler, Braunschweig, Germany.
† Probes obtained from Oncogen Science, Seattle, Washington; T4 polynucleotide kinase supplied by Pharmacia, Freiburg, Germany.

---

P. Jachimecz, et al.
Reversal of cellular immunosuppression in gliomas

expression of TGF-β1 (2.3 kb) and TGF-β2 (4.1 kb) (Fig. 2). The expression of TGF-β1 was fairly well represented in all three tumors (Fig. 2 left); however, the HTZ-209 tumor cell displayed a faint TGF-β2 signal compared to the remaining tumor cell lines (Fig. 2 right).

Modulation of TGF-β Expression by Treatment With TGF-β-Specific S-ODN’s

The effects of TGF-β-specific S-ODN treatment upon TGF-β1 mRNA and protein expression in glioma cells were analyzed by Northern and Western blot and immunocytochemistry. Northern blot analysis of glioma cells treated with TGF-β1-specific S-ODN’s (48 hours, final concentration 1 μM) yielded inconsistent results; the HTZ-153 cells displayed an increase in TGF-β1 expression, whereas the HTZ-209 and HTZ-243 cells showed no detectable expression following S-ODN treatment (Fig. 3). Western blot analysis revealed a decreased TGF-β1-specific signal for all three tumors after S-ODN treatment (Fig. 1).

Immunostaining of glioma cultures treated with TGF-β1-specific S-ODN’s (48 hours, final concentration 1 μM) revealed a decrease in TGF-β1-dependent immunoreactivity compared to non-sense S-ODN-treated and -untreated controls for all three tumors. Controls with normal mouse serum and human AB-serum were negative for TGF-β1 expression (data not presented).

Characterization of Lymphocytes

Autologous effector lymphocytes employed in the following experiments on tumor-dependent lymphocyte proliferation and glioma cytotoxicity were characterized by conventional lymphocyte differentiation antigens. The characterizations are displayed in Table 1. The cell populations reflect the phenotype of lymphocyte subsets of native (Day 0) and activated (Day 6) effector cells employed in proliferation and cytotoxicity experiments. The percentage of CD3+ cells increased up to 55% during culture. The same was true for CD4+ (up to 80%), CD8+ (up to 18%), and CD25+ (up to 60%) cells: the fraction of CD16+ cells increased to a maximum of 50% in the HTZ-243 cell line during the first 6 days of culture.

Table 1

<table>
<thead>
<tr>
<th>Antigen</th>
<th>HTZ-153</th>
<th>HTZ-209</th>
<th>HTZ-243</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>52</td>
<td>61</td>
<td>54</td>
</tr>
<tr>
<td>CD4+</td>
<td>39</td>
<td>52</td>
<td>44</td>
</tr>
<tr>
<td>CD8+</td>
<td>15</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>CD16+</td>
<td>15</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>CD25+</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>54</td>
<td>70</td>
<td>36</td>
</tr>
</tbody>
</table>

* Autologous native PBMC’s (Day 0) and PBMC’s activated for 6 days with interleukin (IL)-1α and IL-2 (lymphokine-activated killer cells) were stained with monoclonal antibodies to cell-surface antigens using alkaline phosphatase and monoclonal anti-alkaline phosphatase complexes (APAAP technique). Numbers are percentages of stained cells/500 cells counted.

J. Neurosurg. / Volume 78 / June, 1993
Cytotoxicity Experiments

Native PBMC's of the tumor patients investigated in our study expressed low cytotoxicity to autologous targets (below 20% at the target:effector ratio of 1:10, data not presented). Preliminary experiments disclosed that preactivation of autologous effector PBMC's was most effective when cells were incubated with 10 U/ml IL-1α and 100 U/ml IL-2 for 6 days (data not shown). These LAK cells were employed in all further cytotoxicity/proliferation experiments.

At a target:effector ratio of 1:10, LAK cells achieved a cytotoxic activity of up to 25% in the autologous target system (Fig. 4). Preincubation of tumor cells with TGF-β neutralizing antibody (final concentration 100 μg/ml) resulted in a cytotoxicity of 30% to 50% (5% to 30% increase above the untreated controls). When tumor cells were preincubated with TGF-β-specific S-ODN's, cytotoxicity increased in a dose-dependent fashion to a maximum of 79% (5 μM S-ODN's, 25% to 60% increase above untreated controls) and 67% (1 μM S-ODN's, 15% to 45% increase above untreated controls). Non-sense control S-ODN's had no effect upon cytotoxicity of autologous lymphocytes (data not shown). All three effector cell populations expressed NK cell activity as detected by cytotoxicity assay against the K 562 cell line, ranging from 60% to 75% (data not presented).

Proliferation Experiments

Lymphocyte proliferation upon stimulation with autologous tumor cells (MLTC) treated with TGF-β-specific S-ODN's was increased in HTZ-153 (Fig. 5 left) and HTZ-209 (Fig. 5 center) tumor cells; however, no effect was observed in HTZ-243 tumor cells (Fig. 5 right). Non-sense S-ODN's (final concentration 1 μM) did not alter lymphocyte proliferation. Effects of TGF-β-specific S-ODN's were observed in a dose-dependent fashion from 0.1 to 1 μM; higher concentrations (5 μM) displayed nonspecific toxicity toward PBMC's and tumor cells. The proliferation of PBMC's in S-ODN-treated MLTC's was consistently lower for oligonucleotide concentrations above 1 μM. High concentrations of TGF-β neutralizing antibody (100 μg/ml) did not enhance lymphocyte proliferation (data not shown). The TGF-β-specific anti-sense S-ODN's had an inhibitory effect upon proliferation of either cultured lymphocyte populations (marginal effect) or autologous target cells, achieving a maximum of 75% at an S-ODN final concentration of 5 μM. Less profound inhibitory effects were observed with randomized control non-sense S-ODN's (average 20%, up to 40%, final concentration 5 μM).

Discussion

The effect of TGF-β-specific S-ODN's on human T-cell proliferation and cytotoxicity upon stimulation with autologous cultured gliaoma cells was investigated. We demonstrated that TGF-β-derived S-ODN's may specifically inhibit protein expression of TGF-β in gliaoma cells. In addition, TGF-β-specific S-ODN's reverse to a significant degree the immunosuppressive effects of TGF-β upon T-cell proliferation and cytotoxicity.

It has been shown previously that the T-cell response in brain-tumor patients is clearly reduced and that TIL's have only a marginal impact upon tumor progression in individual patients. Isolated TIL's from brain tumors are functionally incompetent:14 these immunosuppressive effects have been attributed to in vitro and in vivo TGF-β expression.2,14,20,23,26,31 Lymphocytes from patients with malignant gliomas will not readily kill autologous tumor targets in conventional cytotoxicity assays;14 we therefore preactivated the lymphocytes in vitro with a number of different cytokines (IL-1, IL-2, IL-4, IL-6, IFN-γ, TNF-α, and phytohemagglutinin, individual data not presented). As the best results in cytotoxicity and lymphocyte proliferation assays were obtained with a combination of IL-1 (10 IU/ml) and IL-2 (100 IU/ml), all further experiments have been performed with this preactivation mode. As may be suggested from the mode of preactivation, effector cell populations are therefore preferentially composed of LAK cells (Table 1, Day 6).12

It is probable that TGF-β has a stronger negative impact upon induction of LAK cells and specific CD8+ cells19,20,29 than upon the cytotoxic effector phase. The function of TGF-β has been blocked previously by the application of TGF-β-specific neutralizing antibodies; however, the effect was incomplete, as has been presently confirmed with regard to cytotoxicity (Fig. 4) and proliferation using high concentrations of TGF-β neutralizing antibody (up to 100 μg/ml). Therefore, it seemed logical to employ anti-sense technology to block TGF-β protein synthesis at the translation level.

P. Jachimczak, et al.
Reversal of cellular immunosuppression in gliomas

Fig. 5. Graphs demonstrating dose-dependent effects of transforming growth factor (TGF)-β2-specific and non-sense phosphorothioate-anti-sense oligodeoxynucleotides (S-ODN’s) on proliferation of lymphocytes, glioma cells, and lymphocytes cocultured with autologous glioma cells in HTZ-153 tumor cells (left), HTZ-209 tumor cells (center), and HTZ-243 tumor cells (right). Peripheral blood mononuclear cells (PBMC’s) were pretreated for 6 days with interleukin (IL)-1α and IL-2 and incubated for an additional 6 days with irradiated (60 Gy) autologous tumor cells and TGF-β2-specific (crosses) and non-sense (diamonds) S-ODN-treated glioma cells. Simultaneously, part of the pretreated PBMC’s (lymphocytes) and glioma cells (tumor) was incubated with TGF-β2-specific S-ODN’s (solid circles and solid squares, respectively) and non-sense S-ODN’s (open circles and open squares, respectively) for 3 days to evaluate putative direct effects of S-ODN’s upon effector or target cells alone. Data are means of triplicate samples, and vertical bars represent the standard error of the means.

Oligodeoxynucleotides designed to hybridize specifically to mRNA or DNA may be used to study gene expression and the function of proteins coded by these genes. Cellular uptake of S-ODN’s may be mediated by receptor-mediated, adsorptive, or fluid-phase endocytosis or diffusion, as described previously. As modified anti-sense oligonucleotides are more stable to enzyme degradation (ribonuclease) than conventional oligonucleotides, we preferred phosphorothioate-modified S-ODN’s for our experiments.

It has already been shown that S-ODN’s are able to specifically inhibit human immunodeficiency virus and herpes simplex type 2 virus replication, c-erb B-2 proto-oncogene, and TGF-β1 expression. When unmodified anti-sense oligonucleotides specific for basic fibroblast growth factor mRNA were used in experiments, growth of transformed human astrocytes was inhibited. In our experiments, we have partly reversed the immunosuppressive effects of glioma-associated TGF-β2 on proliferation and cytotoxicity of autologous effector lymphocytes (Figs. 4 and 5). In addition, TGF-β2-specific immunoreactivity in glioma tissue culture slides (data not shown) and Western blot analysis (Fig. 1) were clearly reduced after a short, single treatment with TGF-β2-specific S-ODN’s. However, in Northern blot analysis, the expression pattern of TGF-β2-specific S-ODN-treated glioma cultures was inconsistent: TGF-β2 mRNA expression was decreased in HTZ-209 and HTZ-243 cells, but increased in HTZ-153 glioma cells (Fig. 3). As TGF-β2 is functionally blocked at the protein synthesis level by S-ODN treatment, two additional regulatory events may be hypothesized. If TGF-β2-specific S-ODN’s interact with DNA, TGF-β2 transcription will be reduced or stopped, resulting in a decrease of TGF-β2-specific mRNA (as demonstrated in HTZ-209 and HTZ-243 cells). If there is no interaction of S-ODN’s with DNA, blocked TGF-β2-specific mRNA may lead to a secondary increase of transcriptional activity of DNA (TGF-β2-specific mRNA), which may result in increased TGF-β2-mRNA (as demonstrated in HTZ-153 cells).

In one malignant astrocytoma cell culture (HTZ-243), proliferation of lymphocytes was not increased after 6 days of treating MLT C’s with S-ODN’s (Fig. 5 right), although this tumor produced TGF-β at the mRNA and protein level. Long-term cultures (data not shown) suggest that S-ODN’s at a 1-μM concentration may be toxic for these lymphocytes. Interestingly, in cytotoxicity experiments these effects were not observed (Fig. 4), probably because of a shorter time exposure of lymphocytes to S-ODN’s (3 days). Nonspecific toxicity of S-ODN’s was observed in all three tumor and lymphocyte experiments with a final concentration of 5 μM and culture time exceeding 3 days.

The increase in cytotoxicity of HTZ-209 effector cells after S-ODN treatment was less profound than that of the other gliomas; this tumor displayed low TGF-β expression, although the TGF-β protein level was similar to the other tumors. In addition, HTZ-209 cells expressed constitutively high levels of HLA-DR antigen (Labtek tissue culture slides; characterization of tumor cells via fluorescence-activated cell sorter analysis, data not shown). The functional deficit of TGF-β in this tumor may be reflected by high HLA-DR expression. It has been shown previously that TGF-β2 may suppress HLA-DR antigen expression in vitro. We hypothesize that this interaction may play a role in vivo: although TGF-β2 was detected in sufficient amounts at the protein level, our data (low message, low effect of specific S-ODN’s on cytotoxicity) indicate a less profound biological function of this protein, which may be due to preferential production of latent TGF-β2 in HTZ-209 cells. Constitutive expression of HLA-DR antigen, associated with a low endogenous TGF-β production or function, may perhaps result in a better clinical prognosis (the patient from whom the HTZ-209 tumor cells were collected is still alive 14 months after surgery).
Conclusions

The significant finding of this study is that TGF-β-mediated immunosuppression in glialoma cell cultures may be overcome by use of modified anti-sense oligodeoxynucleotides. In future studies, it may be useful to examine the influence of TGF-β-specific S-ODN’s on the generation of cytotoxic lymphocyte clones against autologous tumor cells. However, it should not be ignored that specific S-ODN’s have toxic side effects that may vary on an individual basis and depend on time of exposure; the application range of S-ODN’s may therefore be relatively narrow.

Acknowledgments

We thank W. Sebald for donating IL-4 and IL-6, U. Schwulera for donating IL-2, and T. Hübner and K. V. Toyka for critical review of the manuscript. The excellent technical assistance of A. Dekant and B. Hessdörfer is thankfully acknowledged.

References

28. Potts JD, Dagle JM, Walder JA, et al: Epithelial-mesenchymal transformation of embryonic cardiac endodermal cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor β3. Proc Natl Acad...
Reversal of cellular immunosuppression in gliomas

Sci USA 88:1516-1520, 1991

Manuscript received May 8, 1992. Accepted in final form November 16, 1992.
This work was supported by Grant Nos. 0319500 A and 01GA88030 from the Bundesministerium für Forschung und Technologie, Germany.
Address reprint requests to: Ulrich Bogdahn, M.D., Department of Neurology, University of Würzburg, Tumorbiologie Laboratory, Josef-Schneider Strasse 11, D-8700 Würzburg, Germany.