Soluble Decoy Receptor 3 Is Expressed by Malignant Gliomas and Suppresses CD95 Ligand-induced Apoptosis and Chemotaxis

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ABSTRACT

Decoy receptor 3 (DcR3) is a newly identified soluble protein that binds to CD95 ligand (CD95L) and inhibits its proapoptotic activity. Here we report that DcR3 is expressed by the majority of long-term and ex vivo malignant glioma cell lines as well as in human glioblastoma in vivo. Expression of DcR3 correlates with the grade of malignancy: 15 of 18 (83%) glioblastomas (WHO grade IV) but none of 11 diffuse astrocytomas (WHO grade II) exhibited DcR3 immunoreactivity. We also demonstrate that human malignant glioma cells engineered to release high amounts of DcR3 into the cell culture supernatant are protected from CD95L-induced apoptotic cell death. In contrast, DcR3 does not confer protection from the death ligand Apo2 ligand (TRAIL). Importantly, ectopic expression of DcR3 resulted in substantial differences in immune cell infiltration in the 9L rat gliosarcoma model. Thus, the infiltration of CD4+ and CD8+ T cells as well as microglia/macrophages into glioma was substantially decreased in DcR3-producing tumors compared with control tumors. Chemotaxis assays revealed that DcR3 counteracts the chemotactic activity of CD95L against microglial cells in vitro. These findings suggest that DcR3 may be involved in the progression and immune evasion of malignant gliomas.

INTRODUCTION

Glioblastomas are the most common malignant brain tumors. Despite considerable efforts to improve neurosurgery and radiochemotherapy, only few patients survive longer than 18 months. The reasons for the poor response of gliomas to radiotherapy and chemotheraphy are not entirely clear. Diverse cellular mechanisms of resistance to induction of cell death and strategies to suppress immune responses are important for glioma progression (1). Glioma-associated local and systemic immune suppression may be the result of the production of immune modulating factors such as transforming growth factor-β (2). The CD95 (Fas/APO-1) system may be involved in the immune response to malignant glioma as well. Cytotoxic T cells and natural killer cells have the ability to kill tumor cells by membrane-bound CD95L (3). Soluble CD95L may also exert proinflammatory antitumor effects through its chemotactic activity against monocytes and neutrophils (4). However, tumor cells develop strategies to evade the CD95-mediated immune attack. Malignant tumor cells may acquire resistance to CD95L by inhibiting the intracellular CD95-mediated signaling cascade, e.g., by FLIP (5–7). Alternatively, the interaction of CD95 and CD95L can be blocked by shedding of soluble CD95, which binds to and neutralizes CD95L (8). This pathway is unlikely to be of significance in human gliomas (9).

Recently, a soluble decoy receptor for CD95L, DcR3 (also known as TR6 and M68), has been identified (10–12). DcR3 is a 35-kDa protein that lacks a transmembrane domain and is secreted into the extracellular space. DcR3 binds to the ligands CD95L and LIGHT, thereby neutralizing their proapoptotic actions (10, 12). It has been demonstrated that DcR3 frequently is overexpressed by malignant tumors arising from lung and gastrointestinal tract (10, 11). Moreover, Pitti et al. (10) detected a high percentage of DcR3 gene amplifications in colon and lung carcinomas. Thus, it has been postulated that CD95/CD95L interactions might limit cancer growth and that cells expressing higher levels of DcR3 are more likely to escape elimination via the CD95/CD95L system.

In this study, we provide evidence that DcR3 may be involved in the progression of malignant glioma. We demonstrate that human malignant gliomas express DcR3 in vivo, that enhanced expression of DcR3 suppresses CD95L-induced apoptosis in vitro, and that DcR3-expressing glioma xenografts are less prone to immune cell infiltration in vivo.

MATERIALS AND METHODS

Materials. Lomustine (CCNU) was obtained from Medac (Hamburg, Germany), teniposide was provided by Sandoz Pharma AG (Basel, Switzerland), and cisplatin and cycloheximide were from Sigma (Deisenhofen, Germany). Soluble CD95L was obtained from murine CD95L-transfected N2A neuroblastoma cells (13). Purified human Apo2L (TRAIL) was prepared as described (14; Genentech, CA). Rat 9L gliosarcoma and human U251MG and U373MG malignant glioma cells were purchased from American Type Culture Collection (Rockville, MD). Human malignant glioma cell lines LN-229 and LN-18 were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland). N9 cells are mouse microglial cells that have been immortalized with an oncoregic retrovirus (15).

Cell Culture, Transfections, and Cytotoxicity Assays. The glioma cells were maintained as described (13). Doubling times were determined during logarithmic growth in 24-well plates; 103 cells were seeded per well, and cell counts were obtained daily for 7 days by trypan blue exclusion. Acute cytotoxic cell death assays were performed as described (16). Glioma cell sublines stably expressing human DcR3 were generated by cotransfecting the cells with the DcR3-pRK5 plasmid (10) and the pcDNA3 neo control plasmid by the SuperFect method (Qiagen, Hilden, Germany). The cells were selected with G418 (500 µg/ml), starting 48 h after transfection. All experiments were carried out with pooled transfectants to avoid cloning or selection artifacts. Cell culture supernatants were harvested after incubating the cells for 24 h with serum-free DMEM medium. For some experiments, the conditioned medium was concentrated in centrifugal filter devices (Millipore, Eschborn, Germany). One unit of DcR3 activity was defined as the amount of DcR3-containing supernatant that neutralized the effects of one unit of CD95L, defined as the concentration required to induce half-maximal (50%) cell death in LN-18 cells (13).

Preparation of Primary Glioma Cell Cultures. Human brain tumors were obtained from patients with glioblastoma who underwent surgery for tumor resection. After tumor removal, the tissues were placed immediately in Petri dishes, minced mechanically, and digested enzymatically using collagenase (1 h, 37°C). Subsequently, the dissociated cells were filtered through 100 µm Cellstrainer (Falcon, Becton Dickinson, Heidelberg, Germany).
μm cell striainers to remove tissue debris. After centrifugation and lysis of erythrocytes by treatment with hypotonic water, the glioma cells were washed and resuspended in full medium (DMEM). Conditioned medium was harvested after no more than five passages.

**Animal Studies.** All animal work was carried out in accordance with the NIH guidelines “Guide for the Care and Use of Laboratory Animals.” F344 (Fischer) rats (Charles River, Sulzfeld, Germany) were anesthetized by i.p. injection of 7% chloral hydrate before all procedures. For intracranial implantation, the rats were placed in a stereotactic fixation device (Stoelting, Wood Dale, IL), and a burr hole was drilled in the skull 2.5 mm lateral to the bregma. The needle of a Hamilton syringe (Hamilton, Darmstadt, Germany) was introduced to a depth of 5.5 mm. Glioma cells (5 × 10^6 cells in 5 μl of PBS) were injected into the right striatum as described (17). Subsequently, the rats were observed at regular intervals, and all rats were sacrificed 14 days after the implantation of tumor cells. The brains of sacrificed rats were removed and quickly frozen on liquid N2 and stored at −80°C. Cryostat sections (10 μm) were stained with H&E or, alternatively, with thionine according to standard protocols. For the assessment of tumor volume, cryostat sections were obtained at regular intervals, routinely stained with H&E, and subjected to an analysis of tumor volumes by MCID software (Imaging Research Inc., Ontario, Canada).

**Immunoblot Analysis.** Glioma cells (5 × 10^6) were cultured in serum-free DMEM for 24 h. The supernatants were harvested and subsequently concentrated by centrifugal filter devices (Millipore). The supernatants of freshly isolated ex vivo glioma cells were prepared accordingly. Proteins (10 μg/lane) were separated on polyacrylamide gels (15%) and blotted onto nitrocellulose standard procedures. The membranes were washed, incubated with primary antibody (anti-hDcR3; 2 μg/ml), washed, and incubated with secondary antibody (antimouse IgG; Amersham, Braunschweig, Germany). Enhanced chemiluminescence reagents (Amersham) were used for detection. Several monoclonal antibodies were raised against DcR3 by immunizing mice with a human DcR3-Fc fusion protein. Specific interaction with DcR3 was confirmed by immunoblot with purified recombinant soluble polyhistidine-tagged human DcR3 and by confirming similar patterns of immunoreactivity obtained with the different clones of DcR3 antibody on immunoblot, and flow cytometric and immunohistochemical analysis of glioma cells.

**Flow Cytometric Detection of DcR3 Expression.** For flow cytometric analysis of DcR3 expression, the glioma cells were detached from culture flasks by incubation with cell dissociation buffer (Life Technologies, Inc., Grand Island, NY), and washed in PBS VIVO. 10^6 cells were then permeabilized with 1 ml of 75% ethanol for 10 min at 4°C and washed with PBS. Samples were resuspended in 150 μl of flow cytometry buffer [PBS (pH 7.4), 1% BSA, 0.01% sodium azide] containing 10% sheep serum and blocked for 20 min at 4°C. After two washes, the samples were incubated with different monoclonal DcR3 antibodies (10 ng/ml) or control mouse IgG (10 ng/ml). After two additional washes with PBS, the samples were incubated with sheep antiserum IgG-FITC (1:160) for 20 min at 4°C. Flow cytometric analysis was performed using a Becton Dickinson FACScalibur cytometer and CellQuest Software.

**Immunohistochemistry.** The surgical specimens of brain tumors [11 diffuse astrocytomas (WHO grade II) and 18 glioblastomas (WHO grade IV)] were obtained from patients treated in the Department of Neurosurgery, University Hospital of Zurich, Switzerland. Tumors were fixed in buffered formalin and embedded in paraffin. Pathological diagnosis was made according to the WHO classification. After deparaffinization, sections were heated and boiled for 15 min (three times 5 min each) in a microwave oven in 10 mM sodium citrate (pH 6.0) buffer. The sections were incubated for 1 h at room temperature with monoclonal antihuman DcR3 antibody at a dilution of 1:200 (50 ng/ml) in antibody diluent (Tris-HCl buffer containing carrier proteins and 0.015 M sodium azide; DAKO, Glostrup, Denmark). The reactions were visualized using a Vectastain ABC kit and diaminobenzidine (Vector Laboratories, Burlingame, CA). Control experiments without primary antibody or with irrelevant primary antiserum did not result in immunoreactivity (not shown). The sections were counterstained with hematoxylin.

**Immunohistochemistry for rat brain sections was performed as follows:** Cryostat sections (16 μm) were cut, air dried, and stored at −20°C. Sections of all tumors were routinely stained with H&E for morphological analysis. For immunocytochemical staining, frontal brain sections containing tumors were thawed, air dried, and fixed in 4% paraformaldehyde. Endogenous peroxidase activity was blocked by incubation in 0.3% H2O2. Nonspecific Fc binding was abrogated by preincubation with 10% normal goat serum and 1% BSA in PBS. Sections were incubated with monoclonal antibodies (Serotec, Kidlington, United Kingdom) directed against ED1 (membrane antigen of microglia/macrophages), OX4 (CD4+ T cells, perivascular microglia), or OX8 (CD8+ T cells).

Following incubation with the primary antibody (1:100) at 4°C overnight, sections were washed and reacted with a secondary biotinylated antiserum (1:200 in PBS/BSA; Dianova, Heidelberg, Germany) at room temperature for 30 min. Antibody binding was detected by incubation with an avidin-biotin-peroxidase complex (Vector Laboratories), according to the manufacturer’s instructions. Sections were processed with 3,3-diaminobenzidine (0.05%) and H2O2 (0.015%) in 10 mM Tris buffer for 3–5 min, and the reaction product was visualized under a Zeiss microscope (Dienhoffen, Germany). Immunolabeled cells within individual tumors were counted in four to six high-power fields of at least four sections per tumor (∼40 lens). Data are given as mean ± SD. Statistical significance was assessed using a t test. Control sections treated in the same way, but omitting incubation with the primary antibody, showed no specific labeling. Rat thymus, lymph nodes, and spleen were used as positive controls for lymphocytes, monocytes, and macrophages. Sections were counterstained with DAPI (17, 18), and nuclear morphology was analyzed on the same sections using epifluorescence.

**Chemotaxis Assay.** The migration of malignant glioma cells through 8 μm pores was assessed using a 48-well microchemotaxis chamber (Neuro Probe Inc., Bethesda, MD) as described previously (19). CD95L-containing medium (30 μl) in the wells of the bottom chamber served as the chemottractant. Medium lacking CD95L was used as a control. The filter membrane was placed between the top and bottom chambers and equilibrated for 30 min at 37°C. N9 microglial cells (5 × 10^5/well) in the absence or presence of DcR3 were applied to the upper wells and were allowed to migrate through the membrane at 37°C in humidified air with 5% CO2. After 24 h, the membrane was removed, and the nonmigrated cells were scraped off with a wipe blade. Migrated cells on the bottom side of the membrane were fixed in methanol and stained in thiazine/ceinusing DiffQuick (Dade Behring AG, Düdingen, Switzerland). Cells migrated through the membrane pores were counted using a microgrid. The monoclonal anti-CD95 antibody SM1/23 that was used in some control experiments was purchased from Alexis (San Diego, CA).

**RESULTS**

**Human Malignant Glioma Cells Express DcR3 in Vitro and in Vivo.** We first assessed DcR3 protein expression in glioma cells in vitro. Twelve human malignant glioma cell lines were incubated with serum-free medium for 24 h. Subsequently, the supernatants were concentrated and subjected to immunoblot analysis. Fig. 1A shows that 8 of 12 cell lines produced significant amounts of DcR3 protein. Two cell lines, U251MG and LN-308, exhibited prominent DcR3

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K. J. Kim and A. Ashkenazi, unpublished data.
expression. A comparison with our previous studies (20) revealed that in this panel of cell lines, the expression of DcR3 and CD95L or CD95 did not correlate as determined by Spearman rank-order correlation analysis. A comparison of DcR3 expression in the 12 cell lines with their sensitivity to CD95L did not disclose an association between DcR3 expression and chemosensitivity as determined by ANOVA (data not shown).

We also investigated whether freshly prepared ex vivo glioma cells produce DcR3. Human malignant glioma cell lines were established from surgical glioblastoma specimens, and conditioned medium was harvested after no more than five passages. Immunoblot analysis of the concentrated supernatants showed that four of four cell lines produced DcR3 (Fig. 1B).

We next investigated whether human astrocytic brain tumors express DcR3 in vivo and whether the expression differs in low- and high-grade gliomas. DcR3 expression was detected in 15 of 18 (83%) glioblastomas (WHO grade IV) but in none of 11 diffuse astrocytomas (WHO grade II; low-grade gliomas). DcR3 expression was observed predominantly in areas surrounding large ischemic necrosis, which are a hallmark of high-grade gliomas (Fig. 2). Typically, positive staining for DcR3 was observed in the cell membrane and cytoplasm of neoplastic cells. DcR3 was not detected in normal brain tissue surrounding the tumors.

**DcR3 Protects Glioma Cells from CD95L-induced Apoptosis.** Prompted by these results, which suggested a role for DcR3 in malignant glioma progression, we investigated the effects of ectopic DcR3 expression in glioma cells *in vitro*. We stably transfected several human (LN-18, U251MG, LN-229, and U373MG) and rat (9L) glioma cell lines with the DcR3-pRK5 plasmid and pcDNA3 vector (cotransfection) or with only the empty pcDNA3 vector, which harbors a neomycin resistance gene (neo). Stable expression of the DcR3 transgene was verified by immunoblot analysis (Fig. 3B) and flow cytometry (Fig. 3B). To further demonstrate the specificity of DcR3 immunostaining, we performed immunoblot analysis with three, and flow cytometry with four, different clones of monoclonal anti-DcR3 antibodies. All experiments resulted in similar patterns of immunoreactivity (data not shown). We then examined whether ectopic DcR3 expression renders glioma cells resistant to CD95L-induced cell death. Glioma cells were cultured for 12 h in DMEM, thereby allowing the release of DcR3 into the supernatant. CD95L was then added in increasing concentrations to the cell cultures (Fig. 4, A–C). Compared with neo control cells, DcR3 transfectants were significantly protected from CD95L-mediated apoptosis. In contrast, there was no protection from Apo2L/TRAIL-induced cell death, another member of the same death ligand family (Fig. 4, D–F). Importantly, protection from CD95L-induced apoptosis was also achieved by endogenously produced DcR3. Supernatants of DcR3-expressing wild-type cell lines (e.g., U251MG and U373MG) were concentrated 14-fold to reach significant protection (20–30%) compared with equally concentrated supernatants of cell lines lacking endogenous DcR3 expression (e.g., LN-18 and LN-229; data not shown).
To confirm the specificity of anti-CD95L activity of DcR3, we took advantage of an antagonistic monoclonal anti-DcR3 antibody. LN-18 neo glioma cells were treated with CD95L at a concentration that produced 80% cytotoxicity. Complete protection of glioma cells was obtained by adding DcR3-containing cell culture supernatant of DcR3-transfected U373MG cells (10 units/ml) or a specific anti-DcR3 antibody (DcR mAb; 3 μg/ml). bars, SD.

**DcR3 Does Not Interfere with Cytotoxic Drug-induced Cell Death and Does Not Participate in the Inhibition of Glioma Cell Fratricide.** The involvement of the CD95 system in cytotoxic drug-induced apoptosis is a controversial issue (21, 22). If drug-induced cell death is mediated by CD95/CD95L interactions, enhanced DcR3 expression should confer protection against this type of cell death. We therefore compared the effects of cytotoxic drugs on DcR3 transfectants and control cells. DcR3 transfectants and neo control cells of diverse glioma cell lines were cultured for 12 h in DMEM and then treated for 24 h with lomustine (CCNU), teniposide, or cisplatin. Table 1 lists the EC\textsubscript{25} drug concentrations (drug concentrations that led to death of 25% of the cells) in acute cytotoxicity assays. No statistically significant differences between the DcR3-producing cells and neo control cells were observed ($P > 0.05$, t test).

It is also known that glioma cells frequently coexpress CD95 and CD95L on their cell surfaces in vitro and in vivo (23–26). However, there is no evidence that this colocalization of proapoptotic receptor and ligand results in suicide or, in case of high cellular density, fratricide of tumor cells. We speculated that DcR3 might contribute to the inhibition of suicide and fratricide by neutralizing CD95L. We therefore cultured U251MG cells until they were grown to subconfluence. This cell line was chosen because it produces significant amounts of CD95L as assessed by flow cytometry (not shown) and DcR3 (Fig. 1A), but it is nevertheless susceptible to apoptosis induced by exogenous CD95L. Before U251MG cells reached confluency, the cells were treated with high concentrations of antagonistic anti-DcR3 antibody (10 μg/ml). To ensure sufficient antibody concentrations, anti-DcR3 antibody was added at 6-h intervals to the glioma cells, and cell death was assayed at different times between subconfluence and tight confluence of U251MG cells. However, we did not observe increased induction of cell death in anti-DcR3 antibody-treated glioma cells (data not shown).

**Suppression of Immune Cell Infiltration in Glioma Xenografts by DcR3 Expression.** Because neutralization of CD95L may predominantly affect functions in the immune system, we investigated DcR3-mediated effects on glioma growth in vivo. DcR3 transfectants and neo control cells of the rat gliosarcoma cell line 9L were stereotactically implanted into the brains of immunocompetent F344 rats. At 14 days after inoculation of tumor cells, the animals were sacrificed, and cryosections of the brains were produced. Histological analysis of H&E-stained sections showed large, solid tumors, with rather distinct margins toward the surrounding brain tissue. There was no diffuse infiltration of tumor cells into the surrounding brain; however, smaller, solid bundles of tumor cells grew into the surrounding brain at a margin of several hundred μm. There were several small areas of necrosis present in almost every tumor, with surrounding macrophages (not shown). DAPI staining of cell nuclei did not reveal any gross difference in size, shape, or density of tumor cells between DcR3-transfected and control tumors (Fig. 5, A and E).

For the assessment of host cell reactions to the glioma transplants, the sections were stained with antibodies specific to ED1 (membrane antigen of microglia/macrophages), OX4 (CD4\textsuperscript{+} T cells), and OX8 (CD8\textsuperscript{+} T cells). Compared with control tumors, immunocytochemical analysis showed fewer CD4\textsuperscript{+} (Fig. 5, B and F) and CD8\textsuperscript{+} (Fig. 5, C

**Table 1 No modulation of chemosensitivity by ectopic expression of DcR3**

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<th>LN-18</th>
<th>U251MG</th>
<th>LN-229</th>
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<td><strong>Lomustine (μM)</strong></td>
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<td>neo</td>
<td>98 ± 4</td>
<td>335 ± 15</td>
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<td>108 ± 6</td>
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<td><strong>Teniposide (μM)</strong></td>
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<tr>
<td>neo</td>
<td>17 ± 3</td>
<td>110 ± 8</td>
<td>95 ± 5</td>
<td>1.8 ± 0.7</td>
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<tr>
<td>DcR3</td>
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<td>95 ± 7</td>
<td>105 ± 5</td>
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<td><strong>Cisplatin (μM)</strong></td>
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<td>18 ± 2</td>
<td>85 ± 6</td>
<td>110 ± 9</td>
<td>34 ± 6</td>
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<td>21 ± 4</td>
<td>90 ± 4</td>
<td>114 ± 10</td>
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and G) lymphocytes in tumors transfected with DcR3. Likewise, the number of ED1-positive cells was reduced in DcR3-expressing tumors (Fig. 5, D and H). The density of immune cells was higher at the tumor margin compared with the tumor center. Quantitative analysis showed a significant reduction of immune cells in DcR3-transfected tumors compared with controls (P, 0.05). Data derived from CD8+ lymphocytes are shown in Fig. 5I.

We next examined whether the modified immune reaction to DcR3-expressing gliomas was also accompanied by a decrease in glioma growth. Again, 2 weeks after inoculation of glioma cells into the brains of Fischer rats, the animals were sacrificed, and cryosections were stained with H&E. Volumetric analysis of tumor volumes showed that DcR3-producing 9L glioma cells tended to form larger tumors (44.2 ± 9.8 mm³) than neo control cells (32.2 ± 9.3 mm³); however, this difference was not statistically significant as assessed by Student’s t test (P = 0.07; Fig. 5J). Of note, the generation times of these cell lines were also not statistically different in vitro, indicating that ectopic expression of DcR3 did not substantially interfere with growth kinetics or cell cycle regulation (30 ± 3 h for 9L neo, 33 ± 4 h for 9L DcR3).

DcR3 Inhibits the Chemotactic Activity of CD95L against Microglial Cells in Vitro. Prompted by these in vivo findings, we investigated whether CD95L-mediated chemotactic locomotion of microglial cells was inhibited by DcR3. We first examined the chemotactic effects of CD95L-containing cell culture supernatant on N9 cells. N9 cells are transformed mouse microglial cells (15) that resist the proapoptotic effects of CD95L. As depicted in Fig. 6, the migration of microglia was substantially increased after the addition of 100 units/ml CD95L to DMEM medium compared with medium lacking CD95L. In the presence of 150 units/ml DcR3, the increase in motility was abolished. As a control, we incubated the cells with an antagonistic CD95 antibody (1 μg/ml) and assessed the cellular motility after CD95L stimulation. Interference with CD95/CD95L interactions by this antibody also resulted in prominent inhibition of migration.

DISCUSSION

Suppression of systemic and local immune responses has been demonstrated in malignant gliomas and has been attributed a role in their pathogenesis. The mechanisms contributing to the protection of malignant gliomas against the immune-mediated defense are not entirely clear. Soluble immunosuppressive cytokines have been implicated as a cause of anergy in immune effector cells. However, the
CD95 system may also be involved in immune-modulating processes that could promote glioma cell proliferation in vivo. Down-regulation of CD95 expression at the cell surface, shedding of soluble CD95, and inhibition of the intracellular death-signaling pathway are mechanisms that may enable glioma cells to escape a CD95-mediated immune response. In the Apo2L/TRAIL-mediated cell death signaling system, the membrane-bound decoy receptors DcR1 and DcR2 have been shown to protect cells from ligand-induced apoptosis (27). These two receptors bind Apo2L/TRAIL without initiating an apoptotic signaling cascade. Moreover, osteoprotegerin, a soluble decoy receptor, binds to and inactivates Apo2L/TRAIL in the extracellular space (28). Similarly, the newly identified secreted decoy receptor DcR3 neutralizes the biological effects of CD95L (10). DcR3 has been attributed an oncogenic role because it is preferentially produced by malignant tumor cells and thus might counteract CD95L-mediated immune responses. By a similar mechanism, DcR3 may contribute to immune escape of tumors by binding to the ligand LIGHT (HVEM-L), which is highly expressed by activated T cells and induces apoptosis of tumor cells (12).

To investigate whether DcR3 has oncogenic properties in malignant gliomas, we first examined DcR3 expression in long-term human malignant glioma cell lines, in freshly prepared ex vivo glioma cells, and in surgical glioma specimens in vivo (Figs. 1 and 2). DcR3 was detected in the supernatants of the majority of gliomas in vitro. The immunohistochemical examination of DcR3 in gliomas with different grades of malignancy led to the interesting finding that diffuse astrocytomas (i.e., low-grade astrocytomas; Ref. 29) lack DcR3 expression, whereas the vast majority of high-grade gliomas are positive for DcR3 expression. These results confirm and extend the data on preferential expression of DcR3 in malignant tissues (10, 11) and suggest a role for DcR3 in the progression from low- to high-grade glioma. Similarly, the expression of CD95 was shown to correlate with the grade of malignancy of astrocytomas (26). In our study, DcR3 expression was localized mainly in glioma cells in areas surrounding large ischemic necrosis. Interestingly, this expression pattern is similar to the pattern of CD95 expression (30, 31), suggesting coregulation of CD95 and DcR3 expression. DcR3 up-regulation may reflect a protective mechanism from CD95-mediated cell death. Our data are consistent with the findings of Bai et al. (11), who also reported a coexpression of DcR3 and CD95 in colon adenocarcinoma.

Because an oncogenic role of DcR3 would most likely become evident by immune-mediated mechanisms, we investigated the effects of glioma-derived DcR3 secretion on the immune response in an immunocompetent rat glioma model. Interestingly, 9L gliosarcomas with enhanced DcR3 expression exhibited a prominent decrease of immune cell infiltration (Fig. 5, A–H). Immunohistochemical analysis demonstrated that microglia/macrophages, and CD4+ and CD8+ T cells infiltrated neo gliomas to a significantly higher extent than DcR3 gliomas. In general, this finding raises the intriguing possibility that DcR3 might favor glioma growth by suppressing the immune-mediated infiltration of cytotoxic T cells and microglia/macrophages, suggesting a new mechanism of immune evasion of malignant gliomas. It has been shown that CD95L exerts strong chemotactic effects on monocytes and neutrophils (4, 34, 35). Therefore, we have modeled the chemotactic activity of CD95L against microglial cells in a chemotaxis assay in vitro. In fact, DcR3 inhibited the CD95L-mediated locomotion of N9 microglial cells, suggesting that, in vivo, DcR3 may counteract the migration of immune effector cells toward glioma cells by inhibiting the chemotactic activity of CD95L. Our finding is the first evidence that, apart from its antiapoptotic function, DcR3 also inhibits CD95L-mediated chemotaxis. Another possible mechanism of DcR3-mediated inhibition of immune response is suggested by the identification of a second ligand for DcR3, LIGHT (12). LIGHT is expressed by activated T lymphocytes and was characterized as a ligand for HVEM/TR2 and LTβR. Signaling via LTβR triggers cell death in various malignant tumor cells, and thus, DcR3 may act as an inhibitor of LIGHT-induced apoptosis by blocking its interaction with LTβR or HVEM/TR2 (12). Alternatively, DcR3 may weaken the activation of cytotoxic T cells and other immune cells by preventing CD95/CD95L interactions. It has been shown that cytotoxic T cells become activated by reverse signaling when T-cell surface-bound CD95L binds to CD95 on target cells (36). Finally, DcR3 might bind to as yet unidentified ligands that, under normal conditions, promote antitumoral immune responses.

In this study, the decreased immune response against DcR3-
expressing gliomas resulted in only marginally increased tumor growth that did not reach statistical significance. This may be the result of overall resistance of 9L cells to the normal immune response in this animal model. We suppose that, in an immunocompetent animal model with a partially effective immune response to the tumor (xenograft), DcR3 expression would lead to enhanced tumor growth via suppression of the antitumor activity of immune effector cells. To our knowledge, no currently available glioma animal model exhibits the immunological features that would allow reliable testing of this hypothesis. Alternatively, the lack of significant effect on tumor growth may be attributable to a weaker interaction of human DcR3 with rat CD95L compared with human CD95L. This is supported by the observation that human DcR3 binds with lower affinity to murine DcR3: A ROLE IN IMMUNE EVASION OF GLIOMA? CD95L compared with human CD95L. Finally, the lack of a more significant difference between tumor sizes may be attributable to the comparatively weak expression of the DcR3 transgene in the animal model in this study (not shown). As shown for human malignant gliomas (Fig. 2) and human malignant gastrointestinal tumors (11), DcR3 can be strongly expressed in vivo, raising the possibility of accumulation in the tumor interstitium.

In this study, we present further evidence for the promotion of tumorigenesis by DcR3. We observed that DcR3 is expressed by the majority of malignant gliomas in vitro and in vivo. The correlation of DcR3 expression and grade of malignancy of astrocytic tumors suggests a role for DcR3 in glioma progression. The findings that CD95L-induced cell death can be blocked by enhanced DcR3 expression in vitro and that immune cell infiltration is substantially decreased in DcR3-producing gliomas in vivo supports the possibility that this decoy receptor may be involved in immune evasive mechanisms that protect malignant gliomas from an effective antitumor response.

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