Glioma gene therapy with soluble transforming growth factor-ß receptors II and III

ULRIKE NAUMANN[1], PHILIPP MAASS[1,2], ANNE-KATHRIN GLESKE[1,3], STEFFEN AULWURM[1,4], MICHAEL WELLER[1,5] and GUENTER EISELE[1,5]

[1]Department of General Neurology, Hertie Institute for Clinical Brain Research, University of Tuebingen, Tuebingen, Germany

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Abstract. Transforming growth factor-ß (TGF-ß) is abundantly expressed in malignant gliomas and is crucial for the tumor micromilieu. TGF-ß not only enhances migration and invasion of glioma cells but also inhibits an effective anti-glioma immune response. TGF-ß mediates its biologic effects through interactions with TGF-ß receptors (TßR)-I to -III. Binding of TGF-ß leads to the activation of an intracellular signaling cascade and subsequent phosphorylation of Sma and MAD-related proteins (SMAD). Soluble TGF-ß receptors (TßRs) abrogate the TGF-ß effect by competing for the binding of the ligand to its receptor. Here we used adenoviral gene transfer to express TßR-IIs and -IIIs in human glioma cell lines. TßR-IIs reduced SMAD2 phosphorylation and TGF-ß-dependent reporter activity. Furthermore, it enhanced glioma cell lysis by natural killer cells. TßR-IIIs alone were inactive in these assays, but enhanced the effects of TßR-IIs. Transduction of LN-308 cells with TßRs markedly delayed growth of intracerebral xenografts in nude mice *in vivo*. These data commend TßRs for possible experimental therapy of gliomas.

Introduction

Gliomas are highly malignant intrinsic brain tumors. Over-expression of TGF-ß is a typical feature of gliomas *in vitro* and *in vivo*. Elevated concentrations of TGF-ß are detectable in glioma cyst fluids and in the cerebrospinal fluid of glioma patients (1). TGF-ß plays a key role in malignant glioma progression. It blocks the activation of immune cells via downregulation of immunoactivating ligands on glioma cells and induces apoptosis of T and natural killer (NK) cells (2,3). To note, TGF-ß is upregulated with increasing grade of glioma malignancy (4,5).

The TGF-ß receptor signaling cascade consists of a variety of different proteins. Three receptors with a different affinity to TGF-ß subtype -1, -2 and -3 have been characterized so far and named type I (TßR-I), type II (TßR-II) and type III (TßR-III/betaglycan). TßR-I and TßR-II are transmembrane proteins with serine/threonine kinase function (6). Membrane-bound TßR-III has no kinase activity and may function to enrich and to present TGF-ß to the signaling complex (7). Once TGF-ß binds to TßR-II, TßR-I is phosphorylated by TßR-II and propagates the signal via phosphorylation of SMAD proteins which leads to the regulation of a variety of genes.

Antagonizing TGF-ß is a promising strategy to counteract malignant properties of tumors (8). Several approaches have been developed such as small molecule inhibitors (9), antisense strategies (10) or soluble TGF-ß receptors. TGF-ß-neutralizing effects and subsequent anti-tumor activity have been described upon expression of TßR-IIs or TßR-IIIs in a variety of cell lines and animal tumor models for pancreatic, prostate or breast cancer and malignant mesothelioma (11-14). In a rat gliosarcoma model, TßR-IIs led to enhanced immunogenicity of the tumors and enhanced survival (15). These data along with the prominent role of TGF-ß in glioma prompted us to assess whether TßR-IIs or TßR-IIIs are putative candidate therapeutic agents for glioma.

Materials and methods

Cell lines and reagents. U87MG, LNT-229 and LN-308, human malignant glioma cell lines, were kindly provided by N. de Tribolet (Lausanne, Switzerland). CCL64 mink lung epithelial cells and MLEC32, a mink epithelial lung cell line stably transfected with an 800-bp fragment of the human plasminogen activator inhibitor-1 (PAI-1) gene fused to the firefly luciferase (Luc) reporter gene in a p19Luc-neo vector, were a kind gift from D. Rifkin (New York, NY, USA). All

Correspondence to: Dr Ulrike Naumann, Department of General Neurology, Hertie Institute for Clinical Brain Research, Otfried-Müller Strasse 27, D-72076 Tuebingen, Germany
E-mail: ulrike.naumann@uni-tuebingen.de

Present addresses: [2]Max-Delbrück Center, Robert-Rössle-Str. 10, D-13125 Berlin; [3]Institute for Medical Microbiology, Elfriede-Aulhorn-Str. 6, D-72076 Tuebingen; [4]Institute of Immunology, University of Tuebingen, Auf der Morgenstelle 15, D-72076 Tuebingen, Germany; [5]Department of Neurology, University Hospital Zürich, Frauenklinikstrasse 26, CH-8091 Zürich, Switzerland

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cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Lonza, Basel, Switzerland) containing 10% fetal calf serum (FCS), glutamine (2 mM), penicillin (100 IU/ml)/streptomycin (100 μg/ml) and the appropriate antibiotics. CD56-positive human NK cells were prepared from peripheral blood monocytic cells using an indirect magnetic labeling system (Miltenyi Biotec, Bergisch Gladbach, Germany). SD-208 is a selective TßR-I kinase inhibitor kindly provided by Scios Inc. (Freemont, CA, USA) (9). Recombinant human TGF-ß$_2$ (rhTGFß$_2$) was purchased from Peprotech Inc. (Hamburg, Germany).

Construction of adenoviral vectors and infection procedure. Ad-LacZ, Ad-eGFP and Ad-ΔE1 have been previously described (16). Ad-TßR-IIs encode the extracellular domain of the TGF-ß receptor type II, fused to a human Fc peptide (17). For the generation of Ad-TßR-IIIs, the extracellular domain of the human TßR-III was amplified by PCR from pBLUE-TßR-III (18) using the forward primer TTT GTC GAC ATG ACT TCC CAT TAT GTG ATT GCC and the reverse primer TTT AAG CTT TAA GTC CAG ACC ATG GAA AAT TGG TGG. The PCR fragment was cloned into pCRII, completely sequenced, compared to the NCBI database and was found to be correct (GenBank accession no. NM003243). Thereon the fragment was cloned into pTRACK-CMV using the Ad-Easy system provided by B. Vogelstein (Baltimore, MD, USA). Ad-TßR-III additionally codes for eGFP in a second expression cassette. Recombinant adenoviral genomes were transfected into HEK-293 cells (ATCC). The virus was purified and titrated according to standard procedures (16,19). Transgene expression and secretion were verified by immunoblotting. Infection with recombinant viruses was accomplished by exposing cells to different concentrations of adenovirus in serum-free DMEM for 15 min followed by the addition of serum-containing medium for 1-5 days. If cells were double infected, total moiety of infection (MOI) as well as eGFP expression was set equal by adding either Ad-LacZ or Ad-eGFP.

Generation of cellular supernatants containing TßRs. For the generation of supernatants, the cells were infected with the appropriate concentration of virus. Six hours after infection, the cells were washed twice with serum-free medium (SFM), and SFM was added for 24-48 h. Supernatants were harvested and cleared from cellular debris by centrifugation. Secreted proteins were assessed by immunoblotting. Supernatants were stored at -80˚C for further use.

Growth and viability assays. Net cell culture growth was determined by crystal violet staining. Briefly, the cells were stained with crystal violet for 10 min followed by several washes with tap water. Bound crystal violet was dissolved in 50% ethanol/0.1 M sodium citrate, and optical density readings were obtained using an ELISA reader (Thermo Electron Multiskan EX) at 560 nm. To assess viability, the cells were trypsinized and stained with trypan blue.

Immunoblot analysis. The general procedure has been previously described (16). The following antibodies were used: anti-TßR-II (SC1700, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TßR-III (AF-242-PB, R&D Systems, Wiesbaden, Germany), anti-ß-actin (SC-1616, Santa Cruz), anti-phospho-SMAD2 (#3108, Cell Signaling, Danvers, MA, USA) and anti-SMAD2/3 (BD Biosciences, Aalst, Belgium).

Luciferase reporter assay. Cells were seeded in triplets in microtiter plates and transfected with 150 ng pGL3-TP3Luc, expressing firefly luciferase under the control of an artificial TGF-ß-inducible promoter, and 20 ng pRL-CMV as an internal standard. At 48 h after transfection, the cells were incubated with supernatants of glioma cells expressing TßRs in the absence or presence of rhTGFß$_2$. To allow complex formation between TGF-ß and soluble receptors, mixtures were pre-incubated for 30 min at room temperature before adding to the transfected cells. TGF-ß-mediated luciferase activity was assessed as previously described (9).

NK lysis assay. To measure the lytic activity of human CD56-positive NK cells, they were incubated for 24 h with supernatants of virus-infected glioma cells secreting TßRs and a parallel addition of rhTGFß$_2$ (1 ng/ml) or vehicle. To allow complex formation, rhTGFß$_2$ and supernatants were pre-incubated for 30 min at room temperature. A standard ^{51}Cr release assay was performed using 2000 ^{51}Cr-labeled LNT-229 target cells/well. Effector (E) and target (T) cells were incubated at various E:T ratios for 4 h.

Glioma/NK cell coculture. For coculture of TßR-expressing glioma cells and NK cells, LN-308 cells were infected with 150 MOI of the appropriate virus for 24 h followed by irradiation with 30 Gy to block proliferation. After irradiation, CD56-positive human NK cells were added. Lysis of eGFP-positive glioma cells was assessed optically at 21 h after coculture.

Animal studies. CD1$^{nu/nu}$ mice (Charles River, Sulzfeld, Germany) were anesthetized and placed in a stereotactic fixation device (Stoelting, Wood Dale, IL, USA). LN-308 cells ($1x10^5$), infected with 150 MOI of the appropriate virus 6 h prior to inoculation, were injected into the right striatum. The mice were sacrificed when developing neurological symptoms. All animal research was carried out in accordance with the law for treatment of animals in Germany and approved by the local authorities (N3/03, Regierungspräsidium Tuebingen, Germany).

Results

Expression and toxicity of TßRs in glioma cell lines. We first analyzed the expression and secretion of adenovirally encoded TßR-IIs and TßR-IIIs. LNT-229, LN-308 or U87MG cells were infected with increasing MOI of Ad-LacZ, Ad-TßR-IIs or Ad-TßR-IIIs. TßRs were expressed in all tested cell lines as analyzed by RT-PCR and by immunoblotting of cellular lysates (data not shown). To ascertain that TßRs were secreted from infected cells, cellular supernatants were studied by immunoblotting (Fig. 1A and B). Protein levels depended on the cell line and MOI. At 48 h after infection, accumulation of TßR-IIs in supernatants was high in all cell lines when using at least 100 MOI of adenovirus. For TßR-IIIs, secretion was

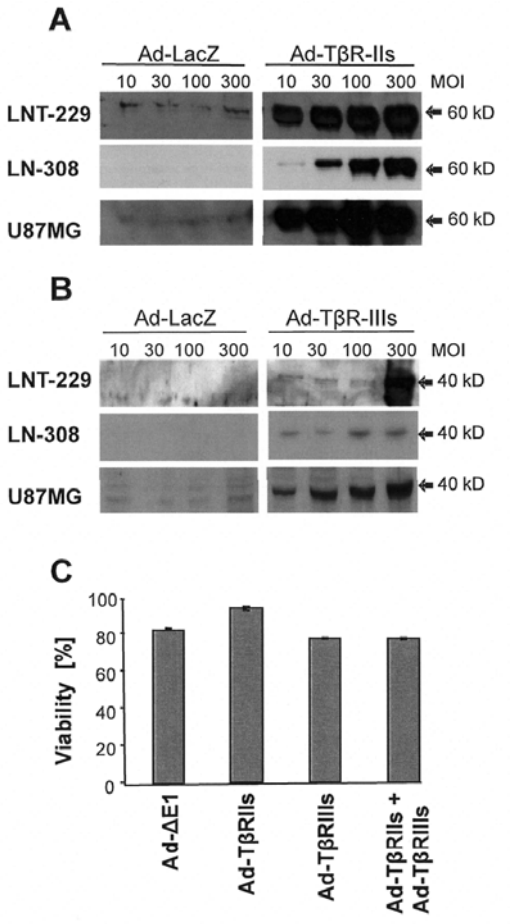

Figure 1. Secretion of TßRs in glioma cell lines. LNT-229, LN-308 or U87MG glioma cells ($1x10^6$) were infected with 10-300 MOI of Ad-LacZ, Ad-TßR-IIs (A) or Ad-TßR-IIIs (B). At 6 h after infection, the cells were washed and SFM was added. Supernatants were harvested 48 h later, and secretion of TßRs was assessed by immunoblotting using antibodies to TßR-IIs (A) or TßR-IIIs (B) (notice the presence of an unspecific protein band in Ad-LacZ-infected cells when using a TßR-II-specific antibody. (C) LN-308 cells were infected with either 300 MOI of Ad-ΔE1, Ad-TßR-IIs, Ad-TßR-IIIs or both. Viability was assessed after 48 h of infection (n=3, SD; one representative experiment is shown).

highest in U87MG and low to moderate in LN-308 and LNT-229 cells. To rule out weak transduction efficiency of Ad-TßR-IIIs, we analyzed if the adenoviral infection was successful. Using 100 MOI of Ad-eGFP or Ad-TßR-IIIs, >95% of the cells were eGFP-positive (data not shown). We therefore used ≥100 MOI for further experiments.

The influence of viral infection on cell proliferation and viability was assessed in parallel. Only a marginal amount of cell death and inhibition of proliferation was detected at 300 MOI of Ad-ΔE1, Ad-TßR-IIs, Ad-TßR-IIIs or both Ad-TßR-IIs and Ad-TßR-IIIs. No cell death was detected using <200 MOI of adenovirus (Fig. 1C for cell line LN-308 and data not shown).

TßRs are functionally active. To assess if TßR-IIs or TßR-IIIs alone or in combination compete for the binding of TGF-ß to cell surface TGF-ß receptors, LNT-229 cells were infected with Ad-LacZ, Ad-TßR-IIs, Ad-TßR-IIIs alone or in combination. At 24 h after infection, rhTGFß$_2$ was added and SMAD2 phosphorylation was assessed. Phosphorylated SMAD2 (P-SMAD2) was reduced in Ad-TßR-II-infected, but not in control or Ad-TßR-III-infected LNT-229 cells. P-SMAD2 was decreased to lowest levels in the cells double-infected with Ad-TßR-IIs and Ad-TßR-IIIs (Fig. 2A). We next analyzed the influence of TßR-IIs and TßR-IIIs on the expression of TGF-ß-responsive genes. The cell line U87MG was chosen for this assay because of the lowest expression levels of endogenous TGF-ß$_1$ and -ß$_2$. U87MG cells were infected with Ad-LacZ, Ad-TßR-IIs, Ad-TßR-IIIs or both. Supernatants were harvested, pre-incubated with rhTGFß$_2$ and added to LNT-229 glioma cells transiently transfected with a TGF-ß-responsive reporter construct. rhTGFß$_2$, pre-incubated with supernatant from uninfected or control virus-infected cells, induced reporter gene activity. This activation was repressed when rhTGFß$_2$ was pre-incubated with supernatants of TßR-II-expressing cells, but not with supernatants of TßR-III-infected cells. Complete repression of TGF-ß-induced reporter gene activity was detected when rhTGFß$_2$ was pre-incubated with supernatant of Ad-TßR-II and Ad-TßR-III double-infected cells (Fig. 2B).

The functional activity of TßRs was also assessed in CCL64 cells (data not shown) and in MLEC32 cells (Fig. 2C), a TGF-ß-sensitive mink lung epithelial cell line stably transfected with a TGF-ß-dependent reporter construct expressing the firefly luciferase under control of the PAI-1 promoter. Again, supernatants of LNT-229 glioma cells containing TßR-IIs and TßR-IIIs reversed the antiproliferative effect in CCL64 cells (data not shown) as well as TGF-ß-induced reporter gene activity of exogenous added rhTGFß$_2$ in MLEC32 cells (Fig. 2C). Expression of sTßR in the supernatants was confirmed by immunoblotting (data not shown).

Expression of TßRs blocks the immunosuppressive effect of TGF-ß on NK cells. TßRs might block the immunosuppressive effects mediated by TGF-ß on NK cells. In the first assay, LN-308 glioma cells were cocultured with NK cells. The cell line LN-308 was selected because of the secretion of large amounts of TGF-ß$_1$ and -ß$_2$. The cells were infected with Ad-LacZ, Ad-TßR-IIs, Ad-TßR-IIIs or both Ad-TßR-IIs + Ad-TßR-IIIs. To uniformly label the glioma cells with eGFP and to equalize MOI of infection, the cells were coinfected with either Ad-eGFP or Ad-LacZ. To avoid further proliferation of glioma cells, the cells were irradiated 24 h after infection and then coincubated with freshly isolated human NK cells. Approximately 90% of Ad-TßR-II-infected glioma cells were eradicated at 21 h after coculture, whereas no such effect was observed with control or Ad-TßR-III-infected glioma cells (Fig. 3A), indicating a rescuing effect of TßR-IIs on the lytic capabilities of NK cells towards the glioma cells. To determine the extent of NK cell-specific glioma cell lysis, human NK cells were cultured in normal medium or supernatants of LNT-229 cells infected with Ad-LacZ or Ad-TßR-IIs and Ad-TßR-IIIs supplemented with vehicle or rhTGFß$_2$. The NK cells were subsequently used as effectors in a standard ^{51}Cr release assay with untreated LNT-229 cells as targets. As expected, addition of rhTGFß$_2$ to control NK cells or to NK cells cultured in supernatant of control virus-infected LNT-229

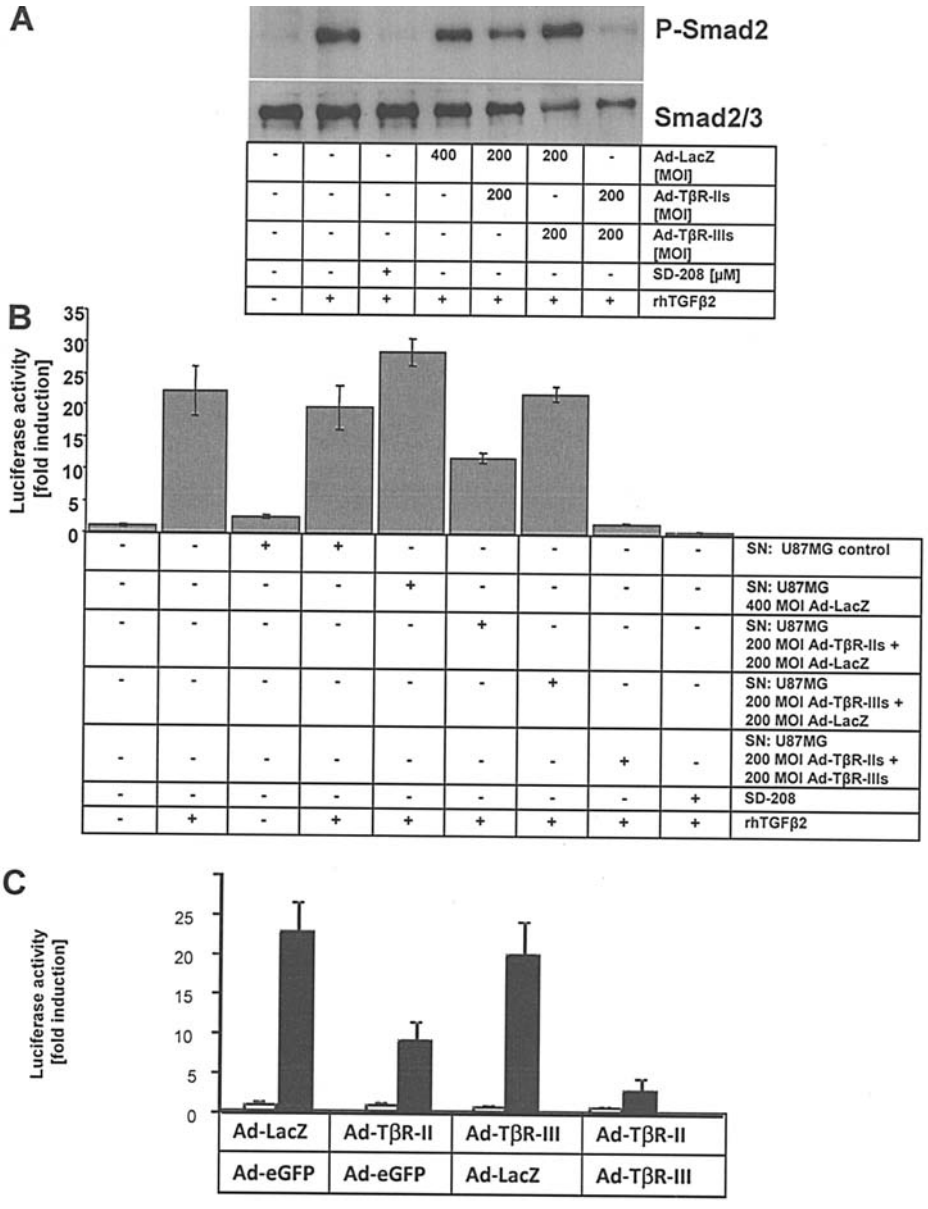

Figure 2. Expression of TßRs inhibits TGF-ß signaling. (A) LNT-229 cells were infected with Ad-LacZ, Ad-TßR-IIs, Ad-TßR-IIIs or both. At 6 h after infection, the cells were washed, and SFM was added. At 36 h after infection, rhTGFß$_2$ (10 ng/ml) or vehicle was added for 15 min. Phosphorylation of SMAD2 was assessed by immunoblotting. (B) LNT-229 cells were seeded in triplets and transfected with pGL2-3TP-Luc and pRL-CMV. At 48 h after transfection, supernatants of infected U87MG cells (200 MOI of each virus, 48 h in SFM), either preincubated with rhTGFß$_2$ (10 ng/ml) or vehicle for 30 min, were added. As a control, LNT-229 cells were pretreated with SD-208. At 24 h after incubation, reporter gene activity was analyzed (n=2, SD, one representative experiment is shown). (C) Supernatants of Ad-Virus-infected LNT-229 glioma cells (200 MOI for each virus, 48 h) were incubated with rhTGFß$_2$ (10 ng/ml, black bars) or vehicle (white bars) for 30 min. Preincubated supernatants were added to MLEC32 cells. TGF-ß-mediated luciferase activity was assessed after an additional 24 h (n=2; SD, one representative experiment is shown).

cells attenuated the lytic capabilities of these NK cells. Again, the immunosuppressive effect of rhTGFß$_2$ was reverted when the NK cells were cultured with supernatants containing TßRs (Fig. 3B). Therefore, TßRs might be able to restore an effective anti-glioma immune response by neutralizing TGF-ß.

Expression of TßRs prolongs survival of glioma-bearing mice. To assess a function of TßRs *in vivo*, LN-308 glioma cells infected either with Ad-LacZ, Ad-TßR-IIs, Ad-TßR-IIIs or both were implanted into the right striatum of nude mice. Survival was assessed in a Kaplan-Meier analysis (Fig. 4). In this experiment, mice receiving a tumor expressing TßR-IIs alone or in combination with TßR-IIIs showed a markedly prolonged survival. Expression of TßR-IIIs alone had no significant effect on prolongation of survival. This is consistent with the *in vitro* data, already indicating a superior effect of TßR-IIs compared to TßR-IIIs.

Discussion

The present study sought to determine the possible value of TßRs as therapeutic agents for malignant gliomas. We showed that adenovirus infection of glioma cells leads to production of TßRs (Fig. 1A and B). TßR-IIs and -IIIs showed

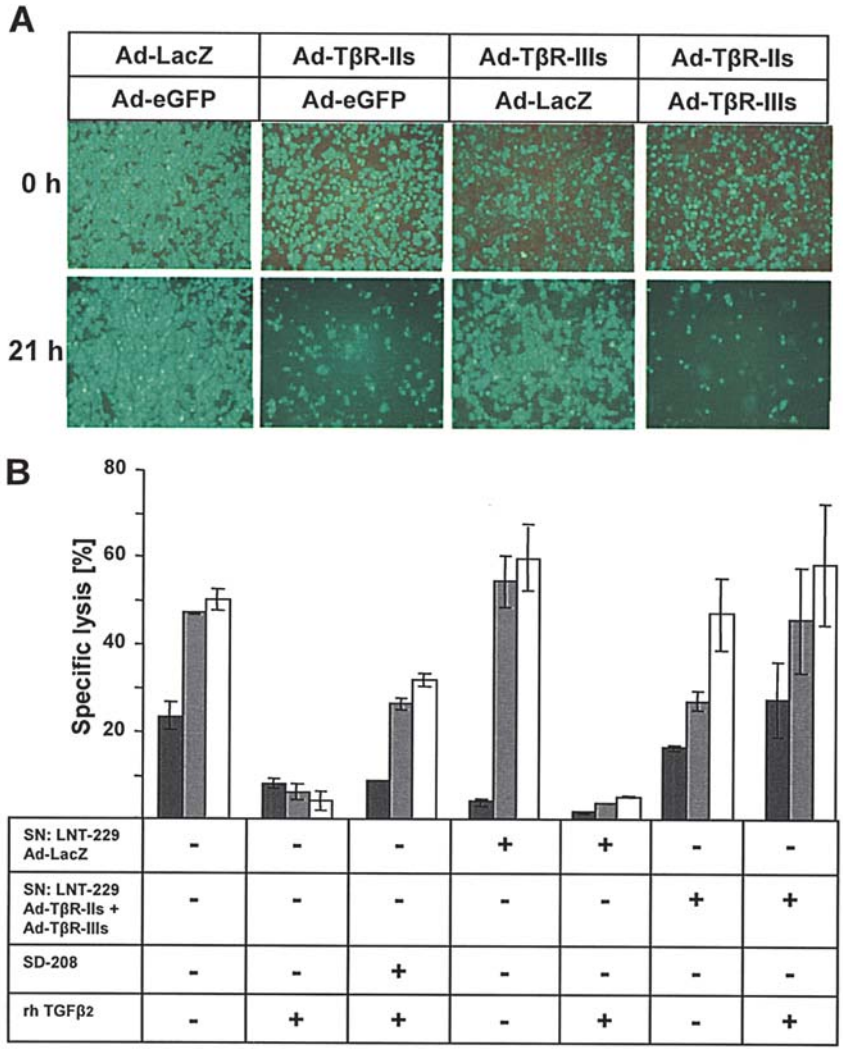

Figure 3. Expression of TßRs represses the inhibitory effects of TGF-ß on the lytic activity of NK cells. (A) LN-308 glioma cells were infected with 150 MOI of the indicated viruses for 24 h and then irradiated with 30 Gy. After irradiation, human NK cells were added. Lysis of eGFP-positive glioma cells was assessed microscopically 24 h after coculture. (B) Supernatants of infected LNT-229 glioma cells (400 MOI Ad-LacZ or 200 MOI Ad-TßR-IIs + 200 MOI Ad-TßR-IIIs) were coincubated with rhTGFß$_2$ for 30 min and then added to human NK cells. After 24 h of incubation, the NK cells were used as effectors in a ^{51}Cr release assay with untreated LNT-229 glioma cells as targets at different E:T ratios (dark gray bars 10:1; light gray bars 20:1; white bars 40:1). (n=3, SD, one representative experiment is shown).

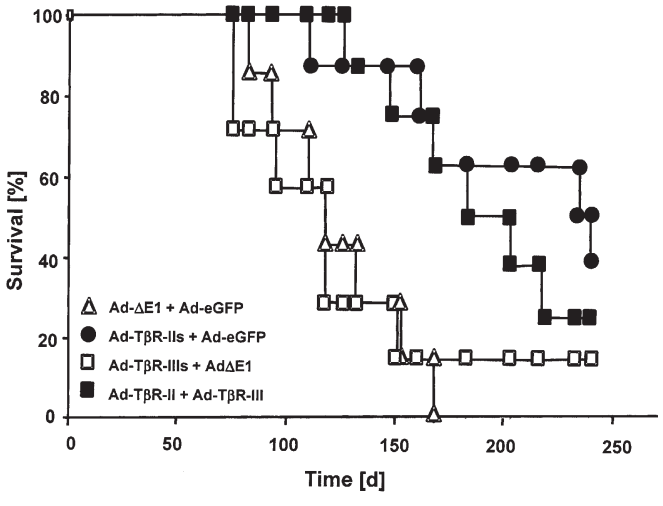

Figure 4. Expression TßR-IIs prolongs the survival of tumor-bearing mice. LN-308 cells were infected with 150 MOI of each virus. At 6 h after infection, 100,000 cells were inoculated into the right striatum of nude mice. Survival was assessed in a Kaplan-Meier-analysis.

different efficacy in counteracting the biological effects of TGF-ß. TßR-IIs inhibited activation of the TGF-ß-signaling cascade when applied alone. In contrast, TßR-IIIs only showed activity when applied in combination with TßR-IIs (Fig. 2A and B). Moreover, the combination of TßR-IIs and -IIIs showed a synergistic functional activity *in vitro* (Fig. 2). Therefore, at least in combination, TßR-IIIs showed functional activity. Nevertheless, it remains unclear why TßR-IIIs were inactive when exposed alone, even though the expression was confirmed by immunoblotting. The synergistic action with TßR-IIs argues against the idea that the expression level was too low. One could hypothesize that TßR-IIIs only act together with TßR-IIs which possibly leads to a stabilized binding of TGF-ß in a complex with both TßRs. TßR-III or betaglycan is known to mediate TGF-ß binding to the TßR-II under physiological conditions on the cell surface (6). Therefore, TßR-IIIs might also act as an enhancer of TGF-ß interception. Consistent with the *in vitro* data, TßR-IIIs alone showed no significant effect *in vivo*. Unlike the *in vitro* results, the combination of TßR-IIs and TßR-IIIs showed no

superior prolongation of survival compared to TßR-IIs alone. This might be dependent on the cell line, the mouse model and the single administration cycle used in this experiment.

The binding affinities of recombinant TßR-IIIs were different for the distinct TGF-ß isoforms (20). Therefore the function of the TßR-IIIs might be dependent on the relative quantities of the TGF-ß isoforms. The LN-308 tumors showed a rather slow progression in this xenogeneic tumor model. Nevertheless, the use of LN-308 appeared to be feasible and might be an alternative to the LNT-229 or U87MG model with the specific feature of slow tumor progression.

The *in vitro* data commend TßRs as an immunotherapeutic tool. NK cells and other immune effector cells are impaired by TGF-ß (21). The lytic functions of NK cells were restored by TßRs (Fig. 3). Again, the action of TßR-IIs was superior to that of TßR-IIIs, and the combination of both showed the best results (Fig. 3B). Witham *et al* used a retroviral vector coding for a truncated form of TßR-II and the rat gliosarcoma model 9L (15). In this paradigm, transduction of 9L cells led to reduced tumorigenicity in a subcutaneous model and enhanced survival after intracerebral inoculation. These effects were shown to be immuno-mediated and dependent on NK cell function. In our research we used an adenovirus instead of a retroviral vector and human glioma cell lines instead of a rat gliosarcoma cell line. Moreover, we investigated for the first time possible synergisms of TßR-IIs and -IIIs in antagonizing TGF-ß effects in the glioma context.

Taken together, adenovirally encoded TßRs can be used for future immunotherapy of gliomas. A major problem remains the route and mode of administration of a possible therapeutic agent to brain tumors. For TßRs, adenoviral gene transfer in a locoregional treatment is feasible. Further studies should also investigate alternative applications such as the use of DNA encoding for TßRs which has shown to be effective in a mouse lymphoma model (22). There are also efforts underway to enhance the antagonistic potency of TßRs by generation of homodimers or heterodimers of TßR-IIs and TßR-IIIs (23). This concept remains to be proven in animal cancer models. A lifelong antagonism of TGF-ß would be desirable in cancer patients. While some argue that chronic TGF-ß-antagonizing strategies may have serious side effects, it is noteworthy that in one study lifetime exposure to TßRs protected mice against metastasis without adverse side effects (24).

One future trend might be the use of an oncolytic virus containing an expression cassette for TßRs. This combines the virtue of an oncolytic virus to replicate exclusively in glioma cells and to follow invading glioma cells with the immune therapeutic effects of TßRs. Such an attempt has been used in a breast cancer model with promising results for further investigation (25).

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