An imbalance between Smad and MAPK pathways is responsible for TGF-ß tumor promoting effects in high-grade gliomas

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Received August 3, 2006; Accepted October 12, 2006

Abstract. The transforming growth factor-ß (TGF-ß) plays a pivotal role in the pathobiology of human gliomas: during carcinogenesis, it turns from a tumor suppressor to a tumor promoter. The traditional Smad pathway and the more recently discovered MAPK pathway are the most important pathways for TGF-ß related intracellular signal transduction mediating differential pathobiological effects. In this study, we investigated the effects of TGF-ß2 and the TGF-ß2 antisense phosphorothioate oligodeoxynucleotide (PTO) AS-11 on the functionality of both the Smad and MAPK pathways in high-grade gliomas. We aimed to correlate the imbalance between the pathways with differences in the behaviour of high-grade glioma cells. Gene and protein expression studies were used to detect levels of members of the Smad and MAPK pathways under regulation of TGF-ß2 and AS-11. Proliferation and migration assays were functional readouts for effects caused by these regulating tools. Gene arrays were used to detect yet unknown regulators of these functional effects. The Smad pathway was functional in the tested cell lines. Exogenous TGF-ß2 inhibited proliferation but enhanced migration. Smad 2 mRNA expression and activation were significantly reduced by incubation with AS-11. K-ras was reduced both in gene arrays and quPCR under treatment with AS-11, but there was no influence of K-ras down-regulation on the activity of ERK. Ubiquitination-related genes also were specifically down-regulated with AS-11. Our results indicate the involvement of K-ras in TGF-ß signaling in high-grade gliomas. ERK, which is a member of the MAPK pathway, was not influenced and seems to be activated through RAS independent cascades in glioma. These results suggest that combined antagonization of the TGF-ß and MAPK pathways might be a promising approach for glioma therapy. An imbalance between these two pathways might be responsible for TGF-ß switching to a tumor promoter protein in high-grade gliomas.

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Key words: TGF-ß, high-grade glioma, Smad pathway, MAPK pathway

Introduction

The transforming growth factor-ß (TGF-ß) is a multifunctional ubiquitous polypeptide cytokine that initially was named for its ability to induce transformation to a mesenchymal phenotype in cultured epithelial cells (1). In healthy tissue, but also low-grade epithelial tumors, TGF-ß induces growth arrest and therefore is considered as a classical tumor suppressor protein. In later stages of carcinogenesis, this effect turns to the opposite with TGF-ß playing a pivotal role as an autocrine stimulus of growth and dedifferentiation (2). Besides these autocrine effects, various other, mainly paracrine, functions emphasize the role of TGF-ß as a key player in the pathobiology of malignomas: it is a highly potent suppressor of immune reactions (3), an inductor of angiogenesis (4) and a promoter of cell motility and malignant invasive capacity (5; Arslan F, unpublished data). Congruently, TGF-ß secretion is enhanced in various malignant entities, such as malignant gliomas (6,7), pancreatic carcinomas (8,9) and colorectal carcinomas (10-12), turning the cytokine into a promising target for new therapeutical approaches.

In human malignant gliomas, overexpression and secretion of the TGF-ß2 isoform has been described as a hallmark of its pathobiology (13). All three TGF-ß isoforms (-ß1, -ß2 and -ß3) interact with the serine-threonine kinase type TGF-ß-receptor (TGFß-R) types I and II. Commonly, mutations in the TGF-ß signaling cascade are blamed for the switch from tumor suppressive to tumor promoting effects.

Besides the traditional Smad pathway, which has been well characterized as a TGF-ß signal transductor mediating mainly anti-proliferative effects (14), a number of other intracellular signaling cascades have been characterized. Yet, the mitogen-activated protein kinase (MAPK) pathway as inductor of a mesenchymal phenotype seems to be the most important of these (15,16; Henis YI, Proceedings of the 1st German Workshop on TGF-ß, 2005). A dysbalance between the Smad and

MAPK pathways with loss of anti-proliferative effects and induction of dedifferentiation is commonly blamed for the tumor promoting effects of TGF-ß on malignant cell types (28), but is not well described for high-grade gliomas. Understanding the nature of these changes therefore seems to be crucial for finding novel anti-neoplastic therapeutical strategies.

There are three different functional groups of Smad proteins: receptor-regulated R-Smads (Smad 1, 2, 3, 5 and 8), the co-receptor Smad (Co-Smad) 4 and the inhibitory I-Smads (Smad 6 and 7) (14,17). Smad proteins are activated by phosphorylation (14). Smad phosphorylation leads to nuclear translocation, where Smad proteins can interact via the Smad binding element (SBE) either in homotrimeric or heteromeric complexes with other transcription factors to process their target genes (18-20). Smads are known to mediate the anti-proliferative effects of TGF-ß signaling, whereas mutations either of the TGFß-R, the Smads themselves or downstream effectors/cooperators are commonly believed to cause the lack of growth inhibition after malignization of the cells (6,21,22).

The MAPK pathway involves ras, Raf and ERK1/2. Initiating the signaling cascade, ras gets activated by phosphorylation of different effectors, among them the active TGFß-R. Phosphorylated Ras (pRas) activates the down-stream serine threonine kinase Raf, which triggers the phosphorylation of the extracellular signal-related kinase (ERK) (23). Whilst a variety of cellular processes such as differentiation and proliferation are regulated by the MAPK pathway (24), mutations of the Ras gene have been described as pivotal in the carcinogenesis of various tumors (25-27). In addition to Smad pathway mutations, changes in the MAPK signaling cascade are commonly connected to the promotion of invasion by overexpression of extracellular matrix molecules and cytoskeletal rearrangement (28-30).

In this study, we investigated the effects of TGF-ß2 and the specific antisense phosphorothioated oligodeoxynucleotide (PTO) AS-11 (31) on both the Smad and the MAPK pathway with the aim to correlate the imbalance between the pathways with differences in the behaviour of high-grade glioma cells.

Materials and methods

Cell culture. Different types of glioma cell lines and primary cultures were used for *in vitro* experiments. Human high-grade glioma cell lines U87 and A172 were obtained from American Type Culture Collection (Manassas, VA). The gliomas named as 'HTZ' were primary tumor cell cultures derived from surgical specimens of human high-grade gliomas (WHO grade IV) as described before (32). All tumor cells were maintained as standard monolayer cultures in tumor growth medium at 37˚C, 5% CO_2, 95% humidity in a standard tissue culture incubator. Growth medium was comprised of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS).

Phosphorothioate oligodeoxynucleotides. We used the TGF-ß2 specific antisense-PTO AS-11 with the sequence 5'-GTA GTG CAT TTT TTA AAA AA-3' (mRNA target region 171-190) (Sigma-Genosys, Steinheim, Germany) (31). As control PTO, we used AS-11m with 3 mismatch bases (sequence: 5'-GTA ATG AAT GTT TTA AAA AA-3').

AS-11 specific suppression of TGF-ß2 secretion. Cells ($2.0x10^5$ per well) were seeded in a 6-well plate and incubated with 1 ml DMEM supplemented with 5% FCS. Fractions were not treated or treated for 6 days with 1, 10, 15, and 20 μM of AS-11, AS-11m. Media, including AS-11 and AS-11m additions, were changed every 48 h. After 6 days, supernatants were aspirated and a TGF-ß2 ELISA was performed. Lipofectamine transfection was performed in parallel with the same concentrations of oligonucleotide to evaluate the effect of lipofectamine on transfection efficacy and cell toxicity.

Cell proliferation assay. Cells ($2.5x10^5$ per well) were seeded in a 24-well plate and incubated with 1 ml DMEM supplemented with 5% FCS. Fractions were not treated or treated with 10 ng/ml TGF-ß2 and 10 ng/ml bovine serum albumine (BSA) as control, and cells were counted at days 2, 4, 6, 8 and 10. Media including the additions were changed at day 8.

Spheroid assay. Multicell tumor spheroids were initiated by seeding $3-8x10^6$ cells incubated in agar-coated wells in order to inhibit adhesion. Mature spheroids with a mean diameter of 200-250 μm were explanted to uncoated 96-well plates containing no supplement, 10 ng/ml BSA as control, 10 ng/ml TGF-ß2, and 10 and 15 μM AS-11 (Fig. 2). The dose of 10 ng/ml TGF-ß2 was chosen from previous studies showing that this concentration was as effective as higher concentrations concerning induction of proliferation in high-grade glioma cell lines (data not shown). Spheroids were allowed to migrate for 5-7 days. The area covered by cells migrating away from a spheroid was photographed and measured manually using the greatest diameter. Assays were performed in triplicates and repeated twice.

ELISA. For the quantitative determination of activated human TGF-ß2 concentrations in cell culture supernatants, the quantitative sandwich enzyme immunoassay technique was used with a commercially available human TGF-ß2 specific immunoassay kit (R&D Systems, Minneapolis, MN). The minimum detectable dose of TGF-ß2 was less than 7.0 pg/ml. The assay was performed in triplicates and as directed in the manufacturer's instructions.

Western blot analysis. Cell extracts from cultured cells were prepared by PhosphoSafe™ Extraction Reagent (EMD Biosciences, San Diego, CA) following the manufacturer's instructions. After extraction, equal amounts of samples (20 μg) were boiled for 5 min with Laemmli sample buffer and loaded directly into pre-poured Tris-HCl-glycine SDS-PAGE gels along with pre-stained molecular weight standards (Bio-Rad Laboratories, Palo Alto, CA). Electrophoresis was performed in Tris/glycine/SDS running buffer (Bio-Rad Laboratories) at 125-150 V for a suitable migration period. Following transfer to PVDF (polyvinylidene difluoride) membrane (Bio-Rad Laboratories) at 120 mA constant current for 1-2 h, blots were briefly washed in TBST (10 mM Tris, 150 mM NaCl, and 0.5% Tween-20, pH 8.0), and blocked for 1 h at RT in 5% non-fat dry milk. Specific antibodies (PhosphoSmad 2: Calbiochem, Laeufelfingen, Germany; Phospho-ERK1/2: R&D) were used in concentrations as suggested by the manufacturers and immunocomplexes were visualized using a horsedish peroxi-

dase-conjugated antibody followed by chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) detection on photographic film.

Quantitative PCR. Total RNA was extracted from glioma cells with the RNA purification system RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA concentration and purity was determined by measuring optical density at wavelengths of 260 and 280 nm using a standard spectrophotometer. First-strand cDNA generated from 1 μg of total RNA samples by using a reverse transcription kit (Promega, Madison, WI) was used to amplify gene-specific cDNAs from expressed genes.

To precisely quantify mRNA expression, a real-time PCR system (ABI PRISM® 7000 Sequence Detection System, CA) that measures nucleic acid molecules based on the detection of a fluorescent reporter molecule (SYBR Green dye) was used. Target-cDNA specific primers (Sigma-Genosys) were established (sequences: Smad 2 forward, 5'-AGT ATT AAC TCA GAT GGG ATC CCC TTC-3'; Smad 2 reverse, 5'-CTT TAA TTG ATG AGA CCT CAA GTG CTG-3'; Smad 4 forward, TAG 5'-CTC CAG CTA TCA GTC TGT CAG C-3'; Smad 4 reverse, 5'-TCT GCA ATC GGC ATG GCT GGT ATG AAGT AC-3'; K-ras forward, 5'-CCT GCT GAA AAT GAC TGA AT-3'; K-ras reverse, 5'-ATA CAC AAA GAA AGC CCT CC-3'). Annealing temperatures were optimized for each primer pair using the following program: DNA polymerase activation at 95˚C for 5 min, denaturation at 95˚C for 45 sec, annealing at 57-60˚C for 1 min, extention at 72˚C for 45 sec with amplification for 30 cycles. RT-PCR products were analyzed on a 1% agarose gel and visualized with ethidium bromide staining. The housekeeping gene ß-actin was used as a positive control to assess cDNA quality. Serial 2-fold dilutions of cDNA were amplified to construct standard curves for both the target gene and the endogenous reference (ß-actin). Standard curves were used for extrapolation of expression levels for the unknown samples based on their threshold cycle (Ct) values. All amplifications of unknown samples were in the linear range. For each reaction, melting curves were used to verify the identity of the amplification products. The target gene amount was divided by the housekeeping gene amount to obtain a normalized target value. Each of the experimental normalized values was divided by the normalized control (untreated) sample value to generate the relative expression levels in fold.

Gene arrays. U133 Plus 2.0 arrays (Affymetrix, Santa Clara, USA) were used for hybridization following the protocol of the manufacturer. For data analysis, NIH David 2.1 Beta analysis software was used. To classify a gene as changed, a 2-fold increase or decrease of expression in one of the fractions was required. To meet the criteria of 'specific effects', expression after treatment with AS-11 was compared to expression after AS-11m treatment and the untreated control group. 'Unspecific effects' were assumed if gene expression changed compared to the untreated control group, whereas AS-11 and AS-11m treated cells showed similar expression. For further analysis, we used the 'Functional Classification' and the 'KEGG pathway' functions of the program. For 'Functional Classification', 'medium stringency' was chosen as grouping criterion.

Table I. TGF-ß2 concentrations as detected by ELISA in supernatants of 10 high-grade glioma cell lines after 2 days of incubation.[a]

Cell line	TGF-ß2 ELISA (pg/ml)
U87 (ATTC)	Below sensitivity
A172 (ATTC)	594.98±43.95
HTZ-190A	110.46±8.04
HTZ-222	119.39±2.63
HTZ-236	274.57±18.56
HTZ-324	Below sensitivity
HTZ-349	830.86±24.76
HTZ-417	121.88±4.34
HTZ-419	815.11±31.72
HTZ-421	223.41±9.05

[a]Eight of the 10 cell lines produce measurable levels of TGF-ß2.

Luciferase assay. A172 and HTZ-349 cells were seeded $2x10^5$ per well. One fraction was stimulated with 10 ng/ml TGF-ß2 for 24 h while another population remained untreated. We used the Luciferase Assay Sytem (Promega) following the manufacturer's protocol for transfection of the cells. CAGA served as Smad-pathway specific reporter. Luciferase activity was measured as baseline before treatment and after stimulation and was compared to Renilla activity to calculate the specific fold of induction after treatment.

Results

Cultured glioma cells secrete TGF-ß2 in vitro. To select suitable cell lines for further assays, we tested expression of TGF-ß2 at the protein level in a series of human high-grade glioma cell lines. In 8 out of 10 cell lines tested, we detected measurable TGF-ß2 concentrations in the supernatants. For the TGF-ß2 secreting lines, concentrations ranged between 110.46 pg/ml and 830.86 pg/ml (Table I).

Antisense-PTO AS-11 specifically suppresses TGF-ß2 protein. To test the capability of the 20 oligomer TGF-ß2 specific Antisense-PTO AS-11 to regulate TGF-ß2, we performed dose-finding pre-assays with 4 cell lines (A172, HTZ-349, HTZ-417 and HTZ-419). We observed a nearly linear dose-dependent suppression of TGF-ß2 concentrations in medium supernatants (data shown for A172, Fig. 1A). The assays were performed in parallel with lipofectamine-enhanced transfection. However, transfection efficacy was only slightly enhanced, and lipofectamine was shown to be toxic on high-grade glioma cells. Therefore, all further transfections were performed without lipofectamine.

For further experiments, A172 and HTZ-349 were chosen because of their growth and regulation patterns. Both were incubated with the TGF-ß2 specific Antisense-PTO AS-11 and AS-11m as mismatch control at a concentration of 15 μM. AS-11 caused an average TGF-ß2 suppression of 36% in A172 cells, while AS-11m caused an unspecific average reduction

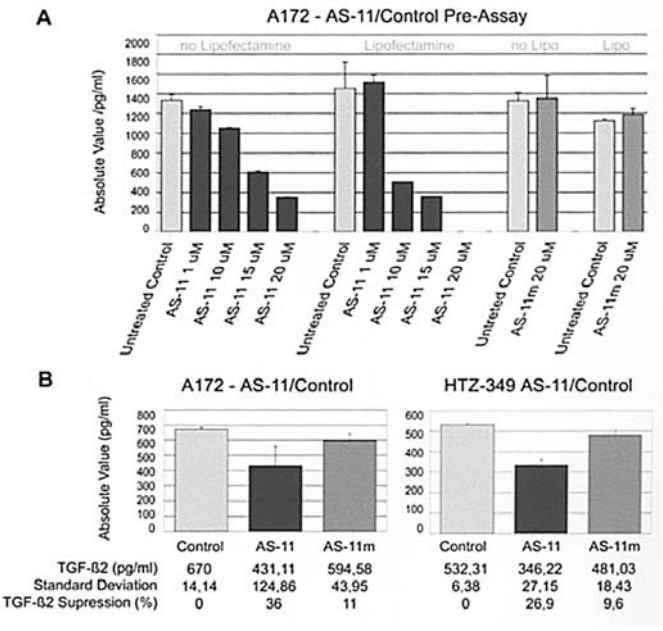

Figure 1. Dose-dependent suppression of TGF-ß2 as detected by ELISA in supernatants of cell lines HTZ-349 and A172 after 6 days of incubation with AS-11. The use of lipofectamine as an enhancer of transfection produces similar transfection efficacies with enhanced toxicity. Transfection of the mismatch control PTO AS-11m is not able to induce significant down-regulation of TGF-ß2.

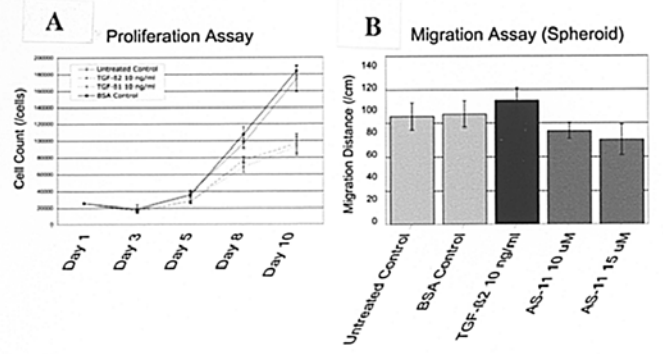

Figure 2. TGF-ß-dependent proliferation and migration (as tested with a spheroid assay) of HTZ-349 cells. A172 showed similar effects in a less pronounced manner. Stimulation with both isoforms of TGF-ß caused anti-proliferative effects. Migration was significantly enhanced by exogenous TGF-ß2. Antagonization of TGF-ß2 by AS-11 showed a dose-dependent suppression of migration. Incubation with BSA as control protein mixture had no impact on proliferation or migration.

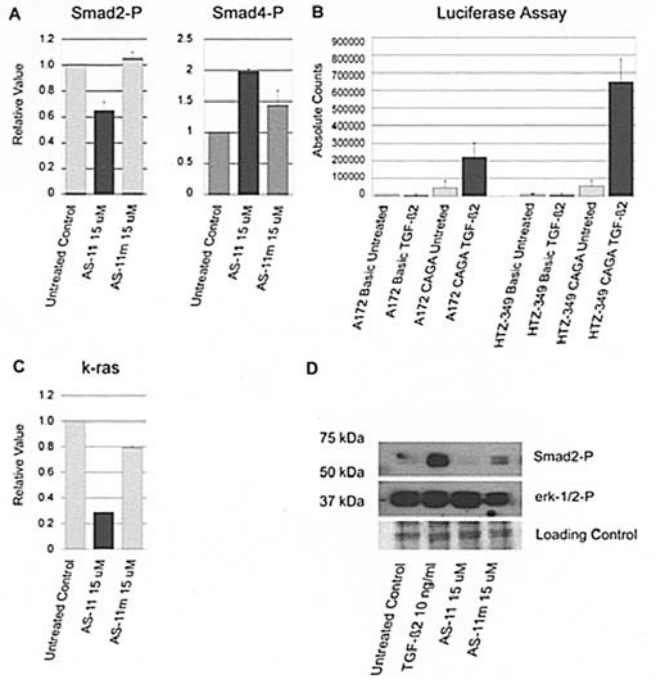

Figure 3. (A), Regulation of Smad 2 and 4 as detected by quPCR in cells incubated with AS-11 and AS-11m and an untreated control group (6 days). The AS-11 treated group showed significantly lowered Smad 2 and elevated Smad 4 levels (results shown for HTZ-349 cells, A172 cells showed analogous results with lower significance). (B), Luciferase assay with A172 and HTZ-349 cells. Both cell lines are stimulated with 10 ng/ml TGF-ß2. The Smad-specific reporter shows an increase of signal activity after TGF-ß2 stimulation. (C), Regulation of K-ras as detected by quPCR in cells incubated with AS-11 and AS-11m and an untreated control group (6 days). K-ras is significantly down-regulated after incubation with AS-11, but not in AS-11m treated cells. (D), Phosphorylation of Smad 2 and ERK 1/2 after treatment with TGF-ß2 and AS-11. In Smad 2-Western, the AS-11 treated cell population clearly shows the weakest, the TGF-ß2 stimulated group the strongest signal. There is little difference in signal intensity between the AS-11m treated group and the untreated control group. There is no regulation of ERK after treatment (results shown for A172 cells, HTZ-349 cells showed similar results with lower significance).

of 11%. In HTZ-349 cells, TGF-ß2 secretion was suppressed by an average of 26.9%, whereas AS-11m caused only 9.6% suppression (Fig. 1B).

TGF-ß inhibits proliferation of A172 and HTZ-349 cells. The Smad pathway is a TGF-ß induced mediator of anti-proliferative effects (52). To investigate the effects of TGF-ß2 on the proliferation of HTZ-349 and A172 cells, we performed a 10-day proliferation assay. TGF-ß2 had anti-proliferative effects on both cell lines, with the most prominent decrease of proliferation after 12 days of incubation. In A172 cells, proliferation was decreased by 44% after 10 days in comparison to untreated control, whereas BSA treated cells showed no significant growth inhibition (data not shown). In HTZ-349 cells, proliferation decreased 32.0% for TGF-ß2 after 10 days. TGF-ß1 had similar effects (Fig. 2A).

TGF-ß enhances migration of A172 and HTZ-349 spheroids, and AS-11 reverts this effect. As inductor of a mesenchymal cell type, MAPK pathway related effects have been described to enhance also the migratory capacity of malignant cells (58). A 4-day migration assay was performed with A172 and HTZ-349 cell spheroids, testing the MAPK mediated TGF-ß2 induced migration of glioma cells. The most prominent impact on migration was observed in the TGF-ß2 stimulated population with migration increased by 15% in comparison to the untreated control group. AS-11 treated cells showed a dose-dependent decrease of migration (10 μM: 13.8%, 15 μM: 21.8%) in comparison to untreated controls (Fig. 2B). A172 cells showed similar results (data not shown).

AS-11 acts as specific antagonist of the TGF-ß2/Smad pathway. We investigated mRNA expression levels of Smad 2 and Smad 4 in A172 and HTZ-349 cells incubated for 6 days with 15 μM AS-11 and 15 μM AS-11m. Smad 2 mRNA levels were down-regulated to 64% in AS-11 treated HTZ-349 cells,

Table II. Regulated ubiquitination-related genes in A172 cells after a 6-day incubation with AS-11 (specific effects).[a]

Ubiquitination related gene	Accession no.	Known functions	Refs.
Fring (ring finger and FYVE-like domain containing 1)	228980_at	Ubiquitination associated protein, caspase-associated Smad-antagonism	(59,60)
Ring finger protein 14	201824_at	Associated with the androgen receptor signaling pathway and with ubiquitin cycle and ubiquitin-like-protein ligase activity	(61,62)
Ring finger protein 12	224047_at	Acts as a negative coregulator for LIM homeodomain transcription factors	(63,64)
Zinc finger protein (C2H2 type) 277	1555193_a_at	Regulation of transcription, DNA-dependent; transcription factor activity	(65)
Membrane-associated RING-CH protein VI (MARCH VI)	201737_s_at	Deletions and mutations in this gene were detected in esophageal squamous cell carcinoma (ESCC), suggesting that this protein may be a potential tumor suppressor	(66)
Hypothetical protein FLJ14281	222850_s_at	Chaperone activity; protein folding	(67)
Ubiquitin protein ligase E3 component n-recognin 1	226921_at	Takes part in ligase activity; ubiquitin cycle; biquitin-protein ligase activity	(68,69)
Tetratricopeptide repeat domain 3	1569472_s_at	Ubiquitin-protein ligase activity, exact biological mechanism unknown	(67,70)
Protein phosphatase 4, regulatory subunit 2	225519_at	Serine-threonine kinase implicated in microtubule organization at centrosomes	(67,71)
Ring finger protein (C3H2C3 type) 6	203403_s_at	Part of protein ubiquitination, exact biological mechanism unknown	(67,72)

[a]Ubiquitination of Smad proteins is known to play an important role in the termination of TGF-ß intracellular signaling activity.

compared to the untreated control. AS-11m had no effect on Smad 2 mRNA levels. Smad 4 mRNA expression was increased almost 2-fold in AS-11 treated cells. Cells incubated with AS-11m showed less increased Smad 4 mRNA levels (Fig. 3A).

To evaluate the extent of TGF-ß2 related signal transduction at the protein level, and since Smad proteins are activated by phosphorylation (14), we performed Western blots for phosphorylated Smad 2 as one of the first steps of the intracellular signaling cascade. AS-11 treated cells showed the lowest amount of phosphorylated Smad 2 (Smad 2-P), whereas in cells incubated with AS-11m, Smad 2-P was nearly as prominent as in the untreated cell fraction. As expected, TGF-ß2 stimulated cells clearly showed the highest amount of phosphorylated Smad 2 (Fig. 3D).

To substantiate the functionality of the Smad pathway, we performed a luciferase assay. Both HTZ-349 and A172 showed an increase of luciferase activity after incubation of cells with TGF-ß2 in comparison with the untreated control group (Fig. 3B), which speaks for the functionality of the Smad pathway in the tested cell lines.

Down-regulation of K-ras mRNA expression by AS-11: impact on the MAPK pathway. A172 and HTZ-349 human malignant glioma cells were treated with AS-11 at a concentration of 15 μM for 6 days. After harvesting, mRNA was isolated and array chip hybridization was performed. In A172 cells (54676 genes on chip, 33627 detectable), 271 genes were specifically, 889 genes were non-specifically altered in their expression. Of the specifically altered genes, 191 were down-regulated, 80 were upregulated. Forty-one genes were only expressed under AS-11 treatment. In HTZ-349 cells (54676 genes on chip, 28611 detectable), 73 genes were specifically, 3164 non-specifically altered. Of the specifically altered genes, 39 were down- and 34 were upregulated.

We used NIH David 2.1. analysis software for functional clustering and pathway analysis of specifically altered gene expression. For A172 cells, upregulated genes were clustered in five functional groups: catabolism, ubiquitination/ligase activity (Table II), serine-threonine kinase activity, mRNA-splicing and processing and gene transcription/DNA-dependent cellular metabolism. Prominently, K-ras was down-regulated in A172 cells due to specific effects. There were no other

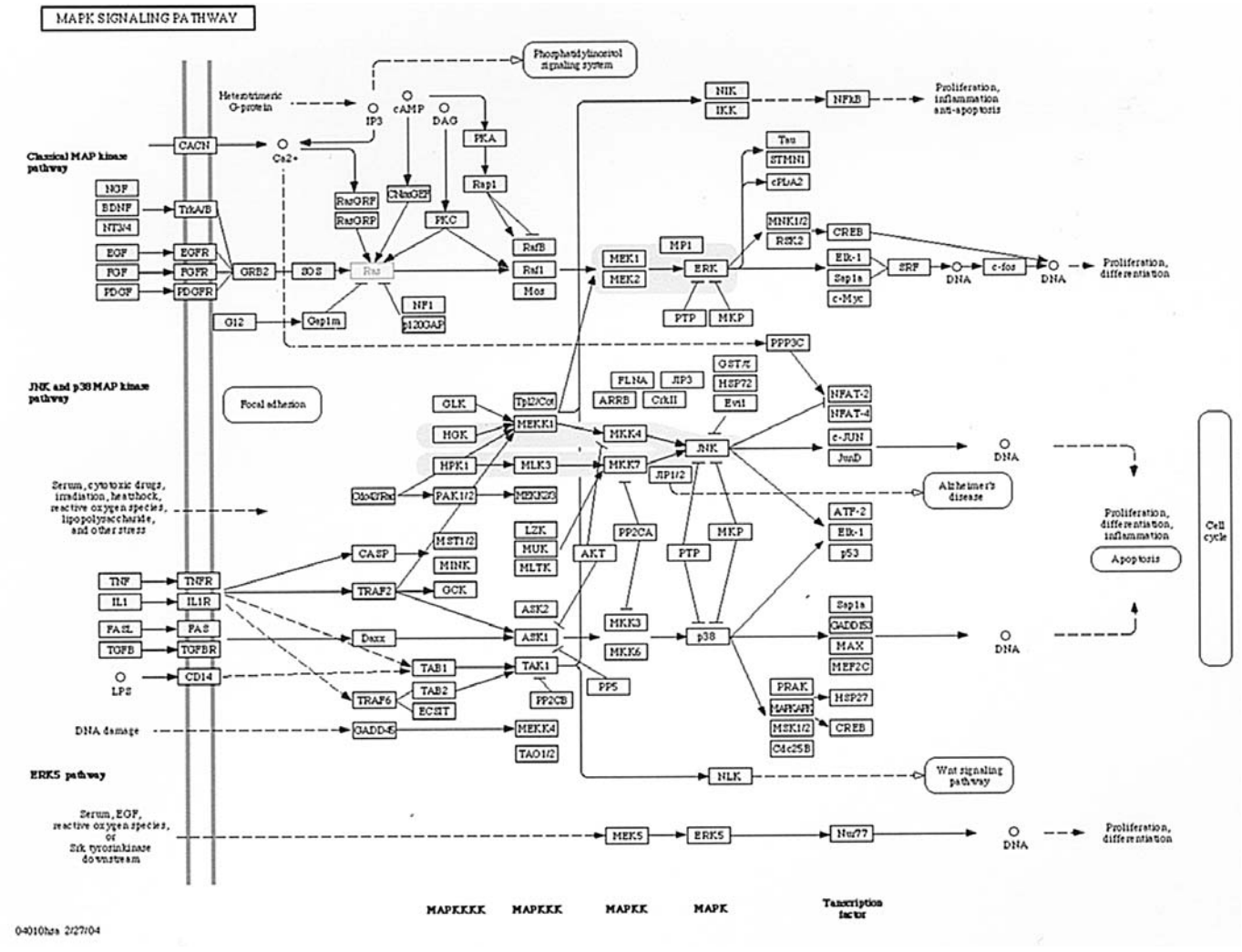

Figure 4. Specific alterations caused by AS-11 in the TGF-ß related pathway after 6 days (NIH David 2.1). Ras was down-regulated in an assay which used specific antisense oligonucleotides against TGF-ß2. There were no other measurable alterations of this pathway as detectable by gene array.

specific effects on gene expression of the TGF-ß2 signaling pathways, but due to unspecific effects, gene expression of downstream Raf was down-regulated, whereas MEK was upregulated also due to unspecific effects (Fig. 4).

To substantiate these results, we performed quPCR on mRNA samples of both cell lines. In both, K-ras was down-regulated significantly due to specific AS-11 effects. Unspecific effects had only little influence on down-regulation of K-ras (Fig. 3C).

Down-regulation of K-ras has no impact on ERK1/2. Since ERK1/2 is a major downstream target of K-ras and a key player in malignant transformation of human cancer cells (33,34), we determined the state of phosphorylation of ERK1/2 by Western blot analysis as an indicator of ERK activation. In contrast to Smad 2 phosphorylation, Antisense-PTO treatment and stimulation by TGF-ß2 had no effect on phosphorylation of ERK (Fig. 3D).

Discussion

TGF-ß2 is known to be secreted by high-grade gliomas both *in vivo* and *in vitro* (3,35,36) and induces a malignant phenotype (13). Imbalance between the Smad and MAPK path-

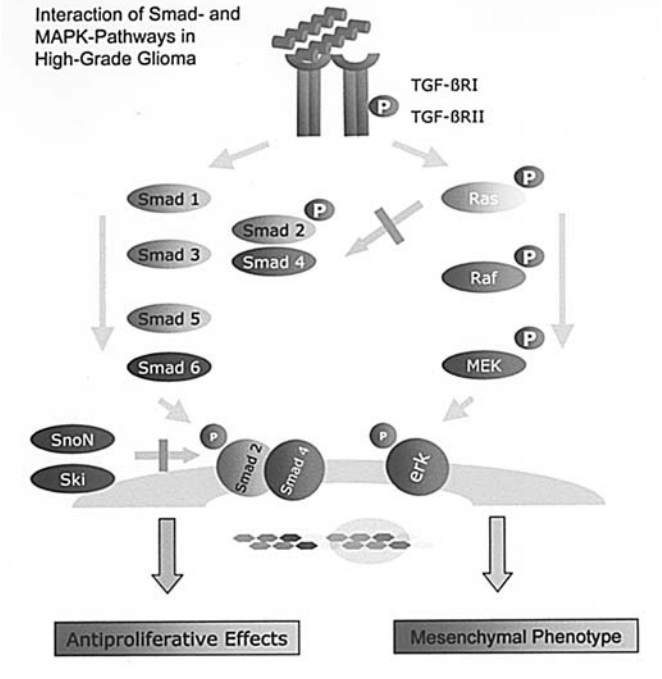

Figure 5. Possible alterations in the Smad- and MAPK pathways leading to TGF-ß tumor promoter activity.

ways may be responsible for the malignant transition of low-grade to high-grade gliomas. In this study, antisense oligonucleotides that selectively inhibit gene expression were used as an approach to study the functional role of TGF-ß2 in glioma cells. After uptake of AS-11 into the cells, which is mediated by receptor-targeted endocytotis (37-39), we were able to specifically suppress the TGF-ß2 secretion. Since Antisense-PTOs have been repeatedly reported to cause effects by non-specific interactions especially with proteins of the serine-threonine receptor group (40-42), the impact of an AS-11 control PTO, AS-11m, on Smad-dependent TGF-ß2 signal transduction was evaluated, but only minor unspecific PTO-related effects were found.

Our results demonstrate that the antisense approach to inhibit TGF-ß2 expression influences both the Smad and MAPK pathways. Smad 2 mRNA was significantly reduced in the AS-11 treated cells, and Smad 2 was inactive in AS-11 treated glioma cells. Smad 2 transcription rates can be used as medium-term indicators for Smad 2 activity, since transcription of Smad 2 and Smad 4 is triggered by post-translational modifications of the two proteins (44). Moreover, Smad 4 transcription was enhanced in the AS-11 treated fractions. This seemingly paradoxical finding can be explained by Smad 4-Ras interactions. Ras has been described as an inductor of Smad 4 degradation by ubiquitination. In the presence of activated Ras, Smad 4 half-life averages at 3 h, whereas successful antagonization of Ras can prolong Smad 4 half-life to 8 h (44). We have also detected specific down-regulation of 10 ubiquitination-related genes with AS-11 in A172 cells, as the ubiquitination of Smad proteins plays an important role in the termination of TGF-ß intracellular signaling activity (45,46).

Consistent with the reduced expression of Ras and ubiquitination-related genes due to specific effects of AS-11, we hypothesize that heightened Smad 4 mRNA levels indicate a reduced Smad 4 degradation. This decreased degradation consecutively might lead to higher Smad 4 transcription due to its dependence on post-transcriptional modifications such as phosphorylation in a Smad 2-like manner. Also gene expression patterns pointed to a reduced Smad 4 degradation.

The second major TGF-ß intracellular signaling cascade, the MAPK pathway, showed decreased activity in AS-11 treated glioma cells. We detected that the expression of K-ras, a brain specific isoform of Ras and the most prominent oncogene of the MAPK pathway (25-27), was down-regulated exclusively in the AS-11 treated populations of both cell lines.

Activation of the MAPK pathway by activated TGF-ß receptor complexes including Ras isoforms has already been described for several cell types (15,16). To the best of our knowledge, this is the first evidence for an involvement of K-ras in TGF-ß dependent signal transduction in malignant glioma cells, although pathological ras-activity has already been observed in glioma cells (47-49).

An imbalance between MAPK- and Smad-dependent TGF-ß signaling might well be the cause for the change of TGF-ß from tumor suppressor to tumor promoter (28). Loss of function in Smad signaling such as nonsense mutations or deletions of the Smad 2 and Smad 4 gene loci have been described as pro-oncogenic for several tumor entities (50). However, the majority of tumors retain a functional TGF-ß signaling system, nevertheless refractory to its growth inhibitory effects. Upregulation of the MAPK pathway during the course of carcinogenesis was blamed for an imbalance of the TGF-ß pathways (51,52). In this context, Ras is thought to play a central role. Ras induces Smad 4 degradation by enhanced ubiquitination and therefore suppresses the anti-proliferative Smad-mediated effects (29). Hyperactivity of Ras therefore might be a key to TGF-ß-related tumor progression. Moreover, Ras leads, via activation of its downstream substrates, to the enhanced transcription of extracellular matrix proteins and to cytoskeletal rearrangement, favouring invasion and migration of the malignant cells (15,29,30). Considering this, down-regulation of K-ras might explain the inhibition of migration with AS-11 in our assays.

It is important to mention that in this model of TGF-ß induced carcinogenesis, the growth inhibitory effects of the Smad pathway are not fully antagonized. Indeed, colon cancer cells lacking the TGFß-R showed slight growth inhibition after reintroduction of a functional receptor, but their invasive and migrative capacity was strongly enhanced (52). These findings are supported by our own data that demonstrated the TGF-ß induced growth inhibition, but increased migration of high-grade glioma cells.

It remains open at this point how the imbalance between Smad- and MAPK-dependent signaling originates (Fig. 5), but our results emphasize that ras hyperactivity can at least be influenced by TGF-ß antagonization. Therefore, a sufficient TGF-ß antagonization might be a promising approach to partially reverse the imbalance of the Smad- and the MAPK-cascades.

Hence, there was little specific effect on gene expression of Ras-specific downstream targets. There was no effect either by TGF-ß2, nor by PTO incubation, on the state of phosphorylation of ERK1/2 as an endpoint of the MAPK cascade.

Activated Ras leads to phosphorylation of the cytosolic serine-threonine kinase Raf, which in turn activates the mitogene-activated protein kinase (MEK), finally leading to activation of ERK (52). Since activated ERK is not only an activator of multiple transcription factors, but also affects parts of the cytoskeleton and various phosphatases, it leads to a more mesenchymal phenotype of the cell and is a pro-migratory factor (54). In this context, ERK becomes important in the course of carcinogenesis (55). In most publications, hyperactivity of the ras-raf-MEK pathway, mainly in the course of ras mutations, is blamed for increased ERK activation (53). Our results indicate that ERK activation is not dependent on the ras-raf-MEK cascade in glioma. This is consistent with previous studies that demonstrated that ERKs are activated through a PKC-raf-MEK-dependent, but ras-independent cascade in high-grade gliomas (56,57). ERK activation has been described as an inhibitor of Smad signaling by blocking Smad translocation into the nucleus (29). It therefore seems to be another key actor in the process of the imbalance between Smad- and MAPK-dependent signaling. Furthermore, recently it has been reported that the ERK pathway regulates glioma cell proliferation, migration and differentiation (58). A combined approach of TGF-ß and MEK/ERK antagonization therefore seems to be a promising step towards a coherent new therapy for high-grade gliomas.

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