Participation of an abnormality in the transforming growth factor–β signaling pathway in resistance of malignant glioma cells to growth inhibition induced by that factor

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Object. Malignant glioma cells secrete and activate transforming growth factor–β (TGFβ) and are resistant to growth inhibition by that factor. Nevertheless, the mechanism underlying this effect remains poorly understood. In this study, the mechanism of the resistance to growth inhibition induced by TGFβ was investigated.

Methods. The authors examined the expression of downstream components of the TGFβ receptor, including Smad2, Smad3, Smad4, and Smad7, and the effect of TGFβ treatment on the phosphorylation of Smad2 and the nuclear translocation of Smad2 and Smad3 by using 10 glioma cell lines and the A549 cell line, which is sensitive to TGFβ-mediated growth inhibition. The expression of two transcriptional corepressor proteins, SnoN and Ski, and the effect of TGFβ treatment on the expression of the SnoN protein and the cell cycle regulators p21, p15, cyclin-dependent kinase–4 (CDK4), and cyclin D1 were also examined.

Expression of the Smad2 and Smad3 proteins was lower in the glioma cell lines than in the A549 cell line and in normal astrocytes. In particular, Smad3 expression was low or very low in nine of the 10 malignant glioma cell lines. Expression of Smad4 was low in four glioma cell lines, and expression of the Smad7 protein was similar when compared with protein expression in the A549 cell line and in normal astrocytes. The levels of Smad2 phosphorylation after TGFβ treatment were lower in glioma cell lines than in the A549 cell line, except for one glioma cell line. Seven of the 10 glioma cell lines exhibited lower levels of nuclear translocation of Smad2 and Smad3, and two cell lines that expressed very low levels of Smad3 protein showed no nuclear translocation. All glioma cell lines expressed the SnoN protein and its expression was unaltered by treatment with TGFβ. Three glioma cell lines expressed high levels of the Ski protein. The expression of the p21<sup>INK4B</sup>, p15<sup>INK4B</sup>, CDK4, and cyclin D1 proteins was not altered by TGFβ treatment, except in one cell line that displayed a slight increase in p21 protein. Overall, the expression of the Smad2 and Smad3 proteins was low in the glioma cell lines, the phosphorylation and nuclear translocation of Smad2 and Smad3 were impaired, and the TGFβ receptor signal did not affect the expression of the SnoN, p21, p15, cyclin D1, and CDK4 proteins.

Conclusions. These results suggest that the ability to resist TGFβ-mediated growth inhibition in malignant glioma cells is due to abnormalities in the TGFβ signaling pathway.

KEY WORDS • transforming growth factor–β • Smad • malignant glioma • Ski • SnoN • p21

Transforming growth factor–β is a multifunctional polypeptide implicated in the regulation of various cellular processes including growth, differentiation, apoptosis, adhesion, and motility. Transforming growth factor–β binds to two different types of serine/threonine kinase receptors, Type I and Type II. The Type I receptor is activated by the Type II receptor on ligand binding and mediates specific intracellular signals (Fig. 1). The activated Type I receptor then causes phosphorylation and activation of downstream signaling components including Smad2 and Smad3. After phosphorylation, Smad2 and Smad3 form heteromeric complexes with Smad4 and then migrate from the cytoplasm into the nucleus. Nuclear Smad complexes bind to transcriptional coactivators or corepressors and regulate transcription of target genes. Smad7 prevents signaling by inhibiting Type I receptor–mediated phosphorylation of Smad2 and Smad3, and then impedes interaction with Smad4 and blocks subsequent nuclear translocation.

The TGFβ signaling pathway is negatively regulated by the transcriptional corepressors Ski and Sno (a Ski-related...
The Ski gene is named for the Sloan–Kettering Institute, where it was identified in the early 1980s as the culprit gene in a virus that causes tumors in chickens. The sequence of Sno, found a few years later, is very similar to the human version of Ski. Four Sno complementary DNAs, representing alternatively spliced forms, have been found in humans, including SnoN, SnoA, SnoI, and SnoN2. Both Ski and Sno are oncoproteins that, when overexpressed induce oncogenic transformation of chicken and quail embryo fibroblasts. Both Ski and Sno also repress the antiproliferative effects of TGFβ. The SnoN protein is degraded by the Smad3 protein and then its expression is induced through a TGFβ signaling cascade. It has been suggested that SnoN participates in the negative feedback control of TGFβ signaling.

Transforming growth factor–β inhibits proliferation of various cell types by inhibiting the activity of CDKs; this leads to the arrest of the cell cycle in the early G1 phase. The mechanism of action of TGFβ in modulating the activity of CDKs involves the regulation of the CDK inhibitors p15Ink4b, p21Cip1, and p27Kip1. Transcriptional induction of the genes for the two CDK inhibitors, p15 and p21, may partially determine the antiproliferating action of TGFβ. Transforming growth factor–β also regulates the expression of cyclin D1 and CDK4.

In our previous studies we found that malignant glioma cells secrete TGFβ, that thrombospondin-1 secreted by glioma cells participates in the activation of latent TGFβ, and that malignant glioma cells are refractory to TGFβ growth inhibition, despite the fact that they express TGFβ receptors. The mechanism underlying this effect, however, remains poorly understood. In the present study we further investigated the mechanism of loss of sensitivity to TGFβ-mediated growth inhibition in malignant glioma cells by examining the expression of the downstream components of the TGFβ receptor, including Smad2, Smad3, Smad4, and Smad7, and the effect of TGFβ, treatment on the phosphorylation of Smad2 and nuclear translocation of Smad2 and Smad3. The expression of the transcriptional corepressor proteins SnoN and Ski, and the effect of TGFβ, treatment on the expression of the SnoN protein and the cell cycle regulators p21, p15, CDK4, and cyclin D1 were also examined.

Materials and Methods

Cell Culture and Surgical Specimens

Ten human malignant GBM cell lines (T98G, U251, A172, KG1C, TM2, YMG1, YMG2, YMG3, YMG4, and YMG5), a human lung adenocarcinoma cell line (A549), and a mink lung epithelium...
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Lial cell line (Mv1Lu) were maintained in RPMI-1640 medium, which had been supplemented with 10% fetal bovine serum and 100 µg/ml kanamycin. The T98G, U251, A172, KG1C, and A549 cell lines were obtained from the Health Science Research Resources Bank, (Tokyo, Japan). The T98G, U251, A172, and KG1C cell lines had been widely used and are characterized by the expression of GFAP and/or S100 protein. The TM2 cell line, which expresses GFAP and S100, was donated by Dr. Masaki Kurimoto (Department of Neurosurgery, Toyama Medical and Pharmaceutical University, Toyama, Japan). The YMG1, YMG2, YMG3, YMG4, and YMG5 cell lines were derived from GBMs or anaplastic astrocytomas and established in our laboratory. All five of these cell lines express S100 and three of them (specifically, YMG1, YMG3, and YMG5) expressed GFAP. The YMG3 and YMG5 cell lines express the p15 and p16 proteins, and the other three do not. A sample of a normal human astrocyte was obtained from Cambrex Bio Science Walkersville, Inc., (Walkersville, MD) and cultured according to the manufacturer’s protocol. The Jurkat cell line (human T-cell leukemia) was obtained by one of the authors (A.N.) and its cell lysate was used as a positive control for Smad7. The sensitivity of Mv1Lu and A549 cells to TGF-β-mediated growth inhibition was evaluated in a previous study.12

Antibodies and Reagents

The primary antibodies used in this study were rabbit anti-Smad2/3 antibody (Upstate Biotechnology, Inc., Lake Placid, NY); rabbit anti–phospho-Smad2 (Ser465/467) antibody (Cell Signaling Technology, Inc., Beverly, MA); mouse anti–Smad4 antibody (BD Biosciences, San Diego, CA); goat anti–Smad7 antibody (Abcam Ltd., Cambridge, United Kingdom); rabbit anti–Smad4 antibody, rabbit anti–SnoN antibody, mouse anti–p21Cip1 antibody, and mouse anti–p15Ink4a antibody (Upstate Biotechnology, Inc.,); rabbit anti–cyclin D1 antibody, rabbit anti–CDK4 antibody, and rabbit anti–actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti–heat shock protein 70 antibody (Transduction Laboratories, Lexington, KY); mouse anti–PCNA antibody (BD Biosciences). The secondary antibodies were alkaline phosphatase–conjugated swine anti–rabbit and rabbit anti–mouse antibodies (DakoCytomation, Carpinteria, CA) and alkaline phosphatase–conjugated rabbit anti–goat antibody (Chemicon, Inc., Temecula, CA). Horseradish peroxidase–conjugated goat anti–rabbit antibody and LumiGLO Reagent were obtained from Cell Signaling Technology, Inc. Recombinant human TGFβ, was obtained from PeproTech (London, United Kingdom). Hela cell lysate was obtained from Upstate Biotechnology, Inc., and used as a positive control for the SnoN and Ski proteins in a Western blot analysis. The HepG2 cell lysate was obtained from Abcam Ltd. and used as a positive control for Smad7.

Growth Inhibition Assay Performed Using TGFβ

The effect of TGFβ on the growth of the Mv1Lu and A549 cell lines and the 10 malignant glioma cell lines was examined in a manner described previously. Briefly, 100-µl aliquots of recombinant TGFβ (5 ng/ml) were placed in a 96-well plate in triplicate experiments and serial twofold dilutions were made in the plate. Fifty-microliter aliquots of each cell suspension (2000 cells) were added to each well. After incubation for 72 hours at 37°C in 5% CO2, the medium was decanted, and the cells were stained for 5 minutes with 0.5% crystal violet dissolved in 20% methanol. After washing, the dye was eluted with 50% ethanol, and the absorbance at 570 nm was determined with the aid of a spectrophotometer. The value of the optical density correlates with the number of cells in a well.12

Treatment of Cells With Recombinant TGFβ

Malignant glioma cells, A549 cells, and normal human astrocytes were cultured in 6- or 9-cm dishes until they were subconfluent. The cells were incubated for 30 minutes with TGFβ (5 ng/ml) for an analysis of Smad2 phosphorylation and SnoN expression and for 12 hours with TGFβ (5 ng/ml) for an analysis of the effect of TGFβ on the expression of SnoN, p21, p15, cyclin D1, and CDK4 proteins. In preliminary studies, p21 protein expression reached the maximal level at least 12 hours after TGFβ treatment (data not shown). The cells were washed twice with phosphate-buffered saline, and cell lysates were prepared by lysing cells in a RIPA lysis buffer (Upstate Biotechnology, Inc.).

Nuclear/Cytosol Fractionation

The 10 malignant glioma cell lines and the A549 cell line were treated with TGFβ (5 ng/ml) for 60 minutes to examine the translocation of Smad2 and Smad3 proteins from the cytoplasm to the nuclear compartment and the expression of SnoN protein. Cell lysates were prepared as described earlier. The cell lysates were separated into nuclear and cytoplasmic fractions by using the Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA) according to the manufacturer’s protocol. The fractionated samples were subjected to a Western blot analysis to detect Smad2, Smad3, and SnoN. The fractionated samples from untreated malignant glioma and A549 cells were also analyzed for Smad7. The efficacy of the fractionation was examined using antiactin and anti–heat shock protein 70 antibodies as internal controls in the Western blot analysis.

Western Blot Analysis

The cell lysates from each sample were separated on 6, 10, or 16% sodium dodecyl sulfate polyacrylamide gels (TREFO, Tokyo, Japan) and electroblotted on a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were treated with 5% nonfat dry milk or 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 for 1 hour at room temperature or overnight at 4°C. After the membranes had been washed, they were incubated with primary antibodies for 1 hour at room temperature or overnight at 4°C. The membranes were again washed and incubated with secondary antibodies for 1 hour at room temperature. Following this, each membrane was incubated with CDP-Star substrate (Roche, Mannheim, Germany) to develop the signal. The immunoblot signals were visualized by chemiluminescence and recorded with the aid of an image analyzer (LAS1000 Lumino Image Analyzer; Fuji Photo Film, Tokyo, Japan). For the horseradish peroxidase–conjugated secondary antibody, LumiGLO agent (Cell Signaling, Technology, Inc.) was used to develop the signal, which was also visualized by chemiluminescence. As internal controls for protein content from whole cell lysate and nuclear protein, the lysates were analyzed using antiactin and anti–PCNA antibodies, respectively.

Results

Effect of TGFβ on the Growth of Malignant Glioma Cells

The growth of the Mv1Lu and A549 cell lines was inhibited by TGFβ in a dose-dependent manner (Fig. 2). The A549 cell line demonstrated some resistance, even to high doses (5 ng/ml) of TGFβ, compared with the Mv1Lu cell line. Six of the 10 malignant glioma cell lines displayed resistance to high doses (5 ng/ml) of TGFβ, whereas three cell lines (U251, A172, and YMG3) showed weak sensitivity and one cell line (YMG2) demonstrated moderate sensitivity (Fig. 3). Expression of Smad Proteins

The expression of the Smad2 and Smad3 proteins was variable and low in malignant glioma cell lines compared with the TGFβ-sensitive A549 cell line and normal astrocytes (Fig. 4). Six (T98G, U251, YMG1, YMG2, YMG3, and YMG5) of the 10 glioma cell lines demonstrated weaker expression of Smad2 than the A549 cell line. Nine glioma cell lines displayed lower expression of Smad3 than the A549 cell line, and six of these cell lines showed very low levels of Smad3. Only the YMG2 cell line exhibited a level of Smad3 comparable to those of the A549 cell line.
and normal astrocytes. In four glioma cell lines (T98G, U251, KG1C, and YMG2), the expression of the Smad4 protein was lower than that in the A549 cell line and was comparable to the expression observed in the other six cell lines (Fig. 4). The expression of the Smad7 protein in glioma cell lines was comparable to the expression of that protein in A549 cell lines and normal astrocytes (Fig. 4).

**Cellular Localization of Smad7**

Western blotting was used to analyze the cytoplasmic and nuclear fractions of glioma cell lysates for Smad2, which prevents signaling by inhibiting the phosphorylation of Smad2 and Smad3, because Smad7 is located in the nucleus in many types of cells and in the cytoplasm in certain cells. The Smad7 protein was mostly localized in the cytoplasm in the malignant glioma and A549 cell lines (Fig. 5).

**Effect of TGFβ on the Phosphorylation of Smad2**

To examine whether malignant glioma cells have an ability to phosphorylate Smad2 by means of the TGFβ receptor complex, we analyzed the phosphorylation of Smad2 after TGFβ treatment. Only the KG1C cell line displayed a level of phosphorylated Smad2 that was comparable to that in normal astrocytes and A549 cell lines (Fig. 6). The levels of phosphorylated Smad2 in the other cell lines were lower than levels observed in A549 cells and normal human astrocytes.

**Effect of TGFβ on Translocation of Smad2 and Smad3 to the Nucleus**

Phosphorylated Smad2 and Smad3 form heteromeric complexes with Smad4 and move from the cytoplasm to the nucleus. The levels of Smad2 and Smad3 decreased in the cytoplasm and increased in the nucleus after TGFβ treatment in the TGFβ-sensitive A549 cell line (Fig. 6). Only the YMG2 cell line displayed a comparable level of translocation of Smad2 and Smad3. Seven glioma cell lines (T98G, U251, A172, YMG1, YMG3, YMG4, and YMG5) exhibited small changes in Smad2 and Smad3 levels, and two glioma cell lines (KG1C and TM2) showed no changes. Interestingly, the KG1C and TM2 cell lines were completely resistant to TGFβ (Fig. 3) and exhibited very low levels of Smad3 protein (Fig. 4). Although the KG1C cell line displayed a high level of Smad2 phosphorylation, no obvious

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**Graphs**

Fig. 2. Line graphs showing the effect of TGFβ on the growth of the Mv1Lu and A549 cell lines, which are sensitive to TGFβ-induced growth inhibition. Transforming growth factor-β (5 ng/ml) was placed in a 96-well plate in triplicate and serial twofold dilutions were made in the plate. The cell suspension (2000 cells) was added to the 96-well plates and cultured for 72 hours. The values of optical density correlate to the number of cells in each well. The values are expressed as means ± standard deviations.

Fig. 3. Bar graph demonstrating the effect of TGFβ on the growth of 10 malignant glioma cell lines and the A549 cell line. The glioma cell lines (2000 cells/well) were cultured in 96-well plates with or without serially diluted TGFβ. The values are expressed as means ± standard deviations.
translocation of Smad2 was observed from the cytoplasm to the nucleus.

Expression of the Transcriptional Corepressors Ski and SnoN

The SnoN and Ski proteins are transcriptional corepressors and negatively regulate the TGFβ signaling pathway. The anti-Sno antibody used in the present study recognizes a carboxyl terminus of SnoN (684 amino acids) and SnoN2 (638 amino acids), because the amino acid sequences of SnoN and SnoN2 are identical in the carboxyl terminus. In human tissues, SnoN2 is the less abundantly expressed isoform. In HeLa cell lysate used as a positive control for SnoN, a thick band (nearly 77 kD) would be SnoN and a thin band below the thick band would be SnoN2. The expression levels of the SnoN protein were high in all 10 malignant glioma cell lines, in the A549 cell line, and in normal astrocytes (Fig. 7). The Ski protein was highly expressed in three glioma cell lines (TM2, YMG2, and YMG5) and weakly expressed in the other glioma cell lines, the A549 cell line, and normal astrocytes.

Effect of TGFβ, Treatment on Expression of the SnoN Protein

We examined the effect of TGFβ treatment on the expression of the SnoN protein, because SnoN is rapidly degraded by the nuclear accumulation of Smad3. After TGFβ treatment, the SnoN protein reaches its lowest level by 30 minutes and an increase in SnoN messenger RNA occurs at 1 to 2 hours and lasts for 24 hours. The levels of SnoN expression in whole-cell lysates did not change 30 minutes and 12 hours after TGFβ treatment in the A549 cell line and in normal astrocytes (Fig. 8A). The levels of SnoN expression in the nuclear fraction did not decrease 60 minutes after TGFβ treatment in A549 cell line and in the 10 glioma cell lines (Fig. 8B).

Effect of TGFβ, on the Expression of the p21CIP1, Cyclin D1, and CDK4 Proteins

Expression of the p21 protein was low in seven of 10 glioma cell lines compared with expression of this protein in the A549 cell line and in normal astrocytes (Fig. 9). Expression of the p21 protein increased after TGFβ treatment in the A549 cell line and in normal astrocytes. Among glioma cells, only the YMG2 cell line exhibited a slight increase in p21 expression; the other nine glioma cell lines showed no increase. This is in agreement with the finding that the YMG2 cell line showed moderate sensitivity to
TGFβ-mediated growth inhibition (Fig. 3). Expression of cyclin D1 increased after TGFβ treatment in the A549 cell line, but the expression of CDK4 was not affected (Fig. 9). Expression of cyclin D1 and CDK4 proteins did not change in the malignant glioma cell lines or in normal astrocytes.

Effect of TGFβ on the Expression of p15INK4B
In preliminary studies, only three (TM2, YMG3, and YMG4) of the 10 malignant glioma cell lines expressed the p15 protein. The effect of TGFβ treatment on p15 expression was evaluated in these three cell lines, the A549 cell line, and normal astrocytes. The T98G and U251 cell lines, which did not express p15, were also used. The U251 cell line is known to lack p15 genes. Treatment with TGFβ did not alter p15 expression in any of the glioma cell lines, the A549 cell line, or normal astrocytes (Fig. 10). The expression level of the p15 protein in normal astrocytes was lower than that in the TM2, YMG3, and YMG4 cell lines.

Discussion
The present study led to the identification of several abnormalities in the TGFβ signaling pathway in the 10 glioma cell lines under investigation. First, the expression of both Smad2 and Smad3 proteins was variable and lower in glioma cell lines than in the A549 cell line and on normal astrocytes. Second, the level of phosphorylation of Smad2 after TGFβ treatment was lower in the malignant glioma cell lines than in the A549 cell line and in normal astrocytes. Third, translocation of Smad2 and Smad3 from the cytoplasm to the nucleus was impaired in the glioma cell lines. Fourth, the expression level of SnoN was not affected by TGFβ treatment in the glioma cell lines. Finally, induction of p21 and p15 proteins by TGFβ treatment was suppressed in the glioma cell lines.

Six of the 10 glioma cell lines expressed low levels of the Smad2 protein and nine expressed low levels of the Smad3 protein; none of the glioma cell lines displayed the levels of expression of Smad2 or Smad3 seen in the A549 cell line and normal astrocytes. Four of the glioma cell lines were observed to have low levels of Smad4 protein as well. These results agree with findings that messenger RNA expression of Smad2, Smad3, and Smad4 is decreased in human gastric cancer cells restored induction of p21 and p15 gene expression, and growth inhibition in response to TGFβ. These reports and the present results suggest that the expression of Smad3 is important in TGFβ signaling in malignant glioma cells.

Overexpression of Smad7 has been reported to be present in some cancers. Enhanced expression of Smad7 may be one of the mechanisms of TGFβ resistance in human gastric carcinomas and pancreatic cancers. In the present study, the glioma cells expressed levels of Smad7 that were comparable to those identified in the A549 cell line and in normal astrocytes. In many types of cells, Smad7 is located in the nucleus, and in certain cells in the cytoplasm. The present study revealed that the presence of Smad7 was mostly localized in the cytoplasm of glioma cells, suggesting the capability of Smad7 to inhibit phosphorylation of Smad2 and Smad3 by TGFβ receptors.

The phosphorylated form of Smad2 is an indicator of...
cells responding to bioactive TGFβ and nuclear translocation is a marker of activation of Smad2 and/or Smad3 in the TGFβ/Smad signaling cascade. In the present study we examined the expression of phosphorylated Smad2 after treatment with TGFβ. Nine of the 10 glioma cell lines expressed lower levels of phosphorylated Smad2 after TGFβ treatment than the A549 cell line or normal astrocytes. This finding is in agreement with the report that TGFβ induces phosphorylation of Smad2 in GBM cells. Nevertheless, the present results also suggest impairment of levels of Smad2 phosphorylation by TGFβ stimulation in glioma cells. We also found that translocation of Smad2 and Smad3 from the cytoplasm to the nucleus was impaired in nine glioma cell lines; the YMG2 cell line was the exception. Based on results showing that expression of Smad7 was not increased, but expression of Smad2 and Smad3 was low in glioma cell lines, we suggest that the relative abundance of Smad7 expression over that of Smad2 and Smad3 may result in impaired phosphorylation of Smad2 and thus in decreased translocation of Smad2 and Smad3. There were exceptions, however. The KG1C cell line displayed a comparable expression of phosphorylated Smad2 to that of the A549 cell line, but no obvious translocation of Smad2 to the nucleus. In contrast, YMG2 cells exhibited a low level of phosphorylated Smad2, but a similar level of translocation of Smad2 and Smad3 to the nucleus when compared to the A549 cell line. This difference between the two cell lines remains to be clarified.

Transforming growth factor–β upregulates the transcription of the CDK inhibitors p21 and p15, which inhibit CDK phosphorylation of the retinoblastoma protein and thus halt cell-cycle progression in the G1 phase. In the present study, the expression of p21 protein after TGFβ treatment was upregulated in the A549 cell line and in normal astrocytes and was slightly increased in only one glioma cell line (YMG2); there was no increase in the other glioma cell lines.

![Figure 7](image1.png)

**Fig. 7.** Results of a Western blot analysis of the transcriptional corepressor proteins SnoN and Ski in 10 malignant glioma cell lines, the A549 cell line, and normal astrocytes. Hela cell lysate was used as a positive control for SnoN and Ski. Forty micrograms of protein was loaded onto each lane.

![Figure 8](image2.png)

**Fig. 8.** Results of a Western blot analysis of the effect of TGFβ on the expression of SnoN protein. A: Normal astrocyte A549 cells were treated with (+) or without (−) TGFβ (5 ng/ml) for 0.5 or 12 hours and whole-cell lysates were examined for SnoN. B: Ten types of malignant glioma A549 cells were treated with (+) or without (−) TGFβ (5 ng/ml) for 60 minutes and the nuclear fractions were examined for SnoN. The protein content of PCNA was examined as an internal control for the nuclear fraction.
cell lines. Furthermore, the expression of p15 protein was not affected by TGFβ/H9252 treatment in glioma cell lines and in normal astrocytes. The transcriptional induction of p21 and p15 is mediated by Smad3 and Smad4 through Sp1 binding sites in the promoter of CDK inhibitor genes. The Smad3 protein is involved in the induction of p21 and p15 expression. The present results showed that nine of the 10 glioma cell lines displayed low or very low levels of Smad3 protein. This suggests that the low levels of Smad3 failed to induce p21 and p15 expression in the malignant glioma cell lines. Interestingly, the glioma cell line (YMG2) whose level of Smad3 was comparable to that of the A549 cell line demonstrated some sensitivity to TGFβ/H9252-mediated growth inhibition. These observations suggest an important role of Smad3 in TGFβ signaling.

The expression of cyclin D1 and CDK4 after TGFβ treatment was also examined because TGFβ regulates the expression of cyclin D1 and CDK4. Only the A549 cell line was found to have upregulation of cyclin D1 after TGFβ treatment; the 10 glioma cell lines and the normal astrocytes showed no response. These results were similar to the response of p21 and p15 expression to TGFβ treatment. Transforming growth factor–β does not affect the level of cyclin D1 in Smad3-deficient colonocyte cells. In the present study, however, the YMG2 cell line, which expresses Smad3, responded weakly to TGFβ, and showed a slight increase in p21 protein, did not show any change in the level of cyclin D1 protein after TGFβ treatment. These results suggest that the role of Smad3 in the induction of cyclin D1 after TGFβ treatment is still unclear.

The expression of SnoN and Ski proteins has not been previously examined in malignant gliomas. In the present study, the levels of SnoN expression in glioma cell lines were comparable to those in the A549 cell line and in normal astrocytes, and overexpression of Ski was found in three glioma cell lines. Both Smad3 and Ski/Sno form a complex that specifically binds DNA in response to TGFβ and overexpression of Ski/Sno proteins represses transcriptional activation by TGFβ. Interestingly, nuclear accumulation of Smad3 is important for degradation of the corepressor proteins Ski and SnoN. The SnoN protein is rapidly degraded by the nuclear accumulation of Smad3, allowing the activation of TGFβ target genes. In the present study, most glioma cell lines displayed low levels of Smad3 and a very small amount of Smad3 translocated to the nucleus after TGFβ treatment. This may be the reason why SnoN expression was not altered by TGFβ treatment in the glioma cell lines. The present results also suggest that the relative abundance of SnoN over Smad3 inhibited the activation of the TGFβ target genes p21, p15, cyclin D1, and CDK4.

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Conclusions

In the present study, we found that glioma cell lines had multiple abnormalities in the TGFβ signaling pathway from Smad2 phosphorylation by the TGFβ receptor complex—which is the initial event of TGFβ signaling—to expression of the TGFβ target genes p21, p15, cyclin D1, and CDK4, which constitute the final events. These results suggest that the escape from TGFβ-mediated growth inhibition in malignant glioma tissues is due to abnormalities in the TGFβ signaling pathway. Given the present results, we need to investigate whether the same abnormalities in the TGFβ signaling pathway exist in vivo in malignant glioma tissues. This approach may provide a potential therapeutic window to try to modulate the growth of malignant gliomas.

References

10. Feng XH, Lin X, Derynck R: Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-β. EMBO J 19:5178–5193, 2000

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