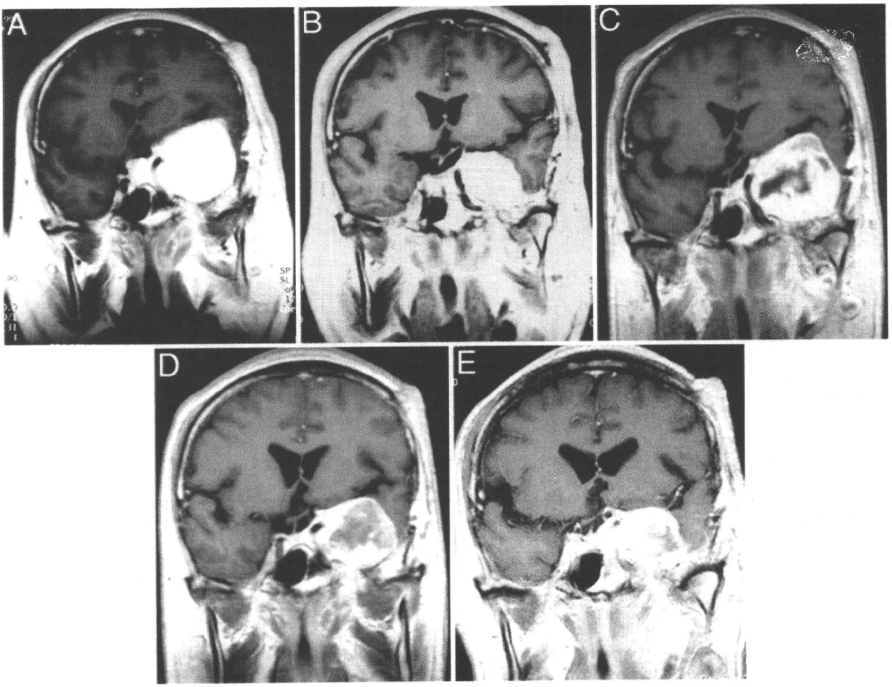

FIG. 1. Case 14. Sequential contrast-enhanced coronal T1-weighted MR images obtained before the second surgery and radiation therapy (A), 31 months after treatment (B), 60 months after these treatments but before GKS (C), and 12 (D) and 84 months after GKS (E).

Discussion

Since the first patient with a CS hemangioma who underwent successful treatment with GKS was described (Case 27 in this article),[5] 16 cases have been reported in the literature (Table 4).[5,6,10,12,13,18] These 16 cases have included 11 female and 5 male patients with a mean age of 48 years (range 14–79 years) at the time of GKS, and a mean tumor volume of 6.0 cm^3 (range 1.5–11.1 cm^3). Selected doses at the tumor periphery have ranged from 12.0 to 19.0 Gy, with a mean and median of 14.8 and 15.0 Gy, respectively. Magnetic resonance images obtained 6–60 months (mean 30, median 27 months) after GKS demonstrated tumor shrinkage in 14 patients and no change in 2. Excluding 2 patients in whom the tumor volume on the most recent MR imaging studies was not available, postradiosurgical volume reduction rates ranged from 14 to 101% (mean and median of 45 and 40%, respectively). Among the 14 of these 16 patients with cranial nerve impairments prior to GKS, complete resolution was achieved in 1 patient, improvement in 7, and in 6 these impairments remained essentially unchanged. No additional symptoms occurred in any of these 16 patients reported in the literature.

In these previously reported cases, however, postradiosurgical follow-up periods were not sufficiently long: the follow-up period was ≤ 36 months in 12 (75.0%) of the 16 patients, and the maximum was 60 months. In the present study of 30 patients, 19 (63.3%) underwent post-GKS follow-up for 3 years or longer, and 10 (33.3%) for 5 years or longer, with a maximum of 138 months. Even in the group of patients with a longer follow-up period, good control of tumor growth was obtained.

We analyzed dose-treatment responses based on 38 cases: 27 of the 30 cases we reported here (the 2 with staged GKS and 1 with partial coverage were excluded) plus 11 previously reported cases. Duplicate citations were avoided.[5,6,10,13] As shown in Fig. 5, there was a tendency for remarkable tumor shrinkage (volume reduction rates of ≥ 50% relative to those before GKS) in the tumors receiving higher doses. Remarkable shrinkage was demon-

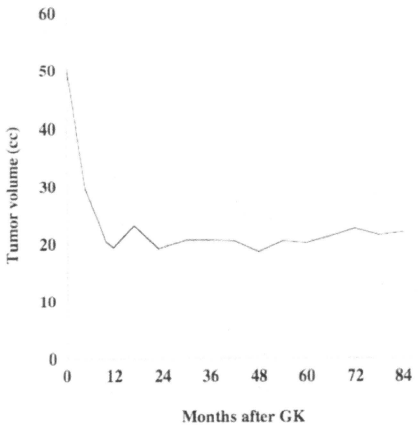

FIG. 2. Case 14. Graph showing postradiosurgical volume changes in this patient.

strated in 15 (83.3%) of 18 tumors that received radiation doses of ≥ 15 Gy, and in 10 of the 20 lesions that received < 15 Gy (not statistically significant; $p = 0.0377$). On the other hand, remarkable tumor shrinkage was demonstrated even in tumors irradiated with relatively low doses: 2 (66.7%) of 3 tumors that received 10.0 Gy, and 3 (60.0%) of 5 that received 12.0 Gy showed shrinkage. We divided these 38 cases into 3 groups based on treatment response shown on the most recent MR images available. Remarkable shrinkage was seen in 25 cases, some shrinkage in 10, and no change in 3. The doses delivered to the tumor periphery differed little among the 3 groups (no statistically significant difference). It can be concluded that a peripheral dose of 14–15 Gy is sufficient to control the growth of CS hemangiomas and that a dose of 10.0–12.0 Gy is the threshold level for tumor growth control.

Although fractionated radiosurgery, also known as stereotactic radiotherapy, is commonly performed for benign intracranial lesions at facilities using a linear accelerator–based radiosurgery system,[4] it is performed only rarely at GKS facilities. Debate continues as to whether stereotactic radiotherapy and staged radiosurgery are effective and safe for benign lesions. In the 2 patients we have described in the present study who underwent staged radiosurgery, good control of tumor growth was observed at 26 and 66 months using a 2-stage GKS technique with doses at the tumor periphery of 11.0 and 8.0 Gy, respectively. On the other hand, as we have described in detail, only the lower half of the tumor was irradiated with a dose of 15.0 Gy in 1 patient, and tumor growth has been well-controlled for 84 months to date. This technique has been applied to relatively large meningiomas,[19] and the treatment concept assumes that the blood supply from the tumor base can be reduced, allowing tumor growth to be controlled (radiosurgical thrombolization). According to a hypothesis proposed by Linskey et al.,[8] most small CS hemangiomas are supplied with blood by the meningeal tributaries of the intracavernous carotid artery, and in cases in which the tumors extend toward the middle fossa, there is an additional blood supply from the middle meningeal and accessory middle meningeal arteries. Though we have only 1 such case, the achievement of tumor growth control using radiosurgical thrombolization can be considered to support this hypothesis. Although a final conclusion awaits further experiences, either staged radiosurgery or radiosurgical thrombolization can be applied to relatively large CS hemangiomas.

Conclusions

The GKS treatment results for CS hemangiomas we report in the present study are more favorable than those previously reported after surgical removal.[9,14,21] Therefore, if a tumor shows clear neuroimaging characteristics of CS hemangioma, and the lesion is small, without evidence either of meningioma or schwannoma, GKS can be performed as the primary treatment procedure.

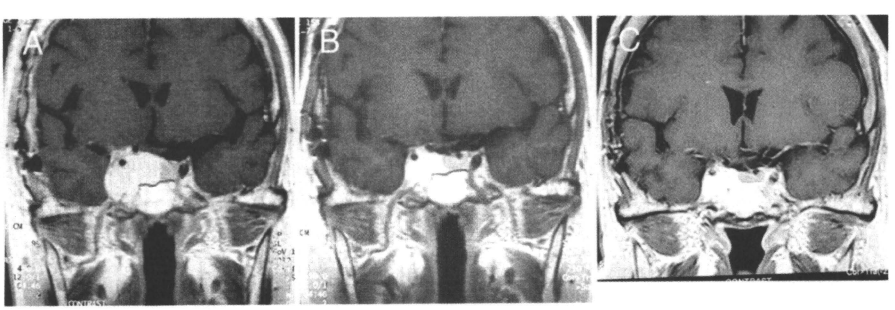

FIG. 3. Case 15. Sequential contrast-enhanced T1-weighted coronal MR images obtained before (A), and 12 (B) and 74 months after GKS (C).

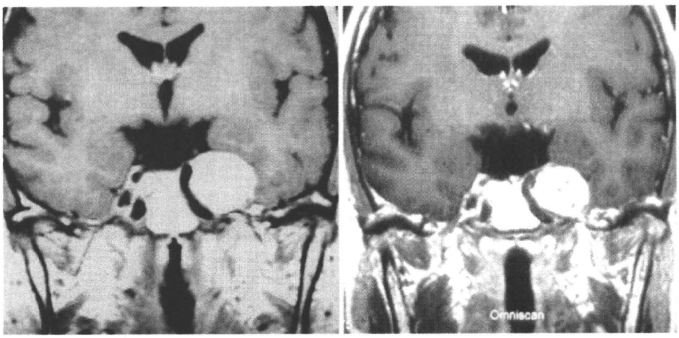

FIG. 4. Case 16. Sequential contrast-enhanced T1-weighted coronal MR images obtained before *(left)* and 64 months after GKS *(right)*.

Disclaimer

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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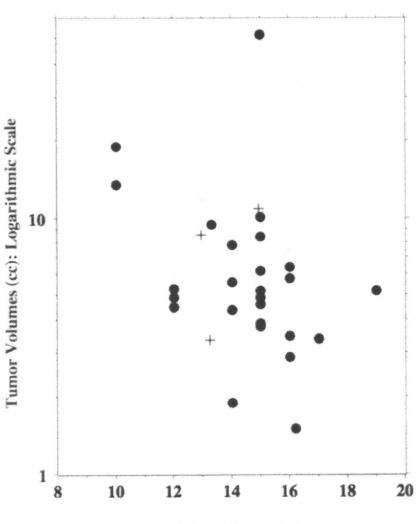

FIG. 5. Scatter plot showing dose, tumor volume, and treatment response. *Circles* indicate remarkable shrinkage, *triangles* indicate some shrinkage, and *plus signs* indicate no change.

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トピックス

悪性脳腫瘍に対するウイルス療法

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はじめに

遺伝子治療に使用されるウイルスは，多くの場合標的細胞に治療用遺伝子を導入するためのベクターとして使用されてきた．細胞に感染し導入遺伝子を発現したところでベクターは役割を終え，ウイルスそのものが細胞内で複製することや，細胞傷害を与えることはない．一方，腫瘍細胞内で選択的にウイルスを複製させれば，ウイルスの本来の細胞傷害性によって感染を受けた腫瘍細胞が死滅し，さらに周囲の腫瘍細胞にウイルスが拡散して同様の効果を及ぼしていくことが期待できる．このようにウイルスで腫瘍細胞を直接破壊する治療法をウイルス療法と称し，そのために開発されたウイルスを腫瘍溶解性ウイルス（oncolytic virus）と呼ぶ（図）．遺伝子工学技術の発達とウイルス学の知見の融合により，この分野は近年急速に進歩した．悪性腫瘍の実用的な治療法の1つとなりうるものとして期待されており，悪性脳腫瘍のほか，前立腺癌，頭頸部癌，乳癌，肝癌，悪性黒色腫などさまざまな悪性腫瘍に対して精力的に研究開発が進められている．

臨床応用のためには，人体に投与しても無害無毒で安全であることが不可欠である．正常組織を傷つけず，腫瘍細胞選択的に細胞傷害性を発揮させるために，さまざまなアプローチで研究が行われてきた．遺伝子組み換えウイルスを設計する場合の基本戦略は，腫瘍細胞のみで活性化あるいは低下している蛋白質（酵素，シグナル蛋白，転写因子，表面受容体など）をウイルス複製の分子機構に結びつけて，その制御に巧みに利用することである．一方，ヒトのウイルスの自然弱毒株や，元来はヒトを宿主とせず病原性を持たないが，腫瘍細胞においてはヒトでも殺細胞効果を現すようなウイルスも使用されている．悪性神経膠腫を対象とするものに限定しても，単純ヘルペスウイルス1型（HSV-1）[1, 2]，アデノウイルスをはじめ，レオウイルス，ニューカッスル病ウイルス，麻疹ウイルスを使用したものなど，多数が欧米を中心に臨床試験に進んでいる．

1．G207とG47Δ

遺伝子組換え型の腫瘍溶解性HSV-1は，いずれも腫瘍細胞選択的な複製のために，ウイルスの*γ34.5*遺伝子やDNA合成関連酵素遺伝子に変異を加えている．一般に，正常細胞ではウイルス感染が起こると二本鎖RNA依存性プロテインキナーゼ（PKR）のリン酸化を介して蛋白合成が遮断され，ウイルス複製が阻止される．HSV-1の場合，細胞に感染後，*γ34.5*遺伝子がリン酸化PKRに拮抗してウイルス複製を可能にするが，*γ34.5*遺伝子を欠失した変異型HSV-1は，それができず正常細胞では複製できない．しかし，腫瘍細胞では元来リン酸化PKRが低く，*γ34.5*遺伝子が働かなくてもウイルス複製が可能となる．リボヌクレオチド還元酵素やチミジンキナーゼはHSV-1のDNA合成に必要な酵素であり，これらの遺伝子が不活化された変異HSV-1は正常細胞ではウイルス複製ができない．しかし，増殖の盛んな腫瘍細胞においては細胞のDNA合成関連酵素の活性が上昇しているため，それを利用して変異HSV-1も複製することができる．

G207は二重変異を有する遺伝子組換えHSV-1で，*γ34.5*遺伝子の欠失と*ICP6*遺伝子の不活化がなされている．段階的用量増加による第Ⅰ相臨床試験が，米国にて再発悪性神経膠腫の患者を対象に行われ，脳腫瘍内直接投与の安全性が確認された[1]．その後第Ib相試験として，再発膠芽腫患者の腫瘍切除後の摘出腔壁などへのG207注入が行われ，この投与法

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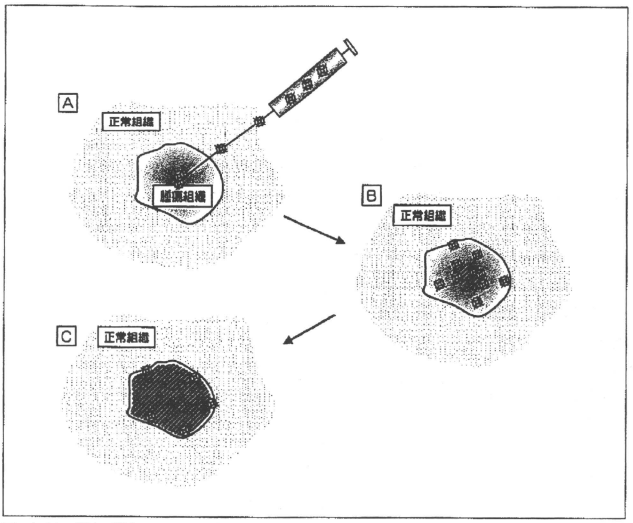

図　ウイルス療法の概略
A投与された腫瘍溶解性ウイルスは，腫瘍細胞内で選択的に複製し，感染した腫瘍細胞を破壊する．B複製したウイルスはさらに周囲の腫瘍細胞内で複製しそれらを破壊する．Cウイルスは正常細胞では複製しないため，正常組織を傷害しない．

での安全も示されている[2]．

G47ΔはG207のウイルスゲノムからさらに*α47*遺伝子を欠失させた三重変異HSV-1であり，G207の安全性を維持しつつ，腫瘍細胞に対する殺細胞作用と特異的免疫誘導が増強するように設計された[3]．*α47*遺伝子は，ウイルスが感染した細胞の抗原呈示関連トランスポーター(TAP)を阻害し，その細胞表面のMHC Class Ⅰの発現を抑制する．*α47*遺伝子の欠失により感染細胞のMHC Class Ⅰ発現が維持され，免疫細胞に対する刺激が向上すると期待される．また，*γ34.5*欠失HSV-1においてはウイルス複製能が減弱しているが，G47Δにおいては*α47*遺伝子の欠失変異の結果，隣りの後期遺伝子*US11*が最早期に発現するようになり，腫瘍細胞に限ってウイルス複製能が復元する．

2．併用療法による効果増強

放射線や化学療法剤は腫瘍細胞のDNAに障害を与え，growth arrest DNA damage 34(GADD34)遺伝子を誘導する．これがG207やG47Δなど*γ34.5*遺伝子を欠損したHSV-1の抗腫瘍効果増強をもたらす[4]．また，HSV-1によるウイルス療法は腫瘍血管新生抑制作用も期待できる[5]．

ネズミの脳腫瘍モデルにおいて，ウイルス投与時にシクロフォスファミド併用投与などで一過性に免疫抑制を行うと腫瘍溶解性HSV-1の感染性と複製能が向上し，抗腫瘍効果の増強が得られると報告されているが，他方，抗腫瘍免疫の惹起による抗腫瘍効果の増強に関しては，免疫増強関連遺

伝子発現の併用は正に作用し，ステロイドの併用は負に作用するとの報告がある．

3．今後の展望

ウイルス療法の臨床試験は，国内では遺伝子治療と同じ手続きで申請を行い審査される．また，品質・安全性・有効性に関する国際規準・規制が整備されてきており，臨床試験に使用するためのウイルス製剤はそれらを充足する必要がある．多くのハードルを越え国の承認を得て，G47Δを用いたウイルス療法が[5]，再発膠芽腫を対象に開始された．

ウイルス療法は，悪性脳腫瘍治療をはじめ広く癌の治療に適用できること，化学療法や放射線治療など従来の治療法と併用が可能で相乗効果も期待できること，腫瘍免疫の作用による腫瘍制御が期待できることなど，実用的な特徴を有している．将来，がん治療における重要な選択肢の1つとなると期待される．

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神経膠腫の遺伝子治療

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神経膠腫に対する遺伝子治療は1990年代に始まり，自殺遺伝子治療や免疫遺伝子治療などが試された．近年は増殖型ウイルスで直接腫瘍細胞を破壊するウイルス療法が注目されている．国内でも，厚生労働大臣の認可を受けて国内初の増殖型遺伝子組み換えウイルスを用いた再発膠芽腫に対する第Ⅰ・Ⅱ相臨床研究が始まった．本稿では臨床試験を中心に，これまでの脳腫瘍に対する遺伝子治療を顧みるとともに，現在注目されている臨床試験について解説する．

Key Words: glioma, glioblastoma, gene therapy, oncolytic virus, herpes virus, clinical trial

Ⅰ．はじめに

神経膠腫の発生頻度は原発性脳腫瘍のなかで25.6%と，約1/4を占める[38]．特にそのなかでも，われわれ脳神経外科医が遭遇する機会が最も多く，最も悪性である膠芽腫（glioblastoma: GBM）の治療成績は，近年テモゾロミド（TMZ）の出現により若干延長したものの，集学的治療（手術＋放射線治療＋TMZ）によっても5年生存率は9.8%と，依然として満足のいくものではない[37]．この治療困難な腫瘍を克服するためにこれまでさまざまな治療法が試みられてきており，特に近年では細胞障害性化学療法，分子標的治療，免疫療法など種々の治療開発が活発に行われている[44]．なかでも，新しいジャンルの治療法として遺伝子治療が期待を集めている．

本稿では誌面の都合上，基礎研究の解説は必要最低限とし，神経膠腫に対する遺伝子治療の臨床研究を総括したうえで，特に近年注目を集め，わが国でも臨床研究が開始された腫瘍溶解性ウイルスによるウイルス療法（oncolytic virus therapy）に焦点を当てる．

Ⅱ．遺伝子治療

A）定義と概念

アメリカ遺伝子細胞治療学会の定義によれば，遺伝子治療とは「遺伝的，もしくは後天的疾病の治療として患者の細胞を変化させるために遺伝子学的物質を使用すること」であり，わが国の文部科学省・厚生労働省の定めた「遺伝子治療臨床研究に関する指針」でも「疾病の治療を目的として遺伝子または遺伝子を導入した細胞を人の体内に投与すること」と定義されている．

先天性疾患への遺伝子治療は，おもに正常遺伝

子を体内へ導入することで機能回復を図る遺伝子補充療法であり，一方「がん」に対する遺伝子治療は正常細胞を傷害することなく腫瘍細胞のみを選択的に死滅させることを目的とする．がんに対する高い殺細胞効果を得るためには，標的細胞へ遺伝子を効率的に到達させることが必要となる．しかしながら，実際には生体内で標的細胞へ遺伝子導入を高効率に行うことは困難を伴い，その効率を上げるため，さまざまな方法が用いられてきた．

遺伝子の導入方法としては，古くから非増殖型のウイルスをベクターとして用いることが多かったが，それ以外にもプラスミドDNAをそのまま用いる方法やリポソームを用いたトランスフェクション，また，近年では幹細胞に遺伝子を導入してベクターとして利用する方法などが試みられている[11]．脳腫瘍に対してこれまで報告されている遺伝子導入方法を表1に示す．

B）遺伝子治療の臨床試験

遺伝子治療は近年の遺伝子工学の発展に伴い，急速に発展した．1970年代後半に遺伝子組み換えDNAが設計できるようになったことを契機に基礎研究がさかんに行われた．それに並行して臨床応用も進められ，1990年にアメリカでアデノシンデアミナーゼ欠損症の女児に対して世界で初めてのヒトに対する遺伝子治療が行われた[1, 19]．

その後もさまざまな疾病に対して臨床応用が進められたが，1999年にオルニチントランスカルバミラーゼ欠損症に対するアデノウイルスを用いた遺伝子治療を受けた18歳の男性が，ウイルス投与後4日目に多臓器不全で死亡した[34]（実際には投与量に問題があり，大量に投与されたウイルスに対する過剰な免疫反応により死亡したと考えられている[33]）．このことは当時大きな社会問題になり，遺伝子治療の臨床試験は一時停滞した．また，1999年からフランスで行われた，X連鎖重症複合免疫不全症（X-SCID）に対するレトロウイルスベクターを用いた遺伝子治療を受けた患者15名のうち，約3年後に3名が白血病を発症し，そのうち1名が死亡したことが報告された[3]．

このように，今まで人類が遭遇したことのなかった副作用が明らかになる一方で，確実に治療効果を認める症例も報告され続け，決して平坦な道

表1　脳腫瘍治療に用いられる遺伝子導入方法・戦略

Viral vectors
- Non-replicating（非増殖型ウイルス）
  - Retrovirus
  - Adenovirus

Cell-based vectors
- Stem cells（SC）
  - Neural SC
  - Embryonic SC-derived astrocytes
  - Mesenchymal SC
  - Bone marrow-derived SC
- Progenitor cells（PC）
  - Neural PC
  - Endothelial PC
- Fibroblasts

Synthetic vectors
- Nanoparticles
  - Liposomes（RNAi, siRNA）

New strategies
- Replicating vectors（増殖型ウイルス）
  - Oncolytic viruses
- Delivery 'enhancers'
  - CED, ultrasounds
- Combined approaches
  - Immune-modulation plus viral delivery
  - Enhancers: cytokines
  - Inhibitors: cyclophosphamide
  - Cell-based plus viral delivery

（文献11より改変）

のりではないものの，現在では難治性疾患に対する遺伝子治療の有効性・将来性は疑いようのないものとなっている．その後も厳しい審査を受けながら世界中で臨床試験は着実に進められており，2009年末までに世界中で登録された臨床試験の総数は1,579件となっている．このデータは'The Journal of Gene Medicine'のホームページで適時更新され，誰でもアクセスできる（http://www.wiley.co.uk/genetherapy/clinical/）．

Ⅲ．脳腫瘍に対する遺伝子治療の臨床試験

神経膠腫に対して登録されたものだけ見ると約70件ほどあり，そのほとんどがWHO grade Ⅲ以上の悪性神経膠腫に対するものである．**表2**に，これまで臨床で悪性神経膠腫の遺伝子治療に用いられたベクターの内訳を示す．レトロウイルスベクターが歴史的に古くから研究されてきたこともあり最も多く，次に単純ヘルペスウイルスが続く．**表3**には，脳腫瘍に対してこれまでに行われた代表的な臨床試験を示した．Phase Ⅲまで到達したものは4件である．

以下，各論について述べる．

A）自殺遺伝子治療

脳腫瘍患者に対する遺伝子治療の始まりは，複数のグループにより1990年代に始められた自殺遺伝子治療であり，単純ヘルペスウイルスのチミジンキナーゼ遺伝子（HSV-*tk*）をレトロウイルスベクターによって腫瘍へ導入した後にガンシクロビル（GCV）を全身投与した[29]．GCVはHSV-*tk*によって特異的にリン酸化を受けるため，遺伝子を導入された腫瘍細胞でのみGCV一リン酸となり，さらにヒトチミジンキナーゼによりリン酸化を受けて細胞毒性を有するGCV三リン酸となる．このGCV三リン酸はDNAポリメラーゼを阻害するため，DNA複製を起こす分裂細胞はアポトーシスに陥り死滅する．さらにGCV三リン酸はgap junctionを介して隣接する細胞へ広がり，直接HSV-*tk*遺伝子が導入できなかった隣接細胞も死滅させる（bystander effect）．

表2　これまで登録された悪性神経膠腫の遺伝子治療臨床試験におけるベクターの内訳

vector	number of trials
Retrovirus	20
HSV	13
Adenovirus	9
Naked/Plasmid DNA	8
Lipofection	3
RNA transfer	3
Lentivirus	1
Measles virus	1
Newcastle disease virus	1
Poliovirus	1
Semliki forest virus	1
total	61

レトロウイルスベクターを用いて多くのphase Ⅰ，Ⅱ studyが行われた[7, 10, 15, 16～18, 25, 26, 28, 30, 31, 35, 36, 42]．2000年にRainovらは248人のGBM患者に対するphase Ⅲ trialの結果を報告したが，標準治療群に比べ生存期間，無増悪生存期間ともに有意差を認めなかった[30]．これはレトロウイルスの感染効率が低いことが大きな因子と考えられ，感染効率を上げることが重要と考えられた．

その後，レトロウイルスに代わってアデノウイ

表3　悪性神経膠腫に対する代表的な遺伝子治療臨床試験の一覧

year	country	phase	gene, name of virus	gene type	vector	PI or Author	No.of Pt.	indication	reference
1997	Spain	I	HSV-tk	Suicide	Retro	Izquierdo	7	GBM	Izquierdo, 1997 [17]
2000	USA	I	HSV-tk	Suicide	Retro	Packer	7	MG	Packer, 2000 [25]
1999	Italy	I	HSV-tk, IL-2	Suicide	Retro	Palu	4	GBM	Palu, 1999 [16]
2000	USA	I	HSV-tk	Suicide	Retro	Griffith R. Harsh	5	MG	Harsh, 2000 [13]
2001	Germany	I	HSV-tk	Suicide	Retro	F. W. Floeth	27	MG	Floeth, 2001 [7]
1997	USA	I / II	HSV-tk	Suicide	Retro	Ram et al	12	GBM	Ram, 1997 [31]
1998	France	I / II	HSV-tk	Suicide	Retro	Valery CA	12	GBM	Klatzmann, 1998 [18]
1999	Switzerland	I / II	HSV-tk	Suicide	Retro	Shand N	48	GBM	Shand, 1999 [36]
2003	USA	I / II	HSV-tk	Suicide	Retro	John C. Van Gilder	30	GBM	Prados, 2003 [29]
2000	International	III	HSV-tk	Suicide	Retro	Nikolai Rainov	248	GBM	Rainov, 2000 [30]
2000	Finland	I	HSV-tk LacZ	Suicide	Adeno or Retro	Immonen A	21	GBM	Sandmair, 2000 [35]
2004	Finland	I / II	HSV-tk LacZ	Suicide	Adeno	Immonen A	36	GBM	Immonen, 2004 [16]
2008	International	III	HSV-tk LacZ: Cerepro®	Suicide	Adeno	Ark Therapeutics	250	MG	Ram, 2009 [48]
2000	USA	I	HSV-tk	Suicide	Adeno	Todd Trask	12	MG	Trask, 2000 [42]
2003	USA	I	HSV-tk	Suicide	Adeno	Frank Lieberman	11	GBM	Germano, 2003 [10]
2003	USA	I	p53	Tumor suppressor	Adeno	Frederick F. Lang	15	MG	Lang, 2003 [20]
2004	USA	I	HSV-tk: ONYX-015	Oncolytic virus	Adeno	E. Antonio Chiocca	24	MG	Chiocca, 2004 [4]
2006	Israel	I / II	NDV-HUJ	Oncolytic virus	NDV	Eitan Galun	11	GBM	Freeman, 2006 [9]
2000	UK	I	1st gen oHSV HSV1716	Oncolytic virus	HSV	Roy Rampling	9	MG	Rampling, 2000 [32]
2002	UK	I	1st gen oHSV HSV1716	Oncolytic virus	HSV	Roy Rampling	12	MG	Papanastassiou, 2002 [27]
2004	UK	I	1st gen oHSV HSV1716	Oncolytic virus	HSV	Roy Rampling	12	MG	Harrow, 2004 [12]
2006~	UK	III	1st gen oHSV HSV1716	Oncolytic virus	HSV	Gareth Cruickshank	recruiting	GBM	
2000	USA	I	2nd gen oHSV G207	Oncolytic virus	HSV	James Markert	21	MG	Markert, 2000 [21]
2009	USA	I b	2nd gen oHSV G207	Oncolytic virus	HSV	James Markert	6	GBM	Markert, 2009 [22]
2009~	Japan	I / II	3rd gen oHSV G47 Δ	Oncolytic virus	HSV	Tomoki Todo	recruiting	GBM	
2004	Japan	I	IFN- β	Cytokine	Lipofection	Jun Yoshida	5	MG	Yoshida, 2004 [46]
2008	USA	I	IFN- β : Ad.hIFN-beta	Oncolytic, Cytokine	Adeno	E. Antonio Chiocca	11	MG	Chiocca, 2008 [5]
2008	USA	I / II	REOLYSIN®	Oncolytic virus	Reovirus	Peter Forsyth	12	MG	Forsyth, 2008 [8]
2006~	USA	I	CEA: MV-CEA	Oncolytic, Antigen	Measles virus	Evanthia Galanis	recruiting	GBM	Myers, 2008 [24]
2009~	USA	I / II	CMV pp65-LAMP	Antigen	RNA transfer	Duane A. Mitchell	recruiting	GBM	
2007	International	II b	TGF- β 2 antisense	Antisense	Naked	Antisense Pharma	145	MG	Bogdahn, 2009 [2]
2008~	International	III	TGF- β 2 antisense	Antisense	Naked	Antisense Pharma	recruiting	AA	

PI=principal of investigator, Pt.=patients, HSV=herpes simplex virus, tk=thymidine kinase, Retro=retrovirus, GBM=glioblastoma, MG=malignant glioma, IL=interleukin, Adeno=adenovirus, gen=generation, oHSV=oncolytic herpes simplex virus, IFN=interferon, AA=anaplastic astrocytoma

ルスがベクターとして臨床試験に用いられるようになった．製剤への動きとしてはArk Therapeutics社が開発を進めてきたCerepro®を用いたphase I[35]，phase I / II study[16]が行われ，2008年にphase III studyが終了した[48]．2009年に欧州医薬品庁（EMEA）へ悪性神経膠腫治療薬として

承認申請を行ったが，追加試験が必要とされ，今年申請を取り下げている．

B）免疫遺伝子治療

がん免疫の理解が進むなか，がん患者は免疫抑制状態にあることや腫瘍細胞は，種々のサイトカインを産生し宿主免疫から逃れていることが知られるようになった[6]．免疫遺伝子治療はインターロイキン12やインターフェロンβ（IFN-β）などの免疫を刺激する遺伝子や，種々の抗原の遺伝子を腫瘍細胞や抗原提示細胞に導入して，抗腫瘍免疫を惹起する治療法である．前項の自殺遺伝子療法や後に述べる腫瘍溶解性ウイルス療法との相乗効果を狙った組み合わせ治療も近年積極的に研究され，臨床試験が行われている[5, 20, 24, 46]．

日本でも，2000年に名古屋大学においてカチオニックリポソームをベクターとして利用し，IFN-β遺伝子を再発GBM患者に投与するphase Ⅰの遺伝子治療が行われた[43, 46]．

C）ウイルス療法（oncolytic virus therapy）

自然界に存在する野生株のウイルスががん細胞でよく増殖することは古くから知られていた．ウイルス療法とは，がん細胞を破壊することを目的に増殖型ウイルスを用いる治療法で，治療に用いるウイルスは腫瘍溶解性ウイルス（oncolytic virus）と呼ぶ．導入した遺伝子産物を介することなく，ウイルス複製に伴うウイルスそのものの殺細胞作用によって抗腫瘍効果を得る点で厳密には遺伝子治療とは異なるが（図），広義の遺伝子治療に含んで語られることが多い．

レオウイルスやニューカッスル病ウイルス（Newcastle disease virus: NDV）など，人を宿主としないウイルスを用いる場合と，単純ヘルペスウイルス1型（herpes simplex virus type 1: HSV-1），アデノウイルス，ワクシニアウイルスなど，おもに人を宿主とするウイルスを用いる場合がある．後者は，通常ウイルスゲノムを遺伝子組み換え技術によって改変し，正常組織を傷害することなく，腫瘍細胞のみで増殖できるようにデザインされた遺伝子組み換えウイルスを用いる．2005年には，E1B領域を欠失させた遺伝子組み換えアデノウイルス（H101）が世界初のウイルス療法製剤として頭頸部癌を対象として中国で認可された[17]．悪性脳腫瘍に対してはHSV-1や，アデノウイルス，NDV，レオウイルス，麻疹ウイルスなどが臨床試験に用いられている[4, 5, 8, 9, 12, 21, 22, 24, 27, 32]．

D）腫瘍溶解性HSV-1開発の経緯と臨床試験

HSV-1はウイルス療法の開発を行ううえで種々の有利な点を持ち合わせている[40, 41]が，ウイルス自体の免疫原性が比較的低く殺細胞作用が強いなど，アデノウイルスよりも脳腫瘍治療に対して治療効果が高いとの報告もある[14]．遺伝子組み換えHSV-1には複数種類があるが，脳腫瘍に対する臨床試験に用いられているものとしては，そのゲノムに1カ所のみの変異を持つ第1世代のHSV1716，二重変異を有する第2世代のG207，そして三重変異を持つ第3世代であるG47Δが主たるものである．

HSV-1の改変には幾つかの代表的なウイルス遺伝子が利用される．*γ34.5*遺伝子は正常細胞内でのウイルス複製に必要な遺伝子であり，HSV-1の病原性の原因となる遺伝子である．正常の細胞ではウイルス感染に呼応して2本鎖RNA依存性プロテインキナーゼ（PKR）のリン酸化が誘導され，

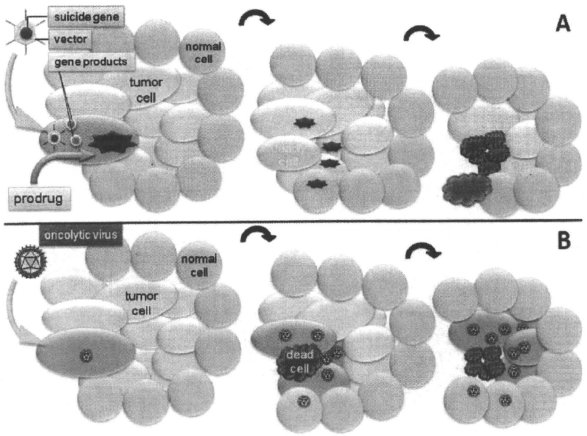

図　自殺遺伝子を用いた遺伝子治療と腫瘍溶解性ウイルスを用いたウイルス療法

A：ベクター（主に非増殖型ウイルスベクターが用いられる）によって腫瘍細胞内へ導入された自殺遺伝子は遺伝子産物の酵素を産生し，全身性に投与されたプロドラッグを特異的に代謝して細胞傷害性物質へと変化させる．この代謝産物は gap junction を介して周囲の細胞へ広がり周囲の細胞も死滅させることがあるが（bystander effect），基本的には遺伝子導入を受けた細胞が死滅する．

B：増殖型の腫瘍溶解性ウイルスは感染した腫瘍細胞内で複製し，宿主となった腫瘍細胞を破壊しながら周囲の細胞へ広がって再び感染する．そこでウイルス複製→腫瘍細胞破壊→拡散→感染を繰り返しながら抗腫瘍効果を現す．しかし正常細胞内では感染してもウイルス複製は起こらないため，正常組織は傷害されない．

細胞内でのタンパク合成が遮断されるためにウイルス複製が阻害される．ウイルスの *γ34.5* 遺伝子産物はリン酸化 PKR に拮抗する作用を有する．したがって，*γ34.5* 遺伝子欠失 HSV-1 は正常細胞内で複製できないが，腫瘍細胞はもともと PKR 活性が低いため，*γ34.5* 遺伝子が欠失しているウイルスでも複製が可能となる．また，*ICP6* 遺伝子がコードするリボヌクレオチド還元酵素（ribonucleotide reductase: RR）や，チミジンキナーゼ（*tk*）はウイルスの DNA 合成に必須であるため，これらをコードするウイルス遺伝子を失活させると正常細胞では複製できない．しかし腫瘍細胞では宿主の酵素活性が高く，欠落したウイルス酵素を代償するためウイルス複製が可能となる．

HSV1716 は *γ34.5* 遺伝子のみを欠失した第 1 世代 HSV-1 で，比較的強力な殺細胞効果を維持する反面，正常脳組織への病原性もある程度残存している．phase I study はイギリスで再発悪性神経膠腫を対象に行われ[32]，1×10^5 pfu（plaque forming units）までの安全性が確認された．続いて

phase Ⅱの結果が2002年[27]と2004年[12]に報告され，ウイルス投与に関連する重篤な有害事象の出現を認めず，現在phase Ⅲが行われている．病原性を生じるため投与できるウイルス量に制限があり，また単一の遺伝子欠損しか持たないため野生株に戻る危険性が払拭できないという欠点がある．

G207は*γ34.5*遺伝子欠失に加え，*ICP6*遺伝子を不活化することで野生株にきわめて戻りにくくなると同時に腫瘍特異性が高まるなどの特徴を示した[23]．さまざまな非臨床安全性試験を経た後，米国ジョージタウン大学とアラバマ大学バーミンガム校で21例の再発悪性神経膠腫患者を対象にphase Ⅰ studyが行われた．$1 \times 10^6 \sim 3 \times 10^9$ pfuまで3症例ずつ段階的に増量して単回の定位的腫瘍内投与を行った結果，安全性が確認された[21]．また，phase Ⅰb studyでは6名の再発GBM患者に複数回のG207投与が行われ，ウイルス投与と関連が明らかな重篤有害事象は見られなかった．効果に関しては，再増大までの中央値は3カ月で，ウイルス投与後からの生存期間の中央値は6.6カ月であった[22]．これらの結果，安全性は確認されたものの，その抗腫瘍効果については改良の余地が残った．

G47ΔはG207の2カ所の変異からさらに，*α47*遺伝子とそれに重なる*US11*プロモーター領域を欠失させた三重変異を有する第3世代HSV-1である．この変異により，感染細胞表面のMHC Class Ⅰの発現を強化して宿主免疫による抗腫瘍免疫を増強させるとともに，*γ34.5*遺伝子欠失によって減弱したウイルス複製能を腫瘍細胞内に限って復元した[30]．これによって安全性を維持したまま殺細胞効果を高めることができ，実際に多くの腫瘍細胞でG47ΔはG207の約10倍のウイルス複製能を示す一方，HSV-1に感受性のあるA/Jマウスの脳内投与試験では同等以上の安全性を示した．

厚生労働省の承認を得て，2009年11月にG47Δを用いた臨床試験が東京大学にて開始された．この試験は再発GBM患者を対象としたphase Ⅰ-Ⅱ studyであり，first-in-manの臨床研究である．1回投与量3×10^8 pfuから3症例ずつ段階的に3×10^9 pfuまで投与量を増量して定位的に2回脳腫瘍内投与を行い，おもに安全性を確認する．

E）その他の注目の臨床試験

現在欧州を中心に，再発退形成性星細胞腫（anaplastic astrocytoma: AA）に対してphase Ⅲが行われているTGF-βのantisense compoundであるAP12009（trabedersen）は，厳密な意味での遺伝子治療ではないが，有望な治療法として期待を集めている[45]．145名の再発AA，GBM患者に対して行われたphase Ⅱb studyの結果は昨年のアメリカ臨床腫瘍学会（ASCO）で発表され，AAに限ってみるとtrabedersen（投与量10 μM）治療群は対照治療群（テモゾロミドまたはブロカルバジン）に比べて2年生存率が83.3% vs 41.7%と有意に延長した[2]．企業治験であり結果の評価は慎重に行わねばならないが，近年脳腫瘍幹細胞はTGF-βを阻害することで著しく幹細胞性を失うことも示されており[15]，phase Ⅲの結果が待たれるところである．

Ⅳ．おわりに

悪性脳腫瘍に対する遺伝子治療の臨床試験について，特にウイルス療法に焦点を当てて述べた．分子標的薬がしばしば話題になるなか，脳腫瘍の

病態を分子生物学的に理解することはきわめて大切であり，その知見を着実に治療へ結びつける意味で遺伝子治療は今後ますます重要な位置を占めていくと考えられる．

国内では「遺伝子治療臨床研究に関する指針」に則って，厚生労働大臣の認可の下，臨床研究が行われているが，これまでに実施された遺伝子治療臨床研究はまだ20件に満たない．脳腫瘍に限れば，G47Δの臨床試験がようやく2件目である．神経膠腫に対する新しい治療法のニーズが高いことを鑑みると，今後，より活発な遺伝子治療開発の進歩が期待される．

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Cell Stem Cell
Article

Autocrine TGF-β Signaling Maintains Tumorigenicity of Glioma-Initiating Cells through Sry-Related HMG-Box Factors

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SUMMARY

Despite aggressive surgery, radiotherapy, and chemotherapy, treatment of malignant glioma remains formidable. Although the concept of cancer stem cells reveals a new framework of cancer therapeutic strategies against malignant glioma, it remains unclear how glioma stem cells could be eradicated. Here, we demonstrate that autocrine TGF-β signaling plays an essential role in retention of stemness of glioma-initiating cells (GICs) and describe the underlying mechanism for it. TGF-β induced expression of Sox2, a stemness gene, and this induction was mediated by Sox4, a direct TGF-β target gene. Inhibitors of TGF-β signaling drastically deprived tumorigenicity of GICs by promoting their differentiation, and these effects were attenuated in GICs transduced with Sox2 or Sox4. Furthermore, GICs pretreated with TGF-β signaling inhibitor exhibited less lethal potency in intracranial transplantation assay. These results identify an essential pathway for GICs, the TGF-β-Sox4-Sox2 pathway, whose disruption would be a therapeutic strategy against gliomas.

INTRODUCTION

Glioblastoma multiforme (GBM), the most malignant form of glioma, is one of the most aggressive human cancers with a 5 year survival rate of less than one out of ten (Surawicz et al., 1998). Despite past huge efforts, this statistic has not markedly improved over the past years. Excessive proliferation, diffuse infiltration into surrounding brain tissue, and suppression of antitumor immune surveillance contribute to the malignant phenotype of glioblastomas.

Cancer-initiating cells (cancer stem cells, CSCs) are rare tumor cells characterized by their ability to induce tumorigenesis and to self-renew. Recent concepts for cancer suggest that a minority population of CSCs may determine the biological and pathological characters of tumors. Similar to other tumors, glioma-initiating cells (glioma stem cells, GSCs) have been isolated from human glioma tissues and several glioma cell lines (Singh et al., 2004; Kondo et al., 2004; Hirschmann-Jax et al., 2004). GSCs are characterized by the expression of neural stem cell (NSC) antigens and the ability to grow as nonadherent spheres termed "neurospheres" or "glioma spheres" when cultured in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) under serum-free condition. Thus, GSCs share several characteristics with normal NSCs (Vescovi et al., 2006).

According to the concept of CSCs, failure to cure cancer may be attributed to the current therapeutic strategies, which have been aimed at the tumor bulk without significantly affecting CSCs. Like other CSCs, GSCs have been reported to be resistant to conventional radiation and pharmacological therapies (Bao et al., 2006; Liu et al., 2006a). Although elimination of CSCs has been regarded as a prerequisite for the development of successful therapeutic strategies, it has not still been fully elucidated how their stemness is maintained. To establish therapeutic strategies against glioma, in vitro and in vivo models that faithfully recapitulate the stem cell component of gliomas have been developed. Among these models, glioma spheres cultured in serum-free media supplemented with EGF and bFGF are considered to reflect biological and pathological characters of primary glioma tissues, have ability to self-renew, and mimic original tumors after intracranial transplantation (Singh et al., 2004; Lee et al., 2006).

Although transforming growth factor (TGF)-β suppresses proliferation of certain carcinoma cells and is well known to be a tumor suppressor, it promotes proliferation of tumors of non-epithelial origin, including glioma and osteosarcoma, through induction of PDGF-BB (Bruna et al., 2007; Matsuyama et al., 2003). TGF-β binds to type I and type II serine/threonine kinase receptors and transduces intracellular signals principally through Smad proteins (Derynck and Zhang, 2003; Massagué, 2008; Miyazawa et al., 2002). Upon phosphorylation by type I receptors, receptor-regulated Smads (R-Smads; Smad2 and -3) form heteromeric complexes with common-partner Smad (Co-Smad; Smad4), translocate into the nucleus, and regulate expression of various target genes. In addition to induction of proliferation, TGF-β pathway has also been implicated in invasion, metastasis, and intratumoral angiogenesis of glioma. These multiple roles of TGF-β in glioma progression have promoted the development of therapeutic agents based on the inhibition of the TGF-β pathway (Golestaneh and Mishra, 2005).

Here, we report that autocrine TGF-β signaling induces Sox2 expression, one of the crucial factors for maintenance of NSCs,

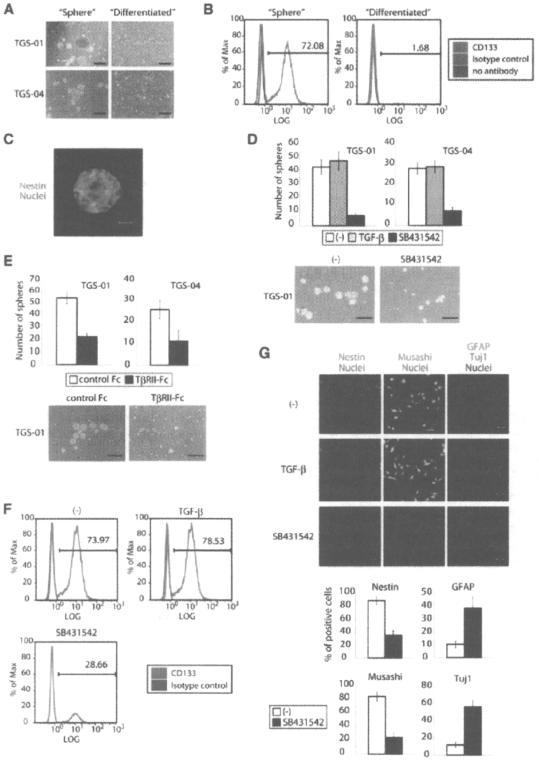

Figure 1. TGF-β Signaling Maintains Stemness of Glioma-Initiating Cells

(A) Representative images of glioma spheres TGS-01 and TGS-04 cultured in serum-free neurobasal media with EGF and bFGF ("Sphere"), and glioma cells derived from the same pathological samples as spheres but cultured in media containing 10% fetal bovine serum ("Differentiated"). Scale bars, 100 μm.

(B) $CD133^+$ ratio of "Sphere" cells (TGS-01) and "Differentiated" cells was determined by flow cytometry.

(C) TGS-01 spheres were stained with Nestin. Scale bars, 20 μm.

(D) TGF-β inhibitor deprives glioma-initiating cells of sphere-forming ability. Glioma-initiating cells were dissociated into single-cell populations and cultured with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 7 days. The data are presented as the number of glioma spheres formed (means ± SEM of five fields). Scale bars, 100 μm.

(E) Glioma-initiating cells were dissociated into single-cell populations and cultured with human TGF-β RII/Fc chimera (1 μg/ml) or control IgG_1 Fc (1 μg/ml) for 7 days. The data are presented as the number of glioma spheres formed (means ± SEM of five fields). Scale bars, 100 μm.

(F) Effects of TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) on $CD133^+$ subpopulation of TGS-01 cells were determined by flow cytometry.

(G) Immunostaining of TGS-01 cells. Spheres were disaggregated, seeded on poly-L-ornithine and fibronectin-coated slide glasses, and cultured in serum-free medium with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 7 days. Quantification of Nestin-, Musashi-, Tuj1-, or GFAP-positive cells was shown in the bottom graphs. Scale bars, 50 μm.

and plays essential roles in maintenance of stemness of the glioma-initiating cells. We also demonstrate that another Sry-related high-mobility group (HMG) box-containing gene, Sox4, is a crucial mediator of TGF-β-induced Sox2 expression. Notably, glioma-initiating cells pretreated with TGF-β signaling inhibitor were less aggressive and showed less lethal potency in intracranial transplantation assay. These findings open the way to depriving GSCs of the tumorigenic activity and will offer new therapeutic possibilities.

RESULTS

Inhibition of TGF-β Signaling Deprives Glioma-Initiating Cells of Tumorigenic Activity

To study the mechanisms of how stemness of glioma-initiating cells is maintained, we have used glioma tissues obtained from two patients with GBM. They were cultured in serum-free medium and termed TGS-01 and TGS-04 (Figure 1A), both of which have the ability to self-renew and mimic the original tumor after transplantation into the brains of immunocompromised mice (Lee et al., 2006). Profiles of the patients and properties of the GBM cells are described in Figure S1 (in Supplemental Data available online). Expression of phosphatase and tensin homolog (Pten) was lost in TGS-01 and TGS-04 "sphere" cells. CD133 (Prominin-1) was reported to be a marker for GSCs (Singh et al., 2004). We confirmed that the $CD133^+$ subpopulation is enriched in these glioma spheres compared to cells derived from the same patient but cultured in media containing 10% fetal bovine serum ("Differentiated" or "Adherent") (Sphere; 72.0%, Differentiated; 1.6%, Figure 1B). In addition, tumor spheres derived from each of tissue samples could be passaged serially and expressed Nestin (neural precursor cell marker), confirming that these are clonogenic and self-renewing cells (Figure 1C). We also validated enrichment of glioma-initiating cells in TGS-01 and TGS-04 "sphere" cells by an intracranial transplantation assay (M.T., Y.I., and T.T., unpublished data).

To test a possible role of TGF-β signaling in glioma-initiating cells, we first examined the effect of inhibition of TGF-β signaling

on their biological characters. After treatment with TGF-β type I receptor (ALK5) kinase inhibitor SB431542 (Inman et al., 2002), glioma-initiating cells were drastically deprived of sphere-forming ability (Figure 1D). Similar results were obtained using three other glioma-initiating cells from patients with GBM (Figures S1 and S2). As SB431542 can inhibit TGF-β type I receptor (ALK5) signaling, as well as activin/nodal type I receptor (ALK4 and ALK7) signaling, we also examined the effect of TGF-β receptor II/Fc chimera (TβRII-Fc) on glioma-initiating cells to assess the role of TGF-β signaling definitely. Glioma-initiating cells treated with TβRII-Fc formed glioma spheres with lower efficiency (Figure 1E). Similar results were obtained with other TGF-β signaling inhibitors, A-78-03 (Tojo et al., 2005) or LY364947 (Sawyer et al., 2003) (Figure S3A), as well as infection of adenovirus carrying cDNA of Smad7, an endogenous negative regulator of TGF-β signaling (Figure S3B). Moreover, preformed sphere cells lost their spherical growth pattern and became attached in the presence of SB431542 (Figure S3C). Decreased number of glioma spheres formed by glioma-initiating cells with depleted TGF-β signaling suggests impaired self-renewal. In agreement with the suggested effect of TGF-β signaling depletion, treatment of glioma-initiating cells with SB431542 for 7 days prior to the sphere-forming assay without the inhibitor also reduced the number of spheres (Figure S4). We also investigated the effects of TGF-β inhibitor on the $CD133^+$ subpopulation. SB431542 decreased the size of $CD133^+$ pool in glioma-initiating cells (Figure 1F). Next, to examine the expression of neural precursor or differentiation markers in each cell, spheres in serum-free medium were disaggregated and seeded on poly-L-ornithine and fibronectin-coated slide glasses. Inhibition of TGF-β signaling decreased the number of cells positive for Nestin or Musashi (neural precursor cell markers) and increased that for GFAP (astrocyte differentiated marker) or Tuj1 (βIII-tubulin, neuronal marker) (Figure 1G). Taken together, these findings suggest endogenous TGF-β signaling maintains tumorigenicity and stemness of glioma-initiating cells. Conversely, we failed to observe striking effects of addition of TGF-β ligand on sphere-forming ability, $CD133^+$ ratio, or marker expression of glioma-initiating cells (Figures 1D, 1F, and 1G). It may be because glioma-initiating cells express all the major components of TGF-β signaling pathway and secrete TGF-β1 and -β2 proteins (Figures S5A and S5B), producing sufficient autocrine TGF-β signaling to maintain their stemness (Figure S5C).

TGF-β is reported to work as a proapoptotic or an antiapoptotic factor in a cell-context-dependent manner (Sánchez-Capelo, 2005; Ehata et al., 2007), but we failed to observe any significant effect of TGF-β ligand or inhibitor on apoptosis of glioma-initiating cells (Figure S6A). TGF-β is also known to control cell proliferation via regulating $p15^{INK4b}$, $p21^{WAF1}$, $p27^{KIP1}$, and *c-myc* (Massagué, 2008), but in glioma-initiating cells, stimulation or inhibition of TGF-β signaling did not markedly affect their expression levels at 3 or 24 hr except for only a slight increase of $p21^{WAF1}$ 24 hr after inhibition of TGF-β signaling (Figure S6B).

Sox2 Expression Is Induced by TGF-β in Glioma-Initiating Cells to Maintain Their Stemness

To elucidate the mechanism by which stemness of glioma-initiating cells is maintained by TGF-β signaling, we next examined the effect of TGF-β or SB431542 on expression of various markers for stemness. mRNA expression of Sox2, a member of HMG-box factors, was induced by TGF-β but suppressed by SB431542 after 24 hr treatment (Figure 2A) and kept at the low levels for at least 7 days (data not shown). In contrast, expression levels of Oct4, Nanog, LIF, or other pluripotent stem cell-related molecules were not significantly affected by TGF-β ligand or inhibitor in our glioma-initiating cells in TGS-01 and -04 cells (Figure S7), although Nanog and LIF were reported to be induced by TGF-β stimulation in some types of cells (Xu et al., 2008; Bruna et al., 2007). Induction of Sox2 by TGF-β was clearly suppressed in the presence of siRNA against Smad2 and Smad3 (Figure 2B), indicating that Sox2 expression is regulated by TGF-β-Smad signaling. We also confirmed regulation of Sox2 protein expression by TGF-β and SB431542 (Figure 2C). Knockdown of Sox2 expression by siRNA (Figure S8) resulted in drastic reduction of sphere-forming ability and self-renewal capacity of glioma-initiating cells (Figures 2D and 2E) and decreased size of $CD133^+$ subpopulation (75.1% to 29.3% or 35.9%; Figure 2F). Drastic reduction of sphere-forming ability by knockdown of Sox2 was also observed in four other glioma cells (Figure S9). Moreover, the number of Nestin-positive cells was reduced and that of GFAP-positive cells was increased by Sox2 knockdown (Figure 2G). These findings indicate that Sox2 is an essential factor for maintenance of stemness of glioma-initiating cells and that downregulation of Sox2 expression as early as 24 hr after treatment with SB431542 appears to be the cause, rather than the result, of deprivation of stemness of glioma-initiating cells.

Downregulation of Sox2 Is a Crucial Step for Differentiation of Glioma-Initiating Cells Induced by TGF-β Inhibitor

For further study of the role of Sox2 in maintenance of stemness by TGF-β, we examined the effect of TGF-β inhibitor on glioma-initiating cells infected with adenovirus encoding Sox2 cDNA. SB431542 only weakly deprived Sox2-overexpressed glioma-initiating cells of sphere-forming ability compared to LacZ-overexpressed cells (Figure 3A). Moreover, SB431542 failed to reduce the number of Nestin-positive cells or to increase the number of GFAP-positive cells in Sox2-overexpressed glioma-initiating cells (Figure 3B). These data suggest that deprivation of stemness of glioma-initiating cells by TGF-β inhibitor is due to downregulation of Sox2, which maintains stemness of glioma-initiating cells.

Sox4 Is a TGF-β Target Gene, which Is Highly Expressed in Glioma-Initiating Cells

Induction of Sox2 expression by TGF-β was observed 24 hr, but not 3 hr, after stimulation (Figure 2A) and attenuated in the presence of cycloheximide, an inhibitor of protein synthesis (Figure 4A). These findings indicate that Sox2 expression is not directly induced by TGF-β but regulated through other factor(s) that are induced by TGF-β. We, thus, searched candidate genes that mediate TGF-β-induced Sox2 expression to play important roles in retention of stemness of glioma-initiating cells. For this purpose, we used microarray data of public resources (Beier et al., 2007; Bruna et al., 2007; Günther et al., 2008; Lee et al., 2006; Tso et al., 2006). Criteria for selection were as follows: (1) genes with higher expression in glioma-initiating cells compared

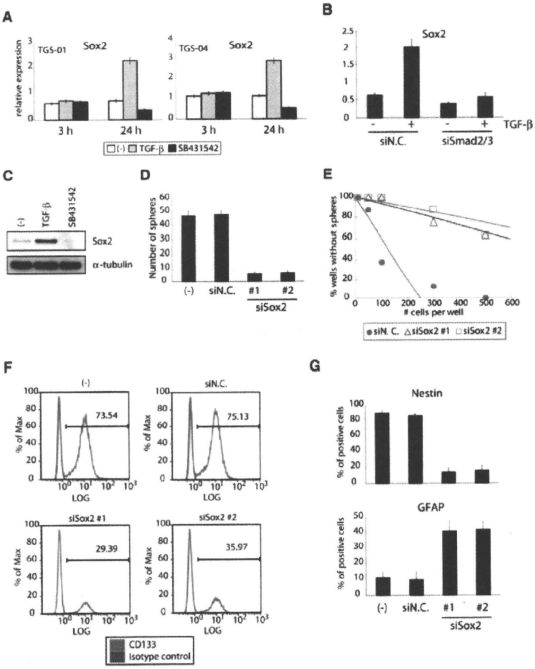

Figure 2. TGF-β Induces Expression of Sox2, an Essential Factor for Retention of Stemness of Glioma-Initiating Cells

(A) Expression of Sox2 mRNA was determined after treatment with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 3 or 24 hr. Values were normalized to that of GAPDH mRNA. Error bars represent SEM.

(B) TGS-01 cells were transfected with siRNA oligonucleotides and incubated for 24 hr. Cells were treated with TGF-β ligand (100 pM) for 24 hr. Values were normalized to that of GAPDH mRNA. Error bars represent SEM.

(C) Expression of Sox2 protein in TGS-01 cells was determined after treatment with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 24 hr. α-tubulin was used as a loading control.

(D) TGS-01 cells were dissociated into single-cell populations, transfected with control (N.C.) or Sox2 siRNA duplex, and cultured for 7 days. The data are presented as the number of glioma spheres formed (means ± SEM of five fields).

(E) Knockdown of Sox2 expression by siRNA in TGS-01 cells resulted in decrease of self-renewal capacity in limiting dilution assay.

(F) Effects of Sox2 knockdown on CD133$^+$ subpopulation of TGS-01 cells were determined by flow cytometry.

(G) Quantification of Nestin-positive or GFAP-positive cells among total cells. Differentiation of TGS-01 cells by Sox2 knockdown was analyzed 7 days after transfection of control (N.C.) or Sox2 siRNA duplex. Error bars represent SEM.

to bulk tumor cells; (2) genes directly induced by TGF-β and suppressed by TGF-β inhibitor in glioma cells; and (3) genes whose expression levels are correlated with that of Sox2 in glioma cells. Among genes highly expressed in glioma-initiating cells, we identified a transcription factor Sox4 as a TGF-β target gene.

We observed higher expression levels of Sox4 in TGS-01 and TGS-04 cells than in matched "differentiated" cells (Figure 4B). We checked whether Sox4 expression is regulated by TGF-β signaling (Figures 4C and 4D). Sox4 mRNA expression was immediately induced after TGF-β stimulation and inversely downregulated by TGF-β inhibitor in TGS-01 and TGS-04 cells. To examine whether Sox4 is a direct target gene of TGF-β, we performed chromatin immunoprecipitation assay using antibody against Smad2/3, DNA-binding mediators of TGF-β signaling. Smad complexes directly bound to Sox4 promoter in response to TGF-β stimulation, and this binding was clearly suppressed by SB431542 (Figure 4E). Moreover, induction of Sox4 by TGF-β was not significantly affected by cycloheximide (Figure 4F). These findings indicate that Sox4 is a direct target gene of TGF-β signaling.

Sox4 Associates with the Sox2 Enhancer Region and Promotes Its Expression

Next, we studied the effect of Sox4 on Sox2 expression. Sox4 overexpression in glioma-initiating cells resulted in upregulation of Sox2 expression (Figure 5A). In contrast, Sox2 expression was suppressed by Sox4 knockdown (Figure 5B). We confirmed that Sox2 mRNA expressed under the control of cytomegalovirus (CMV) promoter was not downregulated by siSox4 (Figure S10), indicating that Sox2 mRNA is not a direct target of siSox4. These results indicate that Sox2 expression is positively regulated by Sox4 at the transcriptional level. To examine whether this regulation is direct, we performed chromatin immunoprecipitation assay using antibody against Sox4. It has been demonstrated that the enhancer element located at the 3′ flanking region of Sox2 gene is important for regulation of Sox2 expression (Chew et al., 2005; Tomioka et al., 2002). The region contains the consensus binding motif for Sox4, "CATTGTA" (Liao et al., 2008). Recruitment of Sox4 to the enhancer element was increased 24 hr after TGF-β stimulation, and such recruitment was clearly suppressed by SB431542 treatment (Figure 5C). These results appear to be due to regulation of Sox4 expression by TGF-β ligand or inhibitor. In addition, TGF-β could induce Sox2 expression only weakly under the condition that Sox4 was knocked down (Figure 5D). Altogether, we concluded that Sox4 directly induced by TGF-β