

Figure5 大脳(海馬CA1)-TUNEL法

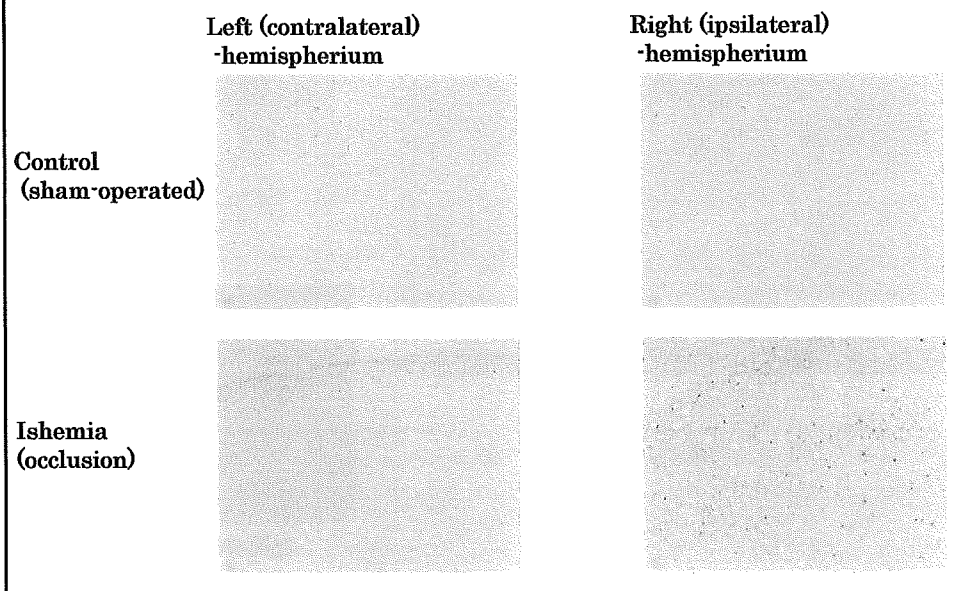


Figure6 大脳(海馬CA1)-免疫染色Anti MMP-9

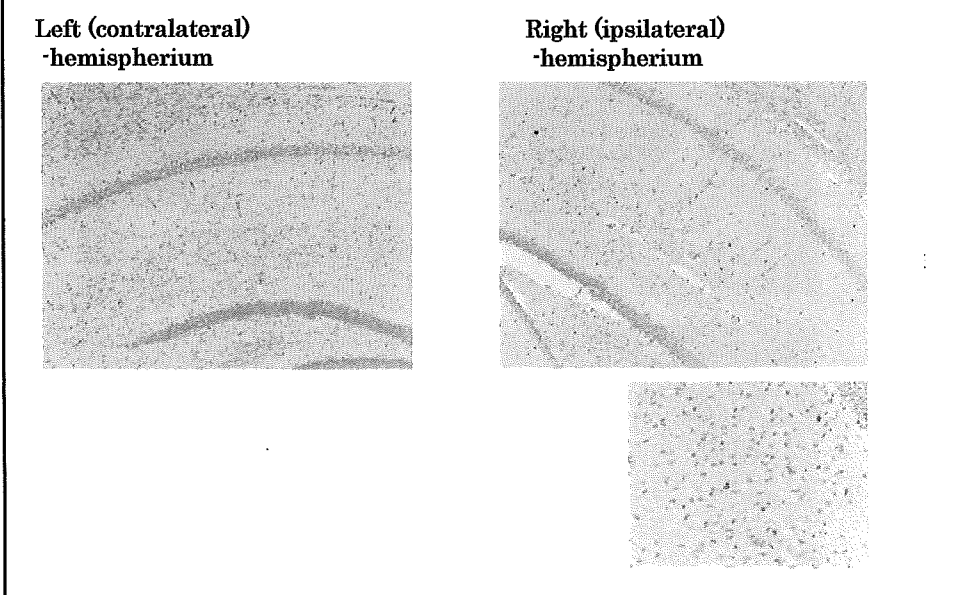
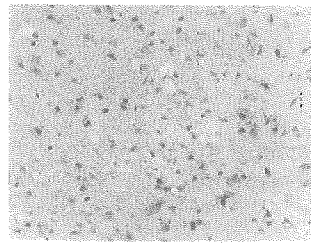
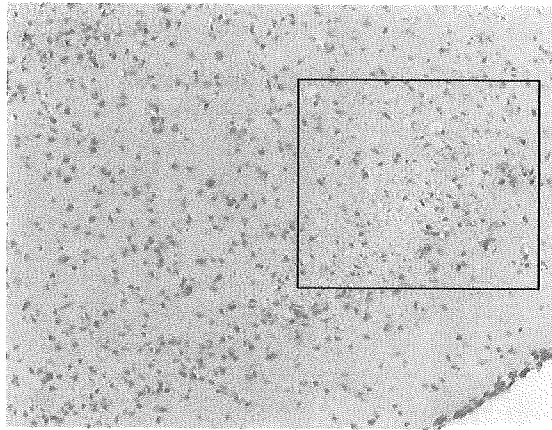


Figure7 大脳-免疫染色Anti TIMP-3



左図枠内の拡大像

Figure8 大脳-免疫染色Anti TIMP-3

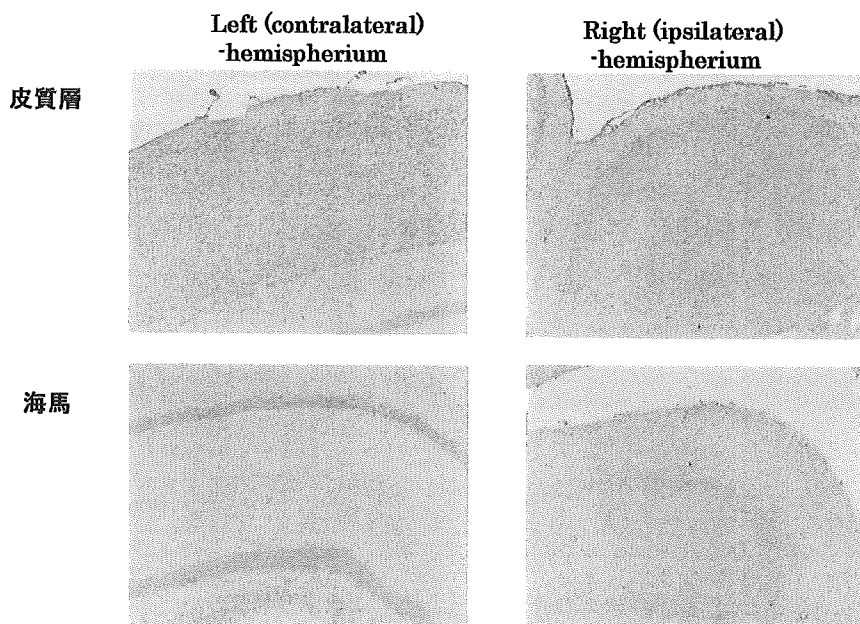
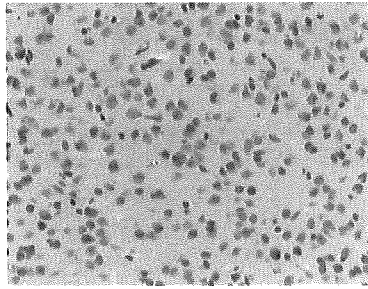
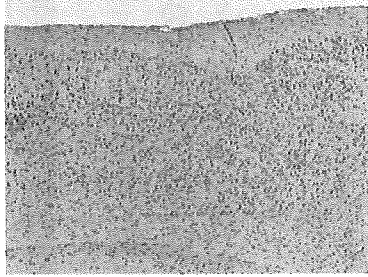
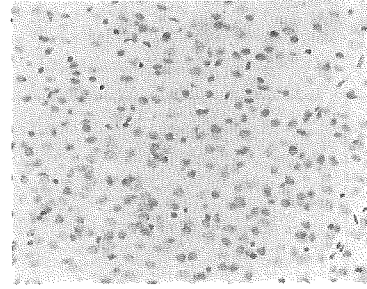
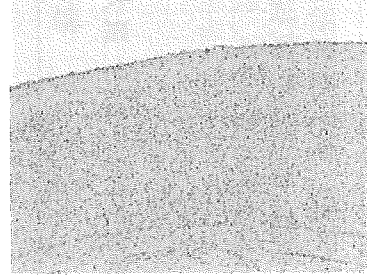


Figure9 大腦-免疫染色Anti TIMP-3

Occlusion contralateral cortex



Sham-operation contralateral cortex



ヒトゲノム・再生医療等研究事業

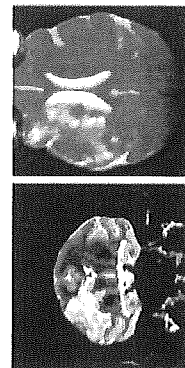
「霊長類を用いた脳梗塞モデルの遺伝子治療研究 (H16-遺伝子-006)」

研究目的: ヒトに近縁な霊長類を用いてラクナ梗塞、中大脳動脈梗塞、全脳虚血モデルを作成し有効な遺伝子治療法を開発する

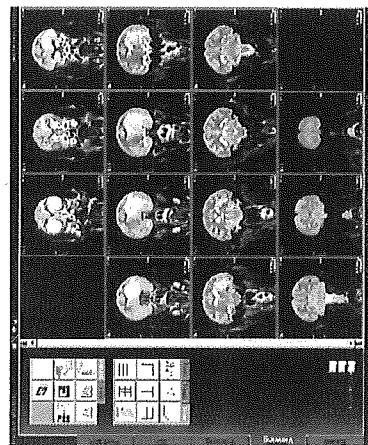
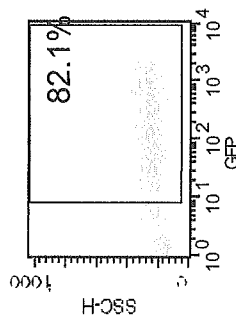
研究成果:

- ① 我が国で開発されたセンダイウイルスベクターの改良とデリバリー4世代目のベクター改良、血液単球へのex vivoでの治療遺伝子導入
- ② サル類での3種類の梗塞モデル作成
20頭のカニクザルを用いた3種類の脳梗塞、脳虚血モデル条件の確立
- ③ 脳梗塞、脳虚血時の治療用新規遺伝子の検索
脳梗塞部位、脳脊髄液のパワープロット、プロテオーム解析
- ④ 梗塞モデルの基礎的・臨床的評価
高次認知機能、MRI、運動機能試験、食物回収試験評価法の開発

研究概要:



ラクナ梗塞モデル



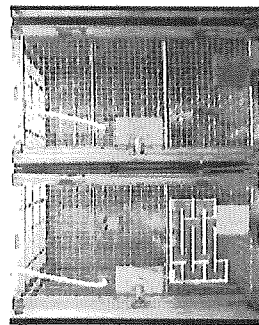
中大脳動脈梗塞モデル

治療遺伝子の組み込み

とデリバリー

有効性評価

全脳虚血の海馬



研究期間: H16 ~18年
各年度予算額: H16 年度4750万円
H17年度43545万円

実施体制: 東大医学部脳神経外科・農学部獣医・新領域創成科学、筑波霊長類センター、ディナベック、九州大学医学部等

プロジェクトリーダー: 東大農学生命科学 教授 吉川泰弘

今後の計画: 標的を明確化する

・ラクナ梗塞は白質の持続性グリア反応の低減をはかる治療法の開発・評価

・中大脳動脈梗塞はペランブラの神経細胞壊死の保護のための治療法の開発・評価

・全脳虚血は海馬の神経細胞の選延性アポトーシスの阻止を目的とした治療法の有効性・安全性評価

モデルを用いた評価結果が必要

霊長類を用いた脳梗塞モデルの遺伝子治療研究 中間評価(H180313)

東京大学農学生命科学 吉川泰弘

研究目的

ヒトに近縁な霊長類を用いてラクナ梗塞、中大脳動脈梗塞、全脳虚血モデルを作成し有効な遺伝子治療法を開発する

研究方法

①我が国で開発されたセンダイウイルスベクターの改良

ウイルスベクター使用、ex vivoでの導入

②サル類での3種類の梗塞モデル作成

③梗塞の診断マーカー、治療用新規遺伝子の検索

パワープロット、プロテオーム解析

④梗塞モデルの基礎的・臨床的評価

高次認知機能、MRI、運動機能試験、食物回収試験

研究組織

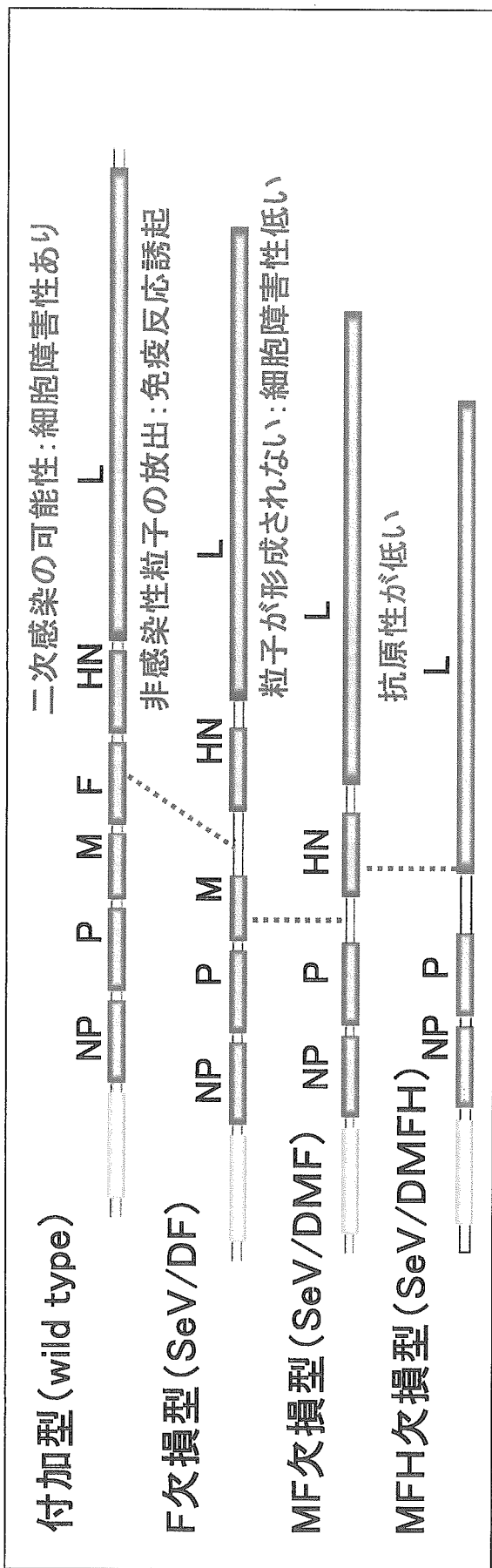
医師、獣医師、基礎科学者の共同研究
東大医学部脳神経外科・農学部獣医・新領域創成科学、
筑波霊長類センター、ディナベック、九州大学医学部など

期待効果

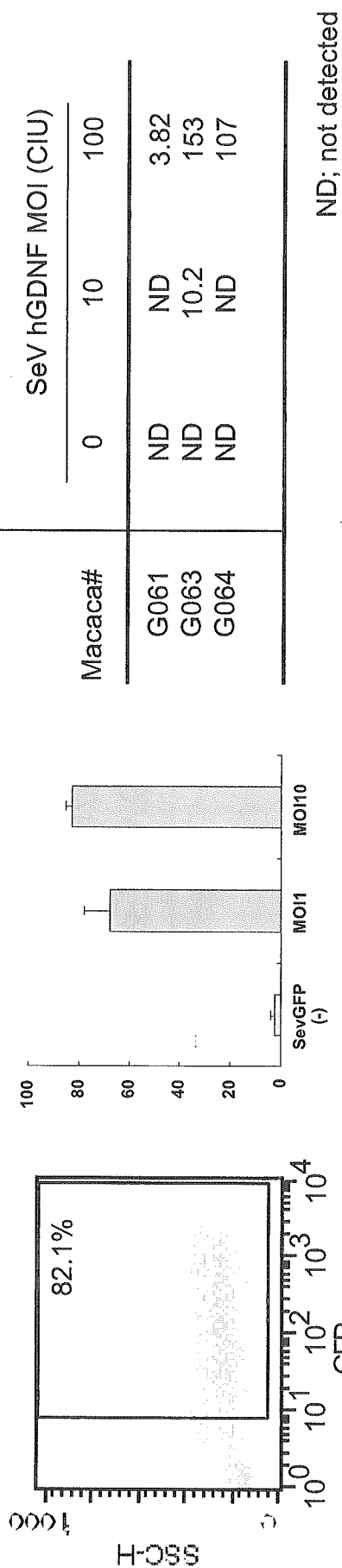
臨床も含めた研究チームであり、トランスレーションナリサーチの1つ。
研究成果はヒトの臨床研究につながる可能性が大きく、極めて有用
性が高い。

①我が国で開発されたセンダイウイルスベクターの改良 ウイルスベクター使用、ex vivoでの導入

センダイウイルスベクターの改良（安全性、有効性の評価を進めている）



血球系細胞への遺伝子導入: ex vivo (GFP, GDNF)



カニクイザル単核球にSeV-GFP, SeV-GDNFを感染させ、FACS、GDNF産生を測定

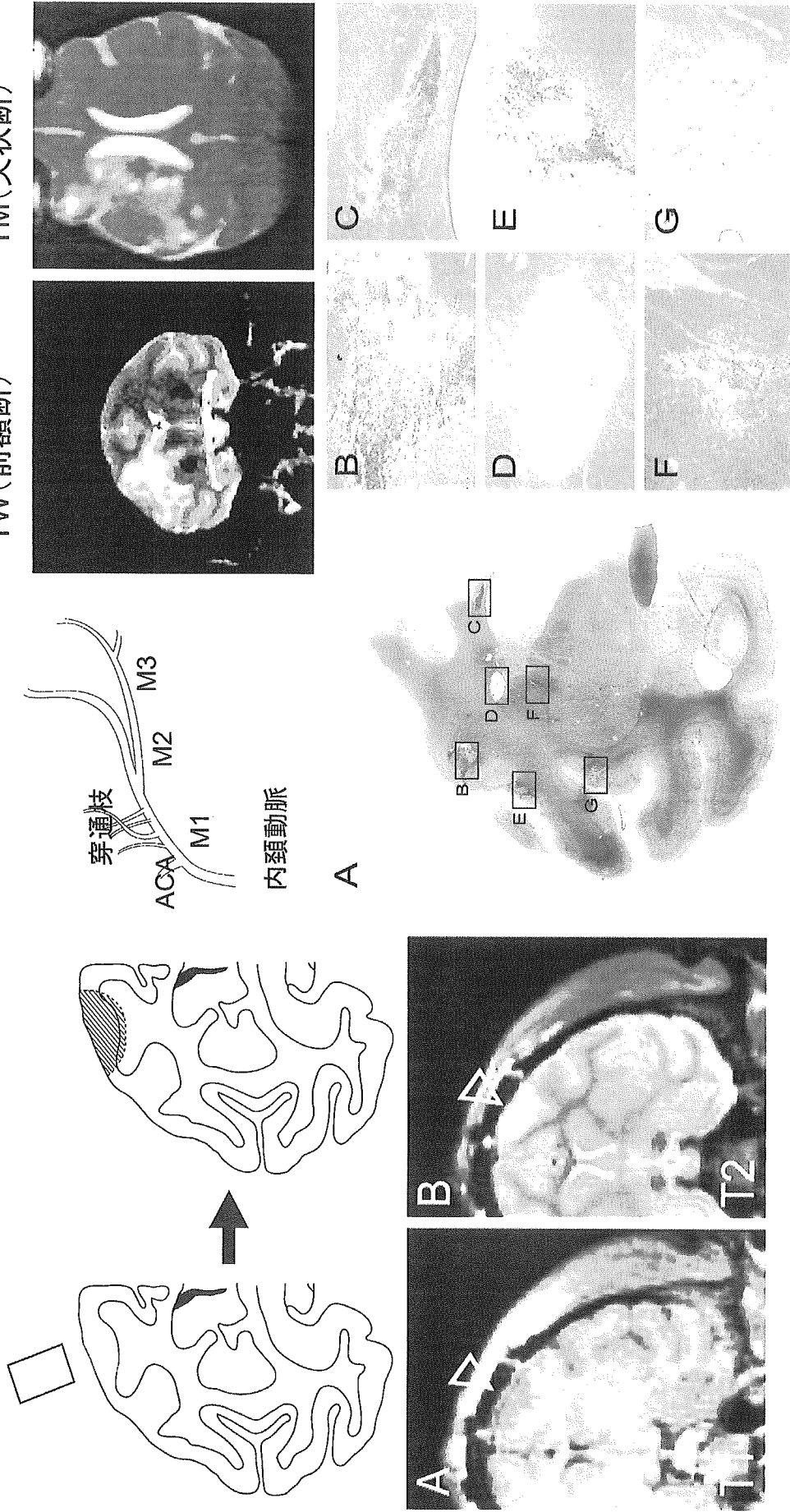
②サル類での3種類の梗塞モデル作成

所属	動物番号	実験番号	体重	性別	年齢	手術日	死亡日	方法	転帰	術後	備考(現在)
川原	1218906063	004	2.97	♀	16	2005/08/02	2005/08/02	全脳虚血	死亡	0	20分虚血、起立不能
吉川	1320003013	G059	4.52	♂	5	2005/08/23	2005/08/30	全脳虚血	実験殺	7	20分虚血、症状軽微
川原	1020502014	011	3.46	♂	3	2005/10/25	2005/10/28	全脳虚血	死亡	3	20分虚血、症状重度、術後2日目のMRI後死亡、循環障害、浮腫
川原	1220010057	005	4.05	♂	5	2005/11/29	2005/11/30	全脳虚血	死亡	1	14分虚血、症状重度
吉川	1118211320	G001	4.50	♀	23	2004/06/14	2004/06/15	中大脳動脈閉塞	死亡	1	永久閉塞、起立不能
川原	1229401003	001	5.60	♂	11	2005/01/19	2005/01/20	中大脳動脈閉塞	死亡	1	6時間閉塞、沈鬱
吉川	1319911100	G060	3.08	♀	6	2005/02/14	2005/02/17	中大脳動脈閉塞	死亡	3	3時間閉塞
川原	1219212255	002	3.52	♀	13	2005/03/01	2005/03/02	中大脳動脈閉塞	死亡	1	3時間閉塞
川原	1229111238	003	4.97	♂	14	2005/04/19	2005/04/20	中大脳動脈閉塞	死亡	1	3時間閉塞、大腿部内出血に気づき止血
吉川	1020407045	G051	3.70	♂	4	2005/06/21	2005/11/01	中大脳動脈閉塞	実験殺	130	3時間閉塞、右側閉塞、無症状、左側再手術、右手、右足多動、症状軽微
吉川	1020502021	G064	2.85	♂	3	2005/11/22	生存中	中大脳動脈閉塞	生存中		症状軽度、梗塞部位は明確
久恒	1320005031	008	5.44	♂	5	2005/01/17	2005/01/18	マイクロビーズ	死亡	1	大腿部内出血
吉川	1020407044	G050	4.28	♂	4	2005/05/23	2005/07/29	マイクロビーズ	実験殺	66	右半身麻痺
吉川	1229912084	G068	3.88	♂	6	2005/08/29	2005/10/07	マイクロビーズ	実験殺	40	右半身麻痺
吉川	1229909054	G069	3.78	♂	6	2005/12/02	2005/12/03	マイクロビーズ	死亡	1	死亡11/27マイクロビーズ、不整脈、冠状動脈周囲変化
久恒	1229912085	001	4.16	♂	5	2004/08/10	2004/08/20	ローズベンガル	実験殺	10	材料採取
吉川	1020502013	G062	2.34	♂	3	2005/06/03	2005/06/08	ローズベンガル	死亡	5	6/7、2回目手術、1日後死亡、全身の染色重度
吉川	1020209148	G026	5.48	♂	6	2005/07/11	2005/07/12	ローズベンガル	死亡	1	沈鬱、全身の染色重度
吉川	1020407046	G052	4.05	♂	4	2005/07/28	2005/08/05	ローズベンガル	実験殺	8	材料採取、1d、8d
吉川	1220104027	G067	3.78	♂	4	2005/08/16	2005/08/17	ローズベンガル	実験殺	1	材料採取

- 2005年、合計20頭のカニクイザルを用いてモデル作成を行った
- げっ歯類と異なり、サル類は脳梗塞に関して致死性が高い(技術的問題もあった)
- いずれのモデル系も梗塞後の生存可能条件が明らかにならなかった

②サル類での梗塞モデル(微小血管梗塞:ラクナ梗塞)

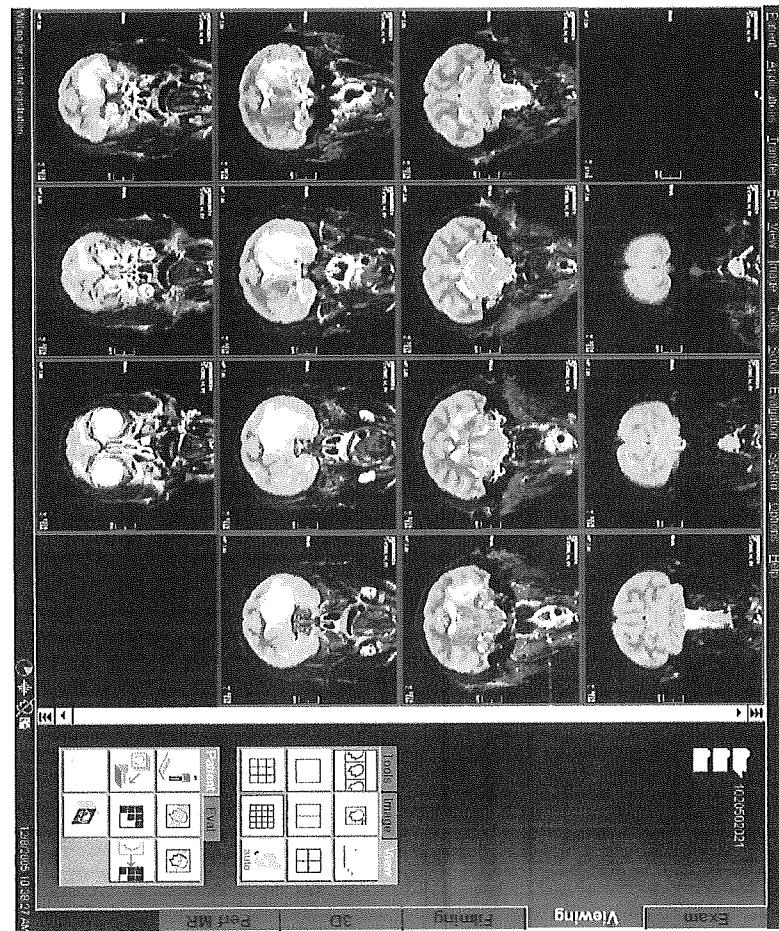
MRIのT2画像
1W(前額断) 1M(矢状断)



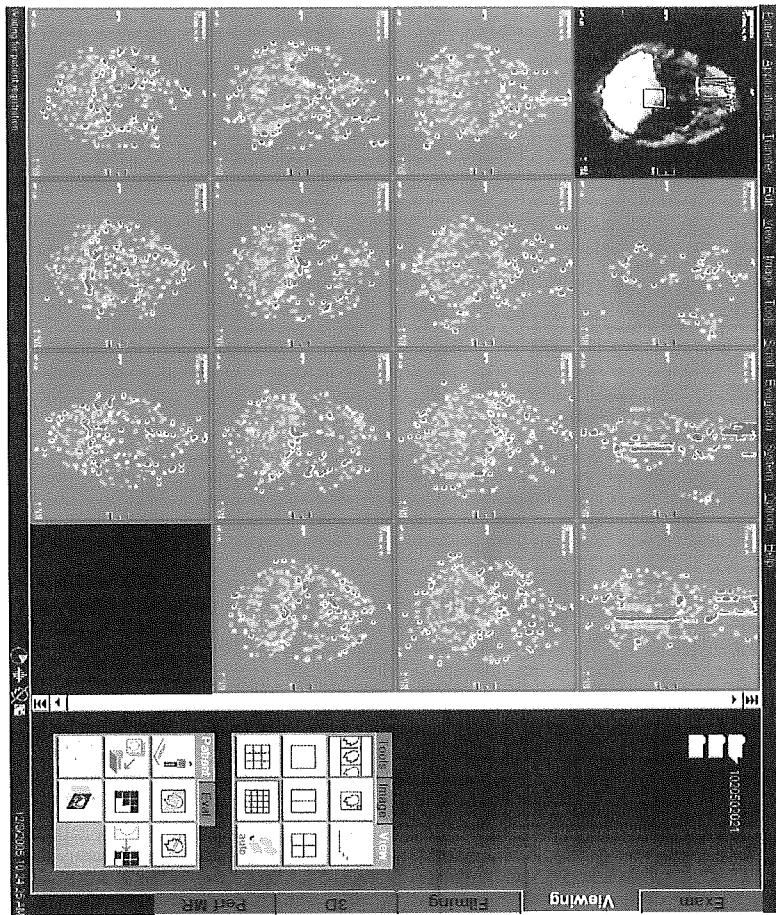
- ・ローズベンガル+レーザー光による微小血管梗塞モデルの病理解析・高次認知機能評価
- ・大腿動脈からマイクロカテーテルを挿入、中大脳動脈へアプローチ、マイクロサイズを注入し、分枝する穿通枝を閉塞(ヒトのラクナ梗塞に類似):ピーズのサイズ、量の決定

②サル類での梗塞モデル(中大脳動脈梗塞)

#64における再還流後2時間のMRI T2強調画像



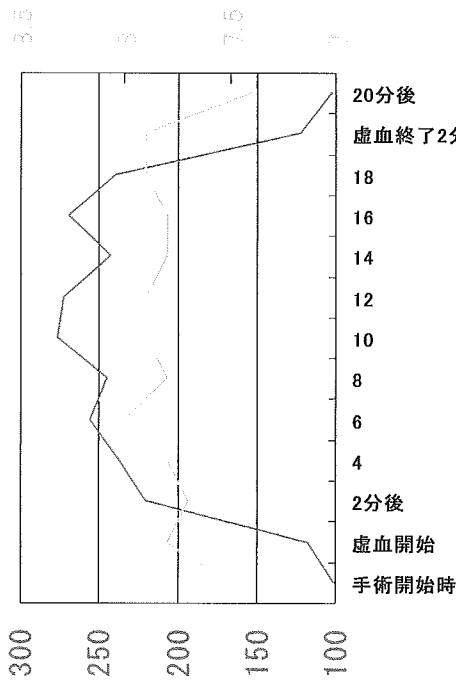
#64: 再還流後2時間のPerfusion 解析所見



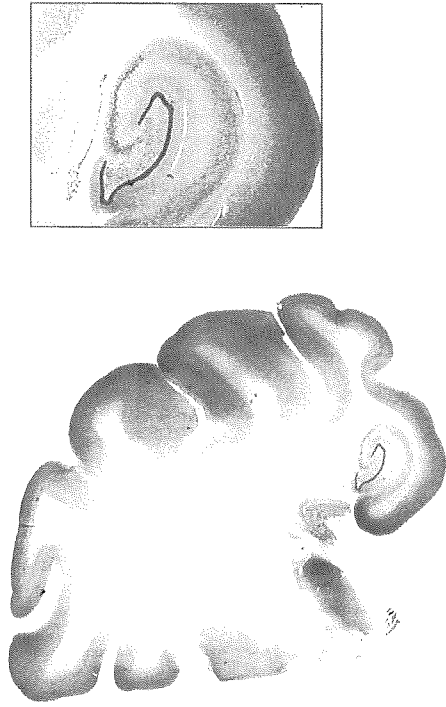
- ・マイクロカテーテルを用いた中大脳動脈閉塞モデル作成を試みた(7例)
- ・梗塞後明らかな臨床症状とMRIによる梗塞像が確認
- ・最初の5例では広範な梗塞部位とともに重度の脳浮腫を伴い死亡
- ・梗塞時間(3時間後再還流)、全身ヘパリン化処置等により2例において生存モデル
- ・MRIによる梗塞評価、食物回収試験による記憶力評価等有効な評価系を確立

②サル類での全脳虚血モデル

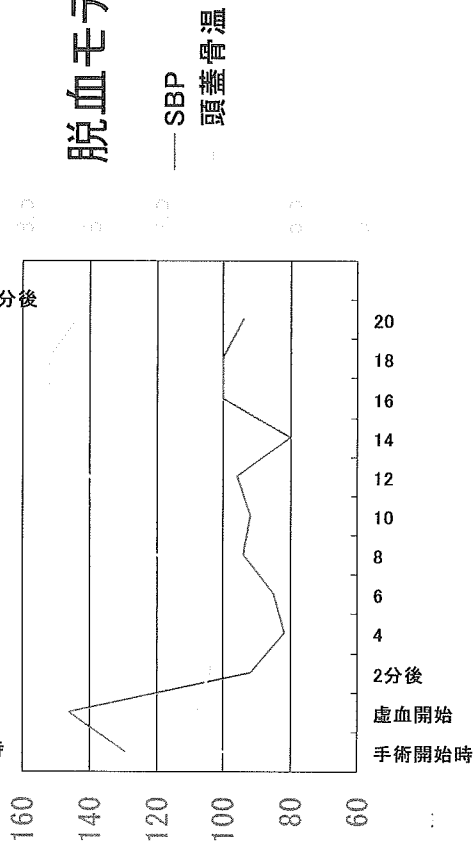
虚血中の著しい血圧上昇が生じた(虚血18分)



海馬の神経細胞は正常

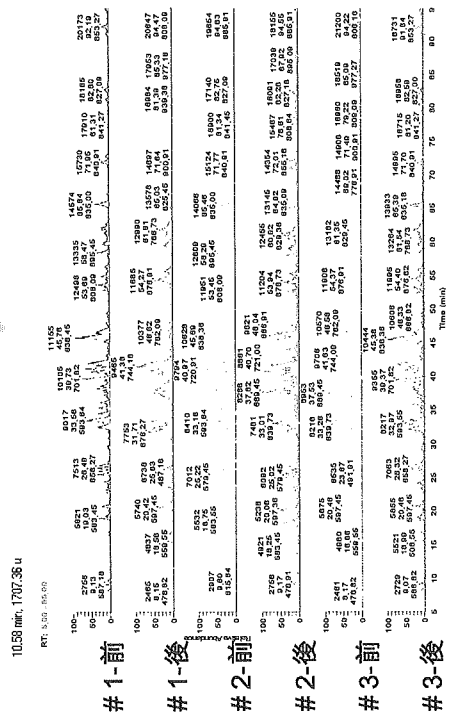
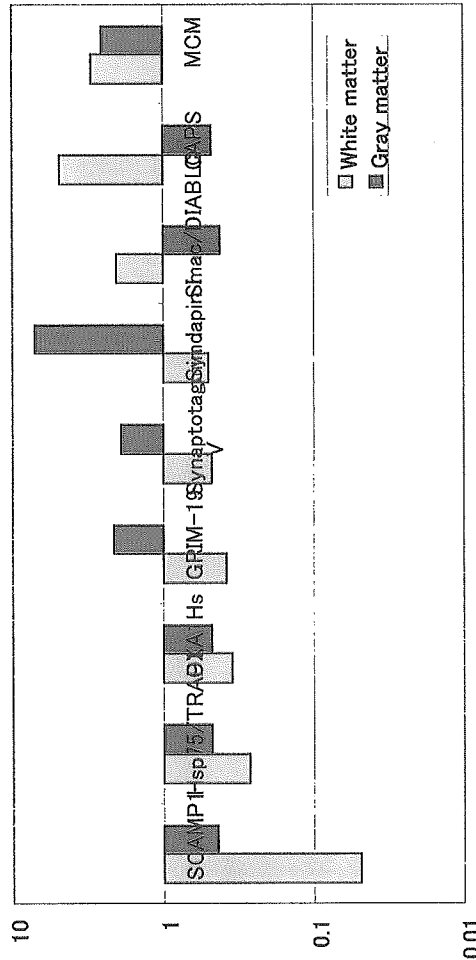
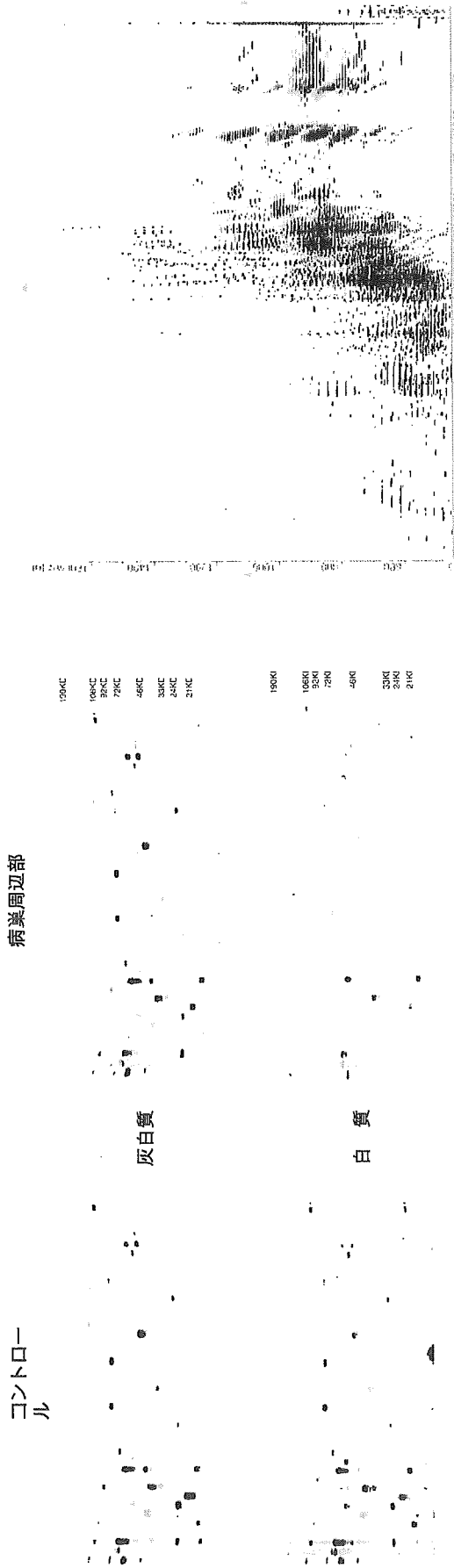


脱血モデル



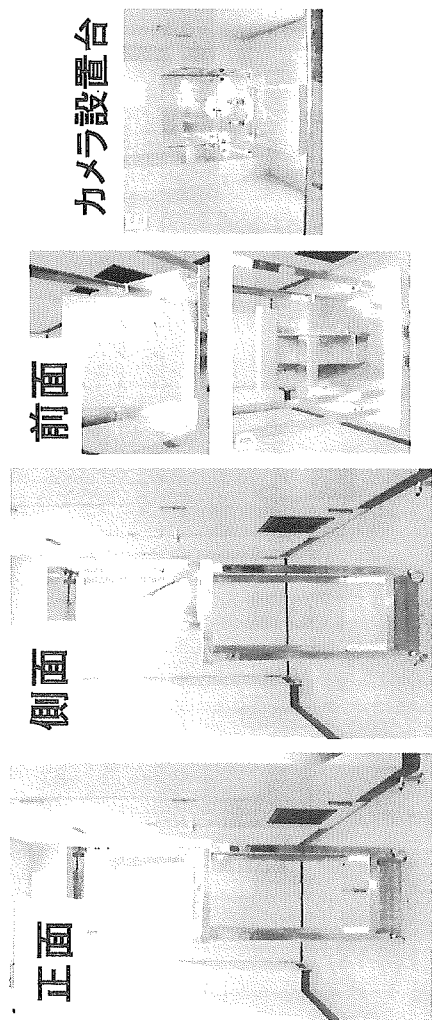
- 従来のサル全脳虚血モデルでは血管閉塞中の血圧コントロールを行っていない(虚血影響ない)
- 虚血中、脱血による厳密な血圧コントロールを行った結果、非常に短時間の血管閉塞でよい
- 必要な虚血時間(5~8分)はヒトでの低酸素脳症に近づいてきている

③ 梗塞の診断マーカー、治療用新規遺伝子の検索 パワープロット、プロテオーム解析

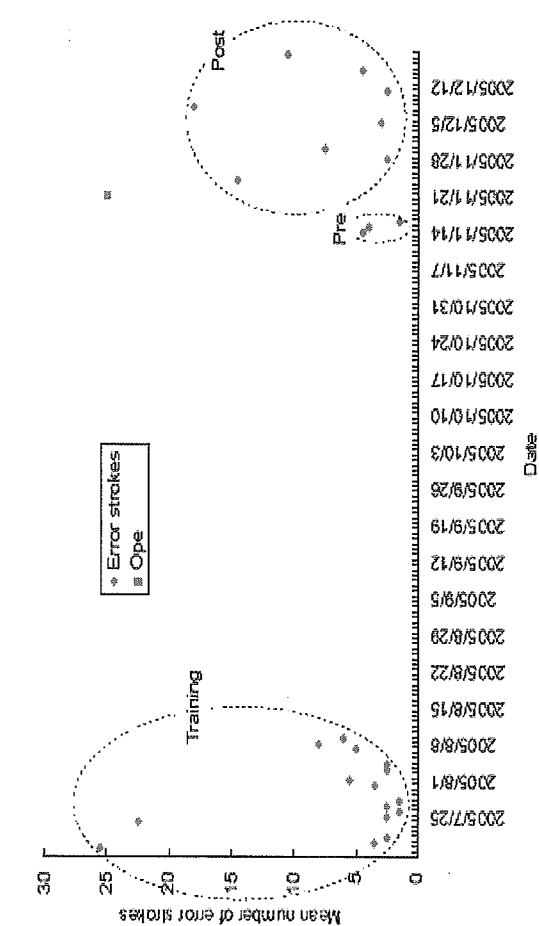
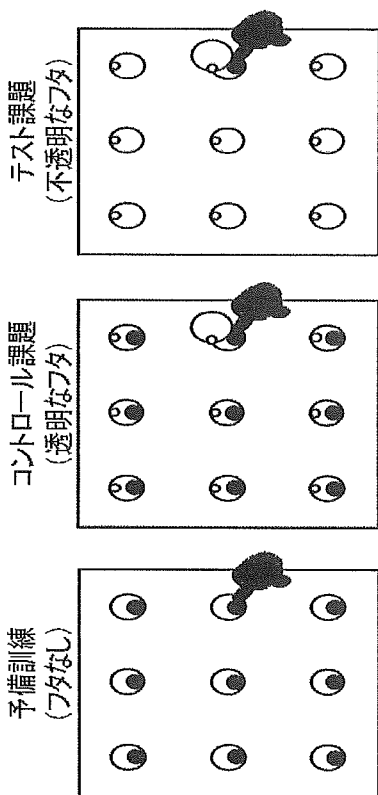


- ・梗塞モデル(ラクナ梗塞)における病巣部、周辺部、正常部の発現蛋白の網羅的解析
- ・ミトコンドリア関連蛋白で変動が見られた(白質、灰白質)
- ・梗塞モデル(中大脳動脈梗塞)における髄液のプロテオーム解析を進めた

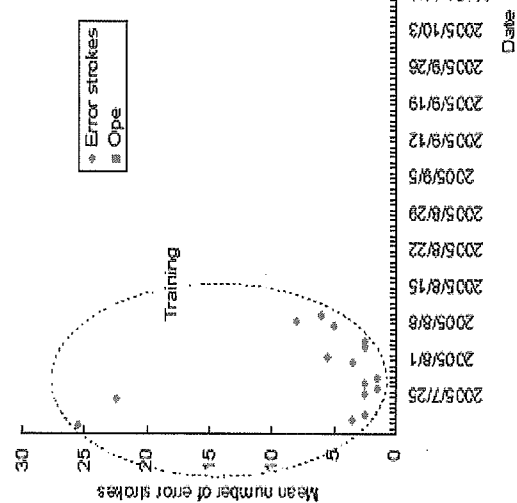
④梗塞モデルの基礎的・臨床的評価 手続き記憶試験



カメラ設置台



#64 誤ストロークの出現位置



#64 トレーニング、手術前、手術後のセッション：平均誤ストローク数

4段指迷路 (高次認知機能)、動画差分による運動機能評価に加えて、食物回収試験を組込む

中間評価の反省

指摘事項：楽観して行ったが、とても厳しかった！

- 疾患モデル開発か？ 遺伝子治療か？
- 診断マーカーとは何か？ MRIで診断可能。

目的を絞るべき。モデルが多い。欲張りすぎではないか

ラクナ梗塞ではマーカーを探る意味はあるのでは。

▪ 標的を明確化する

ラクナ梗塞は白質の持続性グリア反応の低減？

中大脳動脈梗塞はペランブラの神経細胞壊死の保護？

全脳虚血は海馬の神経細胞の遅延性アポトーシスの阻止？

- モデルを用いた評価結果が必要！

資料・業績

Recombinant Sendai Virus Vector Induces Complete Remission of Established Brain Tumors through Efficient *Interleukin-2* Gene Transfer in Vaccinated Rats

Yasuo Iwadate,¹ Makoto Inoue,⁴ Takashi Saegusa,¹ Yumiko Tokusumi,⁴ Hiroaki Kinoh,⁴ Mamoru Hasegawa,⁴ Masatoshi Tagawa,³ Akira Yamaura,¹ and Hideaki Shimada²

Abstract Purpose: Sendai virus (SeV), a murine parainfluenza virus type I, replicates independent of cellular genome and directs high-level gene expressions when used as a viral vector. We constructed a nontransmissible recombinant SeV vector by deleting the *matrix* (*M*) and *fusion* (*F*) genes from its genome (SeV/ Δ M Δ F) to enhance its safety. We also estimated the therapeutic efficacy of the novel vector system against a rat glioblastoma model.

Experimental Design: We administered the recombinant SeV vector carrying the *lacZ* gene or the *human interleukin-2* (*hIL-2*) gene into established 9L brain tumors *in vivo* simultaneous with peripheral vaccination using irradiated 9L cells. Sequential monitoring with magnetic resonance imaging was used to evaluate the therapeutic efficacy.

Results: We found extensive transduction of the *lacZ* gene into the brain tumors and confirmed sufficient amounts of interleukin 2 (IL-2) production by hIL2-SeV/ Δ M Δ F both *in vitro* and *in vivo*. The magnetic resonance imaging study showed that the intracerebral injection of hIL2-SeV/ Δ M Δ F brought about significant reduction of the tumor growth, including complete elimination of the established brain tumors. The ⁵¹Cr release assay showed that significant amounts of 9L-specific cytotoxic T cells were induced by the peripheral vaccination. Immunohistochemical analysis revealed that CD4⁺ T cells and CD8⁺ T cells were abundantly infiltrated in the target tumors.

Conclusion: The present results show that the recombinant nontransmissible SeV vector provides efficient *in vivo* gene transfer that induces significant regression of the established brain tumors and suggest that it will be a safe and useful viral vector for the clinical practice of glioma gene therapy.

Glioblastoma is the most common malignant brain tumor. It is considered incurable despite multimodal approaches of therapy, including surgery, radiotherapy, and chemotherapy (1). The therapeutic application of the gene transfer technique has been expected as a new therapeutic option for glioblastoma (2). The efficacy, however, has been limited by low levels of transgene transduction and poor distribution of the target molecules throughout the tumor tissues (3). The successful gene therapy in clinical practice will depend on the develop-

ment of novel vector systems capable of wide distribution and efficient transduction of the target genes into the tumor cells.

Recent advances to manipulate the genome of negative-strand RNA viruses have led to the development of a new class of viral vectors for gene transfer approaches (4). Sendai virus (SeV) is a murine parainfluenza virus type I belonging to the family Paramyxoviridae and is a single-stranded RNA virus. The genome of SeV is a linear and nonsegmented negative-strand RNA of ~15.4 kb. It contains six major genes that are arranged in tandem on its genome; it is tightly encapsidated with the nucleoprotein (NP) and is further complexed to phosphoprotein (P) and large protein (L; the catalytic subunit of the polymerase). This viral ribonucleoprotein complex constitutes the internal core structure of the virion. The viral envelope contains two spike proteins, hemagglutinin-neuraminidase (HN) and fusion (F), which mediate the attachment of virions and the penetration of ribonucleoproteins into infected cells, respectively. Matrix (M) protein functions in virus assembly and budding.

For the use of gene therapy vectors, the promising characteristics of SeV are as follows: (a) an exclusively cytoplasmic replication cycle without any risk of integration into the genomic DNA, (b) transduction efficacy that is not dependent on the cell cycle of target cells, (c) no homologous recombination between

Authors' Affiliations: Departments of ¹Neurological Surgery and ²Academic Surgery, Graduate School of Medicine, Chiba University; ³Division of Pathology, Chiba Cancer Center Research Institute, Chiba, Japan; and ⁴DNAVEC Research, Inc., Tsukuba, Ibaraki, Japan

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Requests for reprints: Yasuo Iwadate, Department of Neurological Surgery, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, 260-8670 Chiba, Japan. Phone: 81-43-226-2158; Fax: 81-43-226-2159; E-mail: iwadatey@faculty.chiba-u.jp.

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different SeV genomes or to wild-type virus, (d) the remarkably brief contact time that is necessary for cellular uptake, (e) a high and adjustable expression of virally encoded genes in a broad range of host cells, and (f) the lack of association with any disease process in humans (4). Indeed, the SeV vector has been shown to produce 2 to 3 logs higher transfection efficiency than the adenoviral vectors or lipofection (5, 6), and high gene expressions have been noticed in a broad range of tissues, including the airway epithelial cells, vasculature tissues, skeletal muscle, activated T cells, stem cells, and neural tissues (5–12). In addition to these biological features of the SeV vector, we have constructed a nontransmissible recombinant SeV vector by deleting the M and F genes from its genome to enhance its safety (13–15).

We previously verified the efficacy of the intracerebral (i.c.) transplantation of interleukin 2 (IL-2)-producing cells in the rat brain tumor model (16, 17). For clinical application of the cytokine gene therapy strategy, however, the cell-mediated therapy needs a large amount of cytokine-producing cells to cover the whole tumor areas in the human brain. The SeV vector-mediated strategy would be superior in the wide distribution of the transgene products achieved by a small amount of viral solution, and it is expected to be especially suitable for delivery of the secreted proteins, such as cytokines. In the present study, we examined the therapeutic potentials of the nontransmissible recombinant SeV vector carrying human IL-2 gene in a cytokine gene therapy against brain tumors.

Materials and Methods

Cells and animals. Rat 9L gliosarcoma, C6 glioma, and rhesus monkey LLC-MK₂ kidney cell lines were maintained in DMEM supplemented with 10% FCS in a humidified atmosphere of 5% CO₂. Male Fisher 344 rats, weighing between 200 and 240 g (7–8 weeks old), were used as indicated in the experiments. These animals were maintained in a specific pathogen-free environment in accordance with the Laboratory Animal Resources Commission Standards.

Recombinant Sendai virus vector. Genome order of the SeV full-length genome used was as follows: the leader (ld) at the 3'-end followed by viral genes, nucleocapsid (NP), phospho (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large proteins (L). Finally, a small trailer (tr) sequence was placed at the 5'-end (Fig. 1). We utilized both the M and F genes-deleted SeV vector (SeV/ΔMΔF) in the experiment. F protein is essential for viral infection and M protein functions in virus assembly and budding (13, 14). Therefore, SeV/ΔMΔF is nontransmissible with loss of particle formation from infected cells (15). SeV/ΔMΔF carrying human IL-2 gene (hIL2-SeV/ΔMΔF) and lacZ gene (lacZ-SeV/ΔMΔF) were constructed as previously described (13). In brief, human IL-2 (accession no. U25676) cDNA was amplified with a pair of NotI-tagged primers

that contained SeV-specific transcriptional regulatory signal sequences, 5'-ACTTGCGGCGCGTTTAAACGCGCGCCATGTACAGGATGCAACTCCTGTC-3' and 5'-ATCGCGGCGCGGATGAACCTTACCCCTAAGTTTTCTTACTACGGATTTAAATGGCGGCCA-3'. The amplified fragment was introduced into the NotI site of the parental pSeV18+/ΔMΔF. Thus, the cDNA of hIL2-SeV/ΔMΔF (phIL2-SeV/ΔMΔF) was constructed. The cDNA of lacZ-SeV/ΔMΔF (placZ-SeV/ΔMΔF) was constructed in similar manner using the amplified fragment of lacZ (18). phIL2-SeV/ΔMΔF and placZ-SeV/ΔMΔF were transfected into LLC-MK₂ cells after infection of the cells with vaccinia virus vTF7-3 (19), which expresses T7 polymerase. The T7-driven recombinant hIL2-SeV/ΔMΔF and lacZ-SeV/ΔMΔF RNA genomes were encapsulated by N, P, and L proteins, which were derived from their respective cotransfected plasmids. The recovered SeV vectors were propagated using both M and F protein-expressing packaging cell lines (15). The virus titers were determined using infectivity and were expressed in cell infectious units. The SeV vectors were stored at -80°C until use.

Kinetic analysis of interleukin-2 production. LLC-MK₂ cells (10⁶) grown in six-well plates were infected at a multiplicity of infection of 10 for 1 hour with hIL2-SeV/ΔMΔF and incubated in serum-free MEM at 37°C. The culture supernatants were collected every 24 hours, with immediate addition of MEM to the remaining cells. IL-2 protein in the supernatant was quantified by ELISA using the human IL-2 ELISA kit (Biosource International, Inc., Camarillo, CA).

Brain tumor model and treatment. The animals were anesthetized and placed in a stereotaxic apparatus. A burr hole was made at 4 mm posterior to bregma and 3 mm right to midline. A 25-gauge needle was inserted to the point of 3 mm ventral from dura where 1 × 10⁵ syngeneic 9L tumor cells in 10 μL medium were slowly injected. Treatment was started 3 days (day 3) after i.c. inoculation of 9L tumor cells (day 0). The animals received i.c. administration of hIL2-SeV/ΔMΔF or lacZ-SeV/ΔMΔF and/or s.c. vaccination with irradiated wild-type 9L tumor cells. For i.c. administration, 1 × 10⁷ cell infectious units of SeV vector in 10 μL PBS were used in the same stereotaxic coordinates. For s.c. vaccination, wild-type 9L cells were irradiated at 30 Gy and 1 × 10⁶ cells in 100 μL medium were injected into the lower abdominal quadrant (16, 17). The animal experimentation was reviewed and approved by the Institutional Animal Care and Use Committee of Chiba Cancer Center Research Institute.

Magnetic resonance imaging study. To estimate i.c. tumor volume sequentially, all the animals were examined with magnetic resonance imaging (MRI) every 7 days started on day 7 after the tumor inoculation. Rats were anesthetized with 50 mg/kg pentobarbital and injected with 0.2 mL gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA, 0.8–1.0 mL/kg). Coronal T₁-weighted images (TR 500 milliseconds, TE 11 milliseconds, 3 mm thickness, gapless) were obtained with a 1.5-T MR device (Signa Advantage, General Electric, Milwaukee, WI). Tumor volume (mm³) was calculated as the sum of the Gd-DTPA-enhanced portion of each MR-imaged area (mm²) times the imaged thickness. The estimated tumor volumes on MRI have a linear correlation with actual tumor weights obtained immediately after the imaging study (20).

Immunohistochemistry. Tumor-bearing rats were perfused through the ascending aorta with 4% paraformaldehyde and brains were

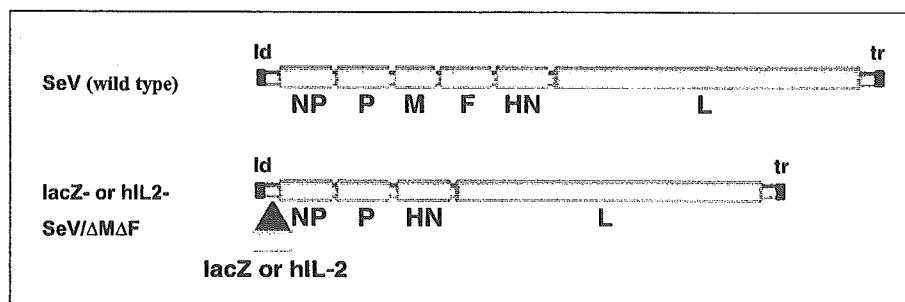
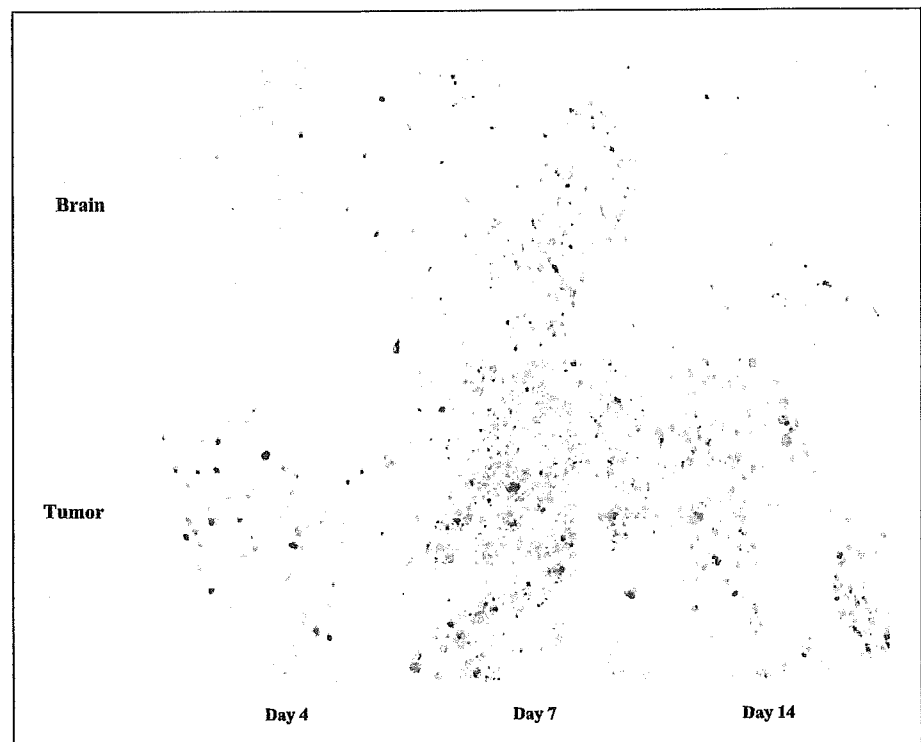


Fig. 1. Schematic genome structures of wild-type SeV and the both M and F gene-deleted SeV vector carrying lacZ or human IL-2 gene. The open reading frame of the lacZ or human IL-2 gene was inserted with the SeV-specific transcriptional regulatory signal sequences, end and start signals between the leader (ld) and the NP gene.

Fig. 2. 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside staining of rat brain tissues (*top*) and 9L brain tumors after 7 days of growth in the brain (*bottom*) administered *in situ* with lacZ-SeV/ Δ MAF. Four, seven, and fourteen days after administration of the vector, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside staining was done ($\times 200$ magnification). Maximal expression or accumulation of β -galactosidase was obtained on day 7 after injection of the vector, and the expression was maintained on day 14 both in the brain tissues and in the brain tumors.



removed. Frozen tissue sections of the brain specimens at 10 μ m thickness slices were reacted with anti-CD4 antibody (W3/25, Serotec, Oxford, United Kingdom), anti-CD8 antibody (OX-8, Serotec), and anti-human IL-2 antibody (R&D Systems, Minneapolis, MN). Tissues were then reacted with horseradish peroxidase-conjugated goat anti-mouse IgG and stained with 3,3'-diaminobenzidine tetrahydrochloride (Nichirei, Tokyo, Japan). β -galactosidase expression was detected using β -Galactosidase Staining Kit (Mirus, Madison, WI).

CTL assay. To test lymphocytes for their antitumor cytotoxicity, a standard 4-hour ^{51}Cr release assay was done. Spleen cells (1×10^6 cells) were harvested from rats on day 14 after treatment and were cultured in RPMI 1640 supplemented with 10% FCS and 5×10^{-5} mol/L 2-mercaptoethanol. They were stimulated *in vitro* with irradiated 9L cells for 5 days. Syngeneic 9L or allogeneic C6 cells were used as ^{51}Cr -labeled targets and were cultured with the spleen cells at various effector-to-target cell ratios. After 4-hour incubation, radioactivities in the culture supernatants were counted with an automatic γ -counter. Specific cytotoxic activity was calculated as follows: $100 \times \frac{[\text{experimental counts per minute} - \text{spontaneous counts per minute}]}{[\text{maximal counts per minute} - \text{spontaneous counts per minute}]}$. The maximal counts per minute were released by adding 1% NP40 to wells in experiments.

Statistics. Comparison of tumor volumes in each treatment group was done with the unpaired *t* test. The Kaplan-Meier method was used to estimate the survival rates and the Cox-mantel log-rank test was used to compare the survival differences in each treatment group. All of the statistical analyses were done with the StatView software (SAS Institute, Inc., Cary, NC).

Results

Sendai virus vector-mediated transduction of β -galactosidase gene into glioma tissue. The efficiency of i.c. transduction of the β -galactosidase gene by SeV vector was examined in brain tumors and normal brain tissues removed 4, 7, and 14 days after administration of lacZ-SeV/ Δ MAF. When injected into the brain

tumor, the typical appearance of the vector-injected tissues was scattered colonies of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside-positive cells, which were composed of transduced tumor cells from the injected lacZ-SeV/ Δ MAF (Fig. 2). Nontransduced tumor cells were seen between the scattered 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside-stained colonies. Maximal expression or accumulation of

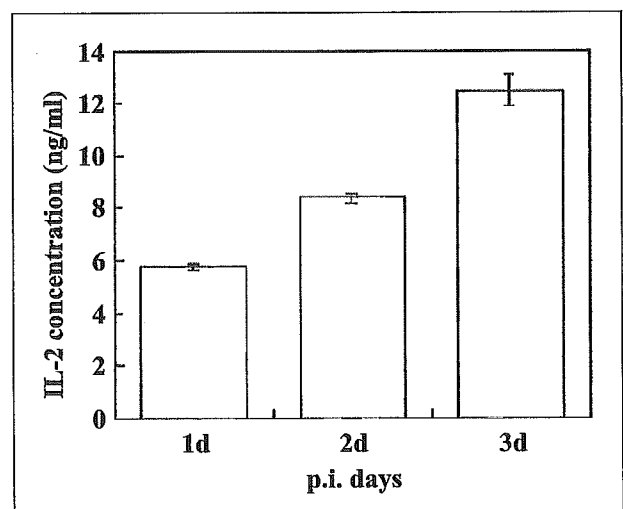


Fig. 3. Kinetics of the expression of IL-2 protein in the cells infected with hIL2-SeV/ Δ MAF *in vitro*. LLC-MK₂ cells (10^6) were infected at a multiplicity of infection of 10 with hIL2-SeV/ Δ MAF. The culture supernatants were collected every 24 hours and the amounts of IL-2 protein were quantified by ELISA. Substantial amount of IL-2 was secreted even at 1 day after the infection. The maximum production of 12.5 ng/mL was observed at day 3.

β -galactosidase was obtained on day 7 and the expression level was maintained on day 14 after injection of the vector. Transduction of normal brain surrounding the tumor was scarcely detected with the exception of the choroid plexus. When injected into the normal brain tissues, transductions of neurons and glial cells were also observed at lower efficiency compared with the case of intratumoral injection. Ependymal cells were not transduced by the intraparenchymal injection of the vector.

In vitro kinetics of interleukin-2 production by cells infected with hIL2-SeV/ Δ M Δ F. To clarify the transgene expression induced by hIL2-SeV/ Δ M Δ F, the amount of IL-2 production by the cells infected with hIL2-SeV/ Δ M Δ F was investigated by ELISA. Sufficient amount of IL-2 protein (5.8 ng/mL) was detected at day 1 after the vector infection and increased to 12.5 ng/mL at 3 days after the infection (Fig. 3).

Antitumor effects of i.c. administration of hIL2-SeV/ Δ M Δ F. All the naive rats inoculated with 9L cells in the brain developed progressive tumors. We examined the therapeutic effect of i.c. administration of hIL2-SeV/ Δ M Δ F combined

with s.c. vaccination by measuring the tumor volumes with serial Gd-enhanced MRI (Fig. 4A). The tumor volumes on day 21 in the rats treated with the i.c. administration of hIL2-SeV/ Δ M Δ F and the vaccination ($86.5 \pm 63.8 \text{ mm}^3$, $n = 10$) were significantly smaller than the following groups: untreated ($286 \pm 51.2 \text{ mm}^3$, $n = 10$, $P < 0.0001$); vaccination alone ($197 \pm 48.9 \text{ mm}^3$, $n = 10$, $P = 0.0005$); i.c. administration of lacZ-SeV/ Δ M Δ F combined with the vaccination ($233 \pm 73.2 \text{ mm}^3$, $n = 6$, $P = 0.0012$); and i.c. administration of hIL2-SeV/ Δ M Δ F alone ($256 \pm 53.2 \text{ mm}^3$, $n = 6$, $P = 0.0001$; Fig. 4B). When treated with the combination strategy, all the inoculated tumors became visible by MRI on day 21 and the established brain tumors were completely eliminated in 3 of 10 rats (Fig. 4A). There was no difference in the tumor volumes on day 21 between the three cured tumors ($81.6 \pm 74.9 \text{ mm}^3$) and the other seven tumors ($88.6 \pm 67.1 \text{ mm}^3$). Accordingly, the lifetime of the rats treated with i.c. administration of hIL2-SeV/ Δ M Δ F vector combined with the vaccination was significantly prolonged compared with the untreated control rats or the rats treated otherwise ($P < 0.05$, log-rank test; Fig. 4C). All the cured animals completely rejected

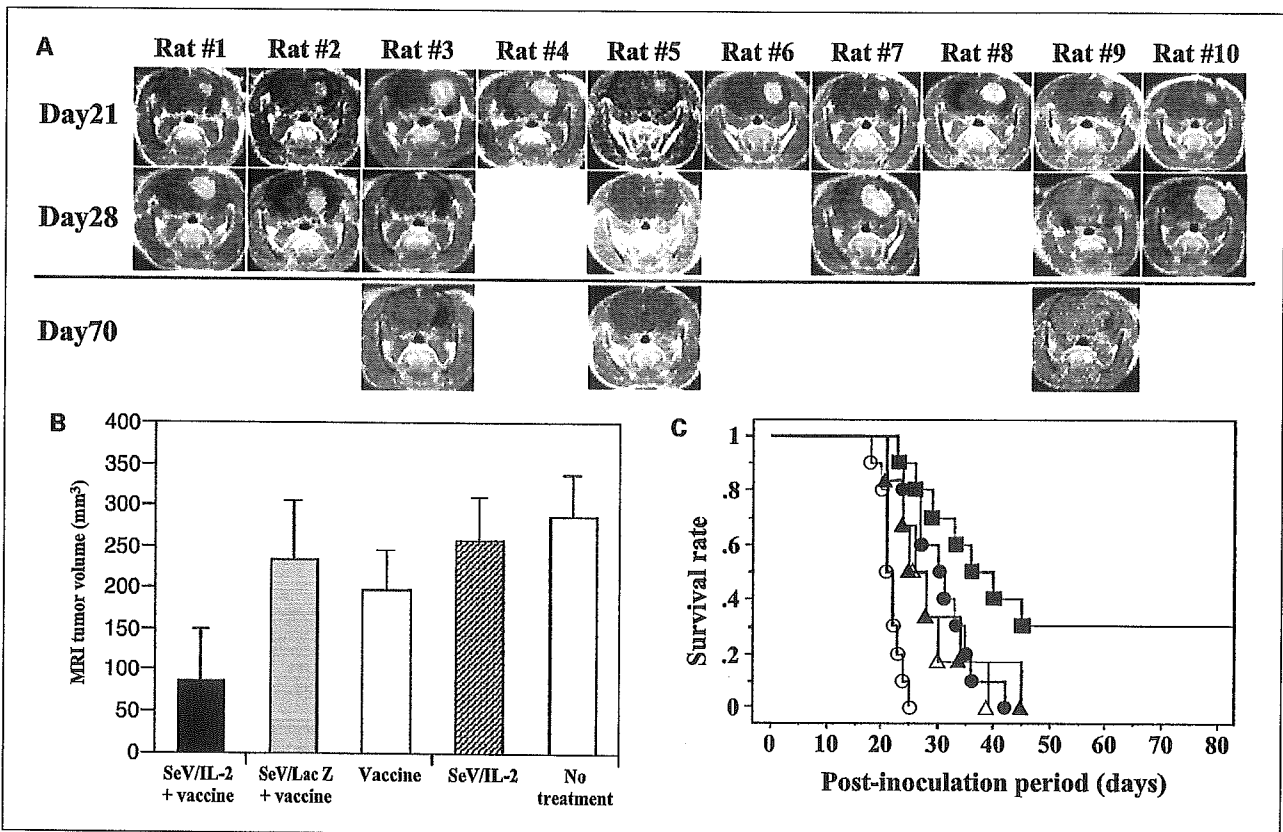


Fig. 4. Antitumor effects of i.c. administration of hIL2-SeV/ Δ M Δ F. **A**, Gd-DTPA – enhanced MRI images of all the 9L brain tumors treated with i.c. administration of hIL2-SeV/ Δ M Δ F and s.c. vaccination of irradiated wild-type 9L cells. All the animals were examined with MRI every 7 days, starting on day 7 after tumor inoculation. When treated with the combination strategy, all the i.c. tumors became visible at latest on day 21. The established brain tumors completely disappeared on day 28 in 3 of 10 rats examined (rats 3, 5, and 10). There was no difference in the tumor volumes on day 21 between the three eliminated tumors ($81.6 \pm 74.9 \text{ mm}^3$) and those of the seven other tumors ($88.6 \pm 67.1 \text{ mm}^3$). **B**, mean volumes of the 9L brain tumors measured by Gd-enhanced MRI on day 21. i.c. administration of hIL2-SeV/ Δ M Δ F combined with the vaccination ($86.5 \pm 63.8 \text{ mm}^3$, $n = 10$) resulted in significantly smaller volumes than the following groups: untreated ($286 \pm 51.2 \text{ mm}^3$, $n = 10$, $P < 0.0001$), vaccination alone ($197 \pm 48.9 \text{ mm}^3$, $n = 10$, $P = 0.0005$), i.c. administration of lacZ-SeV/ Δ M Δ F combined with the vaccination ($233 \pm 73.2 \text{ mm}^3$, $n = 6$, $P = 0.0012$), and i.c. administration of hIL2-SeV/ Δ M Δ F alone ($256 \pm 53.2 \text{ mm}^3$, $n = 6$, $P = 0.0001$). Columns, mean; bars, SD. **C**, Kaplan-Meier survival analysis of the tumor-bearing rats. O, untreated; ●, treated with the vaccination alone; ▲, treated with i.c. administration of lacZ-SeV/ Δ M Δ F and the vaccination; △, treated with i.c. administration of hIL2-SeV/ Δ M Δ F alone; ■, treated with i.c. administration of hIL2-SeV/ Δ M Δ F and the vaccination. Statistical analysis with log-rank test showed that the rats treated with i.c. administration of hIL2-SeV/ Δ M Δ F and the vaccination survived significantly longer than the other treatment groups ($P < 0.05$).

a second challenge of wild-type 9L inoculation in the brain and manifested no adverse effect during the 3-month follow-up period.

Induction of tumor-specific cytotoxic T cells. We used the standard ^{51}Cr release assay to evaluate the cytotoxic activity of spleen cells from the treated rats or those from naive rats against syngeneic 9L cells or allogeneic C6 cells. The cytotoxicity to 9L targets was strongly induced by the spleen cells from the rats treated with s.c. vaccination of the irradiated wild-type 9L cells (Fig. 5). In contrast, the cytotoxicity of the same effector cells to the C6 targets was not observed. The spleen cells from the tumor-bearing rats that were treated with i.c. administration of hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$ alone did not induce significant cytotoxicity against the 9L cells. These data suggest that the irradiated wild-type 9L cells were sufficiently immunogenic in the peripheral tissue and that the s.c. vaccination with these cells could induce 9L-specific cytotoxic T cells.

Immunohistochemical analysis. To ascertain the gene expression at protein level, we analyzed the immunoreactivity of IL-2 protein in the hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$ -injected tumors and confirmed that IL-2 protein was diffusely expressed in the treated tumors (Fig. 6A). We also immunohistochemically examined the presence of CD4^+ T cells and CD8^+ T cells in the tumors. Diffuse and dense infiltrations of CD4^+ T cells and CD8^+ T cells were observed in the tumors that were treated with i.c. administration of the hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$ and concurrent s.c. vaccination (Fig. 6B). In the tumors treated either with i.c. administration of the lacZ-SeV/ $\Delta\text{M}\Delta\text{F}$ and the vaccination or i.c. administration of the hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$ alone, infiltrations of these cells were sparsely detected.

Discussion

We herein first showed that the nontransmissible recombinant SeV vector could transfer genes efficiently into the glioma cells *in vivo*, and this directly correlated with the therapeutic efficacy against the established brain tumors. Even complete elimination of the established brain tumors could be achieved in some cases by the gene therapy strategy using i.c. administration of SeV vector carrying human IL-2 gene with s.c. vaccination. The SeV vector provided substantive expression of IL-2 protein in the glioma tissues, which would have reached a level necessary to induce significant proliferation and expansion of the peripherally activated tumor-specific T cells.

We have previously reported that transplantation of the IL-2-producing cells into glioma tissues could eliminate approximately half of the established brain tumors in animals immunized with an irradiated whole tumor cell vaccine (16). Although the amount of IL-2 produced by the cells transduced with hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$ was several times greater than that of the IL-2-producing cells utilized in the previous experiment, the cellular infiltration and the cure rate obtained with the hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$ treatment were comparable with those of the cell-mediated therapy. This result would partly be explained by the kinetics of the IL-2 expression in the animals treated with hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$. The *in vivo* protein expression would have reached its peak 4 days or later after injection of the vector. However, because the tumor doubling time of human glioblastoma is presumed to be longer than the experimental 9L gliosarcoma model, the time lag to reach

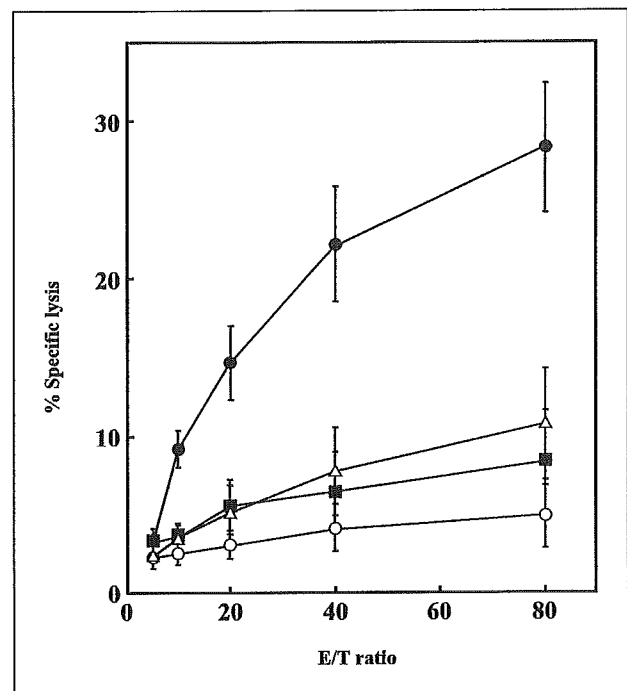


Fig. 5. Cytolytic activity by spleen cells from the treated rats was assessed with a standard ^{51}Cr -release assay against syngeneic 9L or allogeneic C6 targets. ○, spleen cells from naive rats against 9L targets; ●, spleen cells from the rats s.c. vaccinated with irradiated 9L against 9L targets; ■, spleen cells from the vaccinated rats against C6 targets. △, spleen cells from rats treated with i.c. administration of hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$ alone. The vaccination with irradiated 9L cells could induce 9L-specific cytotoxic T cells.

maximum IL-2 production is not considered to be a critical factor in clinical practice (21). In contrast, the main advantage of virus vector application would be the wide distribution of the transgene products achieved by a small amount of viral solution to be i.c. injected compared with the cell-mediated therapy that needs a large amount of therapeutic cells to cover the whole tumor areas in the human brain.

For the clinical application of this strategy, it would be important to verify the reasons for the difference in the therapeutic efficacy of hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$ among individual animals. This experimental brain tumor model using 9L cells has a quite stable property; the animals are 100% fatal between 18 and 25 days after inoculation and all the untreated tumors are visible by MRI on day 7 with a mean size of $8.0 \pm 6.0 \text{ mm}^3$ (16, 17). When treated with hIL2-SeV/ $\Delta\text{M}\Delta\text{F}$ and the vaccination, the observed tumor volumes on day 21 were not different between the eliminated tumors and the other progressing tumors. The pretreatment status of the tumors is not considered to affect the therapeutic outcome. Instead, the presumable differences in the vector injection site (i.e., the center or periphery of the tumor) would affect the efficacy because the stereotactic coordinates used for the tumor inoculation would not necessarily indicate the center of the established tumor. When used in clinical practice, we can precisely identify the center of the brain tumors by computed tomography-guided stereotactic apparatus or navigation system. Another important factor contributing to the difference in therapeutic efficacy may be the expression level of sialic acids.

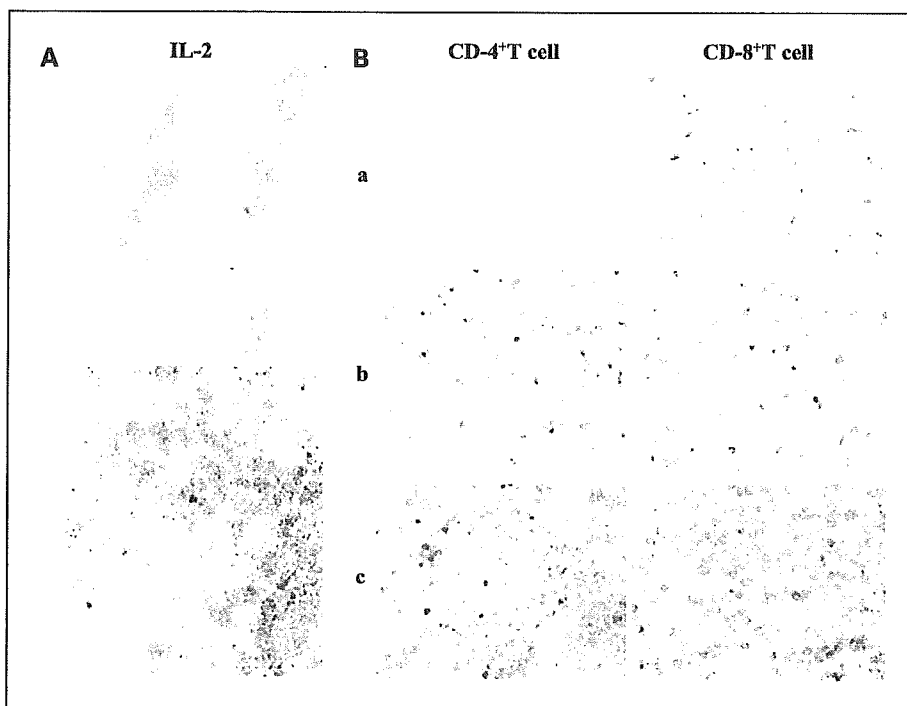


Fig. 6. *A*, immunohistochemical analysis of the expression of IL-2 in the 9L brain tumors treated with i.c. administration of hIL2-SeV/ΔMΔF. IL-2 protein is diffusely expressed. *B*, immunohistochemical analysis of the expression of CD4 and CD8 antigens in rats treated with i.c. administration of lacZ-SeV/ΔMΔF and s.c. vaccination of irradiated 9L cells (*a*), i.c. administration of hIL2-SeV/ΔMΔF alone (*b*), and i.c. administration of hIL2-SeV/ΔMΔF combined with the vaccination (*c*; $\times 200$ magnification). Diffuse and dense infiltrations of CD4⁺ T cells and CD8⁺ T cells were observed in the tumors that were treated with i.c. administration of hIL2-SeV/ΔMΔF and the vaccination.

The receptor for SeV is sialic acid bound to gangliosides, which is present on most cell types including the human glia and glioma cells (22, 23). It may be altered by infiltration of the host cells in the xenograft models (24) and this probably affects the infection efficiency of hIL2-SeV/ΔMΔF and resultant efficacy.

Regarding the safety in clinical application, the biological features of SeV, such as the lack of integration into the cellular genome and the lack of homologous recombination between different SeV genomes, are desirable. Furthermore, we utilized both *M* and *F* genes-deleted SeV (SeV/MF) to enhance its safety. We previously constructed an *F* gene-deleted SeV (SeV/F; ref. 13) and an *M* gene-deleted SeV (SeV/M; ref. 14). *F* gene deletion made the SeV vector nontransmissible and *M* gene deletion worked well to make SeV incapable of forming particles from the infected cells. Although simultaneous deletions of these two genes in the same genome resulted in combining both advantages and contributed to increase the safety of the SeV vector, SeV/ΔMΔF still retains high levels of infectivity and gene expression *in vitro* and *in vivo* (i.e., similar

to the wild-type SeV; ref. 15). These characteristics of SeV/ΔMΔF are considered suitable for the clinical application in gene therapy. In contrast, a theoretical obstacle for the clinical application could be the presence of antibodies against the human parainfluenza virus type I, which are known to cross-react with SeV HN proteins (4). However, it is currently not known whether a respiratory infection with human parainfluenza virus type I in the past will interfere with the transduction process of SeV vector at distant site, such as the central nervous system.

In conclusion, the present study showed that the non-transmissible recombinant SeV vector provided efficient transduction of the target genes into i.c. glioma cells. The i.c. administration of hIL2-SeV/ΔMΔF could induce a substantial production of IL-2 protein to induce the proliferation and expansion of peripherally activated, tumor-specific T cells. The therapeutic efficacy obtained by sufficient gene transfer with wide distribution and the high-grade safety of the non-transmissible recombinant SeV vector warrants clinical trials to evaluate its usefulness for human glioblastoma.

References

- Shapiro WR. Current therapy for brain tumors: back to the future. *Arch Neurol* 1999;56:429–32.
- Hamel W, Westphal M. Gene therapy of gliomas. *Acta Neurochir Suppl* 2003;88:125–35.
- Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000;11:1389–401.
- Bitzer M, Armeanu S, Lauer UM, Neubert WJ. Sendai virus vectors as an emerging negative-strand RNA viral vector system. *J Gene Med* 2003;5:543–53.
- Yonemitsu Y, Kitson S, Ferrari R, et al. Efficient gene transfer to airway epithelium using recombinant Sendai virus. *Nat Biotechnol* 2000;18:970–3.
- Ikeda Y, Yonemitsu Y, Sakamoto T, et al. Recombinant Sendai virus-mediated gene transfer into adult rat retinal tissue: efficient gene transfer by brief exposure. *Exp Eye Res* 2002;75:39–48.
- Shiotani A, Fukumura M, Maeda M, et al. Skeletal muscle regeneration after insulin-like growth factor I gene transfer by recombinant Sendai virus vector. *Gene Ther* 2001;8:1043–50.
- Suzuki S, Li AJ, Ishisaki A, et al. Feeding suppression by fibroblast growth factor-1 is accompanied by selective induction of heat shock protein 27 in hypothalamic astrocytes. *Eur J Neurosci* 2001;13:2299–308.
- Onimaru M, Yonemitsu Y, Tani M, et al. Fibroblast growth factor-2 gene transfer can stimulate hepatocyte growth factor expression irrespective of hypoxia-mediated downregulation in ischemic limbs. *Circ Res* 2002;91:923–30.
- Jin CH, Kusuhara K, Yonemitsu Y, et al. Recombinant Sendai virus provides a highly efficient gene transfer into human cord blood-derived hematopoietic stem cells. *Gene Ther* 2003;10:272–7.
- Okano S, Yonemitsu Y, Nagata S, et al. Recombinant Sendai virus vectors for activated T lymphocytes. *Gene Ther* 2003;10:1381–91.
- Shirakura M, Inoue M, Fujikawa S, et al. Postschismic administration of Sendai virus vector carrying