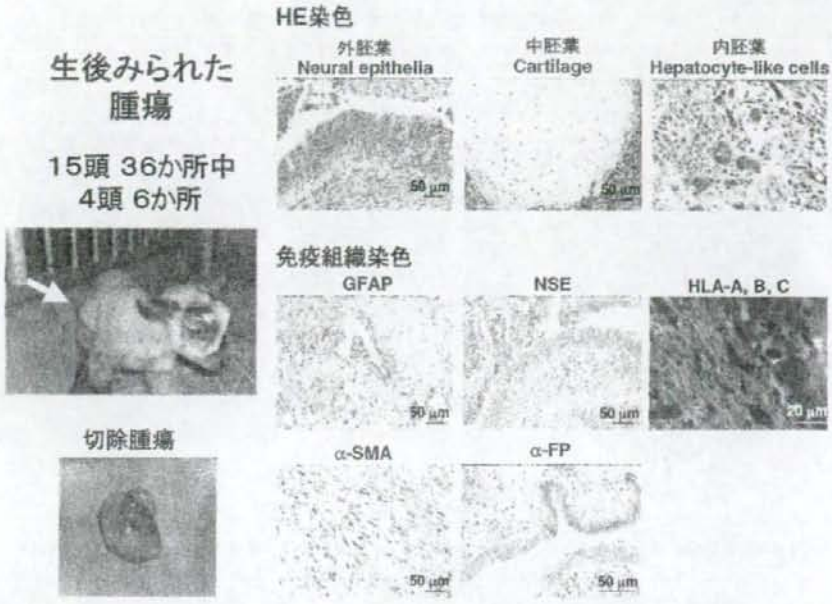

写真1　本当にヒツジにサルの組織を作らせることが可能か，まず未分化のままのサルES細胞をヒツジ胎仔に移植して，サルの組織ができるどうかを調べた。その結果，生後，15頭中4頭で腫瘍（コブ）をもつヒツジが生まれた（図の矢印）。腫瘍は成熟型奇形腫だった（巻頭カラー図版参照）
免疫組織染色法で，神経グリアマーカーのGFAP，ニューロンマーカーのNSE，肝臓マーカーのαFP，平滑筋マーカーのαSMAなどが陽性の組織構造があった。なお，これらはHLA陽性だった。HLA抗体がサルのMHCに交叉したと考えられる。したがって，これら腫瘍は，サルの移植片と考えられた。

ままのサルES細胞を移植したところ，4頭6か所で，生後のヒツジに腫瘍（コブ）の形成が認められた。腫瘍を切除し，切片をHE染色して観察すると，成熟した三胚葉構造をもつ奇形腫（腫瘍の一種，未分化のES細胞は免疫不全マウスに移植すると奇形腫を作る性質をもつが，それと同じもの）だった。このことは免疫組織染色でも確認した（写真1）。たとえば，神経グリアマーカーのGFAP，ニューロンマーカーのNSE，肝臓マーカーのαFP，平滑筋マーカーのαSMAなどが陽性の組織構造があった。しかも，HLAが陽性だった。ヒツジの細胞がHLA陽性になるはずはないから，これはサルのMHCと交叉したものと思われた。したがって，たまたまヒツジの奇形腫が合併したのではなく，サルの奇形腫ができた可能性が示唆された。

ほんとうにサルの組織がヒツジにできたことを証明するために，サルゲノムだけを特異的に認識して，ヒツジゲノムは認識しないプローブを使って，生着した組織を染めたところ，明らかにサルのものだとわかった（写真2）。ただし，栄養血管は，宿主のヒツジ由来だった。生着組織の細胞の核型を調べても，それはサルの核型に一致した。ヒツジに生着した組織は，間違いなくサルの移植片（グラフト）で，ヒツジ血管によって栄養されていたのである。別の実験だが，サルES細胞を造血系に分化させてからヒツジ胎仔に移植して，サルの血液をもつヒツジの作出にも成功している[2]。

免疫能正常のヒツジにサルのグラフトがくっついているわけで，これは，神話でなくて本当のキメラといえよう。この結果の示唆するところは，

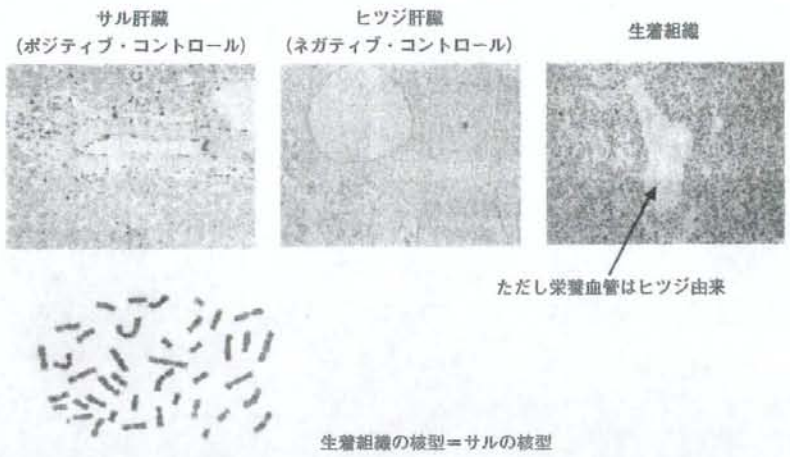

写真2　ヒツジに生着した細胞は，サルに特異的なDNAプローブで染まり，しかも，サルの核型をもっていた。ヒツジの体内に，間違いなくサルの細胞ができたことを示す。ただし，栄養血管はヒツジ由来であった

サルES細胞の代わりに，ヒトES細胞を使えば，ヒトのグラフトをもつヒツジの作出が可能ということである。もっとも家畜からヒトへの水平感染の可能性が否定できないから，本研究の移植医療への応用はまだ先の話で，今は基礎研究の段階であることを強調しておきたい。

3. ヒツジ体内でサル細胞の運命

さて，サルの細胞がヒツジ胎仔に生着するかしないかはどんな条件で決まるのだろうか。まず移植した妊娠日数（満期147日）が問題になるが，妊娠50日を過ぎると全く生着しない。妊娠50日以内が必要条件であった。また，移植細胞数をみると，1か所に百万個より少ないと全く生着しなかった。百万個以上の細胞数が必要であることがわかった。

次に，GFPを発現するサルES細胞を使って，移植後の追跡実験を行った。ファースト・トリメスター（妊娠1/3期，妊娠50日以前）に移植した場合，移植後5日目には，GFP陽性の移植細胞がはっきり検出され，移植2週間後には，そのGFP陽性領域の増大が見られた。一方，ファースト・トリメスター以降（妊娠50日以降）に移植した場合，移植5日目は移植細胞がみられたものの，移植2週後には，GFP陽性領域は消失し，GFPに染まらない宿主由来の肉芽組織に置き換わり，T細胞の浸潤が見られるだけで，移植細胞は見事に拒絶されていた。移植細胞の生着のためには，ファースト・トリメスターに移植することが必要である。

4. 異種細胞が拒絶されない理由

ファースト・トリメスター（妊娠50日以前）に移植したヒツジでは，移植細胞に対する免疫寛容が誘導されたのだろうか？　まず，液性免疫を調べたところ，生下時には移植細胞に対するIgGが検出された。すなわち，液性免疫は誘導されていた。次に細胞性免疫を調べた。サルの奇形腫をもって生まれたヒツジの単核球を使って，混合リンパ球試験（MLR）を行ったところ，生まれた

ヒツジは，移植したES細胞に感作されていることがわかった。つまり，サルの組織が長期間にわたってヒツジに生着しているのにかかわらず，移植したサルの細胞に対して液性免疫も細胞性免疫も検出されたわけである。実際，サル組織が生着しているヒツジに，再度，同じサルES細胞を追加移植しても，さらなる生着はみられなかった。したがって，移植細胞に対する，いわゆる免疫寛容は誘導されていないことになる。

サル組織の生着が見られたヒツジでは，移植直後に宿主（ヒツジ）のCD3陽性のT細胞の浸潤が認められる。しかし，サルのグラフトの破壊を伴っていない。一方，B細胞・マクロファージ・好中球の浸潤はほとんど見られなかった。この組織像を見て，サルのグラフトを囲むT細胞は制御性T細胞（Treg）ではないかと推測した。Tregは，免疫反応を抑えるT細胞である。ヒツジのTregに反応する抗体で染めてみると，移植2週後，案の定，グラフト周囲のT細胞の半分以上がTregだった。移植3か月後の生下時でも，グラフト内のT細胞の10%がTregだった。Tregによって免疫拒絶が抑えられていたと考えられる。生後，免疫能が正常にかかわらず異種動物のグラフトをもつ，すなわち，ギリシャ神話に登場するようなキメラ（またはヌエ）ができたのは，Tregの働きだったらしい。

5. おわりに

百万個以上のサルES細胞を妊娠50日未満のヒツジ胎仔に移植すると，肉眼的なサルのグラフトをもつヒツジの作製が可能である。このサル／ヒツジキメラでは，サルの細胞に対する免疫寛容は誘導されていなかったが，Tregによって免疫拒絶が抑えられ，長期にわたる「肉眼的」サル／ヒツジキメラ状態を可能にした。将来，サルES細胞の代わりにヒトES細胞を使えば，ヒツジ体内にヒトのグラフトを作製しうることを示した。

［謝辞］本研究は，宇都宮大学農学部の長尾慶和准教授，国立成育医療センターの林聡博士との共同研究である。

文　献

1) Y. Tanaka *et al.*, *Stem Cells Dev.*, 17, 367-382 (2008)
2) K. Sasaki *et al.*, *Transplantation*, 79, 32-37 (2005)

<78 頁掲載>

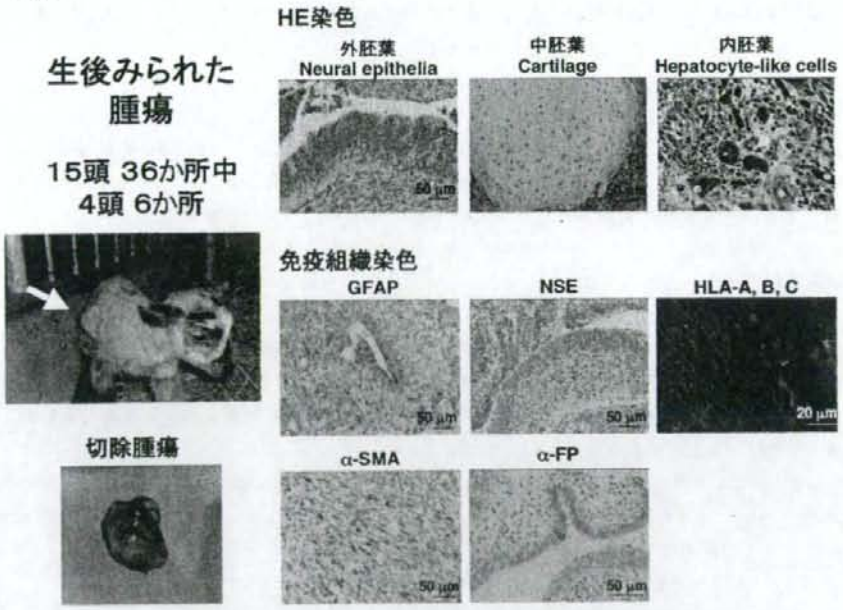

写真1　本当にヒツジにサルの組織を作らせることが可能か，まず未分化のままのサルES細胞をヒツジ胎仔に移植して，サルの組織ができるどうかを調べた。その結果，生後，15頭中4頭で腫瘍（コブ）をもつヒツジが生まれた（図の矢印）。腫瘍は成熟型奇形腫だった。

<83 頁掲載>

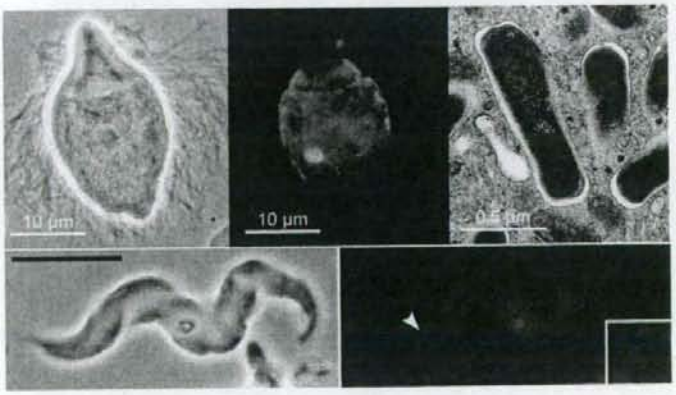

写真2　原生生物のTG1門に属する細胞内共生細菌

ファルマシア

別刷

社団法人
日本薬学会
The Pharmaceutical Society of Japan

Seminar
セミナー

ES/iPS細胞を利用する治療の有効性と安全性

サルの例から考える

花園　豊
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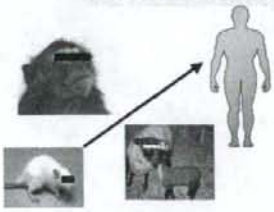
自治医科大学分子病態治療研究センター再生医学研究部教授



1　はじめに

マウスでES/iPS細胞の同種(マウスからマウス)移植実験は実施可能だが，ヒトではそれができない．それでは，これら幹細胞を移植治療に応用する際の有効性と安全性を前もって検証するにはどうすればよいだろうか．ヒトとサルの遺伝的近縁性からいって，サルの細胞をサルへ移植する実験は，ヒトの細胞をヒトに移植する治療の最も忠実な擬似モデルといえる．サルES細胞をサルへ同種移植するという我々の実験例を提示し，その有用性について考察する．また，サル以外の大型動物ヒツジへの移植実験についても触れる．

2　アニッサの事件

1990年，アメリカでいわゆる「アニッサの事件」が起こった．[1] アニッサ・アヤラは，1988年高校2年のとき急性骨髄性白血病と診断されていた．親族に適合者なし．骨髄バンクに適合者1人あるも提供を断わられた．高校の体育館で涙ながらドナーの呼びかけを行った．多くの若者が提供を申し出たが適合者なし．このままでは娘の死が免れ得ないことから両親が一大決心した．もう1人子供を作り，その子の骨髄を移植しようと．適合する25%の可能性に賭けた．妊娠9か月で羊水検査の結果，適合していることが判明．家族は大喜びであった．しかし，これが新聞報道されるやいなや多くの批判を生んだ．「1人の子供を救うために，もう1人子供を作ることは正当化されるか？」(シカゴ・トリビューン)「スペアの臓器を得る目的で子供を作ることは正当化されるか？」(ロサンゼルス・タイムズ)こうした批判ばかりでなく，多くの脅迫やデモ行進がアヤラ家に対して行われた．それでも家族はじっと耐えた．1990年4月2日全米が見守り，数十人の警察官の警護のなか，無事出産．マリッサと命名．マリッサ1歳の時，マリッサからアニッサへの骨髄移植手術を施行し成功した．アニッサの白血病は完全治癒したという．

さて20xx年，アニッサはどのような治療を受けることになるか予想しよう(図1)．白血病に冒されたアニッサを助けるには組織が適合する骨髄移植し

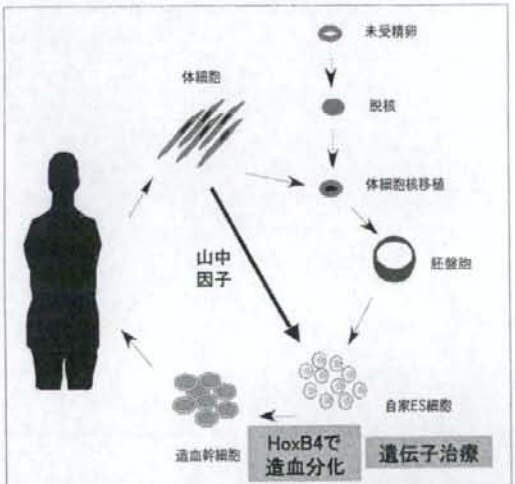


図1　近未来の幹細胞治療(マウスでは成功したが…)

患者の皮膚細胞を取り，その核を提供された卵子に移植する(体細胞核移植)．そこからES細胞を作り出せば，それは患者本人のES細胞である．それを造血幹細胞に分化させて移植する．あるいは，山中教授の技術を使って，いっきに人工ES細胞(iPS細胞)までバイパスする．これらの治療法は，マウスでは既に成功している．いずれも病気の遺伝子に対して，ES/iPS細胞の段階で遺伝子治療を行った上で，*HoxB4*遺伝子で造血分化を行っている．

かないが，適合ドナーはやはり見つからなかった．しかし両親は，もはやもう1人子どもを作る必要はない．ES細胞治療が可能になったからである．まずアニッサの皮膚細胞を取り，その核を提供された卵子に移植する(体細胞核移植)．[2] そこからできる胚盤胞からES細胞を取り出す．適当な方法によってES細胞を造血幹細胞に分化させ，それを移植する．あるいは，山中教授の技術を使って皮膚細胞から人工ES細胞(iPS細胞)を誘導すれば，一気にES細胞までバイパスできてしまう．[3,4] いずれの方法でも，自分の細胞からできた造血幹細胞だから完全に適合する．アニッサは元気になって退院する．

図1の外側の輪(初期胚を経由する方法)を使ったマウスの治療モデル(重症複合型免疫不全症；SCID)は2002年4月 *Cell* 誌に発表された．[5] バイパス路のiPS細胞を利用するマウス治療モデル(鎌状赤血球貧血症)は，2007年12月 *Science* 誌に発表された．[6] いずれも病気の遺伝子に対して，ES/iPS細胞の段階で遺伝子治療(相同組換えによる遺伝子修復)を行い，*HoxB4* 遺伝子で造血幹細胞へ分化を誘導している．

幹細胞治療に関して確かに目指すべきゴールは見えてきた．臨床応用もすぐそこまで来ているように考えられがちだが，本当にそうだろうか？　本稿では，私どもの研究成果をもとに，臨床応用にあたっての問題点を見ていきたい．

3 造血幹細胞遺伝子治療の研究を振り返る

私は1995年アメリカの国立保健衛生研究所(NIH)に留学して以来，造血幹細胞遺伝子治療の研究に従事してきた．造血幹細胞遺伝子治療とiPS細胞治療は，似たところがある．どちらも幹細胞を利用した治療であり，どちらもレトロウイルスベクターで遺伝子を導入した細胞を体内に戻す治療である．したがって，造血幹細胞遺伝子治療の研究は将来のiPS細胞治療(またはES細胞遺伝子治療)を考える上で参考になると思われる．この研究を振り返ってみたい．

1980年代，造血幹細胞を使ってマウスの血液疾患を遺伝子治療するという研究が行われた．これらは大いに成功したため，気をよくしたアメリカは90年代に入ると早速ヒトに応用した．ところが，その結果は芳しくなかった．業を煮やしたNIHは，1995年に「基礎研究重視・サル研究推進」を唱えるレポートを出した．

ちょうどこの時期にNIHに行った私は，この勧告に従いサルを使った研究を始めた．そして，マウスの技術をヒト向けに改良することは，まったく別の技術を作ることだと知った．多くの研究者が地道な基礎研究を重ねた結果，2000年代になってようやく，幾つかのヒト疾患で治療成功例が出始めた．なかでもX染色体連鎖重症複合型免疫不全症(X-SCID)の治療成績は抜群で，骨髄移植以外なすすべのなかった致死性の疾患から子供たちを救った．[7] ところが，

J.Clin.Invest.,**118**,3132-3142(2008)	*J.Clin.Invest.*,**118**,1502-1510(2008)
先天性免疫不全症 (X-SCID)に対する レトロウイルスベクターを用いた 造血幹細胞遺伝子治療後：	*HoxB4*遺伝子を 造血幹細胞に 長期間発現させると：
白血病発症	白血病発症
ヒト　4／10	サル　1／2 イヌ　2／2

図2　マウスでは検出できなかった重大な副作用

マウスでは認められず，ヒト臨床や大型動物試験で初めて重大な副作用(白血病)が見つかる例が相次ぎ，サルのような大型動物を用いた実験の重要性がクローズアップされた．

治療を受けた患児の半数近くに白血病という重い副作用が出た.[8] これはマウスの実験では確認されていない副作用だったので，研究者たちは驚いた.

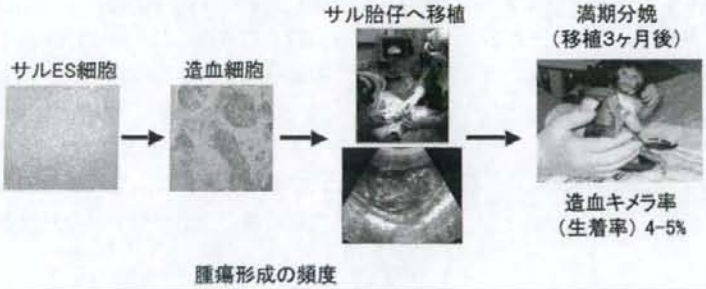
さらに最近，前述のES/iPS細胞の造血分化遺伝子である，*HoxB4* 遺伝子をレトロウイルスベクターで造血幹細胞に導入して移植すると，高率に白血病を起こすという論文が発表された.サル2頭中1頭，イヌ2頭中2頭で白血病が起こったという.[9] これもマウスでは認められていなかった副作用だった.マウスでは認められず，ヒト臨床や大型動物試験で初めて重大な副作用が見つかる例が相次ぎ，サルのような大型動物を用いた実験の重要性がいよいよクローズアップされてきた(図2).

なぜマウスでは白血病を確認できなかったのか？ヒトとマウスでは造血のスケールが全く異なる．例えば，マウスが一生(2年間)かけて作る数の赤血球を，ヒトはたった1日で作っている.[10] これでは，例え100匹のマウスを一生追跡しても，ヒトの100日分にしかならない．これが，大型動物でないと腫瘍を検出しづらい理由の1つかもしれない.

4 サルを用いた模擬実験

私どもは，マウスで成功した上述のES/iPS細胞による個体造血の再生実験を，サルで実施することを試みた．しかし，これらのサル細胞を普通のサルに移植したのでは免疫拒絶されてしまい，実験にならない．免疫不全マウスのような免疫不全動物は，サルでは存在しない．そこで，サル胎仔に注目した(図3)．サル胎仔(特に妊娠1/3期まで)は，胸腺がないから免疫学的に未成熟であり，ヌードマウス(胸腺のない免疫不全マウスには毛がないのでこう呼ばれる)に相当する．実際にサルES細胞をサル胎仔に移植したところ，免疫不全マウスに移植した場合と同様に免疫拒絶が起こらず，期待通り奇形腫(腫瘍の1種)を作った(図3).[11]

ここで重要なのは，腫瘍形成は注射針軌跡上の腹腔及び胸腔に限られ，実質臓器に腫瘍はできないことである．すなわち，漏れた細胞が腫瘍を作る可能性が高い．実は漏れずに移植する技術が腫瘍形成予



腫瘍形成の頻度

移植する細胞	サル胎仔	免疫不全マウス (NOD/SCID)	特記事項
未分化のままのサルES細胞	3 / 3	5 / 5	サル胎仔を使うと，免疫不全マウスと同様に免疫拒絶されない．
サルES細胞由来の造血細胞	3 / 3	2 / 5 *	マウスでは，移植治療後の腫瘍を検出しづらい．

* 腫瘍は小さく，解剖して初めて発見された．

図3 マウスで検出できなかった腫瘍形成をサルで検出できた

サルを用いて同種(サルからサル)移植実験を行うにはどうしたらよいか？ 免疫不全マウスのような免疫不全動物は，サルには存在しない．そこでサル胎仔に注目した．これはヌードマウスに相当する．GFP標識したサルES細胞を造血細胞へ分化させて，それをサルの胎仔に移植した．生まれたサルの体内で移植細胞の運命をGFPを標識にして追跡した．有効性という点では，移植したES細胞由来の造血キメラ率は4~5%で，マウスの80~100%に比べると，まだまだ改善の余地がある．さらに重要なことは，実験に用いたサル全例で腫瘍ができたことである．同じ細胞を免疫不全マウスへ移植する実験ではこれほど高い頻度で腫瘍を検出できない．

防のために重要である．こうしたことは，小型のマウスを使った実験からは知られていなかったことである．

次に，サルES細胞を試験管内で造血細胞へ分化させてからサルの胎仔へ移植し，生まれたサルの体内で移植細胞の運命を調べた．[12] 結果は，期待通り造血系を一部再構築できたものの，移植由来キメラ率は4～5%とマウスの成功例(80～100%)に比べると高くなかった．今後は，マウスで報告されたES/iPS細胞による造血再生技術を，霊長類向けに改良していく必要がある．

さらに問題なのは，実験に使ったサル全例で奇形腫が見られたことで，腫瘍形成リスクは高いといわざるを得ない．分化培養後の細胞を移植したのに，なぜ腫瘍を形成したのか？　実は，サルES細胞を1週間近く分化培養しても，40%ほどの細胞が未分化のままであった．残存した未分化細胞が腫瘍形成の原因である．したがって，腫瘍形成の予防のためには未分化細胞の除去が鍵といえる．霊長類ES細胞の未分化表面マーカーであるSSEA-4の陽性細胞を除去してから移植すると，移植後の造血再生を損なうことなく，腫瘍形成は全く認められなかった．[12]

ところで，サルで全例腫瘍を形成した同じ造血分化細胞を，今度は免疫不全マウスに移植するとどうなるか？　図3に示した通り，マウスを用いた場合は腫瘍形成頻度が低いことから，マウス実験では腫瘍形成リスクを過少評価する可能性がある．安全性の評価には，サルの同種移植実験が望まれる．

5 ヒツジの利用

サル胎仔への移植実験は費用と手間がかかる．私どもは，ヒツジ胎仔への子宮内移植実験も行っている．ヒツジ胎仔へのヒト細胞移植実験は以前から行われている．[13] 動物胎仔でもヒト細胞の受け入れやすさには種差があって，ヒツジは特に良好である．しかもヒツジは流産率が低い．

私どもは，サルES細胞をヒツジ胎仔に移植する実験を行い，サルの様々な細胞を持つヒツジの作出に成功している(図4)．[14,15] ヒツジ胎仔への移植実験は，大型動物を用いた幹細胞の *in vivo* アッセイ系として極めて有用である．それだけでなく，移植用臓器「工場」としての利用への夢がふくらむ．

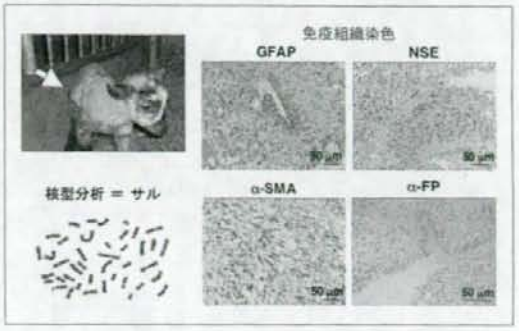


図4　ヒツジの体内にサルの細胞を作る：移植用臓器「工場」へ一歩

サルES細胞を使ってヒツジに様々なサル細胞を作らせた．例えば，神経グリアマーカーGFAP，ニューロンマーカーNSE，肝細胞マーカーαFP，平滑筋マーカーαSMAが陽性の細胞ができた．これらの細胞の核型を調べると，それはサルの核型に一致したから，ヒツジにできたのは間違いなくサルの細胞である．ヒツジの体内にサルの細胞を作ったわけで，これは神話でなくて本当のキメラといえよう．

6 心筋梗塞に対する幹細胞治療

2001年，マウスの心筋梗塞に対して造血幹細胞を局所に移植した結果，移植細胞が血管内皮細胞や心筋細胞へと分化し，心機能の改善が認められたという報告があった．[16] 早速世界各地で，患者自身の骨髄細胞を心筋梗塞局所に移植する治療が行われ，大きな副作用もなく治療効果があったという．

私たちは，いきなり患者に試す前にサルで試し，この治療が本当に効くことを確かめた．しかし問題は，どうして効くのかである．多くの人は，それは移植した幹細胞が内皮細胞や心筋細胞に分化したからだと考えた．しかし私たちが調べてみたところ，意外なことに移植細胞から血管も心筋もできていなかった．実は，移植した細胞(CD 34$^+$細胞)は様々なサイトカインを分泌しており，これが血管新生を促しているらしいことが分かった(図5)．[17] 心筋梗塞の幹細胞治療は確かに効くが，移植した細胞は「幹細胞」として働くのではなく「サイトカイン工場」として働いていたのである．ES/iPS細胞利用の場合も，有効性や機序についてはサルで検証が望ましいと考えている．

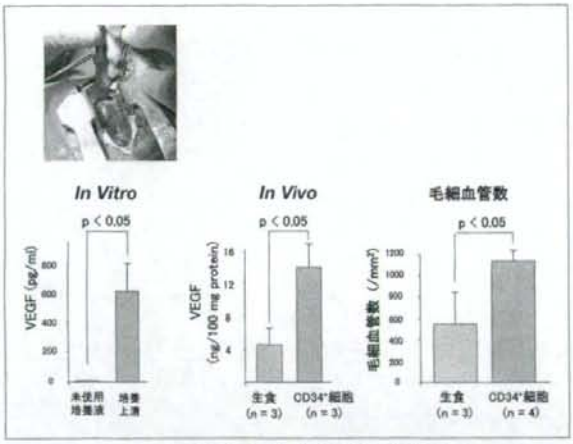


図5　心筋梗塞に対する造血幹細胞治療

サルの心筋梗塞モデルを作製して，造血幹細胞(CD 34⁺細胞)移植が心筋梗塞に効くことを確認した．問題はなぜ効くかである．意外なことに移植細胞から血管も心筋もできていなかった．移植した細胞は，血管内皮増殖因子(VEGF)等のサイトカインを分泌しており，これが血管新生を促しているらしい．つまり移植細胞は，内皮や心筋に分化する「幹細胞」として働いたのではなく，「サイトカイン工場」として働いていた．

7　おわりに

私どもは，大型動物(サル・ヒツジ)を用いた前臨床研究を推進してきた．本稿で述べたことをまとめる．

1) ES細胞の移植によってサルの造血を一部(2〜5%)再構築できたが，腫瘍形成リスクは高い．しかしそれは，未分化マーカーを指標に未分化細胞をあらかじめ除去することによって，予防可能である．

2) マウスを用いる実験では，腫瘍形成リスクを過少評価する．安全性の評価には，サルを用いた移植実験が望まれる．

3) サルES細胞を利用して，ヒツジにサルの細胞を作らせることに成功した．ヒツジは，大型動物の幹細胞 *in vivo* アッセイ系として有用である．

4) 心筋梗塞の幹細胞治療は確かに効くことをサルで示した．しかし移植細胞は，血管や心筋に分化する「幹細胞」として働くのではなく，「サイトカイン工場」として働いていた．

ヒトは決してマウスを大きくしただけではない．今後も，サルやヒツジを用いてマウスからヒトへの「橋渡し」研究を実施し，幹細胞治療の有効性と安全性をしっかり検証しながら臨床応用につなげるための基盤技術を提供していきたい．

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RESEARCH ARTICLE

Newly-developed Sendai virus vector for retinal gene transfer: reduction of innate immune response via deletion of all envelope-related genes

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Abstract

Background Recombinant Sendai virus vectors (rSeV) constitute a new class of cytoplasmic RNA vectors that have shown efficient gene transfer in various organs, including retinal tissue; however, the related immune responses remain to be overcome in view of clinical applications. We recently developed a novel rSeV from which all envelope-related genes were deleted (rSeV/dFdMdHN) and, in the present study, assess host immune responses following retinal gene transfer.

Methods rSeV/dFdMdHN or conventional F-gene deleted rSeV (rSeV/dF) was injected into subretinal space of adult Wistar rats or C57BL/6 mice. The transgene expression and histopathological findings were assessed at various time points. Immunological assessments, including the expression of proinflammatory cytokines, natural killer (NK)-cell activity, as well as SeV-specific cytotoxic T lymphocytes (CTLs) and antibodies, were performed following vector injection.

Results rSeV/dFdMdHN showed high gene transfer efficiency into the retinal pigment epithelium at an equivalent level to that seen with rSeV/dF. In the early phase, the upregulation of proinflammatory cytokines, local inflammatory cell infiltration and tissue damage that were all prominently seen in rSeV/dF injection were dramatically diminished using rSeV/dFdMdHN. NK cell activity was also decreased, indicating a reduction of the innate immune response. In the later phase, on the other hand, CTL activity and anti-SeV antibodies were similarly induced, even using rSeV/dFdMdHN, and resulted in transient transgene expression in both vector types.

Conclusions Deletion of envelope-related genes of rSeV dramatically reduces the vector-induced retinal damage and may extend the utility for ocular gene transfer; however, further studies regulating the acquired immune response are required to achieve long-term transgene expression of rSeV. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords envelope-related genes; innate immune response; recombinant Sendai virus; retinal gene therapy

Introduction

Clinical trials of gene therapy as a new treatment strategy for ocular diseases, such as age-related macular degeneration or retinoblastoma, have demonstrated encouraging results [1,2]. One advantage of the eyes for gene therapy is the anatomical fact of their being relatively small and isolated organs, which enables efficient gene transfer with a limited number of virus vectors and limited systemic exposure [3].

Several vector systems are now available and have made further modifications for successful gene therapy; however, much investigation remains to be done to establish its efficacy and safety.

Sendai virus (SeV) is an enveloped virus with a non-segmented negative-strand RNA genome and is a member of the family *Paramyxoviridae*. It contains six major genes arranged in tandem on its genome: three virus-derived proteins [nucleoprotein (NP), phosphoprotein (P) and large protein (L)] form a ribonucleoprotein complex (RNP) with the SeV genomic RNA, and the RNP acts as a template for transcription and replication. The other three envelope-related proteins [haemagglutinin-neuraminidase (HN), fusion protein (F) and matrix protein (M)] mediate the attachment of virions, penetration of RNPs into infected cells, and virion formation, respectively [4].

SeV makes use of sialic acid residue on surface glycoproteins or asialoglycoprotein present as a receptor [5,6], and SeV-derived vectors (recombinant SeV: rSeV) have been shown to infect various types of mammalian cells, such as airway epithelial, skeletal muscle, ependymal and vascular endothelial cells, and produce two- to three-fold log higher gene transfer efficiency than adenoviral vectors or lipofection [7,8]. The gene transfer and expression take place in the cytoplasm without a DNA phase in the life cycle [9], avoiding possible malignant transformation due to genetic alteration of host cells, which is a safety advantage of rSeV. For use in human gene therapy, we have constructed F-deficient rSeVs (rSeV/dF), which show non-self-transmissible properties, as the first generation of clinically available rSeVs [10]. This type of rSeV still produces efficient gene transfer, and has been employed since 1 February 2006 in an ongoing clinical study of therapeutic angiogenesis for the treatment of critical limb ischemia at Kyushu University after approval by Institutional and Governmental Review Boards.

In ocular tissue, transmissible-type rSeVs exhibited efficient gene transfer to the retinal pigment epithelium (RPE) of the rat retina via subretinal injection; however, the gene expression was markedly decreased 7 days after injection [11]. The transgene expression was prolonged by immunosuppressants, such as corticosteroids or cyclosporine A, suggesting that host immune responses play an important role in eliminating viruses. Despite some advantageous features of rSeV in clinical gene therapy strategies, the related immune responses against SeV and the consequential tissue damage have been too hazardous to expand its utility in some clinical settings, especially for neurosensory tissues such as the retina. Host immune responses remain almost unchanged with the use of rSeV/dF (unpublished data), suggesting that the first generation is still not applicable for retinal gene therapy.

To enhance the safety and efficacy of this procedure, we recently developed a new rSeV vector that is deficient in all the membranous genes (rSeV/dFdMdHN), and succeeded in recovering this vector at high titers. This vector has shown transduction efficacy equal to that of rSeV/dF, and reduced cytototoxicity *in vitro* and *in vivo* [12]. Furthermore, we recently found that rSeV/dFdMdHN significantly reduces host immune responses in pulmonary gene transfer [13]. These vector-related immune responses might be further reduced in retinal gene transfer because the eye is known as an immune privileged site [14,15]. To obtain preclinical information regarding this new generation of rSeV in retinal tissue, in the present study, we assessed the *in vivo* gene transfer efficiency of rSeV/dFdMdHN into rat and mouse retinas and the effect on host immune responses in the early and late phases following vector injection.

Materials and methods

Recombinant Sendai virus vector

F, M and HN gene-deleted SeV vectors (rSeV/dFdMdHN) and F gene-deleted SeV vectors (rSeV/dF) were constructed as previously described [10,12]. The enhanced green fluorescent protein (EGFP) or firefly luciferase gene was inserted between the P and L genes of rSeV/dFdMdHN, and upstream of the NP gene of rSeV/dF. The schematic genome structures of SeV vectors are shown in supplementary Figure 1. The titers of recovered viral vectors were expressed as cell infectious units (ciu) [10]. We repeatedly confirmed the vector titers, and the final products were also highly purified via chromatography.

Animals

Six-week-old male Wistar rats and 8-week-old female C57BL/6 mice were maintained humanely, with proper institutional approval, and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were carried out under approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals by the Committee for Animals, Recombinant DNA, and Infectious Pathogens Experiments at Kyushu University and according to The Law (No.105) and Notification (No.6) of the Japanese Government.

Gene transfer procedures

The subretinal injection of each solution was performed as previously described with minor modifications [11,16]. Briefly, rats or mice were anaesthetized by inhalation ether. The following procedures were then performed using an operating microscope. A 30-gauge needle was inserted into the subretinal space of the peripheral retina in the nasal hemisphere via an external transscleral transchoroidal approach. The vector solution (10 µl)

(rSeV/dFdMdHN or rSeV/dF) or BSS: (137 mM NaCl, 5.3 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4 and 13 mM Tris, pH7.6) was injected, and excess solution from the injection site was washed out using phosphate-buffered saline (PBS). Approximately 3 µl of solution remained in the subretinal space (data not shown). The appearance of a dome-shaped retinal detachment confirmed the subretinal delivery. Eyes that sustained prominent surgical trauma, such as retinal or subretinal haemorrhage or bacterial infection, were excluded from this examination.

Detection and examination of GFP expression using fundus camera

Ophthalmoscopy was performed at various time points after gene transfer (days 2, 4, 7, 10 and 14) as previously described [17]. To detect GFP in the rat retina, we used a fundus camera (TRC-50X; Topcon, Tokyo, Japan), which is widely available as a clinical tool for fluorescein angiography.

Luciferase assay

Enucleated eyes were minced in 500 µl of 1× lysis buffer with a protease inhibitor cocktail, and centrifuged, after which 20 µl of the supernatants were subjected to luciferase assay as previously described [11,18]. Light intensity was measured by a luminometer (Model LB9507, EG&G Berthold, Bad Wildbad, Germany) with 10-s integration. The data are expressed as %RLU (relative light unit) standardized by the mean value of each RLU on day 2 following vector injection.

Histological examination

At each time point after gene transfer (days 2, 7 and 14), the eyes of animals injected with rSeV/dFdMdHN, rSeV/dF or BSS were enucleated, and both paraffin and cryosections were prepared. For paraffin sections, the eyes were fixed with ice-cooled 4% paraformaldehyde in PBS for 1 day at room temperature, and then mounted in paraffin. For cryosections, the eyes were frozen in liquid nitrogen. 5 µm-thick paraffin and cryosections were stained with haematoxylin and eosin, and examined under light microscopy.

Immunohistochemistry

Immunoperoxidase

Infiltrating inflammatory cells in the rat retina were identified by immunohistochemistry. The frozen sections were incubated overnight at 4 °C with primary antibodies: anti-rat CD68 monoclonal antibody (1 : 150, Mouse IgG1, Chemicon, Temecula, CA, USA) for monocytes/macrophages, and anti-rat CD45 monoclonal antibody (1 : 150, Mouse IgG1, Acris GmbH, Hidenhausen, Germany) for leukocytes. Then signals were developed using the avidin-biotinylated peroxidase complex method. For negative controls, the primary antibody was omitted.

Immunofluorescence

The localization of GFP expression was identified by immunofluorescence technique. Deparaffinized sections were incubated overnight at 4 °C with anti-GFP polyclonal antibody (1 : 300, Rabbit IgG, Molecular Probes, Eugene, OR, USA). Anti-rabbit IgG-FITC (1 : 50, Bovine, Santa Cruz, CA, USA) was used as a secondary antibody. All sections were counterstained with DAPI and mounted in Crystal/Mount. Immunofluorescence images were acquired using an Olympus BX51 microscope with a fluorescent attachment (Olympus Corp., Tokyo, Japan). For negative controls, the primary antibody was omitted.

Enzyme-linked immunosorbent assay (ELISA)

The amounts of interleukin (IL)-1β, IL-6, IL-10, interferon (IFN)-γ and tumor necrosis factor (TNF)-α in ocular tissue and serum were determined by ELISA. Commercially available assay systems were used (R&D Systems, Minneapolis, MN, USA). For ocular tissue preparation, conjunctival and muscular tissues were removed from enucleated eyes. The eyes were washed with PBS, minced with scissors in 500 µl of 1× lysis buffer with a protease inhibitor cocktail, and centrifuged at 15 000 r.p.m. for 5 min at 4 °C. The supernatants were subjected to ELISA according to the manufacturer's instructions.

^{51}Cr release assay for cytolytic activity of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs)

Spleen cells were harvested from Wistar rats or C57BL/6 mice on day 2 after rSeV injection for NK cell-lysis assay, and from C57BL/6 mice on day 10 for CTL assay, and contaminated erythrocytes were depleted by 0.83% ammonium chloride. For NK cell-lysis assay, the splenocytes were directly used as NK effector cells. For CTL assay, 4×10^6 splenocytes were cultured with 1 µM SeV-peptide (Sigma, St Louis, MO, USA; H-2^b-restricted peptide) in 1 ml of complete medium in a 24-well culture plate. Two days later, 30 IU/ml human rIL-2 was added to the medium. After 5 days, the cultured cells were collected and used as CTL effector cells. Target cells [YAC-1, SeV-peptide pulsed EL-4, LCMV peptide (H-2^b-restricted peptide) pulsed EL-4 (for third party)] were labelled with 100 µCi $Na_2{}^{51}CrO_4$ for 1.5 h, and were cultured with the spleen cells at various effector-to-target cell ratios. After 4 h of incubation, radioactivities in the culture supernatants were counted with an automatic γ-counter. The percentage of specific ^{51}Cr release of triplicates was calculated as: [(experimental cpm –

spontaneous cpm)/(maximum cpm − spontaneous cpm)] ×100. Spontaneous release was always <10% of maximal Cr release (target cells in 1% Triton X-100).

Anti-SeV antibody

Anti-SeV antibodies in rat serum on day 30 after gene transfer were evaluated by ELISA kit (Wakamoto Pharmaceutical Co., Tokyo, Japan). Serum samples were used for ELISA after appropriate dilutions.

In vitro cytotoxic assay with rSeVs

ARPE-19 cells were seeded in 96-well plates at 3000 cells/well in serum-free Dulbecco's modified Eagle's medium-F12 and, 12 h later, either rSeV/dF or rSeV/dFdMdHN was added to each well at indicated concentrations. Forty-eight hours later, cell viability was assessed by a modified MTT assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Results were calculated as percentage viability standardized by the mean value of control group without rSeV.

TUNEL staining

TUNEL and quantification of TUNEL-positive cells were performed using Apoptosis Detection TACS TdT Kit (R&D Systems) according to the manufacturer's instructions. PE-conjugated streptavidin (1 : 50, BD Biosciences) was used as a secondary antibody. The number of TUNEL-positive cells in the ARPE-19 cells was counted in a masked fashion.

Statistical analysis

All values were expressed as the mean ± SEM. Data were analysed by one-way analysis of variance with Fisher's adjustment. $p < 0.05$ was considered statistically significant.

Results

Transduction efficiency of F, M and HN gene-deleted recombinant SeV

To assess the transduction efficiency, we first injected three different titers (2.5×10^6, 2.5×10^7, 2.5×10^8 ciu/ml) of rSeV/dFdMdHN expressing the EGFP (rSeV/dFdMdHN-EGFP) into the subretinal space of rats, and recorded GFP expression using a fundus camera 48 h after injection. The extent of GFP fluorescence corresponded with the vector-injected area in the middle (2.5×10^7 ciu/ml) and high (2.5×10^8 ciu/ml) titer groups; the expression level was dose-dependent (Figure 1a). Moreover, the GFP expression by rSeV/dFdMdHN was approximately equal to that by rSeV/dF with the same titer (Figures 1a and 1b). A histological assessment demonstrated that the transgene expression was located in the RPE layer (Figure 1c) as previously described [11].

Next, we assessed the time course of transgene expression in vector-injected eyes, monitored by firefly luciferase expression. The data are expressed as %RLU standardized by the mean value of each RLU on day 2 following vector injection. As shown in Figures 2a and 2b, the luciferase expression in rSeV/dF-injected eyes rapidly decreased to approximately 50% by day 4 in each titer group, whereas that in rSeV/dFdMdHN-injected eyes was sustained at this time point ($p < 0.05$, $n = 6–7$ each). However, a decline did occur on day 7, and the luciferase expression by both rSeV vectors had almost disappeared on day 14. We also confirmed the transgene expression in the same eye by a fundus camera, using GFP as a reporter. As with the results of luciferase expression, the GFP expression by rSeV/dFdMdHN was sustained until day 4, then decreased, and disappeared by day 10 following vector injection; by contrast, that by rSeV/dF showed a more rapid decrease (Supplementary Figure 2).

These findings indicate that the deletion of all membranous genes of rSeV might contribute to the delayed vector clearance, but does not result in persistent transgene expression in the rat retina.

Histopathological comparison of retinal tissue treated with rSeVs

To determine whether rSeV/dFdMdHN reduced the immunogenicity in the rat retina, we conducted a histopathological examination of the vector-injected eyes. We injected a middle titer of rSeV/dFdMdHN-EGFP or rSeV/dF-EGFP into the subretinal space and examined the vector-injected site at various time points (days 2, 7 and 14). On day 2 following vector injection, only a slight mononuclear cell infiltrate was seen around the RPE layer in both vector types. On day 7, severe infiltrate of inflammatory cells was found around the RPE and choroid plexus, and the retinal structure was significantly destructed by day 14 in eyes treated with rSeV/dF; by contrast, the retinal inflammation and tissue damage were remarkably reduced with rSeV/dFdMdHN at each time point (Figure 3a). Immunohistochemical studies revealed that both CD68-positive macrophages and CD45-positive leukocytes decreased in number in eyes treated with rSeV/dFdMdHN (Figure 3b). Similar results were obtained in high-titer groups, although the extent of inflammatory cells was more severe in the high-titer groups (Figure 3c).

Moreover, the histopathogical findings of the retinal tissue showed a critical difference between rSeV/dFdMdHN and rSeV/dF. The RPE layer did not break down in the eye treated with rSeV/dFdMdHN, probably due to the reduction of inflammatory responses and vector-related

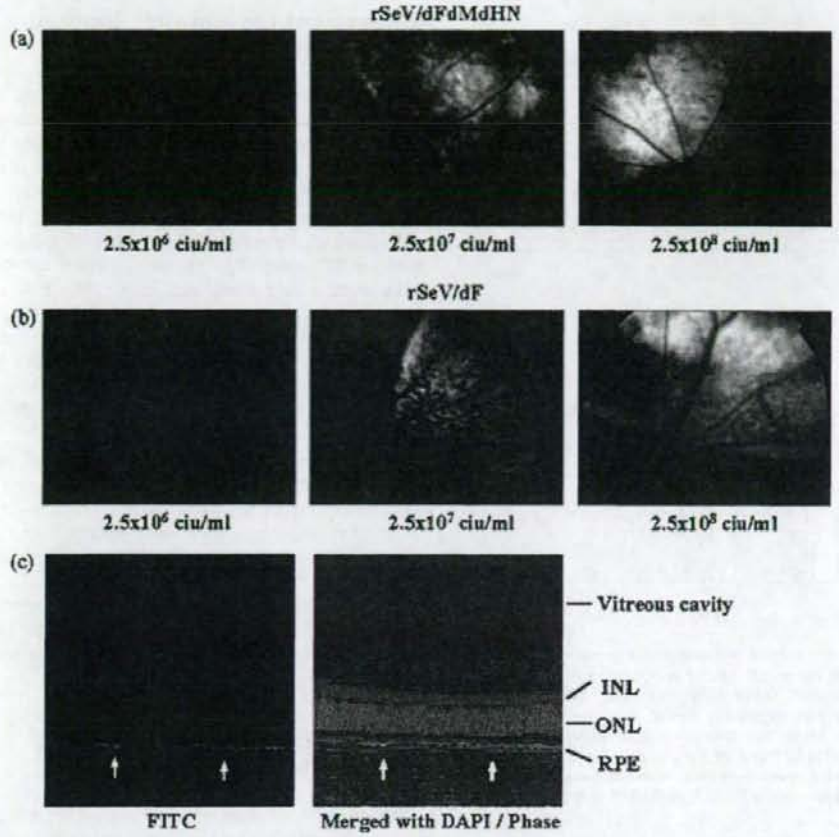

Figure 1. The GFP expression in the retinal fundus (a, b) and corresponding immunohistochemical findings (c) following subretinal injection of rSeV/dFdMdHN or rSeV/dF. (a, b) Three different titers·(2.5· 10^6, 2.5· 10^7 and 2.5· 10^8 ciu/ml) of rSeV/dFdMdHN-EGFP (a) or rSeV/dF-EGFP (b) were injected into the subretinal space of the rat retina, and the GFP expression was assessed by fundus camera on day 2 after injection. rSeV/dFdMdHN showed comparable GFP expression to rSeV/dF with the same titer. (c) The results of immunohistochemical studies against GFP on day 2 after rSeV/dFdMdHN-EGFP injection (2.5· 10^8 ciu/ml). Note the strong staining in RPE (arrows), which was negative for control IgG antibodies (data not shown). INL, Inner nuclear layer; ONL, outer nuclear layer. Original magnification (c) · 200

cytotoxicity (Figures 3a and 3c). As a result, inflammatory cell infiltrate was not apparent over the RPE layer (in the neuroretina), and the structure of the retinal tissue was relatively preserved using rSeV/dFdMdHN (Figures 3a, 3b and 3c).

These findings clearly indicate a significant reduction of host immune responses and vector-induced retinal damage in retinal gene transfer by rSeV/dFdMdHN.

Reduced proinflammatory cytokine production and NK-cell cytotoxicity following gene transfer with rSeV/dFdMdHN

To determine the immune mechanisms underlying the histological differences between rSeV/dF and rSeV/dFdMdHN, we first evaluated the time course of expression of typical proinflammatory cytokines such as IL-1β, IL-6, TNF-α, IFN-γ and IL-10, which are known to be upregulated by respiratory infection with SeV [19]. ELISA revealed that the expressions of IL-1β and IFN-γ were immediately upregulated on day 2 following vector injection and sustained by day 7 in eyes that had been injected with rSeV/dF, whereas these cytokines were markedly reduced in rSeV/dFdMdHN-injected eyes in each titer group (Figures 4a and 4b, $p < 0.05$, $n = 5$–7each). Other cytokines (TNF-α, IL-6 and IL-10) were not detectable in the same samples (data not shown). In the serum, no significant increase of these inflammatory cytokines was seen during the time course (data not shown).

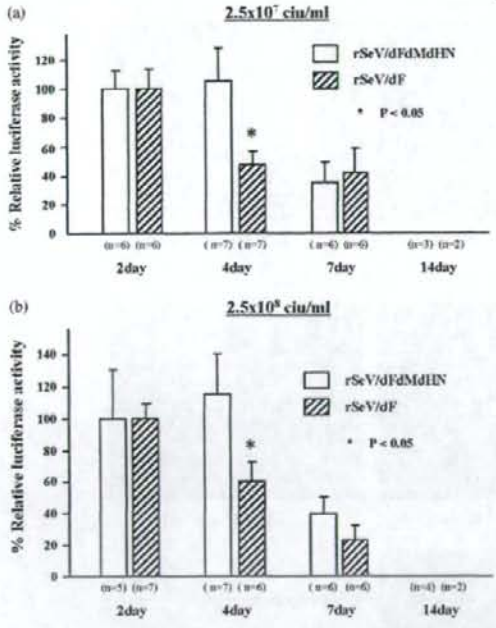

Figure 2. Time course of transgene expression mediated by each rSeV in rat retina. The eyes injected with a middle titer ($2.5 \cdot 10^7$ ciu/ml) (a) or high titer ($2.5 \cdot 10^8$ ciu/ml) (b) of each rSeV vector expressing firefly luciferase were subjected to luciferase assay. The data are expressed as %RLU (relative light unit) standardized by the mean value of each RLU on day 2 following vector injection. Note the delayed reduction of luciferase expression with rSeV/dFdMdHN (day 4, $n \cdot 6-7$ each; $^*p < 0.05$)

Next, we evaluated NK cell activities, which are a crucial component of the innate immune response against virus infection [20,21]. Two days after vector injection or Poly I:C administration, the splenocytes were isolated and used for NK cell activity assay. Strong and significant upregulation of NK cell activity was observed in rats treated with high-titer rSeV/dF, as well as the positive controls (poly I:C), whereas no significant NK cell activity was found in rats treated with rSeV/dFdMdHN (Figure 5a). We also examined NK cell activities in C57BL/6 mice following subretinal injection of each rSeV vector, and found reduced NK cell activity in rSeV/dFdMdHN-treated mice (Figure 5b), as in the case of Wistar rats.

Together with data demonstrated in Figures 2–5, it has been suggested that, in eyes treated with rSeV/dF, expression of proinflammatory cytokines and NK cell activity are immediately increased within a few days, resulting in local infiltration of inflammatory cells and early vector clearance by days 4–7; by contrast, the deletion of membranous genes of rSeV remarkably attenuates these early responses, possibly contributing to the dramatic reduction of retinal inflammation and delay in vector clearance.

Assessment for adaptive immunity against rSeVs

To evaluate the late-phase immune response against rSeVs, we next examined both SeV-specific CTL activity and antibody production. For CTL assay, we used C57BL/6 strain mice (H-2^b), because the target amino acid residue of CTL against SeV has been identified as NP321–336 in this strain. Ten days after vector injection, the splenocytes were isolated, cultured with SeV-peptide and IL-2, and used for CTL assay. By contrast to NK cell cytotoxicity, CTLs from mice treated with rSeV/dFdMdHN showed strong cell lysis activity, which was higher than those from rSeV/dF-treated mice in each titer group (Figure 6a, left panel). As a control experiment, third-party peptide (lymphocytic choriomeningitis virus, LCMV) was used as a target, and no significant release was observed (Figure 6a, right panel).

Next, we evaluated the serum levels of anti-SeV antibody 4 weeks after each vector injection using ELISA. As shown in Figure 6b, no significant reduction in serum levels of anti-SeV antibody was found in both rats and mice treated with rSeV/dFdMdHN in each titer group ($n = 3-5$ each).

These findings suggest that the deletion of membranous genes of SeV have no significant effect on adaptive immune response, leading to the vector clearance by day 14 after injection as shown in Figure 2.

Reduced cytopathic effect of rSeV/dFdMdHN *in vitro*

Infection by rSeV causes some cytopathic effects and induces apoptosis in some types of cells [22]. As a final assessment, we examined the cytopathic effects of each rSeV on ARPE-19 cells, a human RPE-derived cell line. We cultured ARPE-19 cells in the presence of each rSeV and assessed the cellular viability after 48 h of culturing. A mild but significant reduction of cellular viability was found in cells with rSeV/dF in a dose-dependent manner, but not with rSeV/dFdMdHN (Figure 7a, $p < 0.01$, $n = 4$ each). Terminal dUTP-nicked end labelling (TUNEL) stain revealed that infection with rSeV/dF induced apoptosis in $10.9 \pm 1.3\%$ of ARPE-19 cells. By contrast, infection with rSeV/dFdMdHN almost completely suppressed the appearance of TUNEL-positive cells ($1.4 \pm 0.2\%$) (Figures 7b and 7c, $p < 0.01$, $n = 5$ each).

In addition to the effect on inflammatory responses, these data suggest that rSeV/dFdMdHN reduce the cytotoxicity and suppress the apoptosis of infected cells.

Discussion

In the present study we characterized *in vivo* retinal gene transfer using a new-generation Sendai virus vector

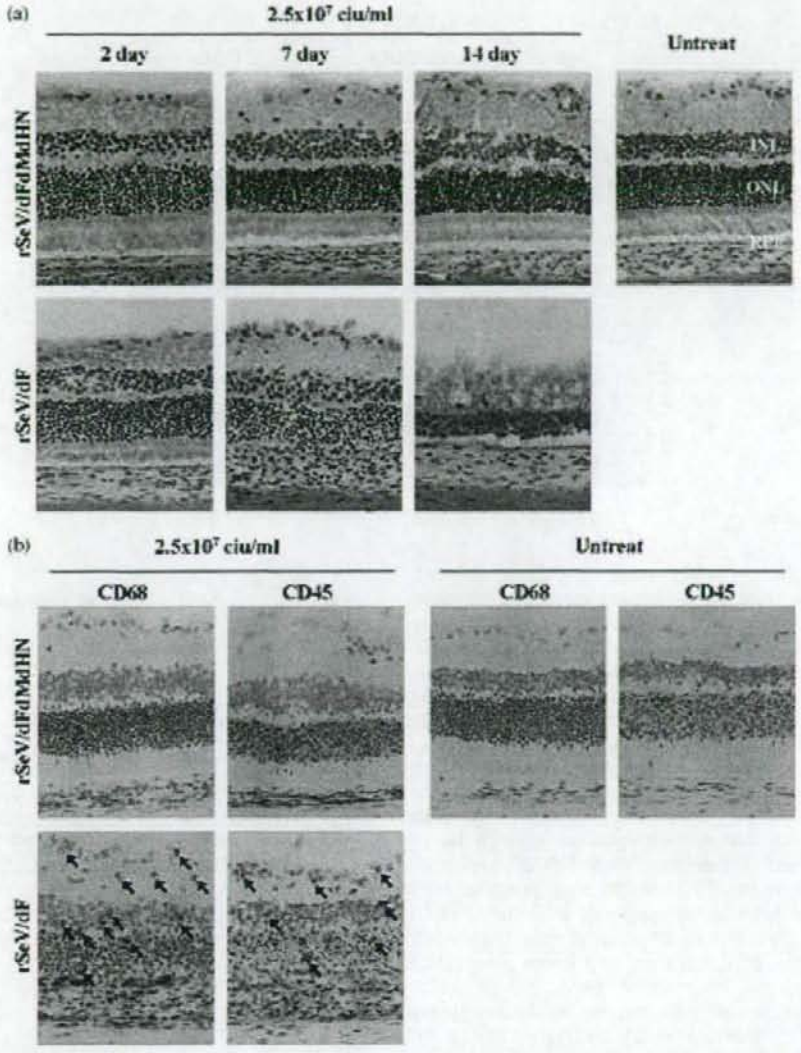

Figure 3. Histopathological (a, c) and immunohistochemical (b) findings of rat eyes injected with each rSeV. (a) Longitudinal sections of eyes injected with a middle titer ($2.5 \cdot 10^7$ ciu/ml) of rSeV/dFdMdHN-EGFP or rSeV/dF-EGFP on days 2, 7 and 14 after gene transfer. The eyes treated with rSeV/dF showed severe inflammation around RPE and choroid plexus peaked on day 7 and tissue destruction on day 14. Note the marked reduction of inflammatory cell infiltrate in the rSeV/dFdMdHN-injected eyes. (b) Immunohistochemical findings in serial sections on day 7. Positive reaction (brown staining) of $CD68^+$ and $CD45^+$ cells are demonstrated. Note that some $CD68^+$ and $CD45^+$ cells infiltrate into the neuroretina in rSeV/dF-injected eyes (arrows) but not in rSeV/dFdMdHN-injected eyes. (c) Histopathological and immunohistochemical findings of rat retina injected with a high titer ($2.5 \cdot 10^8$ ciu/ml) of rSeV vectors on day 7. Arrows indicate the infiltration of $CD68^+$ and $CD45^+$ cells into the neuroretina. Original magnifications (a, b, c) · 200

from which all membranous genes had been deleted, and compared the results with those using rSeV/dF, the presently available clinical vector. The major findings provided by our study were that: (i) subretinal injection of rSeV/dFdMdHN resulted in efficient transgene expression in RPEs, in a dose-dependent manner; (ii) a delay in vector clearance in the retina was observed approximately 4 days after gene transfer, but the transgene expression was diminished by day 14; (iii) histopathologically, the local infiltration of inflammatory cells was decreased, and the RPE layer and retinal structure were well preserved; (iv) both the early production of proinflammatory cytokines

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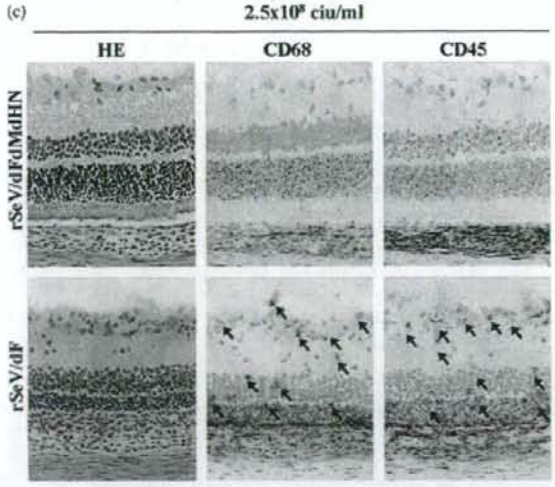

Figure 3. (*Continued*)

and NK cell cytotoxicity were significantly reduced, indicating a reduction of the innate immune response; and (v) significant levels of SeV-specific CTL and antibody were nevertheless induced. The most important advance of this report is that membranous gene-deleted rSeV significantly reduces retinal damage following gene transfer because the retina is a neuronal tissue and its regenerative capacity is limited.

It is of interest that no significant infiltration of inflammatory cells was observed over the RPE layer (in the neuroretina) in rSeV/dFdMdHN-injected eyes (Figures 3b and 3c). By contrast, the retina was extensively inflamed and underwent degeneration with rSeV/dF. We found that the deletion of all membranous genes of rSeV significantly reduced the cytopathic effect in ARPE-19 cells which are human RPE-derived cells (Figure 7a), as reported in other cell types in previous report [12]. In addition, the apoptosis of ARPE-19 cells, which was seen in rSeV/dF infection, was almost completely suppressed in use of rSeV/dFdMdHN (Figures 7b and 7c). Because RPE is part of the blood–eye barrier and limits the access of blood components to the retina [23], one reason for these histological differences might be explained by the difference of cytotoxicity to RPE. Furthermore, RPE cells have many important functions in maintaining homeostasis of the outer retina [24], and so it is necessary to retain the functions in retinal gene transfer targeting RPE. Together with the reduction of local inflammatory responses, the retinal structure is preserved in eyes treated with rSeV/dFdMdHN.

We demonstrated that the deletion of all membranous genes of rSeV resulted in the reduction of proinflammatory cytokine production and NK cell cytotoxicity; however, the cellular and molecular mechanism was not precisely elucidated in the present study. For

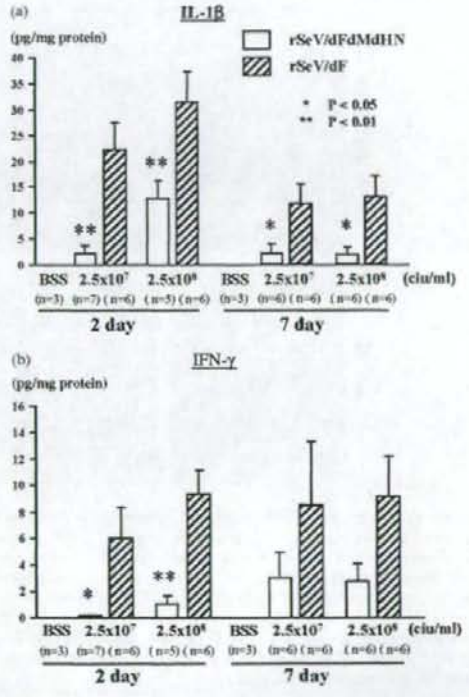

Figure 4. Assessment of proinflammatory cytokines in eyes injected with each rSeV. Protein level of IL-1β (a) and IFN-γ (b) in whole eyes was assessed on day 2 and 7 after vector injection by ELISA. The production of IL-1β and IFN-γ was reduced in rSeV/dFdMdHN-EGFP-injected eyes at each time point (n = 5–7 each; * $p < 0.05$, ** $p < 0.01$, rSeV/dFdMdHN- versus rSeV/dF-injected eyes). No IL-1β or IFN-γ were detected in the BSS-injected eyes

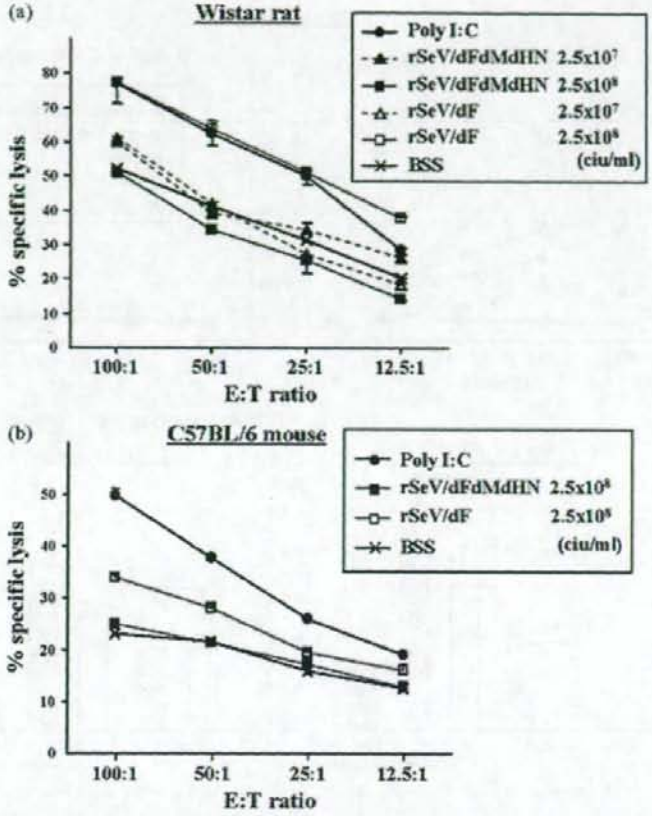

Figure 5. Assessment for NK cell activity in animals treated with each rSeV. Two days after vector injection or poly I:C administration, spleen cells were harvested from rats (a) or mice (b) treated with BSS (•), rSeV/dF-EGFP ($2.5 \cdot 10^7$ ciu/ml) (△), rSeV/dF-EGFP ($2.5 \cdot 10^8$ ciu/ml) (□), rSeV/dFdMdHN-EGFP ($2.5 \cdot 10^7$ ciu/ml) (▲), rSeV/dFdMdHN-EGFP ($2.5 \cdot 10^8$ ciu/ml) (■), or Poly I:C (●). The cytolytic function against ^{51}Cr-labelled YAC-1 targets was assessed by ^{51}Cr release. The animals treated with a high titer of rSeV/dF showed a moderate to strong increase in NK cell activity, whereas no significant increase was observed with rSeV/dFdMdHN. The data are the results from one of three similar experiments

NK cell cytotoxicity, our findings are well-supported by *in vitro* experiments indicating the recognition of the SeV HN protein by NK p44 and p46, the lysis-triggering receptors of NK cells [20,21]. For induction of proinflammatory cytokines, it is well known that host pattern-recognition receptors, such as Toll-like receptors (TLRs), have an important role after viral infection [25]. In paramyxoviruses, several studies have shown that the envelope proteins (e.g. the respiratory syncytial virus F protein and the measles virus haemagglutinin protein) bind TLR2 and TLR4, respectively, and trigger production of a variety of cytokines, such as IL-1β [26,27]. Our findings suggest that there are similar mechanisms in the recognition of SeV envelope proteins. Further studies are required to clarify this point.

The late-phase reduction of transgene expression by rSeV/dFdMdHN, illustrated in Figure 2, probably relates to the development of SeV-specific adaptive immunity, especially the CTL response. Hou *et al.* [28] reported that the CTL activity peaked on day 10 after SeV infection, and CD 8-positive T lymphocytes play a predominant role in virus clearance in the murine lung. We expected the tolerated immune responses against rSeV/dFdMdHN in ocular tissue because tolerance to foreign antigens is induced in the anterior chamber, vitreous cavity and subretinal space [14,29]; however, the present study revealed that a significant level of CTL response against SeV was induced in retinal gene transfer, as well as that seen in lung tissue [13]. In addition, the extent of CTL activity when using rSeV/dFdMdHN was stronger than that using rSeV/dF. Kast *et al.* [30] reported that the major reaction of SeV-specific CTL is directed against the NP protein. Because the gene expression by rSeV/dFdMdHN was sustained by day 4 (Figure 2), the higher CTL response may be explained by the sustained expression

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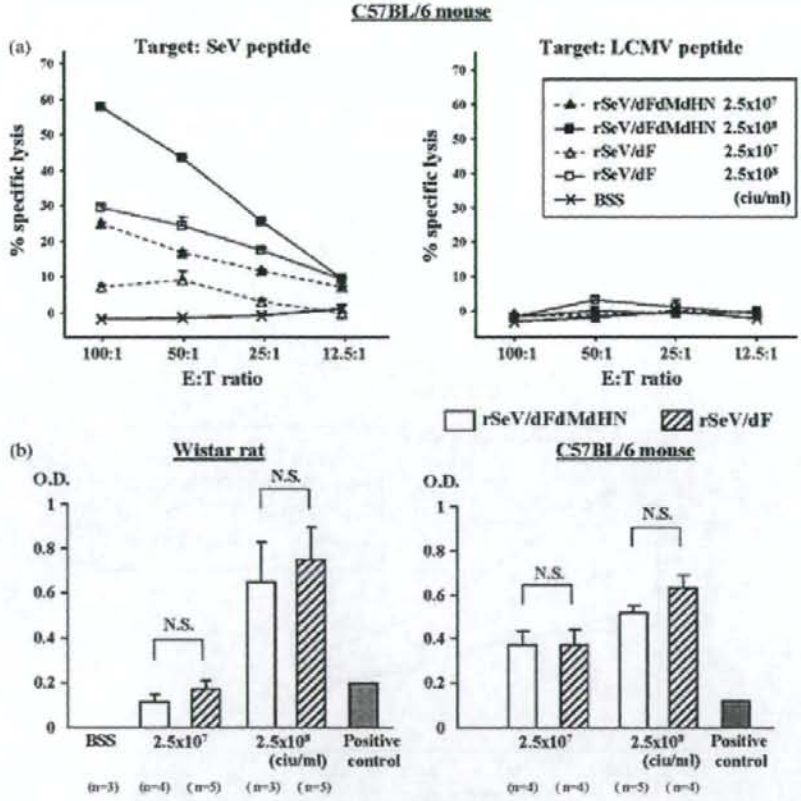

Figure 6. Assessment for SeV-specific adaptive immune response following subretinal injection of each rSeV. (a) The CTL activity of C57BL/6 mice treated with rSeV vectors was assessed with a standard ^{51}Cr-release assay. Ten days after subretinal injection, spleen cells were harvested from mice treated with rSeV/dF-EGFP (2.5 • 10^7 ciu/ml) (△), rSeV/dF-EGFP (2.5 • 10^8 ciu/ml) (□), rSeV/dFdMdHN-EGFP (2.5 • 10^7 ciu/ml) (▲), or rSeV/dFdMdHN-EGFP (2.5 • 10^8 ciu/ml) (■). Controls included BSS-injected mice (•). LCMV peptide was also used as a third-party (right panel). Note the stronger cell lysis activity in rSeV/dFdMdHN-treated mice at the same titer (left panel). The figure shows results from one of three similar experiments. (b) The production of anti-SeV antibodies 4 weeks after vector injection was assessed by ELISA. No significant difference could be found between the two types of vectors (n • 3–5 each)

of NP proteins. For further improvement of this vector system, we are now aiming to introduce mutations on the NP gene to modify the CTL response.

The duration of gene expression by rSeVs is transient in the present design; however, rSeV-mediated gene transfer has demonstrated efficient therapeutic effects in many animal models, including established tumor models. For example, the boost of IL-2 via intracerebral injection of rSeV resulted in a significant antitumor effect on brain tumors combining with peripheral vaccination [31]. In addition, we recently found that rSeV efficiently infects and activates dendritic cells, and the intratumor injection of rSeV-modified dendritic cells (DCs) has been shown to have a strong antitumor effect associated with enhanced CTL responses [32]. These rSeV-based cancer immunotherapies have demonstrated superior antitumor effects over the existing strategies. Thus, we are now assessing whether rSeV-based gene therapy could be an adjuvant therapy for bilateral retinoblastoma because its management is still difficult in some cases despite multimodal treatment approaches [2,33].

In conclusion, the present study showed that membranous gene-deleted rSeV retained efficient gene transfer and remarkably reduced the host innate immune response in ocular tissue. Moreover, vector-related retinal damage found using the previous SeV vector system was dramatically improved. These findings may extend the utility of this new vector system for retinal gene transfer, although the acquired immune response remains to be overcome for long-term transgene expression of rSeV. Therefore, further studies refining this vector system are required for the clinical availability of rSeV for ocular diseases.