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総説

遺伝子治療用ベクターの開発
—最近の話題—

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要旨　既存のベクターによる二つの大きな副作用事例を教訓として，遺伝子治療の安全面での議論が活発になり，有効性向上の工夫と新しいベクターの開発が注目されている。ごく最近，アデノウイルス遺伝子治療製剤が世界で初めて中国政府から承認されたが，この出来事は遺伝子治療製剤の開発と臨床プロトコールの構築に弾みをつけるものとなるだろう。遺伝子治療ベクターは，染色体への組込みの有無あるいはその程度によって分類することができる。完全組込み型で長期遺伝子発現に有利なレンチウイルスを含むレトロウイルスベクター，部分的組込みのあるアデノ随伴ウイルスベクターおよびアデノウイルスベクター，そして新しいクラスのものとして，麻疹ウイルス，ニューカッスル病ウイルスおよびセンダイウイルスなどのように，染色体組込みがなく遺伝毒性のない「細胞質型RNAベクター」である。それぞれの疾病の特徴を生かしたベクターの選択とその改良が行われているが，遺伝子治療を広く普及するためのベクターは，やがてこれらのなかから選択されていくであろう。遺伝子治療ベクターをより早く開発し，患者の治療に提供するために必要なことは，ベクター開発の側では真に安全で有効な遺伝子治療用ベクターを提供すること，また臨床プロトコールをオリジナリティーをもって構築していくことである。

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Recent Progress in Vector Development for Gene Therapy

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Summary

Two highly publicized gene therapy-related side effects have reminded us an important discussion over safety concerns and suggested the value of improving the effectiveness of gene therapy and proposals of new vector systems.

Very recently, the world's first approval of adenovirus vector as a formulation for cancer gene therapy was made by the Chinese government. This event will facilitate the development of many types of vector formulation for gene therapy and encourage the construction of ambitious clinical protocols.

From recent vectorological studies, the gene therapy vectors can be classified into three types according to the presence or absence of integration into the chromosome and to what degree. The first types are retrovirus vectors including lentivirus vectors which are fully integrated vectors advantageous for long-term transgene expression. The second types are those partially integrated, such as adeno-associated virus and adenovirus vectors. As the final classification a new class of vectors we call "cytoplasmic RNA vectors" that may be free from genetic side effects have been recently proposed for use with the measles virus, Newcastle disease virus and Sendai virus. Meanwhile, appropriate vectors will be selected depending upon the characteristics of applied diseases. The gene transfer vector(s) that can lead to widespread use of gene therapy in the medical field will be selected from those in these protocols.

For the rapid development of these vectors and providing such treatment to patients, we the developer

side have to provide truly safe and effective vectors for gene therapy. In addition, the clinical protocols must be constructed with our own originality.

Key words: Gene therapy, Virus vector, Integration, Clinical protocol, Sendai virus
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はじめに

ポストゲノム時代の根治的新規治療法として期待された遺伝子治療であったが，その臨床応用例はここ数年横ばいあるいは減少傾向にある。しかしながら，二度の大きな副作用事例を教訓としてガイドラインの整備と臨床プロトコールの改良，さらに新規ベクターの開発が行われており，実際に遺伝子治療製剤の製造・販売が世界で初めて中国において承認された。また，開発中の遺伝子治療ベクターの多様化も進みつつあり，それぞれのベクターの特性に即した開発と改良が行われている。その有効性および安全性が十分確認された上で，新たな遺伝子治療製剤が世の中に提供されていくものと期待される。

I. 初の遺伝子治療製剤承認と遺伝子治療を取り巻く状況

2003年10月，中国において遺伝子治療製剤の製造・販売が世界で初めて承認になった。そして，2004年4月より「Gendicine（今又生）」という名称で深川賽百諾基因（遺伝子）技術公司（SiBiono GeneTech社，広東省深川）が販売を開始している。この製剤は，癌抑制遺伝子であるp53遺伝子を搭載したアデノウイルスベクターであり，上咽頭癌などの頭頸部の扁平上皮癌に有効だといわれている。また，前臨床試験段階では種々の腫瘍に対する治療効果も示されている。日本を含め各国での臨床研究が実施されたものと類似の製剤ではあるが，本治療製剤の開発成功のポイントは，従来の放射線治療との併用による高い治療効果を示したことにあると考えられる。このように，遺伝子治療製剤が許認可機関（この場合は中国国家食品薬品管理監督局）の承認を得て市場にでたことは，今後の遺伝子治療製剤の可能性を大きく前進させる出来事になった。この事例に続く形で，米国Introgen Therapeutics社（テキサス州Austin）が，2004年12月に遺伝子治療製剤「ADVEXIN」（p53遺伝子を搭載したアデノウイルスベクター）の頭頸部癌に対する治療を目的に，米国食品医薬品局（FDA）に対して新規生物製剤の認可の分割申請（Rolling BLA）を行ったことが発表されている。

医薬品市場に遺伝子治療製剤の第1号がでた一方で，多くの遺伝子治療用ベクターは開発の途上にあり，あるいはその問題点を克服すべく改良がなされている。歴史的にみると，遺伝子治療は1990年にアメリカで行われたアデノシンデアミナーゼ（ADA）欠損症の治療から始まり，2004年までに世界中で約1,000種の遺伝子治療臨床プロトコールが提案され，4,000人以上の患者ボランティアが実際に遺伝子治療を受けている。年次変化を図1に示した。対象疾患としては癌に対するものが多くを占めているが（約66%），慢性疾患やワクチンへの応用など用途は拡大している。実施地域としては，約95%は欧米で実施されている。しかし，アジア地域でも臨床試験への取り組みは熱心になされており，日本で1%，そして特に中国では実施例が顕著に増加しているといわれている。

遺伝子治療は先端医療の一つであり，一般医薬品とは異なる取り扱いで臨床研究がなされてきたが，以下に述べる二つの大きな副作用事例により，より高い安全性への配慮と慎重なプロトコールの設計・実施が求められるようになった。一つは1999年にペンシルバニア州立大学において，アデノウイルスを大量に投与された患者が死亡した例であり，その後，安全で厳重な評価を実施するために臨床プロトコールなどのガイドラインが充実するとともに，倫理面でも個々の患者に対するインフォームドコンセントの徹底が図られるように

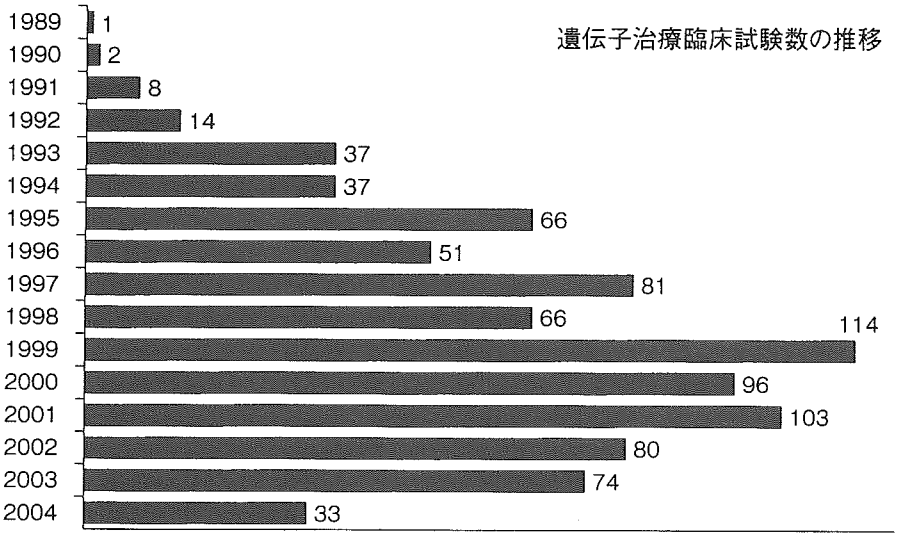

図1　世界の遺伝子治療臨床試験承認および実施数の経時変化（2004年7月1日までの集計）
J. Gene Med.（John Wiley and Sons Ltd.）のwebsite（www.wiley.co.jp/genmed/clinical）のデータを改変して記載。

なった。さらに，2002年10月にフランスのネッカー病院で，X染色体にリンクした重症複合体型免疫不全症（X-SCID）患者に対して，自己のCD34陽性骨髄細胞にIL-2受容体γ鎖遺伝子を搭載したレトロウイルスベクターを用いて遺伝子治療を実施した例において，11例中2例でT細胞性白血病が発症したことが報告された[1]。治療を受けたほとんどの患者において特異的免疫機能が回復し，遺伝子治療による免疫不全症の根本的かつ永続的治療が可能であることが確認されていた重要な試みであっただけに，遺伝子治療分野のみならず社会的にも大きな影響を及ぼした。その原因は，プロトオンコジーンLMO2のプロモーター近傍へのレトロウイルスベクターの組込みにより，LMO2遺伝子の発現増強が生じたためであった。この発症した2名の患者は，その後1人は骨随移植（HLA適合非血縁者間）により，他の患者も薬剤治療（T-ALLに対する化学療法）により回復がみられていたが，うち1名は亡くなったとの報告がなされている。さらに，2005年になって新たに3例目の副作用例が発表され，一時再開が認められていたこの治療法は再び凍結された。X-SCID治療については遺伝子治療による劇的な治療効果があることから，今後の詳細な解析と副作用回避の方策が望まれるが，遺伝子治療の普及のためには，染色体への組込みのない，すなわち遺伝毒性のないベクターの開発が強く望まれている。

II.　染色体組込み型ベクターの改良
—レンチウイルスベクター

染色体組込み型レトロウイルスベクターのなかで最も頻用されてきたのが，哺乳類C型レトロウイルス由来のレトロウイルス（MLV）ベクターであるが，最近はレンチウイルス（HIV，SIV，FIVなど）ベクターの前臨床段階の応用例が非常に多くなっている。このタイプのベクターは，導入遺伝子は宿主染色体に組み込まれて発現するため（図2），長期的な遺伝子発現という観点から非常に有利である。ただし，フランスでのX-SCID治療例と類似の副作用が出現する可能性は残しているため，染色体への導入部位の解析，さらには部位特異的遺伝子導入法の開発も行われている。

レンチウイルスベクターは，神経細胞などの終末分化した非分裂細胞あるいは造血幹細胞など分裂頻度の少ない細胞への遺伝子導入も可能であり，たとえば終末分化したラット色素上皮細胞に，サル免疫不全ウイルス（SIV）ベクターを利用してマーカー遺伝子を導入した場合，1年以上にわたって強い蛋白の発現が確認されている[2]。染色体への導入については，MLVおよびレンチウイルス

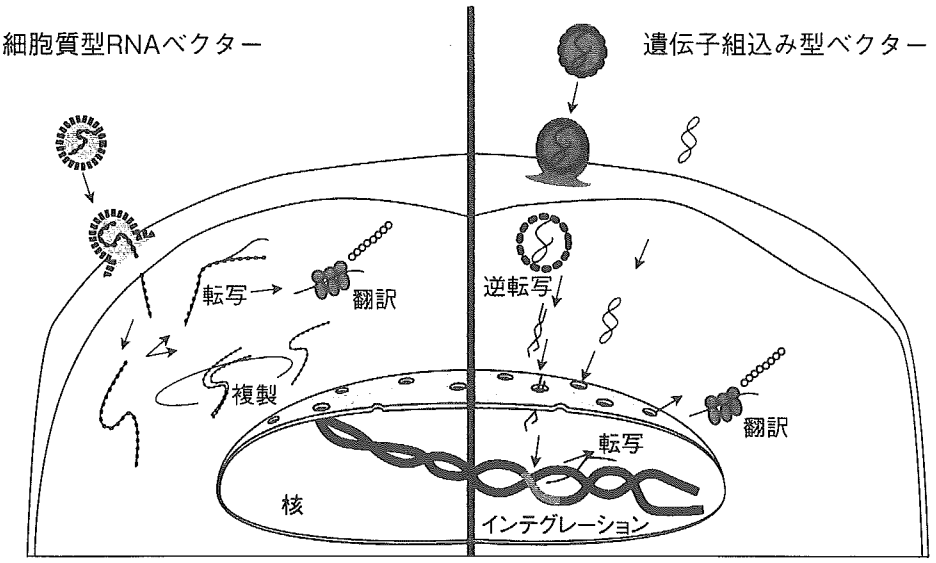

図 2 細胞質型 RNA ベクターと遺伝子組込み型ベクターの遺伝子発現様式
SeV のような細胞質型 RNA ベクターは細胞質内で複製と転写翻訳を行い，核内へは移行しない。また，DNA への変換もない。一方，遺伝子組込み型ベクターは DNA として核内に移行し，染色体にインテグレートされ，遺伝子は転写・翻訳される。

ともに発現が活性化している遺伝子部位への挿入の傾向が強いが，その部位特異性は異なり，MLV の場合には転写ユニットの開始部位近傍（プロモーター近傍）に挿入される傾向が強いのに対して，ヒト免疫不全ウイルス（HIV）の場合はその傾向はなくランダムであることが報告されている[3]。すなわち，レンチウイルスベクター（HIV など）のほうが MLV よりも，染色体への導入による細胞由来蛋白の発現への影響は低いと予想される。しかし，臍帯血中の造血幹細胞に HIV ベクターで遺伝子導入した場合，導入遺伝子陽性細胞の 86 %において遺伝子中へのプロウイルスの挿入を認め，さらにいくつかの遺伝子については白血病関連の遺伝子であったことが報告されている[4]。レンチウイルスベクターを利用する場合も，染色体への遺伝子導入による影響については精査する必要があり，対象とする疾患やプロトコールによっては，これらに配慮した安全対策が必要である。

レンチウイルスベクターの多くの実施例において，ヒト水疱性口内炎ウイルス G 蛋白質（VSV-G）でシュードタイプ化したベクターが生産され，評価に用いられている。このシュードタイプベクターは，生産性が高く高倍率の濃縮に耐え，比較的多くの細胞・組織への導入が可能であるため頻用されている。一方で，このシュードタイプでは粘膜層経由での気道上皮細胞への遺伝子導入が成立せず，嚢胞性線維症（cystic fibrosis）などの呼吸器系疾患への応用ができないという欠点がある。そこで，気道上皮細胞への遺伝子導入が可能なウイルスのエンベロープ蛋白を利用した新型シュードタイプの開発がなされている。エボラ出血熱ウイルス（Ebola）Z 蛋白や重症急性呼吸器症候群（SARS）S 蛋白を利用したものも報告されているが，安全性の面で問題がある。われわれは，センダイウイルス（SeV）のエンベロープ蛋白質である融合蛋白質（F）および赤血球凝集ノイラミニダーゼ（HN）の 2 種の蛋白を有する新型シュードタイプ SIV ベクターの開発に成功し，VSV-G シュードタイプベクターに匹敵する生産システムを構築するとともに，この新しいベクターによりマウスの気道上皮細胞への効率的な遺伝子導入が可能であることを確認した。このベクターによる今後の前臨床試験結果が期待される。

III. 低頻度染色体組込み型ベクターの改良

1. アデノ随伴ウイルスベクター

アデノ随伴ウイルス（AAV）ベクターの特性とされていた第 19 番染色体への部位特異的組込

みはRep依存的であり，Rep遺伝子を欠失した現在のAAVベクターではそれは非常に低頻度になっている。多くのAAVベクターはエピソームとして存在しているといわれているが，低頻度ながら染色体へのランダムな組込みが生じた場合は，活性化領域への導入頻度が高いことが示されており[5]，さらには組込み領域の染色体構造の変化も観察されているため，その遺伝学的影響に関して慎重に考慮していく必要がある。

AAVベクターの特徴は性質の異なる多様なセロタイプの存在にある。最近になって，2種の新規セロタイプ（AAV10およびAAV11）も見いだされ，これで計11種になった。これまで遺伝子治療へ応用されているのは主としてAAV2およびAAV5であるが，AAV2のinverted terminal repeat（ITR）と他のセロタイプのコート蛋白（capsid）を利用するシュードタイプ（ハイブリッド）ベクターも最近多く構築されている。セロタイプにより組織特異性が異なる場合が多く，たとえばAAV1は心筋への遺伝子導入効率が高く，骨格筋へはAAV1，AAV7，AAV8が，神経系にはAAV1，AAV2，AAV5が高い導入効率を示している。また，脳の部位によってセロタイプ選択性がでることが示されている。セロタイプのなかで最も特徴的なのがAAV8であり，組織指向性がなくほとんどの組織に高率での遺伝子導入が可能で，肝細胞へはセロタイプのなかで最も導入効率がよく[6]，さらに血液脳関門（BBB）さえも通過することが示されている。組織指向性のない高率の遺伝子導入は，利点でありかつ欠点でもあるが，そのメカニズム解析が進むことにより，組織および細胞への新規のデリバリー法の開発につながると考えられる。

AAVベクターは長期的遺伝子発現が期待されているので，多くの単遺伝子欠損型の疾患に対しての応用が試みられている。先行したのが，血液凝固第Ⅸ因子遺伝子を搭載した血友病Bを対象にした臨床研究である。動物実験では一過的な免疫反応と17か月以上という長期的な遺伝子発現が確認されたのに対して[7]，ヒトに対する臨床研究での遺伝子発現は，予想に反して数週間で減少し一過的発現に止まった。ヒトは自然感染によって抗AAV抗体を有しているケースが多く，あらかじめ抗体をもつヒトの場合に，より短期間の発現であったとされている。当初，免疫原性が低いといわれていたAAVに対しても免疫反応の影響が出現し，2回目の遺伝子導入は効率が低くなるといわれている。さらに，遺伝的に欠損している蛋白を補った場合の導入遺伝子産物に対する患者の免疫反応も問題になっている。遺伝子欠損型の患者を対象にした長期遺伝子発現の難しさを示している。他の重要で難しい課題への取り組みの一つとして，デュシェンヌ型筋ジストロフィーへの応用がある。原因遺伝子はジストロフィンであるが，AAVベクターは小型ウイルスに由来するベクターであるため搭載可能な遺伝子長に限界があり，AAVゲノム遺伝子をできるだけ削る（分割する），あるいはミニ・マイクロジストロフィンと呼ばれる短鎖型のジストロフィンを構築する[8]，さらにはVEGF蛋白と併用することで血管透過性を高め，非常に高い効率で全身の骨格筋にデリバリーする手法などが開発され，少なくともモデル動物において高い有効性を示している。その他，神経変性疾患あるいは癌への遺伝子治療研究も精力的に行われている。

2. アデノウイルスベクター

アデノウイルスベクター遺伝子は核内にエピソームとして存在し，染色体へは組み込まれないと一般にはいわれていたが，0.001～1％の低頻度ながら，ランダムの組込みが生じることが確認されている[9]。さらにこの確率は過小評価されており，実際には10%近い頻度であるという報告もある。アデノウイルスベクターについても，遺伝毒性という観点からの影響は無視できないのかもしれない。逆に，染色体への組込み効率を上げて長期遺伝子発現をめざす研究もなされている。

世界初の遺伝子治療製剤が，アデノウイルスベクターによる頭頸部癌の治療を対象としているように，当該ベクターの臨床研究の多くは癌を対象にして行われている。特に，制限増殖型・腫瘍崩壊性（replication-selective・oncolytic）アデノウイルスベクターに関する報告がここ数年非常に多いが，その代表的なものとして，E1Bによってコードされる55 kDaのp53結合蛋白（E1B55K）遺伝子を欠損し，p53経路に欠陥のある癌細胞のみで増殖するように加工されたONYX-015（dl1520）

がある。phase Ⅰ/Ⅱまで順調に結果が報告されたが，開発していたOnyx Pharmaceuticals社（カリフォルニア州Richmond）は，2003年1月に，同時に開発中の他の治療製剤（Raf kinase阻害剤）に資源を集中させるとして，ONYX-015の開発を中断すると発表した。ONYX-015と同じベースのベクターを基に，ほとんどの癌細胞で活性化しているhuman telomerase reverse transcriptase（hTERT）のプロモーター制御下にE1A遺伝子を発現するアデノウイルスベクターが開発され，ONYX-015よりも強い抗腫瘍効果があることが示された[10]。その他にも，prostaglandin-endoperoxide synthase 2（PTGS2）の3′untranslated region（UTR）をE1A遺伝子と結合することで，E1AのmRNAをRas活性化特異的に安定化する，すなわちRas活性化依存的に増殖するアデノウイルスベクター，あるいはアデノウイルスの増殖に必要なRNA-activated protein kinase（PKR）の不活性化に必要なvirus-associated（VA）RNAを欠失し，PKR下流のRasが活性化している癌細胞でのみ増殖可能なアデノウイルスベクターなど，全腫瘍の80％で活性化が起こっているRas活性化依存的に増殖する制限増殖型アデノウイルスベクターが開発されている。このようなRas活性化依存的腫瘍崩壊をねらったものは，単純ヘルペスウイルス（HSV）ベクターなどの他の種類の制限増殖型ウイルスベクターでも利用されている。

また，すでに市場にでた遺伝子治療製剤「Gendicine（今又生）」が従来の放射線治療との併用を想定したものであったように，臨床研究段階で他の治療法と併用して効果をみる例が増えている[11]。たとえば，明確にcombination therapyとして治療戦略を取ったものに，前立腺癌のphase Ⅰ臨床試験において，制限増殖型アデノウイルスにcytosine deaminase（CD）とherpes simplex virus thymidine kinase（HSV-tk）の融合遺伝子を搭載し，5-fluorocytosine（5-FC）およびvalganciclovir（GCV）のプロドラッグで治療し，さらに3次元原体照射（three-dimensional conformal radiation therapy，3D-CRT）を実施しているケースもある。最良の治療効果をだすためにも，現実的な放射線治療あるいは化学療法との併用による評価が今後も増えていくものと予想されるが，その一方で，より遺伝子治療自体の効果を上げるためのベクター開発も望まれるゆえんである。

Ⅳ．染色体非組込み型ベクターの開発 —細胞質型RNAベクター

遺伝子治療を一般製剤並みの技術製品として普及させるための一つの方向性として，遺伝毒性のないベクターの開発が必要である。つまり染色体への組込みのない遺伝子治療用ベクターの開発であり，そのための試みも数多く実施されている。代表的なものが「細胞質型RNAベクター」（図2）と呼ばれるベクター群であり，現時点ではそのほとんどが腫瘍崩壊性ベクターとして癌治療へ応用されている。具体的には，mumps virus，Newcastle disease virus，measles virus（MV），vesicular stomatitis virus，influenza virus，reovirusおよびpoliovirusなどで実施されている[12]。このなかで，Newcastle disease virusのワクチン株（PV701）を利用した臨床試験が先行しており，比較的良好な結果が得られているという[13]。また，MVを用いた卵巣癌や多発性骨髄腫に対する臨床試験も，近々に実施される予定である[14]。このベクターについては，癌への応用に特化したベクター改良が行われており，たとえば，癌細胞表面で高発現している蛋白に親和性のある単鎖抗体遺伝子を利用して癌細胞へのターゲッティングを可能にした腫瘍崩壊性ベクター，あるいはヒトthyroidal iodide symporter（NIS）遺伝子を搭載したMVを用いて，ヨウ素123（^{123}I）を癌へ集積し，非侵襲的なimagingと放射線感受性の悪性腫瘍を治療するシステム（radiovirotherapy）も構築されている。現在，ベクターの低生産性という悪い条件があるが，臨床研究へ向けてのGMP生産の準備が行われている。

細胞質型RNAベクターを癌に特化しない一般的な遺伝子治療用ベクターとするために，われわれはSeVゲノムから遺伝子を欠失していく手法をとってきた（図3）。遺伝子を欠失することで安全性を高めるとともに，治療対象疾患に則したベクターへ改良することに成功している。宿主細胞への侵入にかかわる膜融合蛋白質（F蛋白）遺伝子

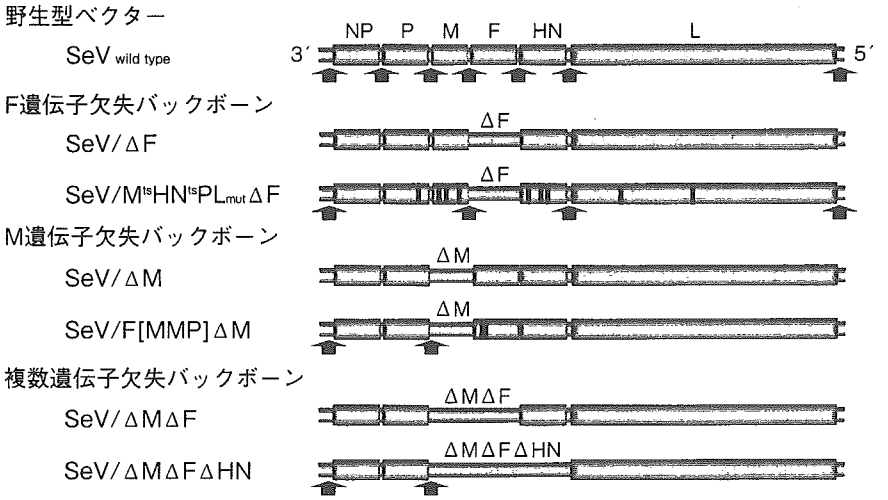

図 3　各種遺伝子欠失型センダイウイルス（SeV）ベクター
目的によって各種遺伝子欠失型ベクターを使い分ける。また，目的遺伝子（GOI）の期待する発現量により，遺伝子搭載位置（⇑）を変更してベクターを構築する。

をゲノムから欠失させることにより，二次感染性のない，非伝播型ベクターへ改良することに成功した[15]。このタイプのベクターを用いて，九州大学附属病院において臨床研究が計画され，現在，厚生労働省での審議が行われている。その計画では，塩基性線維芽細胞増殖因子（fibroblast growth factor 2, FGF-2）遺伝子を搭載したベクターを利用して，慢性動脈硬化症などを原因とする重症の虚血下肢（重症虚血肢）に対する血管新生を目的とした遺伝子治療が実施される。ベクター選択とともに，搭載遺伝子選択にも工夫が凝らされた計画である。この臨床研究は国内開発ベクターによる初めての細胞質遺伝子治療となるが，臨床研究実施に向けてGMP生産にも成功している。つまり，この新しいベクターの臨床レベルの基本技術はできあがっている状況である。また，ウイルス粒子のアセンブリーと出芽の中心的役割を担っているマトリックス蛋白（M蛋白）遺伝子を欠失することで，感染細胞からの粒子放出を原理的に抑え，さらにF蛋白を浸潤転移性癌特異的に発現が亢進しているマトリックスプロテアーゼ（MMP-2, MMP-7など）依存的に活性化するように改変することで，浸潤転移性癌特異的な細胞融合死を生じる新しいタイプの腫瘍崩壊性ベクターの構築に成功した[16]。さらに，複数の遺伝子の欠失にも成功しており[17]，組織傷害性・免疫原性などの減弱を確認している。SeVのヒトへの投与実績として，野生型ウイルスをヒトパラインフルエンザウイルスⅠ型ウイルスへの生ワクチンとして使用した米国での臨床試験が報告されているが，このような野生型SeVをヒトへ投与した場合でも重篤な副作用は検出されておらず[18]，非伝播型SeVベクターであれば，理論的により安全に投与できるものと期待され，臨床研究の実施が待たれる。

おわりに

開発中の遺伝子治療用ベクターは，染色体への組込みという観点からだけでも多種多様である。導入遺伝子の発現期間，免疫系賦活化能，遺伝子導入効率などを含めてそれぞれのベクターの特性が異なる。当面その特性をよく理解し，治療対象疾患に則した改良と臨床研究・臨床試験を推進しなければならない。ベクターの開発・改良が進むことによって，遺伝子治療を臓器移植並みの技術に閉じ込めない，汎用性の高いベクターが取捨選択されていくものと思われる。

遺伝子治療は先端医療であり，基礎研究に根ざして開発されたテクノロジーである。しかし，遺伝子治療が先端医療であるからこその課題も指摘されており，その一つが研究から臨床に上がるまでのdeath valleyの存在である。実際に，350例

の臨床試験を実施するための資金力がなくpendingになっているケースもかつて存在した。最近は，基礎研究の成果を臨床の場に応用し，そこでの治療効果を検定することを推進するための「トランスレーショナル・リサーチ」という言葉が浸透し，その考え方の重要性が認識されるようになってきた。大学などの教育・研究機関においても，そのための取り組みが積極的になってきている。さらなる制度的・研究資金環境の改革によって，基礎科学に根ざした先端医療である遺伝子治療をより早く臨床現場で評価するシステムを構築する必要がある。この時に必要なことは，ベクター開発の側では真に安全で有効な遺伝子治療用ベクターを提供すること，また臨床プロトコールをオリジナリティーをもって構築していくことである。

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RESEARCH ARTICLE

Generation of a recombinant Sendai virus that is selectively activated and lyses human tumor cells expressing matrix metalloproteinases

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Malignant tumor cells often express matrix metalloproteinases (MMPs) at a high level to enable their dissemination and metastasis. Sendai virus (SeV), a nonsegmented negative strand RNA virus, spreads in the target tissues in vivo via cleavage activation of the viral fusion glycoprotein by a tissue-specific, trypsin-like enzyme. By deleting the viral matrix protein, we previously generated a recombinant SeV that does not bud to mature virions, but is highly fusogenic and spreads extensively from cell to cell in a trypsin-dependent manner. Here, we changed the tryptic cleavage site of the fusion glycoprotein of this virus to a site susceptible to MMPs. The resulting recombinant virus was no longer activated by trypsin but spread efficiently in cultured cells supplemented with MMP2 or MMP9 and in human tumor cell lines expressing these MMPs. Furthermore, the virus spread extensively in tumor cells xenotransplanted to nude mice without disseminating to the surrounding normal cells, leading to the inhibition of the tumor growth in the mice. These results demonstrate the selective targeting and killing of human tumor cells by recombinant SeV technology and greatly advance the reemerging concept of oncolytic virotherapy, which currently appears to rely largely upon a natural preference of certain viruses for cancer cells.
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Keywords: *Sendai virus; oncolytic virotherapy; tumor; MMP; matrix protein*

Introduction

The idea of using infectious viruses as antitumor agents is again attracting attention in part because of progress in understanding virus–host interactions and because currently available chemotherapy is not completely satisfactory for many reasons, including the development of drug resistance.[1–3] The oncolytic virotherapy strategies proposed to date rely largely upon the natural preference of certain viruses for cancer cells, as exemplified by Newcastle disease virus (NDV) and vesicular stomatitis virus, which belongs to the family Paramyxoviridae and the family Rhabdoviridae, respectively. Both of these families of viruses are characterized by a nonsegmented negative-strand RNA genome, and thus are also called mononegaviruses. Preclinical studies using viruses in other families have involved extensive efforts to increase selectivity for tumor cells by manipulation of the viruses by recombinant DNA technology. Examples of such efforts include attempts to change adenovirus attachment protein so that it recognizes tumor-specific cell surface molecules and deletion of herpesvirus accessory genes that are indispensable for the viral life cycle in normal cells but likely dispensable in tumor cells.[4,5]

Sendai virus (SeV), also a member of the Paramyxoviridae, infects most mammalian cells and directs high-level expression not only in cultured cells but also in experimental animals of the genes within its genome that have been exploited for therapeutic use and vaccination.[6–10] A strong potential of NDV as a vaccine vector was also demonstrated.[11] SeV and most of the other mononegaviruses replicate independent of cellular nuclear functions and do not have a DNA phase in their lifecycle, and therefore are not considered to present a high risk of cell transformation by integration of the viral genetic information into the cellular genome. Thus, the feasibility of using SeV and other mononegaviruses as a novel class of vector is now increasing.

SeV displays a narrow spectrum of tissue tropism in susceptible hosts, growing in the respiratory tract of mice or in the allantoic cells of embryonated chicken eggs with little appreciable spreading to other tissues in these host organisms, even though its receptor is sialic acid residues, which are ubiquitous throughout the body. This restricted tropism is primarily due to the fact that the specific tissue proteases required for cleavage activation of viral fusion (F) glycoprotein, and thus for infectivity of progeny (the capacity to penetrate into and initiate infection of the next cell), are available only on the surface of those limited types of tissue.[12] This concept

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of protease-dependent tropism of SeV was substantiated by the identification of responsible tissue proteases: the blood clotting factor Xa ectopically expressed by allantoic cells *in ovo* and secreted into the allantoic fluid,[13] and tryptase Clara secreted into the airway epithelium in the lungs of rodents.[14] The cleavage motif for these proteases in the fusion glycoprotein precursor F_0 is the sequence Q–S–R. Most tissue culture cells so far tested do not express SeV-activating proteases, and hence require a low concentration of exogenously added trypsin to allow SeV spreading.[15] The receptor recognition is mediated by another envelope glycoprotein called hemagglutinin-neuraminidase (HN).

The extracellular matrix (ECM) surrounding a tumor cell serves as a barrier that blocks tumor cell migration, infiltration and metastasis. Highly invasive, metastatic cancer cells express high levels of ECM-degrading enzymes such as matrix metalloproteinase (MMPs) and urokinase-type plasminogen activator (uPA).[16,17] We have expanded the plasmid-based reverse genetics technology originally developed to manipulate the full-length SeV genome[18] in various directions, including deletion of certain viral genes, to generate nontransmissible, safer versions of SeV vector for gene therapy.[19–21] Among them, M (matrix protein) gene-deleted SeV (SeV/ΔM) is unique in that it does not bud into a mature particle in infected cells but rapidly spreads from cell to cell in the presence of trypsin, with the induction of massive syncytia followed by rapid cell death throughout the monolayer.[22] We therefore considered it likely that SeV/ΔM would be more potent in killing solid tumor tissue than the wild-type SeV or any other deletion mutants. Here, we used the SeV/ΔM plasmid as the starting material and applied the concept of 'protease-dependent viral tropism' in order to generate a recombinant SeV/ΔM capable of 'tumor-specific protease-dependent oncolysis' at high efficiency.

Results

Cell-to-cell spreading of M gene-deleted SeV

Deletion of the viral M gene from SeV (Figure 1a) almost completely abolished virus maturation into particles and instead greatly facilitated cell-to-cell virus spreading via membrane fusion in the presence of F protein-activating trypsin[22] (also see Figure 2a). Thus, the M gene-deleted SeV (SeV/ΔM) appeared to be more promising for killing the cells in a solid tumor than the wild-type SeV, because the facilitated cell fusion itself is cytotoxic in many cases. In addition, the absence of free infectious particles indicated that the mutant should undergo little undesired spreading to tissues distant from the initially infected cells. Therefore, the SeV/ΔM cDNA plasmid was used as the starting material for all subsequent manipulations to generate oncolytic SeVs. Virus growth and spreading were monitored by the expression of enhanced green fluorescent protein (GFP), whose open-reading frame (ORF) had been inserted into the viral genome in place of the M ORF (see Figure 1a).

Attempt to alter the tryptic cleavage site in the SeV F protein to render it susceptible to MMPs

We initially had to know whether or not it would be possible to alter the tryptic cleavage site of SeV F

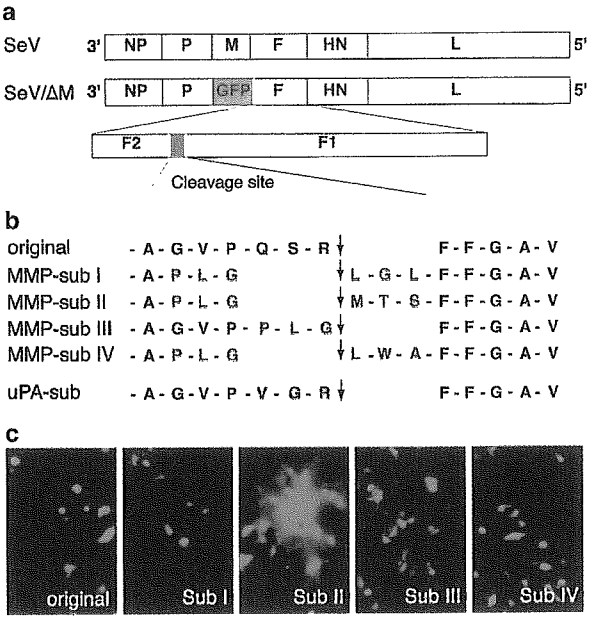

Figure 1 Gene maps of the wild-type SeV and SeV/ΔM (a), amino-acid changes at the F protein cleavage site (b) and the capacity of the resulting F protein mutants to induce cell fusion (c). In SeV/ΔM, the ORF of the M protein was replaced by the GFP ORF. The amino-acid changes made to render the F protein an MMP substrate (subI–subIV) and those made to render it an uPA substrate are shown in red and blue, respectively. Cell fusion was analyzed by cotransfection into MMP-expressing HT1080 cells of the chimeric F/HN genes with the modified cleavage sequences and the plasmid-expressing GFP. Syncytia formation was observed 48 h after transfection under a fluorescence microscope.

glycoprotein to a site susceptible to MMPs. There are a number of known MMPs, and the cleavage sites recognized by the MMPs appear to consist of the tripeptide PLG followed by an additional tripeptide LGL or LWA, according to the findings of studies using synthetic substrates for MMP assays.[23–25] Cleavage takes place between the two (upstream and downstream) tripeptides (Figure 1b). On the other hand, the natural cleavage of SeV F protein into F2 and F1 occurs between the R and F in Figure 1b, and the resulting N-terminal sequence of the F1 begins with FFG. A previous study demonstrated that the carbobenzoxy-FFG oligopeptide could specifically inhibit SeV fusion activity and infectivity in cultured cells,[26] suggesting that the N-terminal FFG was specifically required for fusion capacity. Thus, the addition of LGL or LWA to the natural N-terminal FFG might impair fusion activity. Under these circumstances, the alteration of the tryptic cleavage site to an MMP-specific site without impairing fusion activity would not be easy. We therefore designed numerous sequences and show some of them (designated MMP-subI to MMP-subIV) in Figure 1b. These include MTS (subII), which was newly designed here by changing the MMP2- and MMP9-specific sequence MWS[27] to MTS to accord with the favorable sequence for MMP9 substrates, Pro-X-X-Hy-(Ser/Thr), which was identified by phage display.[28] MTS is less bulky and less hydrophobic than LGL or LWA, and may thus be less obstructive for fusion when it is attached to the N-terminus of F1

after cleavage. Total omission of the downstream tripeptide specific for MMPs was also attempted so that FFG would appear at the N terminus. Designing uPA-specific substrate was relatively easy, because uPA and trypsin generally cleave after an R. Thus, only the two residues Q and S before the R were changed to V and G, respectively, to make the substrate more favorable for uPA (Figure 1b).

These and other modifications of the cleavage site were introduced into a plasmid encoding the F and HN proteins as a fusion polypeptide. Each of the resulting plasmids was transfected, together with a GFP-expressing plasmid, pCAGGS/GFP, into human fibrosarcoma cell line HT1080 and human stomach cancer line MKN28, which expressed and secreted MMP and tissue-type plasminogen activator (tPA), respectively, at high levels.[29,30] tPA has the same cleavage specificity as uPA. As shown in Figure 1c, the F protein with the cleavage motif compatible with MMP-subII was fully fusion competent in the relevant target cell line, HT1080, whereas the other three substrates (subI, subIII and subIV) were not. While the subII mutant was fusion incompetent in the MKN28 cell line, the uPA-sub mutant was highly fusion inducing in this cell line but not in HT1080 (not shown).

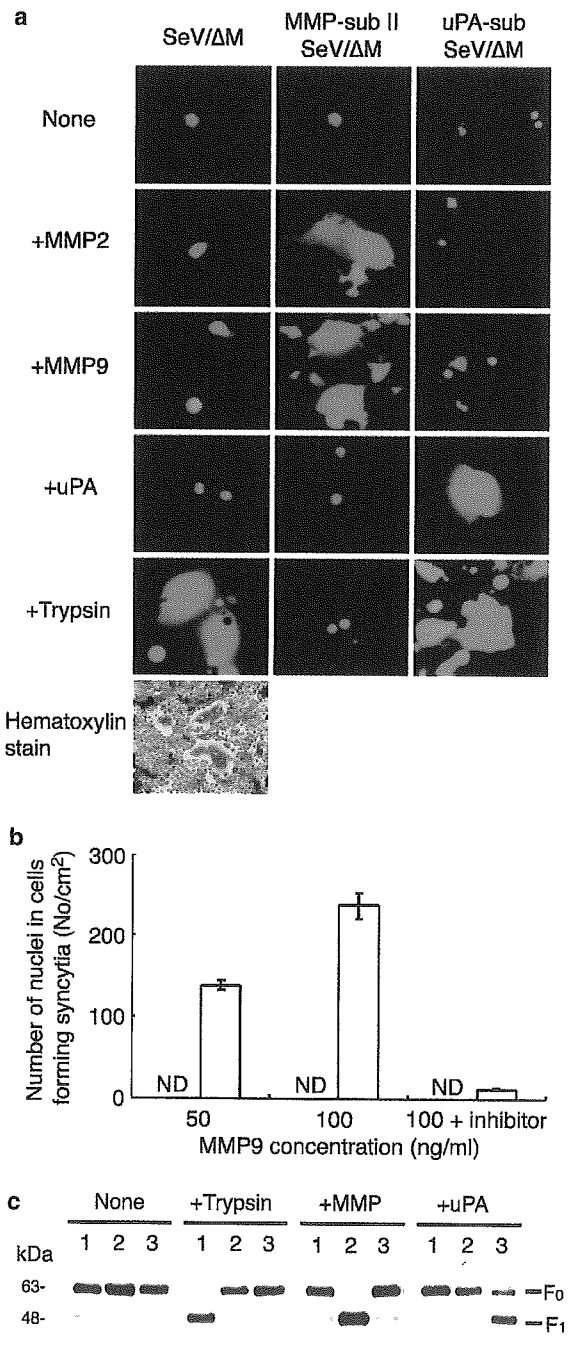

Figure 2 Confirmation of alterations of protease requirement and cell tropism of SeV/ΔM by exogenously added proteases. (a) LLC-MK$_2$ cells infected with parental SeV/ΔM, MMP-subII SeV/ΔM or uPA-sub SeV/ΔM at an MOI of 0.02 were cultured with MMP2, MMP9, uPA (0.1 μg/ml each) or trypsin (7.5 μg/ml). The induction of cell fusion and cell-to-cell viral spreading was monitored by assessing the GFP expressed from each virus under a fluorescence microscope 4 days later. Cells infected with parental SeV/ΔM and cultured with trypsin were also observed after hematoxylin staining. (b) Quantitative analysis of cell fusion of LLC-MK2 cells infected with MMP-subII SeV/ΔM in response to added MMP9. MMP9 was added to the medium (serum-free MEM) immediately after the infection with MMP-subII SeV/ΔM (open bars) or parental SeV/ΔM (closed bars, ND: not detected) at an MOI of 0.1. The number of nuclei in the cells forming syncytia was counted under a microscope 2 days after the infection. (c) Specific cleavage of the modified F proteins by treatment of the virions with proteases in vitro. Virus particles of the parental SeV/ΔM (lanes 1), MMP-subII SeV/ΔM (lanes 2) and uPA-sub SeV/ΔM (lanes 3) were prepared from the culture supernatants of an M protein-expressing helper cell line after infection at an MOI of 3 (CIU/cell) and subsequent incubation for 2 days in the absence of any protease. The virus particles produced were pelleted by centrifugation at 18 500 g for 3 h, resuspended in PBS and incubated with trypsin (7.5 μg/ml), MMP-9 (0.1 μg/ml) or uPA (0.1 μg/ml) for 30 min at 37°C. The controls (none) were incubated similarly in the absence of any protease. The viral proteins were then analyzed by Western blotting using an anti-F$_1$ antibody that recognized both F$_0$ and F$_1$ proteins.

Recovery of SeV/ΔM viruses with modified cleavage site and confirmation of their protease requirements

According to the above results obtained by plasmid-based expression, the SeV/ΔM plasmid was engineered to possess the MMP-subII or uPA-sub cleavage motif, and infectious progeny were rescued in an M protein-expressing helper cell line.[22] The rescued viruses were examined for the protease requirement for the induction of cell fusion and virus spreading in LLC-MK$_2$ cells (Figure 2a). SeV/ΔM with the MMP-subII cleavage motif in the F gene (MMP-subII SeV/ΔM) spread extensively via cell–cell fusion in the presence of MMP2 or MMP9. The number of nuclei per syncytium reached as high as about 100–300, and syncytium formation was almost completely inhibited by the addition of the MMP-inhibitor 1, 10 phenanthroline (Aldrich, Milwaukee, WI, USA) (Figure 2b). The same mutant could neither spread nor induce cell fusion in the presence of trypsin or uPA or in the absence of any protease (Figure 2a). The substrates of uPA and trypsin share an arginine at position −1 relative to the cleavage site, whereas the substrates of the MMPs do not have an arginine at this position. Thus, SeV/ΔM with uPA cleavage motif

(uPA-sub SeV/ΔM) spread in the presence of not only uPA but also trypsin, but not in the presence of the MMPs (Figure 2a). Spreading of the parental SeV/ΔM was possible only in the presence of trypsin. Trypsin might be able to cleave both the natural QSRFFG and artificial VGRFFG motifs (see Figure 1b) because it is a digestive enzyme with a broad specificity, whereas uPA might act only at the latter because it generally causes limited proteolysis with strict substrate specificity.

The highly selective protease dependency of virus-induced cell fusion and virus spreading nicely paralleled the cleavability of the F_0 precursor on the virions into F_1 (Figure 2c) and F_2 (not shown) only by the relevant proteases but not by any irrelevant ones. The F_0 of uPA-sub SeV/ΔM virions was not well cleaved by trypsin under the conditions employed, including 30 min of incubation with the enzyme, but incubation for a longer period (4 h) resulted in complete cleavage (not shown). This may account for the remarkable spreading of this mutant in the presence of trypsin (Figure 2a).

In perfect correlation with the above results obtained with exogenously added proteases, MMP-subII SeV/ΔM spread extensively in a human fibrosarcoma cell line, HT1080, endogenously producing and secreting MMPs, but not in a human stomach cancer line, MKN28, producing tPA, whereas uPA-sub SeV/ΔM did the opposite, namely, spread in MKN28 but not in HT1080 (Figure 3a). Neither of the cell lines was permissive at all for the parental SeV/ΔM.

A human pancreatic epithelioid carcinoma cell line, Panc I, which expresses MMP2 and MMP9 but at lower levels than HT1080 (Figure 3c), was unable to activate MMP-subII SeV/ΔM (Figure 3a). However, Panc I cells became permissive for this mutant (Figure 3a and b) when MMP9 production was enhanced by treating the cells with a phorbol ester, phorbol 12-myristate 13-acetate (PMA) (Figure 3c). In this experiment, SW620 (a human lymph node colorectal adenocarcinoma cell line) served as a control showing no stimulation of MMP production with PMA and no enhancement of virus spreading. Production of both MMP2 and MMP9 in HT1080 cells was enhanced by PMA (Figure 3c), but this enhancement resulted in only slight augmentation of MMP-sub II SeV/ΔM spreading beyond the levels seen in the cells not treated with PMA (Figure 3b). SeV activation by the MMPs thus appeared to be nearly an 'all or none' phenomenon with a critical threshold of the enzyme level. In addition, it should be emphasized that MMP2 and MMP9, together with MMP7, are likely more

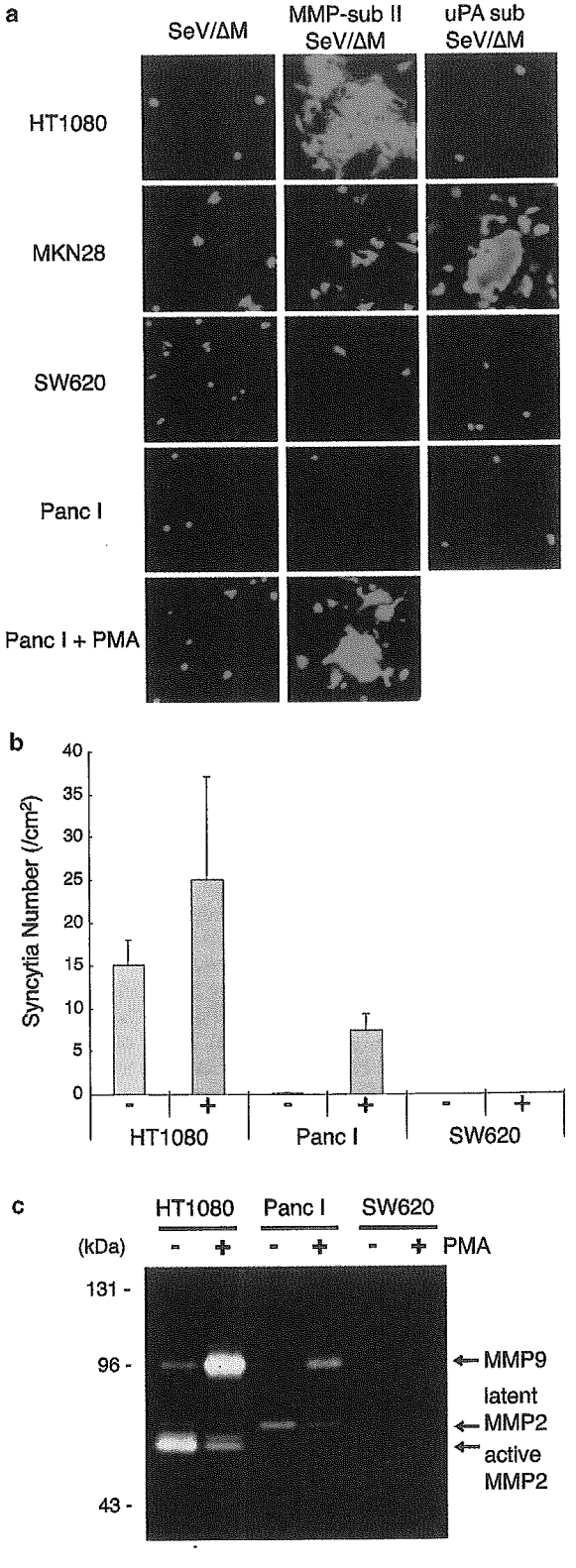

Figure 3 Confirmation of alterations of protease requirement and cell tropism of SeV/ΔM by endogenous proteases. (a) Cell-to-cell spreading dependent on endogenous proteases expressed by tumor cells. Four different tumor cell lines, HT1080, Panc I, SW620 and MKN28, were infected with the parental SeV/ΔM, MMP-subII SeV/ΔM or uPA-sub SeV/ΔM at an MOI of 0.02 and cultured in the medium containing 1% heat-inactivated fetal bovine serum, and cell fusion was observed 4 days later. Panc I cells were additionally analyzed to assess cell fusion following culturing with 20 nM PMA for 1 day. (b) Cell fusion of HT1080, Panc I and SW620 cells was quantified by counting the number of cells forming syncytia as observed under a microscope after infection and incubation for 4 days following culturing with (+) or without (−) 20 nM PMA for 1 day. (c) Gelatin zymography[47] of HT1080, Panc I and SW620 cells incubated with (+) or without (−) 20 nM PMA for 1 day and cultured in serum-free medium for 4 days. Gelatin zymography is a sensitive technique for the estimation of both active and latent types of MMP-2 and MMP-9 content in biological samples. PMA enhanced the levels of MMP9 in HT1080 and Panc I but not in SW620 cells.

important for tumor metastasis than some 20 other known MMP species, since only the former three have the capacity to disrupt basement membranes.[27,28]

Inhibition of HT1080 tumor growth in vivo by MMP-subII SeV/ΔM

MMP-subII SeV/ΔM was injected directly into the center of the mass of a HT1080 tumor that had been subcutaneously transplanted into nude mice and grown to an approximate diameter of 3–8 mm. As control viruses we used not only the immediate parent, SeV/ΔM, of the mutant but also a standard SeV with a full-length genome that harbored the GFP gene before its NP gene. After 2 days, light emission from the GFP protein in tumor cells was observed in the mice placed under a fluorescence microscope. GFP expressed from both of the control viruses was detected as small foci, likely at the injection site. In contrast, GFP expressed from MMP-subII SeV/ΔM spread throughout the entire tumor (× 1 magnification in Figure 4a). Differences between MMP-subII SeV/ΔM and the control viruses were also remarkable at higher magnification (× 10). The boundaries between the tumor cells were not seen in the former, giving a highly amorphous impression, whereas in the controls infection foci were much smaller in size and separated from one another, and cell boundaries were clearly seen (Figure 4a). This strongly suggested vigorous viral spreading of the MMP-dependent mutant in the tumor, leading to efficient tumor lysis. Staining with anti-GFP was much less marked in the central area including the site of inoculation (arrow) than in the peripheral region in the MMP-subII SeV/ΔM-infected tumor, whereas the central area was strongly positive for TUNEL staining and the peripheral region was negative or only poorly positive (Figure 4b). The TUNEL-positive cells are apoptotic ones characterized by internucleosomal DNA fragmentation. This result suggested that the cells in the peripheral area were actively supporting virus growth and viral gene expression, whereas those in the central area, which were probably infected earlier, were now dying via the apoptotic pathway. It should be noted that viral gene expression appeared to remain in the central area following infection by SeV/ΔM, with no strong TUNEL staining in any area (Figure 4b). Furthermore, we quantitatively showed that MMP-subII SeV/ΔM strongly inhibited HT1080 tumor growth in the mice, whereas neither of the control viruses caused marked inhibition (Figure 4c).

The same *in vivo* experiment was performed using the other tumor line, SW620, that is low in MMP expression. After the injection of MMP-subII SeV/ΔM into the SW620 tumor, GFP expressed from MMP-subII SeV/ΔM spread slightly in the tumor (Figure 5a). Also, SW620 tumor growth in the mice was very slightly inhibited by MMP-subII SeV/ΔM injection (Figure 5c). However, immunohistochemical analysis showed that neither GFP- nor TUNEL-positive cells were significantly

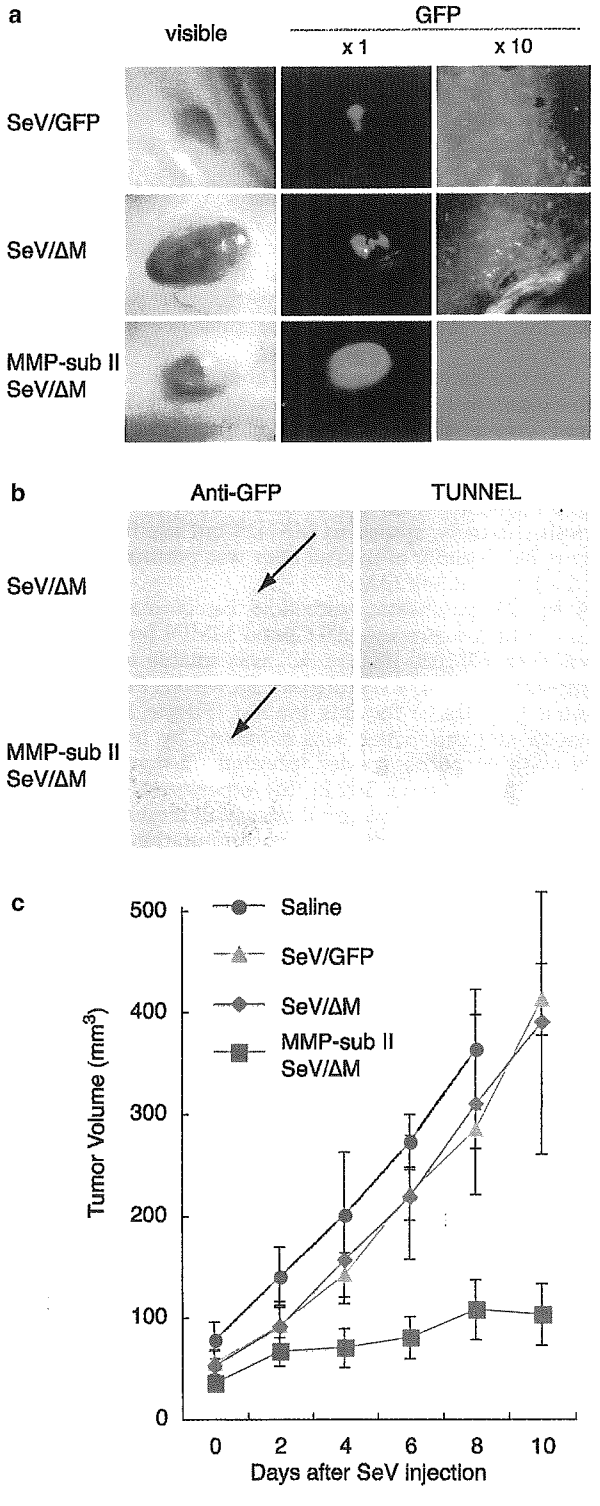

Figure 4 Inhibition of HT1080 tumor growth in nude mice by MMP-subII SeV/ΔM. (a) Wild-type SeV, parental SeV/ΔM and MMP-subII SeV/ΔM (5×10^7 CIU/50 μl, each) were injected once directly into the subcutaneously transplanted HT1080 tumors in nude mice. After 2 days, light emission from the GFP protein in tumor cells was observed externally under a fluorescence microscope. Magnification, × 1 and × 10. (b) TUNEL staining of cells. Arrows indicate the sites of virus injection. (c) Tumor volumes were calculated using the formula (volume = π/6 × abc:(length (a), width (b), height (c)) and were expressed as the average (n = 7). Significantly different volumes were observed between the group administered MMP-subII SeV/ΔM and all other groups (P < 0.05, Student's t-test) at 8 and 10 days after injection.

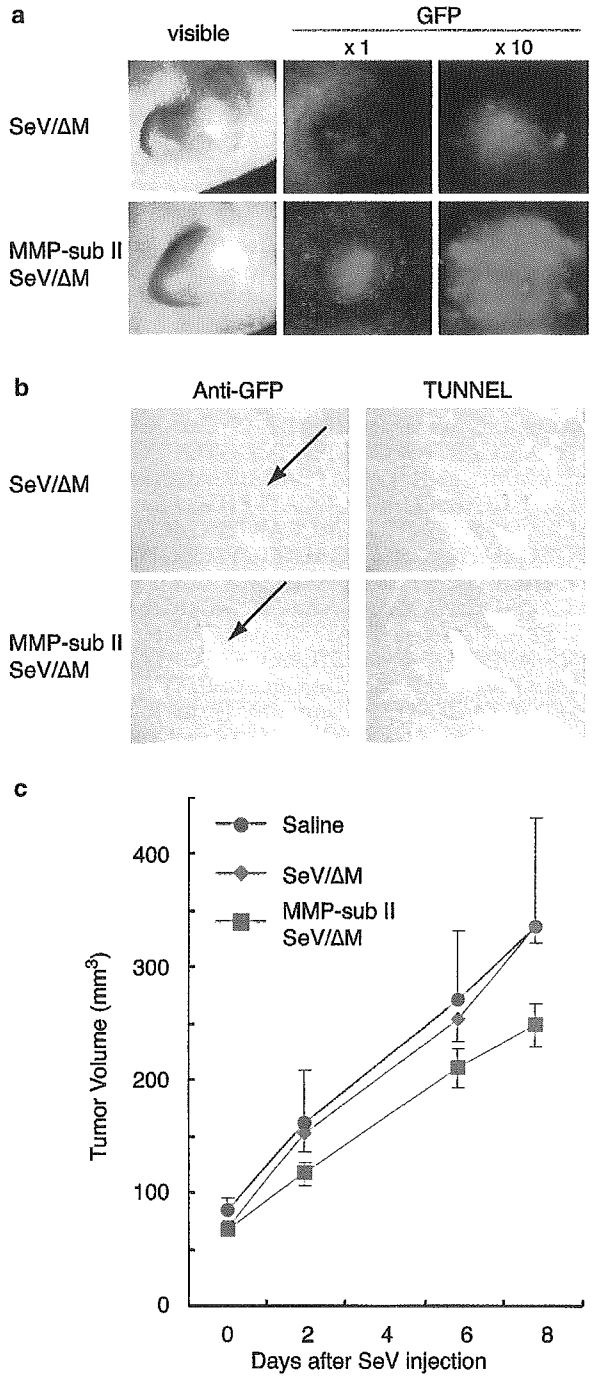

Figure 5 Restricted spreading of MMP-subII SeV/ΔM in the SW620 tumors in vivo. (a) Parental SeV/ΔM and MMP-subII SeV/ΔM (5×10^7 CIU/50 μl, each) were injected once directly into the subcutaneously transplanted SW620 tumors in nude mice. After 2 days, light emission from the GFP protein in tumor cells was observed externally under a fluorescence microscope. (b) TUNEL staining of cells. Arrows indicate the sites of virus injection. (c) Tumor volumes expressed as the average ($n=5$). No significant difference was observed between the respective groups.

increased in either MMP-subII SeV/ΔM- or SeV/ΔM-injected SW620 tumors (Figure 5b). As the expression of MMPs of SW620 cells is enhanced after the engraftment,[31] MMP-subII SeV/ΔM would spread slightly in SW620 *in vivo*. However, viral spreading was limited in SW620 even *in vivo*, as the expression level of MMPs in SW620 was significantly lower than that in HT1080. In addition, no spreading was observed when MMP-subII SeV/ΔM was administered in normal tissues such as lung, trachea, nasal cavity and skin and muscle (data not shown). These results demonstrate the selective spreading of MMP-subII SeV/ΔM in the tumors that are high in MMP expression *in vivo*.

Discussion

We report here the generation of oncolytic recombinant SeVs that selectively target human tumor cells expressing MMP2, MMP9 and uPA by changing the tryptic cleavage-activation signal to a signal susceptible to those tumor-associated proteases. An additional alteration that we made was to delete the viral M gene because this conferred on SeV the capacity to spread extensively from cell to cell with the induction of massive syncytia, and at the same time rendered the virus nontransmissible to distant tissues and thus safer than the wild type.[22] Another M gene-deleted paramyxovirus, measles virus, was reported to propagate efficiently from cell to cell in an animal model (mouse brain).[32] The successful recovery of MMP-subII SeV/ΔM that selectively targeted and lysed MMP2- and MMP9-expressing tumor cells is of particular interest as these proteases represent two of a few proteases that have shown strong association with tumor metastasis among a large variety of MMP protease family members. Further improvement of MMP-subII SeV/ΔM is now being attempted by increasing the fusogenic activity and/or by the construction of vectors carrying therapeutic gene(s) such as immuno- and suicide genes, since MMP-subII SeV/ΔM did not succeed in complete eradication of HT1080 tumors in our experiments.

Johnson *et al*[33] succeeded in targeting the cytotoxicity of a fusogenic viral glycoprotein to an MMP-expressing glioma cell line. The strategy involved conditional inactivation and activation of the gibbon leukemia virus envelope protein. It was inactivated due to an extra peptide attached to its N-terminus with an MMP-cleavable linker and became active (fusion competent and cytotoxic) only when it encountered an MMP-expressing cell that removed the peptide. However, their studies published so far were limited to plasmid-based expression of the engineered glycoproteins and not expression in the context of the viral replicon of either a full-length or defective genome. Thus, their studies and ours are largely different from each other in both conceptual and practical details, although both depend on MMP–substrate interaction. Aside from this, the measles virus was modified for the targeting to tumor cells by linking a single-chain antibody recognizing tumor-cell-specific proteins to its envelope protein.[34,35] Although it affects only targeting and not tumor-cell-specific spreading, such modification (for targeting) could be combined with one of our vectors (for tumor-cell-specific spreading), such as MMP-subII SeV/ΔM.

Our strategy is theoretically and practically applicable to a large number of other paramyxoviruses, including NDV, which is now under extensive clinical trials based on its natural preference for cancer cells, and members of other virus families, such as influenza viruses, because they share the same cleavage-activation mechanism of the envelope proteins for initiation and spread of infection[12,36] and because recombinant DNA technologies have been established for them. The matrix gene deletion from the genome may not always be necessary but would be advantageous when targeting a tumor mass.

A key issue in the application to actual medicine of the findings obtained here using clonal tumor cell lines is to 'individualize'[37] the approach by identifying the protease species in actual tumors of individual patients. There is increasing evidence suggesting that the higher the protease level at diagnosis the worse the prognosis,[27,38] and we have shown here that there would be a critical threshold of the enzyme levels required for virus activation. Thus, not only the species but also the amounts of the proteases involved in the actual tumor will have to be included in 'individualization'. The protease species as well as their levels would be relatively easy to determine by the analysis of biopsy materials from individual patients by conventional immunohistochemistry, ELISA and other assay methods.[39–42] Once they are determined, it would also be easy to generate appropriate protease activation mutants of SeV and related envelope viruses. The safety of recombinant SeVs is another key issue. They so far do not appear to cause any serious problems or symptoms in rhesus monkeys after administration in various ways, including intramuscular injection as well as intranasal inoculation (unpublished data).

Materials and methods

Cell lines

The cell lines HT1080 (human fibrosarcoma), Panc I (human pancreatic epithelioid carcinoma), SW620 (human lymph node colorectal adenocarcinoma) and LLC-MK_2 (monkey kidney) were purchased from ATCC (Manassas, VA, USA). The human stomach cancer cell line MKN28 was purchased from the Institute of Physical and Chemical Research (Tsukuba, Japan).

Construction of plasmids expressing SeV F and HN proteins as a fusion polypeptide with modified cleavage site sequences in the F protein

The F protein is a direct mediator of cell fusion but requires coexpression of the receptor-binding HN protein from a second plasmid for the actual expression of fusion capacity.[43,44] Alternatively, we found that the expression of the F and HN proteins as a single polypeptide connected by 30 unrelated amino acids was also fusion inducing (details will be published elsewhere). The plasmid for this F–HN-coupled expression was constructed by the amplification of the F and HN genes in the plasmid pSeV18+b[18] by polymerase chain reaction and cointroduction of the amplified genes into the mammalian expression vector pCAGGS.[45] The primer pair specific for the F gene was Fu-F (5′-ATCCGAATTCAGTTCAATGACAGCATATATCCAGAG-3′) and Fu-R (5′-ATCCGCGGCCGCCGGTCATCTGGAT TACCCATTAGC-3′), and that for the HN gene was HN-F(+linker) (5′-ATCCGCGGCCGCAATCGAGGGA AGGTGGTCTGAGTTAAAAATCAG GAGCAACGAC GGAGGTGAAGGACCAGAGGACGCCAACGACCCA CGGGGAAAGGGGTGAACACATCCATATCCAGCCA TCTCTACCTGTTTATGGACAGAGGGTTAGG-3′) and HN-R (5′-ATCCGCGGCCGCTTAAGACTCGGCCTTG CATAA-3′). The amplified F and HN genes were digested with *Not*I and *Xho*I (for the F gene) or *Not*I (for the HN gene) and ligated together with pCAGGS digested with *Not*I and *Xho*I. Conversion of amino-acid sequences around the cleavage site of F protein was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The primer pairs used for mutagenesis were pMMP #1-F (5′-CAAAATGCCGGT GCTCCCCcGTtGgGATTCTTCGGTGCTGTGATT-3′) and pMMP #1-R (5′-AATCACAGCACCGAAGAATCc CaACgGGGGAGCACCGGCATTTTG-3′) for MMP-subI; pMMP #2-F (5′-CTGTCACCAATGATACGACACAAA ATGCCccTctTggCatGaCGAGtTTCTTCGGTGCTGTGAT TGGTACTATC-3′) and pMMP #2-R (5′-GATAGTACCA ATCACAGCACCGAAGAAaCTCGtCatGccAagAggGG CATTTTGTGTCGTATCATTGGTGACAG-3′) for MMP-subII; pMMP #3-F (5′-GTCACCAATGATACGACACA AAATGCCccTctTggCctGtgGgcaTTCTTCGGTGCTGT GATTGGTACTATC-3′) and pMMP #3-R (5′-GATAG TACCAATCACAGCACCGAAGAAtgcCcaCagGccAa gAggGGCATTTTGTGTCGTATCATTGGTGAC-3′) for MMP-subIII; pMMP #4-F (5′-CTGTCACCAATGATAC GACACAAAATGCCccTctTggCCtGggGttA TTCTTCGGT GCTGTGATTGGTACTATCG-3′) and pMMP #4-R (5′-CGATAGTACCAATCACAGCACCGAAGAATaaCccCa GGccAagAggGGCATTTTGTGTCGTATCATTGGTGA CAG-3′) for MMP-subIV; and puPA-F (5′-GACACAA AATGCCGGTGCTCCCgtGggGAGATTCTTCGGTGCTG TGATTG-3′) and puPA-R (R: 5′-CAATCACAGCACC GAAGAATCTCccCacGGGAGCACCGGCATTTTGTGTC-3′) for uPA-sub. Lower case letters in the primer sequences described above represent the substituted nucleotides.

Construction and amplification of F-modified SeV/ΔM

The parental plasmid SeV/ΔM, which is pSeV18+/ΔM-GFP with a bacteriophage T7 promoter,[22] was digested with *Sal*I and *Nhe*I, and the resulting fragment (9.6 kb) was subcloned into LITMUS 38 (New England Biolabs. Beverly, MA, USA). Site-directed mutagenesis was performed on this plasmid. Recovery and amplification of F-modified SeV/ΔM from cloned cDNA were performed essentially as described,[19–21] using recombinant vaccinia virus expressing T7 RNA polymerase[46] and the SeV M protein-expressing LLC-MK_2 packaging cell line.[22] Type IV collagenase (5 U/ml) of *Clostridium histolyticum* (ICN, Aurora, OH, USA) and 7.5 μg/ml of trypsin were added to the medium to activate the modified F protein in the cases of MMP-subII SeV/ΔM and uPA-sub SeV/ΔM, respectively. The amplification of recovered viruses was also carried out using the M-expressing LLC-MK_2 packaging cell line.[22]

Cell fusion analysis by transfection of F/HN-expressing plasmids

The above-described expression vector, pCAGGS, carrying the individual F/HN fusion gene with a modified

cleavage sequence in the F protein was transfected together with pCAGGS/GFP expressing the enhanced GFP into MMP-expressing HT1080 cells using the transfection reagent FuGENE6 (Roche, Basel, Switzerland). Syncytia formation was observed 48 h after transfection under a fluorescence microscope (Leica, Wetzlar, Germany).

Cell fusion analysis following infection with F-modified SeV/ΔM

LLC-MK_2 cells infected with the parental SeV/ΔM, MMP-subII SeV/ΔM or uPA-sub SeV/ΔM at an MOI of 0.02 were cultured in serum-free MEM containing MMP2, MMP9 or uPA (0.1 μg/ml, each) or trypsin (7.5 μg/ml). For the inhibition study, the MMP-inhibitor 1, 10 phenanthroline was added to the medium at a final concentration of 12.5 μM. To quantify the fused cells, the number of nuclei in cells forming syncytia was counted after culturing in serum-free MEM containing a series of concentrations of collagenase or MMP-9. Tumor cell lines HT1080, Panc I, SW620 and MKN28, which express the proteases indicated in the text endogenously, were also infected with these viruses at an MOI of 0.02, and cultured in the medium containing 1% heat-inactivated fetal bovine serum. In both of these exogenous and endogenous protease requirement studies, cell fusion was observed under a fluorescence microscope (Leica, Wetzlar, Germany) 4 days after infection.

Western blotting

SDS-PAGE and Western blotting were carried out according to the method described.[22] A rabbit polyclonal anti-F_1 antibody was raised against three mixed synthetic peptides of SeV F protein, that is, (1) FFGAVIGT + Cys, (2) EAREAKRDIALIK and (3) CGTGRRPISQDRS, corresponding to amino acids 117–124, 143–155 and 401–413 of SeV-F, respectively. Immunization was carried out after conjugation of the peptides to keyhole limpet hemocyanin. Incubation of the Western blotting membrane with the anti-F_1 primary antibody was followed by incubation with the second antibody, anti-rabbit IgG conjugated with HRP (ICN, Aurola, OH, USA). The proteins on the membrane were detected by a chemiluminescence method (ECL Western blotting detection reagents; Amersham Biosciences, Uppsala, Sweden).

In vivo study

HT1080 or SW620 cells (5×10^6 cells/mouse) were injected subcutaneously into the right back of Balb/c nude mice (Charls River Co, Kanagawa, Japan). The engrafted tumors developed to 3–8 mm in diameter by 8–9 days after transplantation. The mice were then divided into four groups ($n = 5$ or 7/group) that received intratumoral injection of saline, wild-type SeV, parental SeV/ΔM or MMP-subII SeV/ΔM (5×10^7 cell infectious unit (CIU)) in a 50 μl volume using a 26-gauge needle.

Immunohistochemistry

For anti-GFP antibody staining (Molecular Probes, Eugene, OR, USA) and Apotag peroxidase *in situ* apoptosis detection (Intergen Co., Norcross, GA, USA), tumors were removed, fixed with 4% paraformaldehyde and embedded in paraffin 2 days after injection of each virus. In all, 5-μm-thick sections were used for stainings.

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Sendai virus for gene therapy and vaccination

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Sendai virus (SeV), also known as hemagglutinating virus of Japan (HVJ), is a negative-strand RNA virus and a member of the paramyxovirus family. The capacity of SeV to enhance fusion of lipid vesicles and cell membranes was recognized in the early 1980s, and has since been utilized in the form of HVJ-liposomes. More recently, advances in reverse genetics have allowed the construction of a new class of RNA vector with cytoplasmic expression, namely non-transmissible recombinant SeV. Extensive preclinical studies have shown the therapeutic potential of this vector for use in airways, brain and for ischemic diseases and tumors. In addition, the potential of SeV vector for vaccination has been explored. Data on the use of SeV for gene therapy and vaccination since January 2004 are reviewed and recent improvements in SeV vectorology are discussed.

Keywords Gene therapy, gene transfer, hemagluttinating virus of Japan-liposome, murine parainfluenza virus, Sendai virus, vaccination

Introduction

Sendai virus (SeV) is a rodent pathogen that causes experimental pneumonia in infected animals, but is thought to be non-pathogenic in humans. The virus infects most mammalian cells, including human, and replicates in the cytoplasm. Virus replication is a pre-requisite for gene expression. During its lifecycle, the virus does not go through a DNA intermediate and does not enter the nucleus. Thus, there is no risk of genome integration and insertional mutagenesis, an important feature when considering SeV for human administration.

The SeV genome (15.4 kb) is non-segmented and linear, and contains six major genes arranged in tandem (Figure 1A). The nucleoprotein (NP), the phosphoprotein (P) and the large protein (L) complex the RNA genome into a tight nucleoprotein (RNP) complex. The viral envelope contains two proteins, the hemagglutinin-neuraminidase (HN) and the fusion (F) protein, which are central to virus uptake into cells. The matrix (M) protein is crucial for virus assembly at the cell membrane and for efficient budding. Reporter and therapeutic genes can be inserted at various positions between these genes throughout the genome (Figure 1A). In general, gene expression is higher for genes that are inserted closest to the 3' end of the virus and lower for genes inserted further towards the 5' end. The insertion site-dependent gene expression is termed 'polar effect' and is caused by polymerase drop-off when moving from 3' to 5' along the viral genome. By using this effect, a set of vectors expressing various levels of transgene has been established.

Recombinant SeV efficiently transduces a variety of tissues and cell types *in vivo* and *ex vivo*

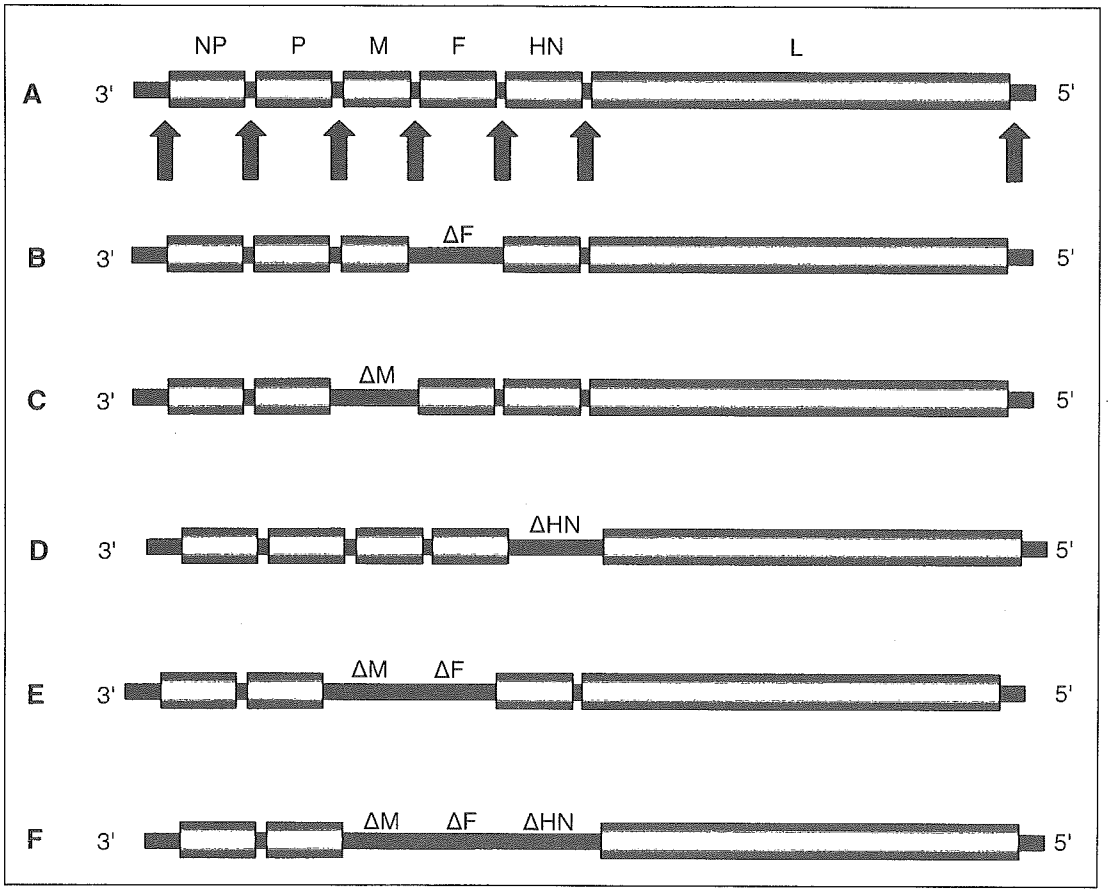
Lung

SeV is the most efficient vector for transfection of airway epithelial cells *in vivo*, largely because of its ability to overcome some of the extra and intracellular barriers. Importantly, in contrast to most other viral gene transfer agents, the receptors for SeV, cholesterol and sialic acid are present at the apical surface of airway epithelial cells and are therefore accessible after topical administration. Most early research was carried out with transmission-competent SeVs, which carried reporter or therapeutic genes in addition to all viral genes (Figure 1A). These primitive-type vectors enter cells and replicate, and virus particles with the capacity to re-infect additional cells bud out. Pinkenburg *et al* demonstrated that transmission-competent SeV is generated after transduction of human bronchial epithelial cells grown at an air-liquid interphase, but that these cells become non-permissive to SeV transfection for a while after first application [1], perhaps due to loss of cellular virus receptors. However, potential virus shedding and transfection of other organs are concerns.

In an attempt to improve the safety potential of SeV for clinical use, DNAVEC Corp described the successful production of second-generation SeV in 2000 [2]. In these viruses, the F protein, essential for virus entry, has been deleted (ΔF/SeV) (Figure 1B), but is supplied *in trans* during virus production in cell culture [2]. ΔF/SeV enters cells and replicates, but budding virus-like particles do not carry the F protein and, therefore, are unable to transduce neighboring cells. More recently, we assessed ΔF/SeV in murine airway epithelium *in vivo* and primary human nasal epithelium *ex vivo* and, importantly, showed that this new generation of ΔF/SeV transduced airway epithelial cells as efficiently as the primitive wild-type virus-based vector [3••]. However, gene expression in the rodent airway was transient (less than 2 weeks) for the primitive type of SeV. Furthermore, as with other viral vectors, there is induction of antibody and T-cell-mediated immune responses in mice [4] and repeated transduction at intervals shorter than several months has not been successful for murine airway epithelial cells [5].

However, in contrast to humans, mice are natural hosts for SeV infections and may therefore respond to SeV differently. SeV-induced immune responses should therefore be studied in animal models that are not natural hosts for SeV. We recently demonstrated that ΔF/SeV transduces sheep lung

Figure 1. Wild-type and modified Sendai virus genomes.



(**A**) Wildtype SeV, (**B**) F gene-deleted (ΔF) SeV, (**C**) M gene-deleted (ΔM) SeV, (**D**) HN gene-deleted (ΔHN) SeV, (**E**) F, M gene-deleted (ΔF, ΔM), (**F**) F, M, HN gene-deleted (ΔF, ΔM, ΔHN) SeV. Arrows indicate positions in which reporter and therapeutic genes can be inserted. **F** fusion protein, **HN** hemagglutinin-neuraminidase protein, **L** large protein, **M** matrix protein, **NP** nucleoprotein, **P** phosphoprotein.

efficiently [6], and repeat administration of SeV is currently being carried out.

Muscle-including angiogenesis

In the past, several research groups, including our own, have assessed the capacity of SeV to transduce skeletal and heart muscle, with the aim of producing recombinant protein for local effects such as angiogenesis mediated by vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF), or to promote muscle growth using insulin-like growth factor (IGF). Muscle has also been used as a factory for secreted proteins such as interleukin (IL)-10.

One outcome of these approaches is that Kyoshu University Hospital has submitted a clinical trial protocol involving the use of ΔF/SeV-FGF-2 in therapeutic angiogenesis for critical limb ischemia (the trial protocol is currently only available in Japanese) [7]. In preclinical studies using mouse and rabbit ischemia models, the recombinant SeV vector was injected into skeletal muscles, resulting in highly efficient rescue of limb loss and recovery of new blood flow. Topical high-level expression of FGF-2 in the muscle induced upregulation of VEGF, hepatocyte growth factor (HGF) and VEGF-A, -C and -D that appeared to lead to functional angiogenesis and lymphogenesis [8]. The therapeutic effects were superior to naked VEGF and HGF in these models [9]. Preliminary agreement to the protocol has been achieved, and the trial will be initiated this year. Furthermore, China's largest pharmaceutical company has licensed this vector to begin a clinical trial for ischemic limb therapy in China [10].

Brain

SeV carrying a green fluorescent protein (GFP) gene transfected ependymal cells efficiently after intra-ventricular injection [11]. In addition, SeV carrying neurotrophic genes prevented delayed neuronal death when administered 4 days before induction of transient ischemia in gerbils [11]. More recently, the same researchers investigated the effects of post-ischemic administration of SeV carrying a variety of neurotrophic factor genes [12]. Importantly, post-trauma administration of SeV, a clinically more relevant approach, also prevented neuronal death in gerbils. The new-generation vectors (ΔF/SeV and the further derivative ΔFΔM/SeV, in which both F and M genes are deleted) showed far lower inflammatory side effects in brain. Thus, these advanced-type vectors might be more suitable for the treatment of central nervous system diseases [13••]. More recently, Iwadate *et al* reported the use of ΔFΔM/SeV to treat