ルートも報告されている[8,9]．これらの感染経路を遮断して，標的細胞特異的に結合するリガンド等をウイルス表面タンパク質のファイバーやヘキソン，protein IX 領域（ヘキソンとヘキソンの間に存在するタンパク質）に付与すれば，ターゲティング能をもったアデノウイルスベクターが開発できる（著者らはこれらの領域に簡便に外来リガンドを挿入する技術も開発済みである[10]）．著者らはファイバーノブ，シャフト，ペントンベースの3領域を同時に改変したトリプル改変ベクターを開発し（図3），このベクターが肝臓をはじめとする *in vivo* での遺伝子発現能をほとんど消失していることを明らかにしている[11,12]．現在このトリプル改変ベクターに，標的細胞特異的に高親和性を示すリガンドを付与することでターゲティング能をもったアデノウイルスベクターの開発を進めている．

3. マイクロ RNA による遺伝子発現制御システムを搭載したアデノウイルスベクターの開発

近年，タンパク質をコードしない non-coding RNA であるマイクロ RNA（miRNA）とよばれる 21-23 塩基の小さな RNA が発生・分化等の過程に多大な影響を及ぼすことが報告され注目されている．miRNA は細胞特異的に発現し，標的遺伝子のメッセンジャー RNA の 3'非翻訳領域に結合することにより，複数（10～100 程度の遺伝子）の標的遺伝子の翻訳を厳密に抑制する．最近著者らは，miRNA による遺伝子発現制御システムを利用して，特定の細胞で目的遺伝子の発

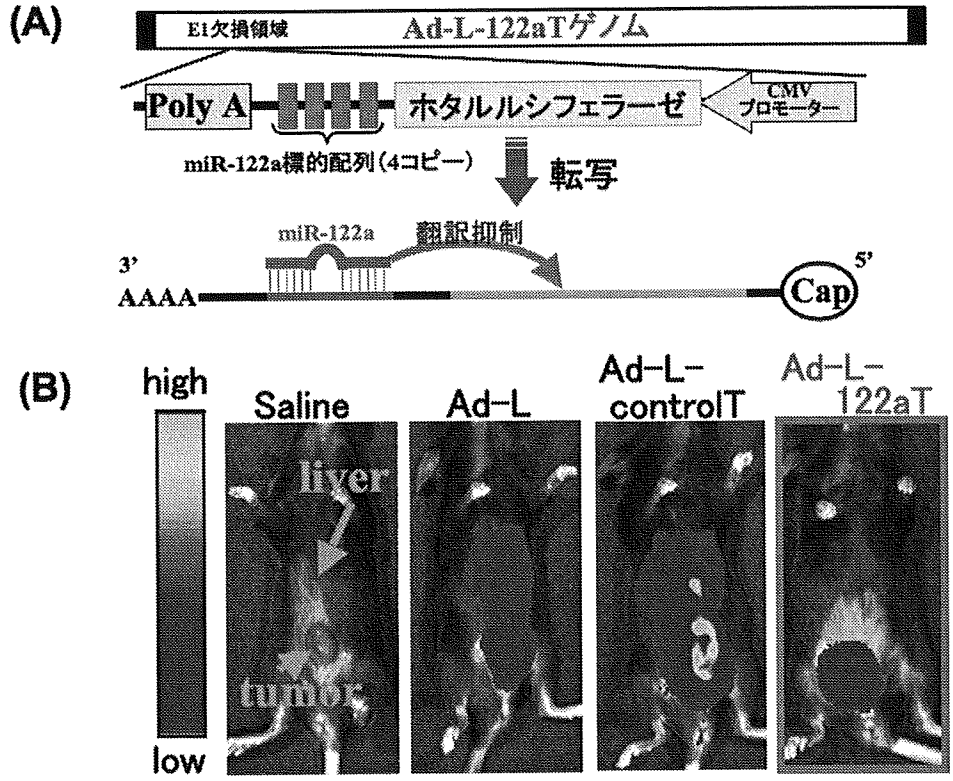

図4　マイクロ RNA による遺伝子発現制御システムを搭載したアデノウイルスベクター

ルシフェラーゼ遺伝子の下流に肝臓特異的な miRNA である miR-199a の標的配列を 4 コピー挿入したアデノウイルスベクター（Ad-L-199aT）(A) は，担癌マウスの腫瘍内に投与した場合，腫瘍特異的な遺伝子発現が認められるが (B)（*in vivo* イメージング装置で遺伝子発現を検討），コントロール配列を付与したベクター（Ad-L-controlT）や miRNA の標的配列を付与していない従来のベクター（Ad-L）は，腫瘍のみならず，（腫瘍から漏れ出たベクターが肝臓に移行して）肝臓での遺伝子発現も認められる．（p.8 カラー図参照）

現をオフにするアデノウイルスベクター発現制御系を開発した[13].

アデノウイルスベクターを全身投与した場合，前述したように肝臓で主に遺伝子発現が認められるが，肝臓特異的なmiRNAであるmiR-199aの認識配列を目的遺伝子の3'非翻訳領域に挿入することにより，肝臓での遺伝子発現を100分の1程度に抑えられることを見いだした[13]（**図4**）．herpes simplex virus thymidine kinase（HSVtk）遺伝子を用いた自殺遺伝子治療においては，ベクターを腫瘍内に投与した場合においても，腫瘍部位から漏れ出たベクターが肝臓をはじめとする正常組織に移行し，組織障害を起こすことが問題となっているが，HSVtk遺伝子の3'非翻訳領域にmiR-199aの認識配列を付与した場合，高い抗腫瘍効果を維持したまま肝障害を劇的に軽減させることが可能であった[13]．目的組織での選択的な遺伝子発現を得るためには，組織特異的プロモーターが汎用されるが（一般的に組織特異的プロモーターは転写活性が低いことが課題となっている），miRNAによる遺伝子発現制御システムは特定の組織・細胞でのみ遺伝子発現をオフにする新しい遺伝子発現制御法であり，遺伝子治療への応用だけでなく，基礎研究分野においても強力な技術になると期待される．

V. 免疫反応の抑制が可能なアデノウイルスベクターの開発

アデノウイルスベクターを生体に投与した場合，①数時間以内に生じる自然免疫，②7～10日以内に生じるベクターにより産生されたウイルスタンパク質（および外来遺伝子産物）に対する細胞性免疫，そして，③ウイルスタンパク質に対する液性免疫の3種類の免疫反応が起こる．これらの免疫反応を克服できるベクターや投与方法（投与戦略）の開発は，安全性や有効性の高い遺伝子治療の実現にとって必要不可欠である．

ウイルスを始めるとする異物を生体に投与すると，生体は即座にインターフェロンやサイトカイン，ケモカイン等を産生し，それらの異物を排除しようとする自然免疫が発動する．アデノウイルスベクターを生体に投与した場合にも自然免疫の活性化は生じ，1999年に米国で起こったオルニチントランスカルバミラーゼ欠損症に対するアデノウイルスベクターを用いた死亡事故では，自然免疫の過剰な活性化が原因と考えられている．アデノウイルスベクターをマウスに全身投与した場合には，IL-6，IL-12等の産生が投与3時間以内に生じる．全身投与されたベクターの90％以上は肝臓（実質細胞とクッパー細胞をはじめとする非実質細胞）に移行するが，多くの炎症性サイトカインは肝臓（のクッパー細胞）ではなく，主に脾臓で分泌される[14,15]．興味深いことに，前述したポリリジンでファイバーを修飾したアデノウイルスベクターでは，脾臓への移行性が減少する結果，血中へ分泌されるIL-6は約1/4にまで減少し（肝臓をはじめとする各臓器での遺伝子発現は従来型ベクターと同等以上に起こる）[14]，安全性が高いことが明らかとなった．アデノウイルスベクター投与に伴う自然免疫に関与する生体側因子やウイルス側因子，シグナル伝達機構の解明は，自然免疫応答の克服に向けて重要な研究課題であり，著者らのグループでは，各種改変アデノウイルスベクターやノックアウトマウス，RNAi技術，マイクロアレイ解析を通して，これらの研究を進めている．

一方，アデノウイルスベクター投与後の細胞性免疫の活性化については，従来のアデノウイルスベクターはE1領域を除去することで，ウイルスタンパク質の産生が生じないように設計されているが，E1非依存的に他のウイルスタンパク質の合成がわずかながら起こり，これが免疫系のターゲットとなることが原因と考えられている．この問題を克服するために，ウイルスコード遺伝子を全て除去したgutted アデノウイルスベクターが開発されており，本ベクターを用いた場合は，通常のマウスにおいても長期間の遺伝子発現が認められる[16]．従来は，高タイターのgutted アデノウイルスベクターの産生が技術的に難しいのが課題点であったが，最近はヘルパーウイルスとパッケージング細胞の改良により，その問題点は一部克服されつつある[17]．

液性免疫の制御に関しては，主要カプシドタンパク質のヘキソンに対する抗体が液性免疫の主体であることから，ヘキソン改変ベクターが，この問題を克服するために開発されている[18]．また，異なった血清型

(11型や35型)[19,20]や異なった種（チンパンジー，イヌ，ヒツジ，トリ，ウシ，マウス等）に属するアデノウイルスベクターが開発されており，これらのベクターでは，抗ヒト5型アデノウイルス抗体存在下でも遺伝子導入活性を示す．これらのベクターの中には，CAR以外の受容体を認識して感染するものもあり，感染域を変えることも同時に可能になる．

VI. その他のアプローチによるアデノウイルスベクターの高機能化

従来のアデノウイルスベクターではE1欠損領域の1ヵ所にしか外来遺伝子を挿入できなかったが，著者らはE1欠損領域に加え，E3（欠損）領域，並びにE4遺伝子と3'ITR（inverted terminal repeat）の間の領域にも同時に外来遺伝子を挿入することが可能なダブル・トリプル遺伝子発現系を搭載したアデノウイルスベクターを開発した[21,22]（**図5**）．これにより，複数の外来遺伝子を単一のアデノウイルスベクターに搭載することが可能となり，複数のベクターの共投与の場合と異なり，作用させるベクター量も最小限で済ませることができるようになった．これらのベクターの応用例として，テトラサイクリン耐性オペロンを利用した遺伝子発現制御系を単一のベクターに搭載させたシステムを開発し，目的遺伝子の発現を，正あるいは負に自在に制御することが可能となった[21,22]（**図5A**）．さらに，E3欠損領域にGFP（green fluorescence protein）発現単位を挿入し，E1欠損領域に目的遺伝子発現単位を挿入することで，遺伝子導入された細胞をGFPで視覚的に同定しながら目的遺伝子の機能を検討する実験系への利用等が可能となった[23]（**図5B**）．

一方，短鎖の二本鎖RNA（small interfering RNA: siRNA）発現カセットをアデノウイルスベクターに搭

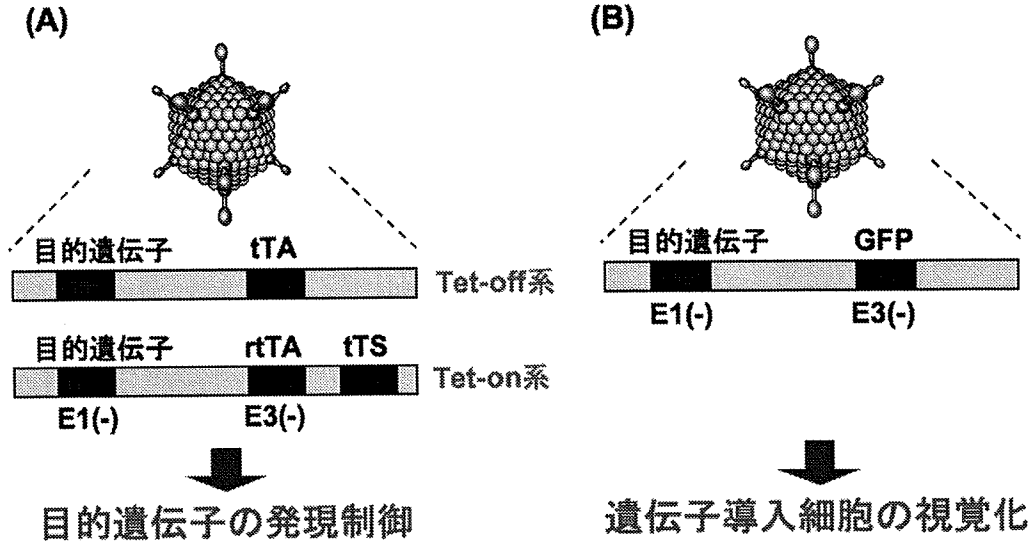

図5　複数の外来遺伝子の搭載が可能なアデノウイルスベクター

(A) E3欠損領域にtet-off系の転写活性化因子tTAを発現するカセットを有し，E1欠損領域にテトラサイクリン誘導性プロモーター下に目的遺伝子を発現するカセットを有したアデノウイルスベクター，あるいはE3欠損領域にtet-on系の転写活性化因子rtTAを発現するカセットを，E4領域と3'ITR領域の間にテトラサイクリン制御性サイレンサーのtTSを発現するカセットを挿入し，E1欠損領域にテトラサイクリン誘導性プロモーター下に目的遺伝子を発現するカセットを挿入したアデノウイルスベクターでは，ドキソサイクリン（テトラサイクリンの誘導体）濃度に依存した目的遺伝子の発現制御が可能になる．

(B) E3欠損領域にGFPを発現するカセットを有し，E1欠損領域に目的遺伝子を発現するカセットを有したアデノウイルスベクターでは，遺伝子導入された細胞を視覚化することが可能になる．いずれのベクターにおいても，ユーザーはE1欠損領域に挿入する遺伝子だけを入れ替えれば使用できる．

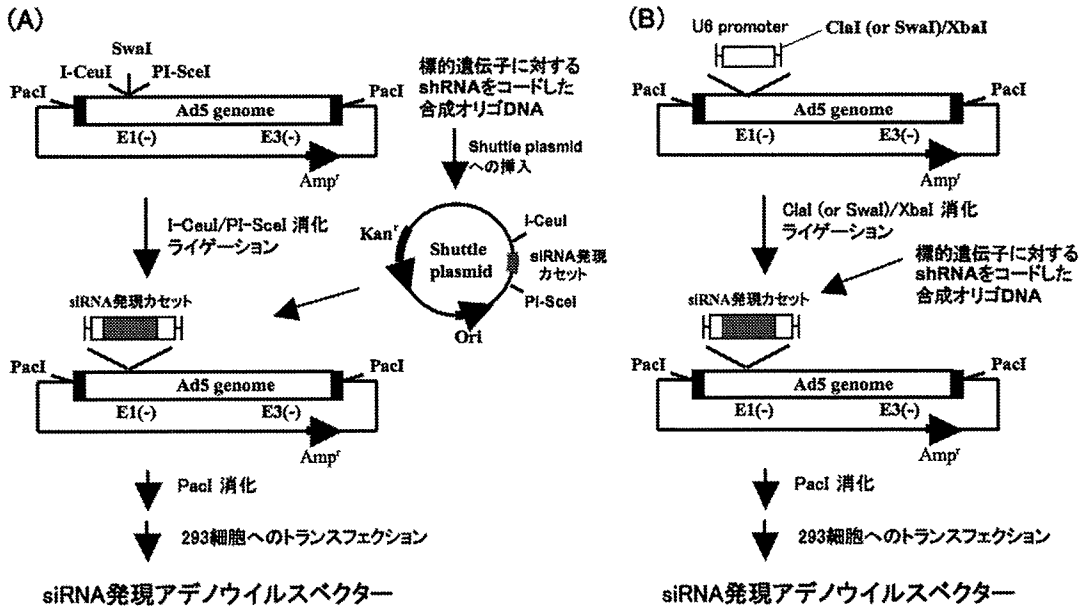

図6　ベクタープラスミドへの直接クローニングによる迅速なsiRNA発現アデノウイルスベクター作製法

従来の *in vitro* ライゲーションに基づくベクター作製法 (A) では，目的遺伝子のsiRNA配列に相当する合成オリゴDNAをまずシャトルプラスミドに挿入し，次にベクタープラスミドに組み換える必要があるが，直接クローニングによるベクター作製法 (B) では，目的遺伝子のsiRNA配列に相当する合成オリゴDNAを直接ベクタープラスミドに挿入でき，極めて簡便である．

載させれば，目的遺伝子の発現を特異的にノックダウン可能になるが，著者らはsiRNA発現アデノウイルスベクターの作製法を飛躍的に簡便化することにも成功した[24]（詳細は**図6**を参照）．新しい方法では，siRNA配列に相当する合成オリゴDNAをアデノウイルスベクタープラスミドにライゲーションし，これを293細胞にトランスフェクションするだけで目的のベクター作製が可能となり，極めて有用である．

おわりに

本稿で紹介したように，遺伝子治療への応用を通して発展してきたアデノウイルスベクターの改良研究は，ベクター作製の簡便化や高機能化により生命科学研究を進めていく上での必須の基盤技術になっている．著者らが開発した新しい遺伝子導入技術が，広く生命科学研究に利用されることを期待している．

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技術

アデノウイルスベクターの作製・増幅・精製法

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実験のコツと注意点

従来はアデノウイルスベクターの作製は煩雑であり一部の研究室でのみ使用できる特殊な技術であったが，現在では，種々の簡便なアデノウイルスベクター作製法が開発され，基本的な分子生物学の知識・技術を習得していれば，アデノウイルスベクターの取り扱いは比較的容易になった．アデノウイルスベクターは，用いるベクターのバックボーン（構造）によりE1やE3領域の欠損サイズが異なっており，それによりベクターを増幅する際のコツも微妙に異なることに注意していただきたい．本稿では，著者らが開発し，現在クロンテック社より市販されている*in vitro*ライゲーションによるプラスミド構築に基づいたアデノウイルスベクター作製法について述べるが，紙面の都合上，ベクターの増殖・精製法（のコツ）に絞って解説する．なお，アデノウイルスベクターの諸性質や，機能面で優れた改良型アデノウイルスベクターの開発研究については，本誌1月号の著者の総説を参照していただきたい．

原　理

従来，アデノウイルスベクターはE1遺伝子を発現するパッケージング細胞である293細胞内での相同組換えを利用して作製されていた（種々のアデノウイルスベクター作製法については，著者らの総説[1]を参照して頂きたい）．即ち，目的遺伝子の発現単位をE1領域の上流と下流のゲノムDNAの一部の間に組み込んだプラスミド（あるいはコスミド）と，E1領域を除くウイルスゲノム全長（あるいはE1領域を除くウイルスゲノム全長を有するプラスミド）を293細胞にコ・トランスフェクションし，E1領域前後の相同な遺伝子配列領域間での相同組換えを期待し，E1領域を外来遺伝子に置き換えるというものである．しかしながら，動物細胞内での相同組換えの効率が良くないことや，煩雑な作業を必要とすること，293細胞の染色体に組み込まれているE1遺伝子とも相同組換えを起こし，高頻度に野生型ウイルスからなるクローンも生成するといった問題点があり，アデノウイルスベクターの作製はそれほど容易なものではなかった．これはアデノウイルスのゲノムサイズが約36 kbと巨大なため単一の制限酵素切断部位を得ることが困難であり，プラスミド構築に基づいて簡単にE1領域を外来遺伝子に置き換えるような方法が開発されていなかったことに起因している．

著者らは，これまであまり用いられることのなかったI-CeuIとPI-SceI（これらの酵素はアデノウイルスゲノムを切断しない）という少なくともそれぞれ9～10，11 bpを認識する制限酵素に着目し，これらの酵素の認識配列をE1欠損領域に組み込むことによって，簡便な1ステップの*in vitro*ライゲーションに基づいたプラスミド構築で外来遺伝子をE1欠損領域に挿入する方法を開発した[2,3]（**図1**）．作製した組換えプラスミ

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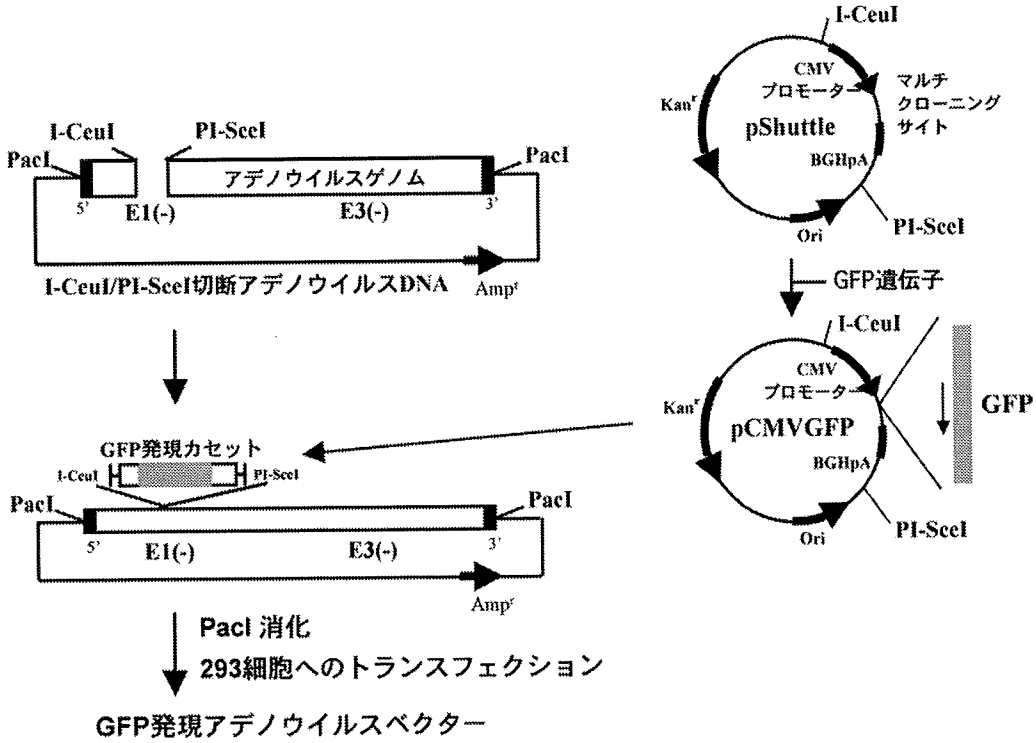

図1　*in vitro* ライゲーションによるプラスミド構築を利用したアデノウイルスベクター作製法の概略

シャトルプラスミドに目的遺伝子〔ここではGFP（Green Fluorescent Protein）を用いた〕を組込み，I-CeuI と PI-SceI. で切断する．これを I-CeuI と PI-SceI で切断したアデノウイルス DNA とライゲーションする．作製した組換えプラスミドをアデノウイルスゲノム両末端に存在する制限酵素部位 PacI で切断し，293 細胞にトランスフェクションとするとアデノウイルスベクターができる．

ドをゲノム両末端に存在する制限酵素 PacI で処理することにより線状にし，293 細胞にトランスフェクションすると，組換えアデノウイルスが生じる．本法は，1）簡便なプラスミド構築に基づいているため，特別な試薬・テクニックを必要とせず，だれでも簡単にウイルス DNA に相当するプラスミドの作製が可能であり，2）相同組換えを必要としないため効率が良い，3）均一なウイルス DNA（に相当するプラスミド）を 293 細胞へ導入することにより組換えアデノウイルスを作製するので，野生型ウイルスからなるクローンを生成する可能性は極めて低く（ベクター増殖中に野生型ウイルスが生成する可能性はある），プラーク精製などを通した目的の組換えアデノウイルスのスクリーニング・精製を必ずしも必要としない，といった長所を有しており，アデノウイルスベクターの作製は格段に簡略化された．本システムはクロンテック社より Adeno-X expression system としてキット化され，広く普及している．

準　備

- 超遠心機（ベックマン超遠心機，SW41 ローター，あるいは日立超遠心機，P40ST ローター）
- 塩化セシウム溶液（0.22 μm のフィルターで滅菌する．オートクレーブ滅菌は比重が変わるので禁句）
  - 1.25 g/cm^3（塩化セシウム 26.99 g を PBS 73.01 ml に溶かす）
  - 1.35 g/cm^3（塩化セシウム 35.18 g を PBS 64.82 ml に溶かす）
  - 1.50 g/cm^3（塩化セシウム 45.41 g を PBS 54.59 ml に溶かす）
- 透析膜（Spectrum laborator 社，Spectra/Por Membrane

MWCO：50,000など）
- 透析バッファー（10 mM Tris（pH7.5），1 mM $MgCl_2$，10 % glycerol：オートクレーブ滅菌）
- −70度以下にセットしたエタノールバス，あるいはエタノールドライアイス，液体窒素など．

プロトコール

目的遺伝子をまずシャトルプラスミド（マルチクローニングサイトの両端にI-CeuIとPI-SceI部位を有している）に挿入し，その後I-CeuIとPI-SceI部位を利用して目的遺伝子をベクタープラスミドに組み換える（**図1**）．作製したプラスミドをアデノウイルスゲノム両末端に存在する制限酵素PacIで切断し，293細胞にトランスフェクションするとアデノウイルスベクターが生じる．なお，PacI切断プラスミドの293細胞へのトランスフェクションの際には，目的遺伝子を挿入したベクタープラスミドの大量調製は必ずしも必要ではなく，制限酵素解析に用いたミニプレップDNAを直接PacI処理，およびトランスフェクションに用いることができ，非常に簡便である．以上の遺伝子組換えのステップはクロンテック社のキットのプロトコール[4]を参照していただきたい．ここでは高タイターのアデノウイルスベクターを得るための増幅法と精製法の“コツ”を中心に述べる．

（注意）現在種々のアデノウイルスベクターが市販されているが，個々のベクター間でE1領域やE3領域の欠損サイズには微妙な違いがみられる（ベクターバックボーンが異なれば欠損サイズは微妙に異なっていることが多い）．このわずかな欠損サイズの違いにより，個々のアデノウイルスベクター間で，高タイターのベクターを得るための“コツ”は異なると考えた方が良い．本稿で述べるコツは，比較的大きなサイズのE3領域の欠損（2.6 kbや3.1 kbのE3欠損）をもつアデノウイルスベクターにあてはまると考えて頂きたい．

1. アデノウイルスベクターの増幅

1) 目的遺伝子を組み込んだアデノウイルスプラスミドをPacI消化し，カチオン化リポソーム試薬などで293細胞（60mm dishあるいは12well dishに70～90 %コンフルエント状態のもの）にトランスフェクションする．
2) 通常の液体培養をする．
3) 約10日後，CPE（cytopathic effect：ウイルス増幅のため，細胞が死滅する現象）が現れるので（293細胞の性質やトランスフェクション効率，アデノウイルスベクターの構造等によりCPEが生じる時期は異なる），細胞をピペッティング等で集める．遠心（1,500～2,000 rpm，5分）にて細胞を集め，上清を1 ml程度だけ残して他は捨てる（あるいは上清を全て捨てて，新鮮な培地を1 ml加える）．
4) 凍結融解（−70度以下→37度）を3～5回行い，遠心後（2,000～3,000 rpm，5分），上清を回収する．一部を−70度で保存する（後のウイルス増幅過程でトラブルがあった際に，この時点から再開できるように）．
5) 5×10^5 個/60 mm dishあるいは$2 \sim 5 \times 10^4$ 個/12well程度の293細胞に，4で回収した上清（ウイルス液）を含む新鮮培地を加えて培養する．なお，3で生じたCPEの程度により1次ウイルス液を作用させる293細胞数は異なる．慣れれば，どの程度の細胞数に作用させれば最適かが習得できる．はじめは上記のように2種類の細胞数を用意し，両者に作用させれば良い．
6) 48時間以内にCPEが起こっている細胞を回収，遠心し（2,000～3,000 rpm，5分），上清を1ml程度だけ残して他は捨てる（あるいは上清を全て捨てて，新鮮な培地を1 ml加える）．凍結融解（−70度以下→37度）を3～5回行い，遠心後（2,000～3,000 rpm，5分），上清を回収する．
7) 回収したウイルス液を含む新鮮培地を5で作用させた細胞数の10～20倍の細胞数の293細胞（70～90 %コンフルエントの状態にする）に作用させ培養する．回収したウイルス液を10～20倍の細胞数の293細胞に作用させると48時間以内に確実にCPEが起こる．
8) 48時間以内に細胞を回収し，6～7を繰り返す（1～2度）．この時点で150 mm dish 3～5枚以上の293細胞からウイルスを回収できているはずであり，$10^{10\text{-}11}$ PFU（plaque forming Unit）以上の

ウイルス液が得られる．

(注意) アデノウイルスベクターの増幅の各ステップで，293細胞にアデノウイルスベクターを作用させてから48時間以内に完全CPEが生じる条件下でウイルスを増幅させることが，高タイターのウイルス液を得るためには極めて重要である．完全CPEが生じるまでに72時間以上を要した場合には，次の増幅のステップではもう一度，同じ細胞数の293細胞に作用させ，48時間以内に完全CPEが生じたウイルス液を増幅させていくようにする．

2. アデノウイルスベクターの精製

アデノウイルスベクターを精製せずに実験に供している場合も散見されるが，未精製のウイルス液は，細胞由来のプロテアーゼ等を含んでいることから，細胞毒性の原因になるばかりでなく，発現させたい目的遺伝子産物も含みシュード・トランスダクションの原因にもなる．特に，アデノウイルスベクターを*in vivo*の個体に用いる場合には，ベクター精製は必須である．アデノウイルスベクターの精製には，塩化セシウム/超遠心を用いた標準的な精製法とカラムを用いた方法（クロンテック社等で発売）がある．アデノウイルスベクターの取り扱いに不慣れであったり，超遠心機を有していない研究者はカラムを用いた方法が簡便である（コストは塩化セシウム/超遠心法に比べ高価である）．塩化セシウム/超遠心法は，ベクター精製に2～3日を要するが（透析を含めて），カラムを用いた方法は1日で精製が完了するという特徴も有する．ここでは，塩化セシウム/超遠心を用いた精製法を紹介する（**図2**）．

1) 150 mm dish 1～5枚から回収したCPEが起こっている細胞を遠心し（1,500～2,000 rpm，5分），上清を捨ててPBS 5 mlを加える．凍結融解（－70度以下→37度）を3～5回行い，遠心後（2,000～3,000 rpm，5分），上清を回収する．

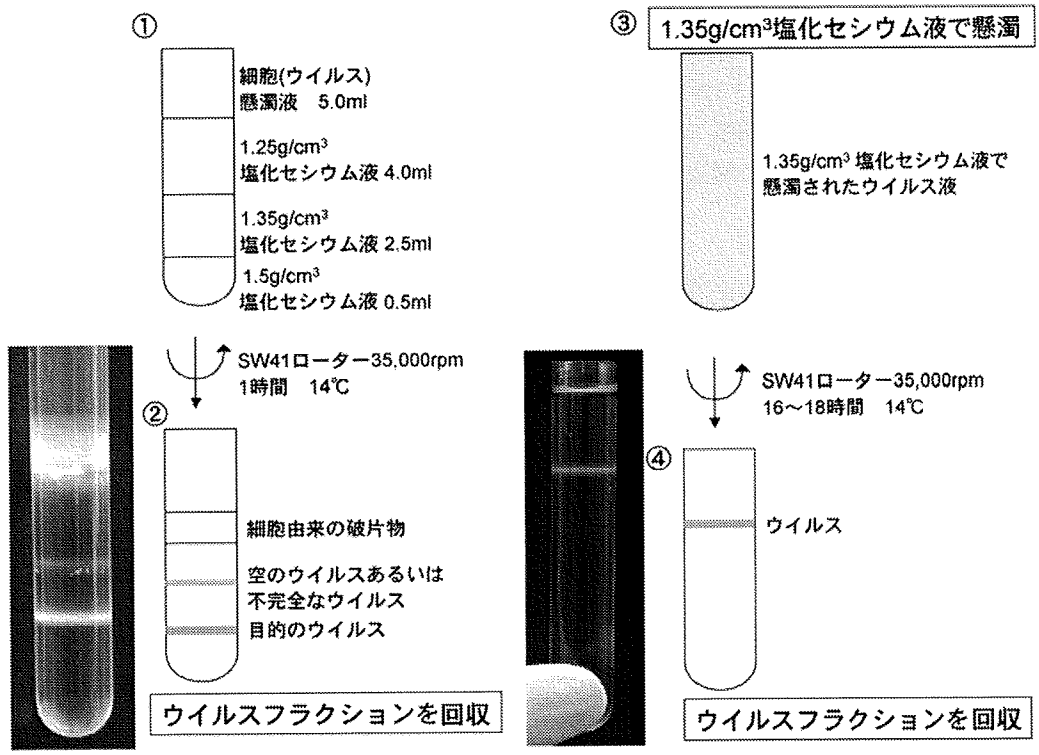

図2 アデノウイルスベクターの塩化セシウム密度勾配遠心法による精製

(p.7カラー図参照)

2) $MgCl_2$（1 M）50 μl，RNaseA（10 mg/ml）100 μl，DNaseI（10 mg/ml）100 μlを加え，37℃で30分間incubateする．本ステップは必ずしも必要ではないが，特に回収した細胞数が多い場合や初心者の場合には本ステップで細胞由来の核酸を断片化しておくと，後の塩化セシウムの密度勾配遠心後のウイルスバンドがきれいにでき，精製が容易になる．
3) 超遠心機のスウィング・ローターのチューブに1.50 g/cm³の塩化セシウム液0.5 mlを入れ，その上に1.35 g/cm³の塩化セシウム液2.5 ml，1.25 g/cm³の塩化セシウム液4.0 ml，そして最後にウイルス液5.0 mlを注意深く重層する．
4) 35,000 rpm，14℃，1時間遠心して，ウイルス分画を回収する．
5) ウイルス液を1.35 g/cm³の塩化セシウム液で懸濁し（最終用量12 ml），35,000 rpm，14℃，16～18時間遠心してウイルス分画を回収する．
6) 透析バッファーで一昼夜，4℃で透析する（バッファー交換を1度行う）．
7) ウイルス液を小分注して－70℃で保存する．
8) タイターを測定し，増殖型アデノウイル（replication competent adenovirus：RCA）の混入がないことを確認した後，実験に供する．

コメント

RCA混入の確認法は理研DNAバンクが開発した方法が簡便である[5]．本方法は，感度は低いが簡便であり，実験室レベルでのRCA混入の確認としては十分である．より高感度にRCA混入を検出したい場合には，我々が開発したinfectivity PCRを用いた方法が便利である[6]．著者らは，アデノウイルスのパッケージングリミットを利用することで，RCAが生じにくい新規アデノウイルスベクター系を開発済みであり，詳細は本誌1月号[7]を参照していただきたい．

アデノウイルスベクターのタイター表記法としては，生物学的タイターと物理化学的タイターがある．生物学的タイター測定法としてはプラーク法やEnd-point dilution法，アデノウイルスのヘキソンに対する抗体を用いた免疫染色に基づく方法（Adeno-X Rapid Titer Kit：クロンテック社）が（**図3**），物理化学的タイター測定法としてはOD_{260}法が用いられる（**図4**）．一般的には生物学的タイターが用いられるが，カプシドタンパク質を改変することで感染域を制御した改良型アデノウイルスベクター[7]の場合には，293細胞への感染性も変わってくるため，物理化学的タイターを用いるのが一般的である．

1）プラークアッセイ
タイターはplaque forming unit（PFU）/mlと標記される．

2）$TCID_{50}$法
96穴プレートを用いて階段希釈したウイルス液を作用させ，
50%細胞変性終末点（$TCID_{50}$）を調べることによる算出法．
タイターは$TCID_{50}$/mlと標記される．

3）その他
Adeno-X rapid titer kit（ヘキソン抗体を用いた免疫染色に基づく）．
タイターはinfectious unit/ml等と標記される．

図3　生物学的タイターの測定法

0.1% SDS（final）を含むTEでウイルス液を懸濁.

5分，ボルテックス.

→ 15,000 rpmで5分遠心.

→ 上清の OD_{260} を測定.

→ 下記の計算式でparticle titerを算出.

OD_{260} 0.1 = 1.1×10^{11} particle titer/ml

図4　物理化学的タイターの測定法

通常，150 mm dish 5枚から回収・精製したアデノウイルスベクターのタイターは約 2×10^{10} PFU/ml ～ 2×10^{11} PFU/ml（約1 ml）程度である.

III おわりに

アデノウイルスベクターは作製法が簡便化されたこともあり，最先端の生命科学研究を行う上での必須の基盤技術として多くの研究室で恒常的に使用されるようになっている．しかしながら，個々の研究者は必ずしもアデノウイルスベクターの取り扱いを熟知しているとは言い難い．本稿および改良型アデノウイルスベクターの開発状況をまとめた著者らの総説[7]が，アデノウイルスベクターの理解に役立ち，正しい技術を習得して本ベクターを使用することに役立てれば幸いである.

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Creation of Novel Cell-Penetrating Peptides for Intracellular Drug Delivery Using Systematic Phage Display Technology Originated from Tat Transduction Domain

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Many biologically active proteins need to be delivered intracellularly to exert their therapeutic action inside the cytoplasm. Cell penetrating peptides (CPPs) have been developed to efficiently deliver a wide variety of cargo in a fully biological active form into a range of cell types for the treatment of multiple preclinical disease models. To further develop this methodology, we established a systematic approach to identify novel CPPs using phage display technology. Firstly, we screened a phage peptide library for peptides that bound to the cell membrane. Secondly, to assess functionality as intracellular carriers, we recombined cDNAs of binding peptides with protein synthesis inhibitory factor (PSIF) to create fusion proteins. Randomly chosen clones were cultured and expression of peptide-PSIF fusion proteins induced, followed by screening of protein synthesis activity in cells. Using this systematic approach, novel and effective CPPs were rapidly identified. We suggest that these novel cell-penetrating peptides can utilized as drug delivery tools for protein therapy or an analytical tool to study mechanisms of protein transduction into the cytoplasm.

Key words cell penetrating peptide; phage display; Tat

Many biologically active compounds, including a variety of large molecules, need to be delivered intracellularly to exert their therapeutic action inside the cytoplasm or within the nucleus or other specific organelles. An important requirement in the use of proteins in this context (ex. kinases, phosphatases, transcriptional factors) is the ability of these molecules to efficiently penetrate across the cell membrane. However, the plasma membrane of cells is largely impermeable to proteins and peptides. Recently, it was discovered that certain short peptide sequences, composed mostly of basic, positively charged amino acids (*e.g.* Arg, Lys and His), have the ability not only to transport themselves across cell membranes,[1—3] but also to carry attached molecules (proteins, DNA, or even large metallic beads) into cells.[4—6] These basic sequences are now commonly known as protein transduction domains or cell-penetrating peptides (CPPs) and have been successfully employed to transport cargo proteins across a variety of cell membranes.[7] Cellular delivery using CPPs has several advantages over conventional techniques; indeed, it is efficient across a range of cell types and can be applied to cells *en masse*.[8]

It has been proposed that the Tat transduction domain of HIV is first endocytosed into a caveola compartment and secondarily released into the cytoplasm, following vesicle disruption.[9] Once CPP binds to the cell surface heparan sulfate proteoglycan (HSPG), the CPP-fused protein is internalized *via* a lipid raft-mediated pathway. Additionally, the mechanisms responsible for CPP mediated cargo internalization estimated with regard to enter the cells *via* macropinocytosis[10] and/or through clathrin-mediated endocytosis,[11] or possibly *via* an unknown alternative mechanism. In spite of some common features of these peptides, particularly their highly cationic nature, their structural diversity has fuelled the idea that the penetrating mechanism is not the same for CPPs of different types. As such, the mechanism(s) of internalization of CPPs has not been resolved yet.[7]

Given the potency of the Tat-derived CPPs in mediating the cellular uptake of small and large macromolecular cargos, as demonstrated within the last few years, a large number of laboratories have exploited this system as a tool for transcellular penetration of cultured cells.[12] Most of these applications are based on the fusion of the protein transduction domain of Tat to the protein of interest, either at the N- or C-terminus, followed by addition of the recombinant fusion protein to the culture medium of the cells of interest. It is clear that CPPs are novel vehicles for the rapid translocation of cargo into cells, and exhibit the properties that make them potential drug delivery agents.[13] However, there are problems in respect to a decrease in the rate and efficiency of translocation for large proteins that has not yet been overcome. Accordingly, a large number of different CPPs have been explored to promote translocation of various types of useful cargo, ranging from small molecules to proteins and large supramolecular particles, with great efficiency and reasonable velocity.

We previously showed that the gene III proteins (pIII) of M13 filamentous phage could be used to display mutant protein, with these modified proteins showing fully functional binding to receptor and consequent biological activities.[14,15] Recently, we established a novel whole cell panning method,

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which selected cell adhesive phage-displayed peptides and, subsequently, a cohort of these peptides having cell penetrating qualities *via* the use of PSIF (protein synthesis inhibitory factor). In this study, we constructed a Tat-based mutant peptide library using this phage display system. Moreover, we demonstrated the direct selection of a unique cell-binding activity utilizing whole cell panning methods and the screening of internalizing peptide using peptide-PSIF fusion protein.

MATERIALS AND METHODS

Cell Line Human epidermoid carcinoma A431 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 5.0% CO_2 at 37 °C. Human adenocarcinoma Hela cells were grown in DMEM supplemented with NEAA and 10% FCS. Chinese hamster ovary (CHO)-K1 cells were grown in Ham's F12K medium supplemented with 2 mM L-glutamine and 10% FCS.

Preparation of Phage Peptide Library Primers shown below and used in library construction were purchased from Hokkaido System Science Inc. The phage-display vector pCANTAB-5E was used as a phagemid vector for the generation of the peptide-pIII fusion gene repertoires (Fig. 1). To construct a DNA fragment library encoding 13 amino acid peptides, primers P-oligo1 and P-ologo2 were annealed and elongated with the Klenow fragment in the presence of nucleotide triphosphates. These cDNA-encoding peptide library products were purified with QIAquick® Gel Extraction Kit (QIAGEN) and used as templates for PCR with primers pCANTAB-Hind and Not I Ext to generate the pIII fusion gene repertoires. The peptide-encoding genes were digested with the restriction enzymes HindIII and NotI, agarose gel-purified, and ligated into pCANTAB-5E, which was cut with the same restriction enzyme. The ligated products were electroporated into *E. coli* TG1 cells, plated on modified LB medium (Invitrogen) containing 2% glucose and 50 μg/ml ampicillin, and then incubated overnight at 37 °C. The clones were scraped off the plates into 2YT medium with 10% glycerol and subsequently stored at −70 °C. P-oligo1; 5′-GAT TAC GCC AAG CTT TGG AGC CTT TTT TTT GGA GAT TTT CAA CGT GAA AAA ATT ATT ATT CGC AAT TCC TTT AGT TGT TCC TTT CTA TGC GGC CCA GCC GGC CAT GGC C-3′, P-oligo2; 5′-CGG CGC ACC TGC GGC CGC SNN SNN CGG SNN SNN SNN CTG SNN SNN SNN SNN SNN ACC GGC CAT GGC CGG CTG GGC CGC ATA GAA AGG-3′, pCANTAB-Hind; 5′-GGA AAC AGC TAT GAC CAT GAT TAC GCC AAG-3′, Not I Ext; 5′-GCG GCC TTG TCA TCG TCA TCC TTG TAG TCT GCG GCC GC-3′.

Rescue of Peptide-Phage To rescue the peptide-phage library, 1 l of 2YT medium, containing 2% glucose, and 100 μg/ml of ampicillin, was inoculated from the glycerol stock library. The culture was shaken at 37 °C until OD600 nm=0.4 and 3.2×10^8 plaque forming units of M13KO7 helper phage (Invitrogen) were added. After 30 min incubation at room temperature with shaking, the culture was centrifuged and the pellet recovered. The pellet was then incubated with 50 μg/ml of kanamycin and 100 μg/ml of ampicillin within 2YT medium and grown for 6 h at 37 °C. The phage was purified by standard polyethylene glycol precipitation and filtration with a 0.45 μm PVDF filter (Millipore). Peptide-phage which did not express the objective peptide were removed by a FLAG panning method, as described previously.

Biopanning Method We used a slightly modified procedure from that found in the literature. Briefly, 1.0×10^6 A431 cells were harvested in 6 well culture plates and incubated for 24 h at 37 °C within a 5.0% CO_2 incubator until the logarithmic phase of growth was reached. The culture plates were washed with PBS 3 times and 2% BSA Opti-MEM® (Invitrogen) added 2 h prior to the addition of the peptide-phage. Cells were incubated with the peptide-phage library for 2 h at 37 °C with shaking every 15 min during the round of panning. Following this, the cells were washed twenty times with PBS at room temperature. After washing, the cells were lysed with 1 ml of 100 mM HCl and neutralized with 0.5 ml of 1 M Tris–HCl, pH 8.0. One-hundred microliters lysate was used

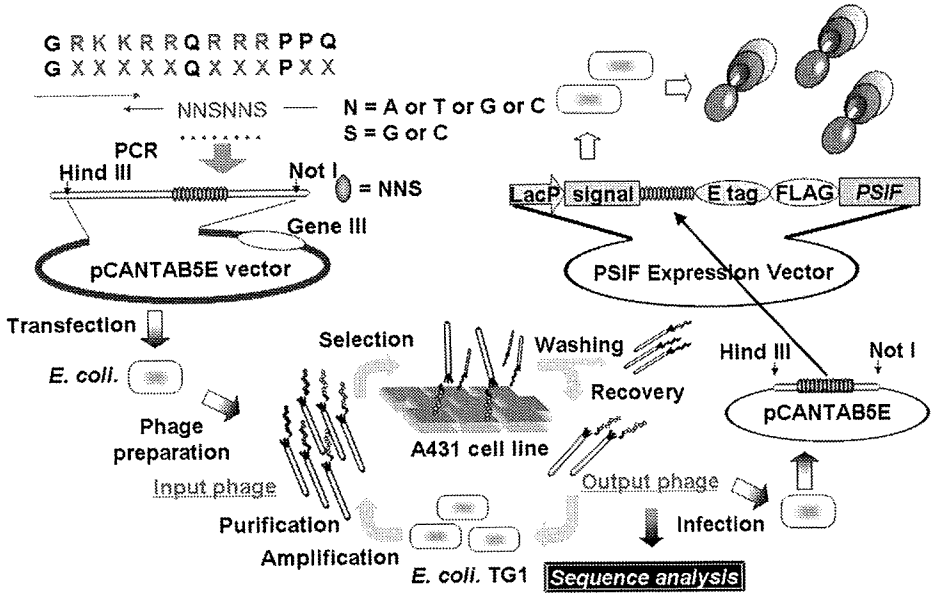

Fig. 1. Schema for Screening CPPs

to infect 0.3 ml of *E. coli* TG1 cells, with phage being rescued as described above and used in the next round of selection.

Expression and Purification of a Peptide-PSIF Fusion Protein Protein synthesis inhibitory factor (PSIF, PE fragment) is an approximately 40 kD fragment of the bacterial exotoxin (GenBank Accession No. K01397) derived from *Pseudomonas aeruginosa* (ATCC strain No. 29260). PSIF lacks its cell binding domain, and is the truncated form of *Pseudomonas aeruginosa* exotoxin, which is a non-toxic protein outside of the cell. One (Dr. Tsunoda) of us cloned the cDNA for PSIF from *Pseudomonas aeruginosa*, Migula by PCR using the primer set 5′-GAT GAT CGA TCg cgg ccg caG GTG CGC CGG TGC CGT ATC CGG ATC CGC TGG AAC CGC GTG CCG CAg act aca aag acg acg acg aca aaC CCG AGG GCG GCA GCC TGG CCG CGC TGA CC-3′ and 5′-GAT CGA TCG ATC act agt CTA cag ttc gtc ttt CTT CAG GTC CTC GCG CGG CGG TTT GCC GGG-3′. The fusion protein, denoted peptide-PSIF, consisted of peptide at the N-terminus and a PSIF at the C-terminus. First, the peptide gene containing phagemid vectors were recovered with QIAprep® Miniprep Kit (QIAGEN) and digested with HindIII and NotI. The peptide gene fragments were then subcloned into PSIF Expression Vector, which is modified from pCANTAB-5E. The fusion proteins were expressed and collected within the supernatant from *E. coli* TG1 cells, with the supernatant being used for cell viability assays.

Cell Viability A431 cells (2.0×10^4) were incubated with 35 μl Opti-MEM® and 10 μl cycloheximide (100 μg/ml) in 96 well plates. Cells were treated with 5 μl peptide-containing supernatant for 24 h and the cell viability was monitored by MTT assay. Twenty-four hours after addition of the peptides, 10 μl of 5 mg/ml MTT (Dojindo) were added to each well and the cells were further incubated at 37 °C for 4 h. Subsequently, the insoluble formazan crystals were solubilized in a solution of 20% SDS containing 0.01 N HCl. Absorbance measurements were taken at $\lambda=595$ nm with background subtracted at $\lambda=655$ nm. Each sample point was performed in duplicate.

FACS Analysis The specific cell binding activities of peptides towards A431, Hela, CHO-K1 cells were measured by FACScan (Becton Dickinson). Cells were grown in tissue culture flasks to late logarithmic phase. Culture medium was renewed 2 h prior to the addition of the peptide-phage. FITC-labeled peptides were purchased from Genenet Co., Ltd. and 1×10^5 cells were incubated with FITC-labeled peptide for 3 h at 37 °C. For the endocytosis inhibitor assays, FACS analysis was performed after pre-treating A431 cell monolayers at 37 °C with 10 mM methyl-β-cyclodextrin (MβCD; caveola-mediated endocytosis inhibitor) or amiloride (macropinocytosis inhibitor) in serum-free Minimal Essential Medium (MEM) for 30 min, followed by a 1-h co-incubation with FITC labeled-peptide. After three washes with PBS, 0.25% trypsin solution (Gibco BRL) was added and incubated for 15 min to digest non-specific binding peptides. After three additional washes, cells were resuspended in PBS/4% paraformaldehyde and analyzed using FACScan.

RESULTS

Construction of Phage Peptide Library and Quality Check The pCANTAB-5E phagemid library used here has previously been screened successfully for mutant protein which binds to receptors.[14,15] Additionally, we previously reported the identification and characterization of a series of cationic peptides, similar to the CPP derived from Tat, which are able to penetrate large protein complexes into a wide variety of cells, including fibroblasts. Here, we made a novel phage peptide library, which altered ten amino acids within the Tat transduction domain (13 amino acids). The library of the TAT-based CPPs was made *via* the annealing and elongation of two mutated primers, followed by PCR amplification and cloning into a phage expression system The peptide-encoding cDNA library was placed into a phagemid vector and expressed as a fusion protein with phage coat protein, pIII. We confirmed the identity and sequence distribution of this phage peptide library by DNA sequencing (Table 1). In this context, eight clones which were sequenced showed independent sequences, highlighting the distribution of this phage peptide library.

Concentration of Binding Peptides with a Cell Panning Method The constructed peptide phage library was selected *in vitro* against A431 cells. Selection was performed as described in the Materials and Methods section, with a view to enriching for peptides displaying cell binding activity. With respect to the phage panning and amplification processes, which were repeated for one to four rounds, the output/input ratio was found to increase in a manner dependent on cycle number (Fig. 2). These results indicated that the peptides having an affinity for A431 cells were enriched gradually by this cell panning approach.

Identification of A431 Cell Binding Peptides Peptide clones that became internalized in A431 cells were isolated by four rounds of selection. In order to select only internalized phage-derived peptides, cells were incubated with super-

Table 1. Random TAT Peptide Library Sequence before Panning

Clone	Sequence
1	GMHINGQSNPPHA
2	GGMHESQSHMPGD
3	GTQAFLQQFEPWI
4	GIKHSPQQISPRW
5	GILCIQQDHQPLG
6	GFKLSSQAVAPLQ
7	GSIRAPQGDSPWP
8	GTRHGIQTQPPNN

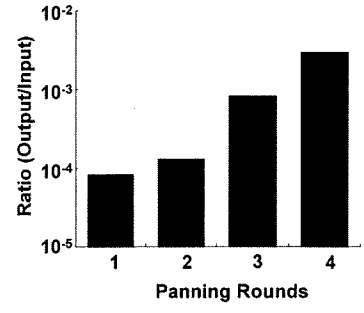

Fig. 2. Enrichment of Phage Clones by Biopanning

A431 cells were incubated with 4×10^{10} titer phage. After washing with PBS, binding phages were recovered and the titer was determined. The index of enrichment was evaluated with input/output ratio.

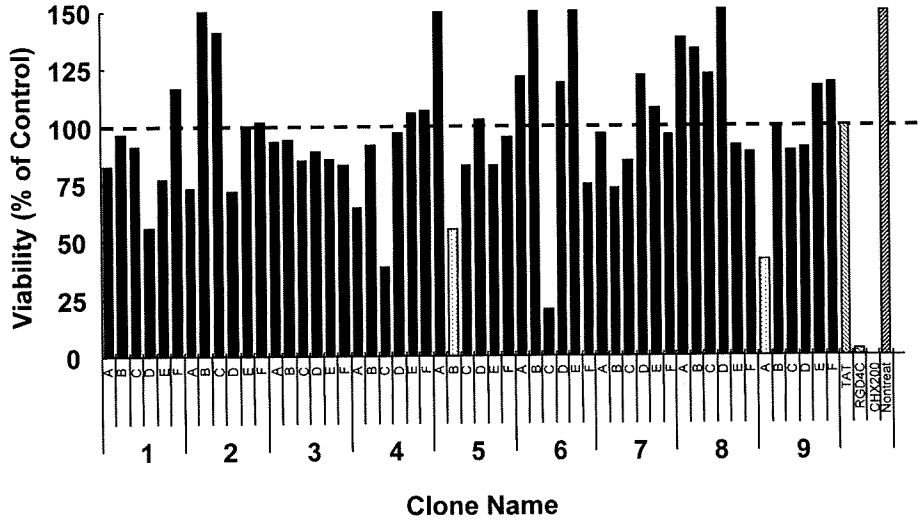

Fig. 3. Measurement of Penetrating Activity as an Index of Cytotoxicity to A431 Cells

PSIF-CPP fusion genes were transformed into TG1 cells, with resulting fusion proteins then recovered from the supernatant. The cytotoxicity of the supernatant was then assessed to examine the activity of the CPPs. Cell viability in response to fusion proteins was compared with that exhibited by exposure to the parent Tat13 peptide (cell viability=100%), and by exposure to 200 μg/ml cycloheximide (CHX; cell viability=0%).

natant collected from *E. coli* cells. After four rounds of selection, an enrichment in the order of 10^5 was obtained. The random insert region of the single-stranded DNA from individual clones, following the fourth round of selection, was sequenced and the amino acid coding sequence deduced. To identify peptides capable of facilitating internalization, we developed a screening method using an M13 peptide phage display library. We incubated 1×10^6 A431 cells, with 4×10^{10} phage from a 13-mer peptide M13 phage display library for 2 h at 37 °C. Following four rounds of screening, we isolated phage from 28 plaques and determined the identity of the encoded peptides by DNA sequencing (Table 2). Twenty-eight of the peptide sequences were found in at least 15 independent plaques and were selected for further analysis.

Evaluation of Cell Penetrating Activity of CPPs Following creation of the peptide library (input phage library), we expressed these Tat-based CPPs as fusion proteins with PSIF. From this, 54 candidates were found that exhibited lower cytotoxic activity than the parent Tat peptide (data not shown). These results indicated that the penetrating activity is remarkably decreased as a consequence of random conversion of amino acids within the Tat transduction domain. In addition, we made the PSIF-fused peptide library after fourth round panning (Fig. 3). In screening this peptide library, a tryptophan-rich (GSSSWWQRWWPPW) peptide was identified (Table 2). However, this peptide did not exhibit cytotoxicity when recombined with PSIF. This result indicated that this tryptophan-rich peptide binds to the cell membrane but does not penetrate through to the cytoplasm. Next, we reconfirmed that fixation of the cells significantly affected the cellular distribution of peptides (Fig. 4). 435B peptide (GPFHFYQFLFPPV) and 439A peptide (GSPWGLQHHPPRT) showed internalization characteristics similar to those of the parent Tat peptide. These FITC-labeled peptides did not show cytotoxicity at a dose of 10 μM. However, the two another clone (434C and 436C peptide) does not TAT-derived peptide , which is not consist of 13 amino acid or occur the flameshift, respectively. So we were excluded these two clones from followed experiment.

Table 2. Random Tat Peptide Library Sequence after 4th Panning

Clone	Sequence
1	GPMESLQAFWPPW
2	GSSSWWQRWWPPW
3	GSSSWWQRWWPPW
4	GVFLLKQVPQPSH
5	GSSSWWQRWWPPW
6	GRLWWLQLFEPGH
7	GLRKVPQSVPPDM
8	GSSSWWQRWWPPW
9	GHFLKPQVLRPTR
10	GQFMMRQYWPPVH
11	GSSSWWQRWWPPW
12	GSSSWWQRWWPPW
13	GSSSWWQRWWPPW
14	GLLKYQQWASPLC
15	GYFWYDQPWQPEQ
16	GRNHYIQRDNPVS
17	GVFHVLQNAIPQY
18	GSSSWWQRWWPPW
19	GTMPNMQHHDPAR
20	GSSSWWQRWWPPW
21	GSSSWWQRWWPPW
22	GSSSWWQRWWPPW
23	GTRYLVQYLFPHL
24	GRPATQQGLTPAR
25	GYIGTYQQWNPPP
26	GSSSWWQRWWPPW
27	GSSSWWQRWWPPW
28	GSSSWWQRWWPPW

Uptake of FITC-Labeled Peptides into Human and Murine Cells To address the question of whether 435B and 439A peptides were more active than the parent Tat peptide, peptides conjugated to FITC were constructed. Cellular uptake of both peptides were judged by flow cytometric analysis on human carcinoma A431 and Hela and CHO cells. Assuming that the surface-adsorbed 435B and 439A peptides were susceptible to tryptic degradation, we washed the cells five times with PBS and treated them with trypsin prior to assessing the amount of the internalized peptide. On A431

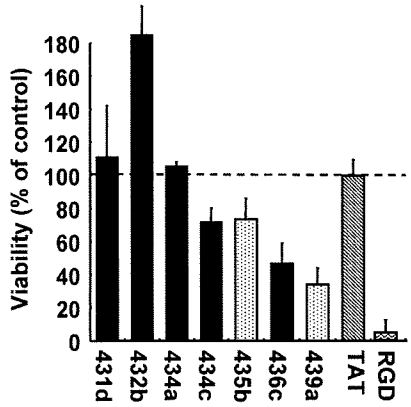

Fig. 4. Evaluation of Cell Penetrating Activity of Individual Clones

Cell penetrating activity was reconfirmed using the same method as that referred to in Fig. 3. Cell viability in response to fusion proteins was compared with that exhibited by exposure to the parent Tat13 peptide (cell viability=100%), and by exposure to 200 μg/ml cycloheximide (CHX; cell viability=0%).

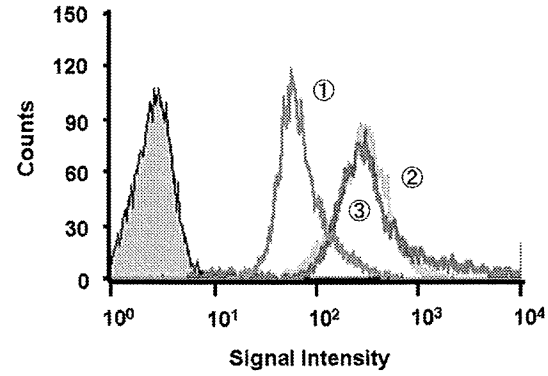

Fig. 5. Intracellular Penetrating Activity of FITC-Labeled CPPs on A431 Cells

Ten micromolar FITC-labeled 439A peptide (①), 435B peptide (②) and parent Tat peptide (③) were added to A431 cells. Gray area showed the distribution of non-treated cells. Following trypsinization, the quantity of penetrating peptide was evaluated in cells according the level of fluorescence intensity.

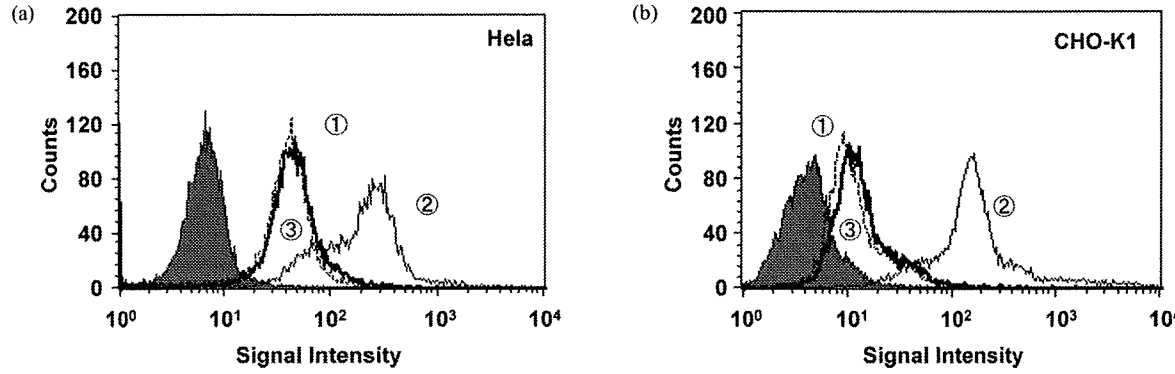

Fig. 6. Intracellular Penetrating Activity of FITC-Labeled CPPs on Human and Murine Cell Lines

Ten micromolar FITC-labeled 439A peptide (①), 435B peptide (②) and parent Tat peptide (③) were added to Hela (a) and CHO (b) cells. Gray area showed the distribution of non-treated cells. Following trypsinization, the quantity of penetrating peptide was evaluated in cells according the level of fluorescence intensity.

cells, the efficiency in terms of cell penetration between the parent Tat peptide and 435B peptide was almost the same, though the penetrating activity of the 439A peptide was decreased about 10 fold compared to the parent Tat peptide and 435B peptide (Fig. 5). However, on Hela and CHO cells, the efficiency of cell penetration between the parent Tat peptide and 439A peptide was almost the same, though the penetrating activity of the 435B peptide was increased about 10 fold compared to the parent Tat peptide and 439A peptide (Figs. 6a, b).

Inhibition of Endocytic Internalization Several studies were done to investigate the involvement of macropinocytosis or caveolae/raft-dependent endocytosis on peptide transduction domain such as TAT. The effect of the specific macropinocytosis inhibitor, amiloride, on TAT peptide penetration was determined. As seen in Fig. 7, treatment with amiloride did not inhibit 435B and 439A pepetration. Additionally, Methyl-β-cyclodextrin (MβCD)-sensitive caveolae/raft-dependent endocytosis of 435B and 439A peptides was detected, internalization of 435B and 439A *via* transduction is significantly affected by MβCD treatment.

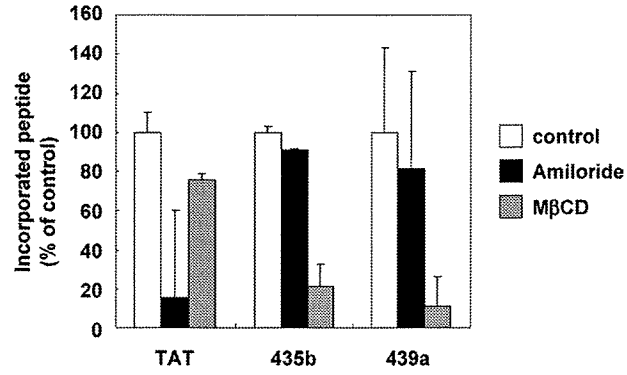

Fig. 7. Inhibitory Effects of Amiloride and MβCD for Peptide Incorporation on A431 Cells

FITC-labeled 435B peptide and 439A peptide were added to cells after amiloride and MβCD pre-incubated. The quantity of penetrating peptide was evaluated by FACS analysis according the mean of fluorescence intensity.

DISCUSSION

In an effort to search for novel CPPs, we have screened an M13 peptide phage display library comprised of CPPs based on the Tat transduction domain. From this screening approach, we have identified peptides that displayed a capability for cell penetration. In particular, a screen using A431 cells resulted in identification of peptide 435B and 439A that is able to penetrate cultured cells *in vitro* efficiently. Thus, this method of screening for CPPs using phage peptide li-

braries can be readily applied to select from a wide variety of possible peptides.

Whether the parent Tat peptide shows the penetrating capability of other known CPPs is uncertain.[12,16] The RGD peptide has long been used to facilitate the transport of bioactive molecules through adsorptive endocytosis.[17] However, comparison of short oligolysine peptides to that of equivalent length polymers of arginine showed that oligoarginine was much more efficient in carrying GFP into cultured cells.[18] Conversely, the ability of short oligolysine peptides, from 6 to 12 residues in length, has been shown to be more efficient than oligoarginine in carrying larger macromolecules (60—500 kDa) into cultured cells. In this study, the 435B and 439A peptides contained hardly any basic amino acids but still displayed an ability to mediate cell penetration more efficiently than the parent Tat peptide. This result indicated that the cell penetrating activity of CPPs is controlled not only by the electrical charge but also by the structural characteristics. Practically, it is known that the amino acid component and associated tertiary structure of peptides are essential for cell penetrating activity.[19] We did not examine our peptides on a structural level; however, the results presented here suggest the importance of hydrophobicity, as hydrophobic amino acids were enriched by sequential biopanning.

It has been theorized that the ionic interaction between positively charged Arg residues in these CPPs and the negatively charged phosphate head group of the membrane lipid bilayer plays a key role in CPP membrane interaction.[20] However, the exact mechanism by which these CPPs operate is still largely unknown. Work performed by several investigators has shown that Tat binds heparin and that this heparin/Tat interaction involves the basic domain of Tat.[21] Meanwhile, previous study showed that the histidine residues of peptide sequence might enhance an endosomal escape of the cargo.[22] In this study, the 435A and 439B peptides did not show enrichment of basic amino acids, but did exhibit an increase in proportion of hydrophobic amino acids. Several numbers of histidine residues were included in 435B and 439A peptide sequence compared to native TAT peptide. Accordingly, it is possible that these peptides do not penetrate through the binding of cell surface receptors such as HSPG but escape from endosome efficiently. Whereas the conformation of these peptides should be examined, our results suggest that the 435B and 439A peptides penetrate the cell membrane independently of cell surface receptor.

In this study, the transduction efficiency was observed to be different between the peptides fused with PSIF and those labeled with FITC. We think there are two ideas to explain this discrepancy. Firstly, the molecular size and structure of the respective cargo is different. Secondly, there is some possibility that the intracellular kinetics is different between the parent Tat peptide and our peptides. It is thought that the parent Tat peptide is transferred to nuclei after penetrating the cell membrane, while our mutant peptide-PSIF conjugate diffuses throughout the cytoplasm. We are currently examining the intracellular kinetics of these peptides in an effort to resolve this issue.

In this study, our peptides have a unique sequence compared to preexisting CPPs. These peptides are able to introduce a large molecule into the intracellular space more efficiently than the parent Tat peptide, the latter which is known to have a high level of transduction ability. Using these peptides, efficient introduction of large molecules to the cytoplasm is accomplished. As such, one could readily conceive of using these peptides to target disease-related proteins, revealed from extensive-omic analysis. Furthermore, our peptides can be used as analytical tools to explore the mechanism(s) of peptide penetration.

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Role of amino acid residue 90 in bioactivity and receptor binding capacity of tumor necrosis factor mutants

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Abstract

We have previously produced two bioactive lysine-deficient mutants of TNF-α (mutTNF-K90R,-K90P) and found that these mutants have bioactivity superior to wild-type TNF (wtTNF). Because these mutants contained same amino acid except for amino acid 90, it is unclear which amino acid residue is optimal for showing bioactivity. We speculated that this amino acid position was exchangeable, and this amino acid substitution enabled the creation of lysine-deficient mutants with enhanced bioactivity. Therefore, we produced mutTNF-K90R variants (mutTNF-R90X), in which R90 was replaced with other amino acids, to assay their bioactivities and investigated the importance of amino acid position 90. As a result, mutTNF-R90X that replaced R90 with lysine, arginine and proline were bioactive, while other mutants were not bioactive. Moreover, these three mutants showed bioactivity as good as or better than wtTNF. R90 replaced with lysine or arginine had especially superior binding affinities. These results suggest that the amino acid position 90 in TNF-α is important for TNF-α bioactivity and could be altered to improve its bioactivity to generate a "super-agonist".

Keywords: TNF; Mutant; Phage display; Lysine residue; TNF receptors; Structure

1. Introduction

TNF-α is an inflammatory cytokine able to mediate tumor regression in experimental and clinical cancers [1–3]. Attempts to use TNF-α for its cytotoxic property led to the development of several strategies that, in some cases, resulted in the use of TNF-α in clinical trials of tumor immuno-chemotherapy [4–6]. However, a frequent administration at high dose of TNF-α was required to obtain anti-tumor effect because of its poor stability and short half-lives, and resulted in severe side-effects [7]. Therefore, clinical application of TNF-α is still limited. More recently, some groups have reported positive results using melphalan in combination with TNF-α for patients with melanoma [8, 9]. These findings suggest that the enhanced anti-tumor effect of melphalan observed after the combination with TNF-α resulted from potentiation of the TNF-induced accumulation of melphalan into tumor accompanied by increased tumor vascular permeability [10]. The improved retention of TNF-α in the vascular space and the resultant decrease in transfer of TNF-α to normal tissues is expected to reduce the side effects of TNF-α therapy [11]. Thus, improving the circulation time of TNF-α may not only enhance its anti-tumor effects but also vascular permeability activity without increasing its side effects, resulting in longer bioavailability.

One of the most useful ways of enhancing the plasma half-lives of proteins is to conjugate them with polyethylene glycol (PEG) or other water-soluble polymeric modifiers [12–14]. The

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covalent conjugation of proteins with PEG (PEGylation) increases their molecular size and steric hindrance, both of which depend on the properties of the PEG attached to the protein. This prevents renal excretion and improves their proteolytic stability while decreasing their immunogenicity and hepatic uptake. We have also reported that optimal PEGylation of bioactive proteins could selectively improve their in vivo therapeutic potency and reduce side-effects [15, 16]. However, the PEGylation of proteins was mostly nonspecific and targeted lysine residues, some of which were in or near an active site. As a result, the PEGylation of proteins was accompanied by a significant loss of their specific activities in vitro [14, 17]. Thus, the clinical application of PEGylated proteins has been limited. To overcome the problems of PEGylation, we attempted to develop a novel strategy for site-specific mono-PEGylation of TNF-α to improve its antitumor potency in vivo [18, 19]. We produced bioactive lysine-deficient mutants of TNF-α (mutTNFs) from phage libraries expressing mutTNFs in which all of the six lysine residues were replaced with other amino acids. Among these mutant proteins, mutTNF-K90R and mutTNF-K90P have superior bioactivity, especially mutTNF-K90R, which has a 60-fold broader anti-tumor therapeutic window than wild-type TNF-α (wtTNF) [18].

Interestingly, these two mutTNFs were identical except for single amino acid changes at amino acid 90. Other lysine residues (amino acid 11, 65, 98, 112, 124) were replaced with alanine, serine, alanine, leucine, threonine respectively. Our previous study discussed the significance of R90 and P90 in the context of the TNF–TNF receptor structure. In the wtTNF structure, K90 forms a hydrogen bond with E135. This interaction likely stabilizes the loop structure containing residues 84 to 89, which is involved in receptor binding according to the model. In the mutTNF-K90R, arginine also is likely to be involved in hydrogen bonding with E135. The interaction may contribute to the stabilization of the loop structure. To confirm this speculation and clarify the importance of amino acid 90, it is necessary to create mutTNF-K90R variants (mutTNF-R90X) by replacing R90 with other amino acid in mutTNF-K90R, and validate their bioactivity, ability to form trimers, and binding affinity toward receptors. Moreover, the fact that only amino acid 90 substitutions were obtained highlights the importance of amino acid 90 as a key determinant between TNF-α and TNF receptor affinity and for its resulting level of bioactivity. This idea raises the possibility that mutTNFs with stronger bioactivity than mutTNF-K90R are created by the substitution of amino acid 90. Therefore, in this study, we evaluated the binding ability and bioactivity of mutTNF-R90X in order to examine the structural importance of the amino acid position 90 and to obtain mutTNFs with stronger bioactivity.

2. Materials and methods

2.1. Random amino-acid substitution of R90 in mutTNF-R90

The *Escherichia coli* library expressing mutTNF-R90 variants (mutTNF-R90Xs) in which R90 is replaced with other amino acids was constructed by the method as shown in Fig. 1. These mutTNF-R90Xs were also lysine-deficient except when X is lysine. pY02-mutTNF-R90 was used as a PCR template, and the arginine codon of mutTNF-R90 was replaced with the randomized sequence 'NNS (where N and S represent G/A/T/C or G/C, respectively) ' by two-step PCR using 4 primers. Oligo-1: 5′-cgG GCC AAG GCT GCC CCT CCA CCC ATG TGC TCC TCA CCC ACA CCA TCA GCC GCA TCG CCG TCT CCT ACC AGA CCN NS GTC AAC CTC CTC TCT GCC ATC-3′, Oligo-2 : 5′-GCC CAG ACT CGG CAA AGT CGA GAT AGT CGG GCC GAT TGA TCT CAG CGC T-3′, Oligo-3 : 5′-TGT ACC TTA TCT ACT CCC AGG TCC TCT TCT CGG GCC AAG GCT GCC CCT C-3′, Oligo-4 : 5′-GCC CAG ACT CGG CAA AGT CGA GAT AGT CGG GCC GAT TGA TCT CAG CGC T-3′. First PCR was carried out using Oligo-1 and -2. The PCR condition was cycled 30 times at 95 °C for 60 s, 57 °C for 60 s, and 68 °C for 60 s. PCR products (251 bp) were purified with QIAquick PCR purification Kits (QIAGEN, Valencia) and used as templates for second PCR. The second PCR was carried out using Oligo-

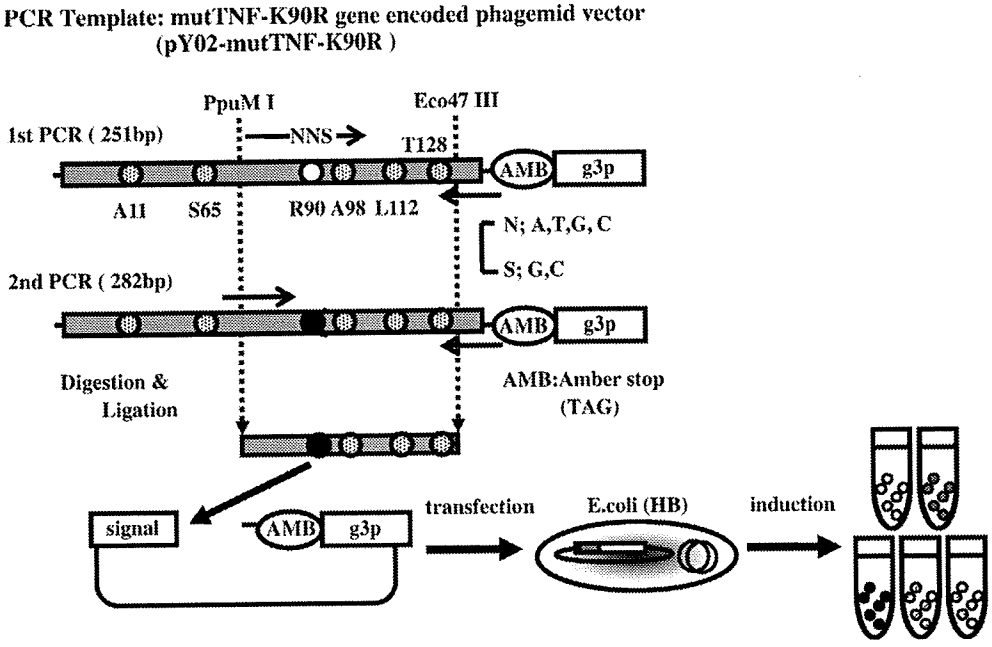

Fig. 1. Construction of mutTNF-R90X *E. coli* library.

3 and -4 under the same cycling conditions. Purified PCR products (283 bp) and pY02-mutTNF-K90R were digested with PpuM I and Eco47III. The resulting PCR products were inserted into pY02-mutTNF-K90R using T4 ligase (Roche Diagnostics, IN) at 16 °C for 16 h. Ligated DNAs were purified and introduced into *E. coli* HB2151 using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, CA). The *E. coli* was then grown by culturing at 37 °C in LB agar medium with ampicillin (100 μg/mL) and glucose (2% w/v).

2.2. Preparation of E. coli culture supernatant

Colonies on LB agar medium were picked and grown by culturing at 37 °C in 2-YT medium with ampicillin (100 μg/mL) and glucose (2% w/v) until the OD_{600} of the culture medium reached 0.4. After centrifugation, supernatants were removed, and fresh 2-YT media with ampicillin (100 μg/mL) was added to *E. coli* pellets. After incubation for 6 h supernatants were collected and used for ELISA and bioassay.

2.3. TNF-α ELISA

Human TNF neutralizing monoclonal antibody (4 μg/mL, R&D systems, US) was coated onto Maxisorb immunoplates (NUNC, Denmark). After blocking, *E. coli* supernatant was then added into the plates and incubated at 37 °C for 2 h. The plates were washed three times with PBS and 0.05% Tween PBS and incubated with 200 ng/mL biotinylated anti-human TNF polyclonal antibody (R&D systems, US) at 37 °C for 1 h. After incubation, the plates were washed three times, and incubated with diluted avidin-HRP (Zymed Laboratories, Inc, US) at 37 °C for 1 h. After washing, TMB peroxidase substrate (MOSS, Inc. US) was added, and the absorbance was read at 450 nm / 650 nm using a micro plate reader.

2.4. Cytotoxicity assay

L-M cells, a cell line derived from L929 cells, were maintained in Eagle's Minimum Essential Medium (MEM, Sigma-Aldrich, Inc. Japan) with 1% bovine fetal serum and antibiotics. L-M cells treated with 1 μg/mL actinomycin D were seeded at 3×10^4 cells/well in 96 well plates, and cultured in the presence of *E. coli* supernatants or serially diluted TNFs. After incubation for 24 h, L-M cells were fixed by 25% glutaraldehyde and stained with 0.05% methylene blue for 15 min. After washing, 0.33 N HCl (100 μl) was added to each well, and the absorbance of released dye was measured at 655 nm /415 nm. Recombinant human TNF (R&D systems, US) was used as a standard.

In the case of HEp-2 cells, cells were maintained in RPMI 1640 medium (Sigma-Aldrich, Inc. Japan) with 10% bovine fetal serum, sodium pyruvate (1 mM), 2-mercaptoethanol (50 μM), and antibiotics. Cells were treated with 100 μg/mL cycloheximide, seeded at 4×10^4 cells/well, fixed after 18 h incubation, and used as described above.

2.5. Purification of recombinant proteins

Purification of recombinant proteins was described previously. Briefly, TNFs were produced in *E. coli* BL21(DE3). TNFs were recovered from inclusion bodies, which were washed in Triton X-100 and solubilized in 6 M guanidine–HCl, 0.1 M Tris–HCl, pH 8.0, and 2 mM EDTA. Solubilized protein (10 mg/mL) was reduced with 10 mg/mL dithioerythritol for 4 h at RT and refolded by 100-fold dilution in a refolding buffer, 100 mM Tris–HCl, 2 mM EDTA, 1 M arginine, and oxidized glutathione (551 mg/L). After dialysis with 20 mM Tris–HCl, pH 7.4, containing 100 mM urea, active trimeric proteins were purified by Q-Sepharose and MonoQ chromatography. Additionally, size-exclusion chromatography (Superose 12, GE Healthcare, England) was performed.

2.6. Surface plasmon resonance assay (BIAcore assay)

Human TNFR1 or TNFR2 Fc chimera (R&D systems, US) was diluted to 50 μg/mL in 10 mM sodium acetate buffer (pH 4.5) and immobilized to a CM3 sensor chip using an amine coupling kit (BIAcore, Sweden), which resulted in an increase of 4000–6000 resonance units (RU). During the association phase, mutTNFs or wtTNF diluted in running buffer (HBS-EP) at 26.1 nM, 8.7 nM or 2.9 nM were individually passed over the immobilized TNFRs at a flow rate of 20 μl/min. During the dissociation phase, HBS-EP buffer was applied to the sensor chip at a flow rate of 20 μl/min. Elution was carried out using 20 μl of 10 mM glycine–HCl. The data were analyzed globally with BIA EVALUATION 3.0 software (BIAcore®, Sweden) using a 1:1 binding model.

2.7. Induction of GM-CSF in PC60-hTNFR2 cells

PC60-hTNFR2, which is a cell line transfected with the human TNFR2, was kindly provided by Dr. Vandenabeele, and induction experiments were performed as previously described. PC60-hTNFR2 cells were cultured in RPMI-1640 supplemented with 10% bovine fetal serum, sodium pyruvate (1 mM), 2-ME (50 μM), and puromycin (3 μg/mL). Cells were seeded at 5×10^4 cells/well in 96 well plates with 2 ng/mL IL-1β (Peprotech, US) and serially diluted mutTNFs and wtTNF. After 24 h incubation, production of rat GM-CSF was quantified by ELISA according to the manufacturer's protocol (R&D systems, US).

3. Results

3.1. Phage library construction and bioactivity of mutTNF-R90X in culture supernatant of E. coli

In the first PCR step, DNA fragments (251 bp) in which the codon of R90 was replaced with an NNS sequence were synthesized. In the second PCR step, the first PCR product was extended to the PpuM I site. This second PCR product was digested with PpuM I and Eco47 III, and ligated with the phagemid vector pY02 to express mutTNF-R90X in culture supernatant of *E. coli*. We confirmed that R90 in clones was randomly replaced with other amino acids by sequence analysis (Fig. 1).

To screen the functional mutTNFs, the binding affinities of mutTNF-R90Xs for anti-human TNF-α neutralization antibody were measured using culture supernatant (Fig. 2). As a result, mutTNF-R90R, R90P, and R90K showed significant binding affinity for the anti-TNF-α neutralization antibody. Next, cytotoxicity of mutTNF-R90Xs against L-M cells was also evaluated using culture supernatant (Fig. 3). As a result, mutTNF-R90R, R90P, and R90K showed significant cytotoxi-

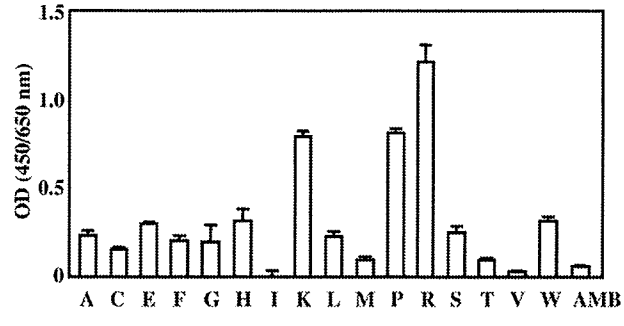

Fig. 2. Selection of clones by ELISA using anti-TNF neutralizing antibody. The reactivity of mutTNF-R90X with anti-TNF antibody was quantified by ELISA using culture supernatant of *E. coli* cells carrying the phagemid vector encoding mutTNF-R90X. Anti-TNF antibody was used as the capturing antibody, and bound mutTNF-R90X was detected with a biotinylated anti-TNF polyclonal antibody followed by addition of HRP-conjugated avidine and substrate reaction. Then the OD (450–655 nm) was measured. Each value represents the mean±SD.