いて，特に注意して解析を行う必要があるが，機能性人工タンパク質の場合は，化学修飾により製造される修飾タンパク質の修飾位置異性体の問題，構造改変により目的以外の生物活性が変化している可能性，抗原性の問題，などが特に留意すべきポイントである。

製造工程でPEG化，デキストランやマンノース等を用いた糖修飾のような化学的改変(化学修飾)を行う場合，人為的に施されるPEG化反応や糖付加反応などはタンパク質の部位特異的に起こるものではないため，1種類あるいは数種類のアミノ酸に，しかも複数ヵ所にPEGあるいは糖などがランダムに導入される場合が多い。したがって，PEG化反応あるいは糖修飾後の機能性人工タンパク質はPEG化あるいは糖修飾された部位，導入されたPEGや糖の分子数などにおいて異なる構造を持つ分子種の混合物となり，分子量などを指標に精製された画分についても，修飾位置異性体の混合物となってしまう。したがって，特性解析においては，得られた修飾タンパク質について，分子量，PEGあるいは糖などの結合分子数，PEGあるいは糖などの結合部位，修飾位置異性体の構成比といった構造・組成や物理化学的性質を最新の分析法を用いて明らかにすると共に，修飾位置異性体ごとの生物学的性質についても可能な範囲で詳細な解析を行う必要がある。修飾位置異性体ごとに作用プロファイルが異なる場合，修飾位置異性体の混合物は機能面(生物活性，体内挙動)から見ても不均一な機能分子の集団となり，そのために修飾位置異性体の構成比が有効性および安全性に影響を及ぼす可能性がある。実際，PEG化インターフェロンでは，PEGの修飾位置異性体ごとに抗ウイルス活性が異なることが報告されている。また，修飾位置異性体の構成比が異なるロット間では体内動態も異なるとされている。修飾タンパク質における修飾位置異性体の解析手法としては，例えば，液体クロマトグラフィーにより異性体を分離し，それぞれのピークについて，ペプチド分析，アミノ酸配列分析，質量分析などを行うことによって，修飾部位を同定することが可能である。また，液体クロマトグラフィーの溶出パターンから異性体の構成比が分かるため，ピーク強度比を規定することで，異性体構成比の一定性が確保できる。

ここで重要なことは，前臨床，臨床試験に供した製品が，どのような不均一な分子種の集団であったか，不純物プロフィール等を明らかにすることである。前臨床，臨床試験を通して不均一性のパターンや不純物プロフィールなどの品質特性の変動がどのような範囲内であったか，その品質特性プロフィールの変動が有効性，安全性にどのように影響を及ぼしたかを精密に観察する必要がある。その結果，有効性，安全性に影響を及ぼすことがなかった品質特性プロフィールの変動の範囲が，以降，維持管理すべき製品の品質特性プロフィールの変動の範囲ということになる。当然，異性体構成比の一定性の確保や目的物質関連物質，主要な不純物に関する試験方法および規格値・判定基準は，製品の規格および試験方法の必須の項目とする必要がある。化学修飾を行う場合に製品に混入する可能性のある不純物については，製造工程由来不純物として，PEG化反応や糖付加反応などの工程で用いられる試薬やその変化物を評価項目に加える必要がある。また，目的物質由来不純物として，非結合型となった遊離のタンパク質，PEGが目的とする分子数以上に結合したdi-あるいはオリゴPEG変異体，O-結合型PEG修飾体，凝集体が混在する可能性を評価して，必要に応じて許容量に関する規格を設定すべきである。目的物質の脱アミド体や酸化体も多くのタンパク質性医薬品では，留意すべきものである。これらが，目的物質に匹敵する生物活性と安全性を有していれば目的物質関連物質として有効成分の一部を構成するが，目的物質に匹敵しない場合は目的物質由来不純物となる。

修飾反応条件が修飾部位異性体の構成比に大き

く影響し，原薬の生物活性にも影響を与える可能性が考えられることから，製品の品質の一定性を確保するためには，PEG あるいは糖など修飾条件，精製工程などは厳密に工程管理されなければならない。また，工程管理の中では，PEG あるいは糖鎖など付加反応に用いられる各種試薬の品質管理なども必要であろう。最終製品の規格および試験方法で不均一性を含む製品の品質特性プロフィール全体をカバーした十分な試験が実施できないときには，修飾条件，精製工程の工程管理や各種試薬の品質管理をより厳密に行うことで総合的に製品の品質とその恒常性を保証する必要がある。

機能性人工タンパク質の生物学的性質に関しては，天然に存在するタンパク質からの構造改変により目的以外の生物活性が変化している可能性があるため，慎重な検討が必要である。改変により意図しない変化が生じた例として，持続型のインスリン改変体であるインスリングラルギンでは，インスリン受容体との親和性はインスリンと差異がないものの，インスリン様成長因子 IGF-1 受容体との結合親和性がインスリンの 6～8 倍であると報告されている[4]。げっ歯類を用いた 24 ヵ月間反復投与の発がん性試験により，インスリングラルギンは発がん性を有さないと判断されているが，IGF-1 受容体への高親和性結合と安全性との関連の全貌が必ずしも明らかにされたとは言えない。このような特性を持つ機能性人工タンパク質で従来の製品より医薬品としてより有用と目されるものの場合には承認を可とされたとしても，市販後安全対策（ファーマコビジランスプランニング）をしっかりとたて，市販後調査などにより安全性を慎重に観察していく必要がある。ちなみに，医薬品として実用化されているものではないが，B 鎖 10 番目の His を Asp に置換し，インスリン受容体との親和性が亢進した改変インスリンでは，ラットで乳腺腫瘍の発生が報告されている[5),6)]。1 アミノ酸の置換により発がん性が生じることを示した典型例であり，改変による生物学的性質の変化が安全性に大きく影響する場合があることを認識する必要がある。

機能性人工タンパク質の免疫原性・抗原性についても注意深い観察が必要である。一般に PEG 化タンパク質の場合には免疫原性・抗原性が減弱すると言われている。一方，アミノ酸置換体などの改変タンパク質の場合に必ず懸念されるのが，免疫原性および抗原性の問題である。タンパク質性医薬品の免疫原性や抗原性は，タンパク質の一次構造上の特徴はもとより，高次構造，製剤中の目的物質の凝集体や製造工程由来不純物，添加剤，あるいは投与経路などにも大きく影響される[7)]。通常のヒト型組換えタンパク質性医薬品でも免疫原性や抗原性が問題になる例があるが[8)]，もともとヒトには存在しない機能性人工タンパク質の場合には，その免疫原性・抗原性により一層の注意を払わねばならない。ただし，がんなどのように宿主の免疫機能が低下している患者への機能性人工タンパク質の適用と，免疫機能が過剰に亢進しているアレルギーやリウマチといった炎症性疾患への適用では，異なった免疫原性・抗原性問題への取組みやその評価基準が必要であると考えられる。またヒトに対する抗原性は一般に動物実験では評価できず，非臨床試験における評価は困難であるため，ヒトでの抗原性の予測についての方法論の確立などが望まれるところであるが，当面は治験中や市販後における注意深い臨床観察がなによりも重要であると考えられる。

5. おわりに

本節では，昨今加速度的に創出されつつある機能性人工タンパク質の品質・安全性評価の観点から，現状と将来展望，課題について論じた。ゲノミクス，トランスクリプトミクスやプロテオミクス，グライコミクス，メタボロミクスといった大規模な網羅的解析および高効率高発現・標的細胞

指向性のある遺伝子導入技術や発現制御技術，特異的評価系などによる個々の遺伝子やタンパク質の機能解析により，疾患の治癒に関わるタンパク質(医薬品シーズ・タンパク質)の探索・同定が進展し，今後益々，機能性人工タンパク質が，種々の難治性疾患に対する有用な治療薬として開発の対象となることが期待される。一方で，ウイルスや細菌のゲノム解析等の進歩も相俟って，より効率よく宿主の免疫機能を活性化したり，メモリー機能を亢進させたりするような新興・再興感染症に対する機能性人工ワクチン(抗原タンパク質)の登場も予想される。機能性人工ワクチンの場合，免疫原性・抗原性そのものが薬効となる一方で，非特異的免疫の活性化や精緻に構築されている生体免疫機構を乱すことによる思わぬ副作用が発現する可能性に十分な注意が必要となる。また，分子特性・品質特性，用法・用量や投与期間など考慮しつつ，必要に応じて上述した機能性人工タンパク質の品質・安全性確保上の課題をクリアする必要があると思われる。

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細胞内薬物導入キャリアとしての細胞内移行ペプチドの応用技術

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要 旨

細胞内移行ペプチド（protein transduction domain：PTD）は，タンパク質や核酸，ナノ粒子といった高分子医薬を効率的に細胞内へと導入可能であることから，細胞内への物質導入技術として注目を集めている。本稿では，実際に筆者らが行ったPTDの特性評価から得られた知見を紹介したうえで，現在世界で試みられているPTDの応用例を概説する。また後半部では，PTDの応用範囲をさらに拡大するための技術として，PTDとfusogenic peptideを併用した際の細胞質内への物質導入の高効率化，ならびに細胞内導入活性に優れた新規PTDの創製研究に関して述べる。

キーワード：細胞内移行ペプチド（PTD），DDS，ファージ表面表示法，ペプチドライブラリー，HIV-1 Tat，fusogenic peptide，オルガネラターゲティング，細胞内薬物治療

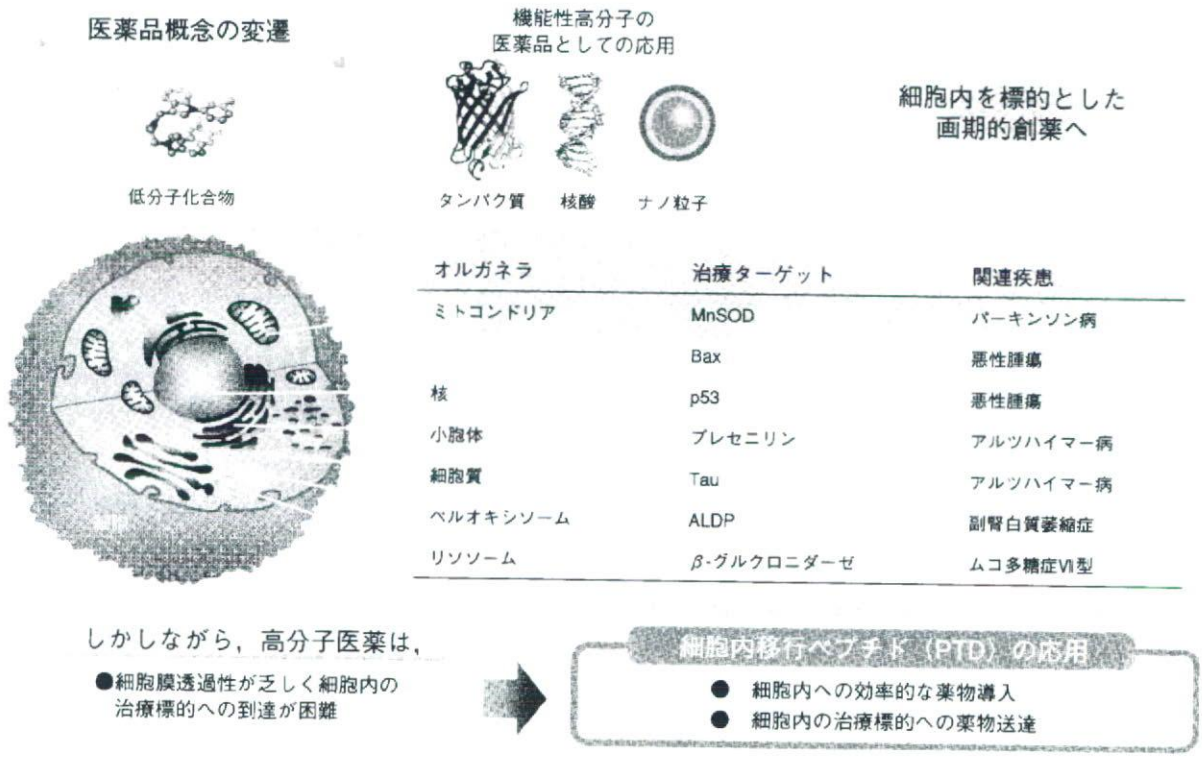
Ⅰ．背景

近年の医薬品概念のパラダイムシフトに伴い，医薬品は低分子有機化合物であるという固定概念はなくなり，癌に対する抗体分子をはじめとする生理活性タンパク質や各種核酸といった機能性高分子医薬が疾病の治療・診断へ応用されている。多くの難治性疾患の発症は，遺伝的あるいは外的要因によってタンパク質の機能や発現に異常が生じ，体内の恒常性が破綻することにより起こるため，それら治療標的タンパク質の機能を直接的に制御可能な上記医薬品は，21世紀型医薬品として，その開発に世界的な期待が寄せられている。しかしながら，上市されているこれらの医薬品の治療ターゲットの多くは「細胞外」の可溶性分子やレセプターに限局されてしまい，「細胞内」への展開はいまだ研究途上であると言わざるをえない。したがって，21世紀型医薬としての機能性高分子の治療適用範囲を「細胞内」へと拡大した新たな機能性高分子製剤の開発が待望されている。

1つの正常細胞を構成するタンパク質は10億にものぼり，その多くが細胞内で機能しているという事実から，一般的に数多くの治療ターゲットが細胞内に潜んでいる可能性が示唆されている。実際に，近年のタンパク質研究の進展に伴い，癌における転写因子や，アルツハイマー病をはじめとする神経変性疾患におけるミトコンドリア酵素など，各種難治性疾患の原因タンパク質が細胞内の特定オルガネラに存在し，その機能を発揮していることが明らかとなりつつある（図❶）。したがって，今後の医薬品開発では，細胞内を標的とした薬物治療戦略がますます注目されていくものと考えられる。しかしながら，このような細胞内疾患関連タンパク質を治療標的とした場合，高分子製剤特有の問題点により，その実用化は極度に制限されてしまう。それは，一般的にタンパク質，核酸などは，高分子量かつ水溶性であるため，脂質二重膜から構成される細胞膜を通過し細胞内へ侵入できないことに起因する。したがって，細胞内タンパク質をターゲットとした新規治療戦略を実現するためには，タンパク質，核酸などの高分子医薬を，活性を保ったまま細胞内へと導入できる技術が必要不可欠である。

このような背景の下，近年，protein transduction domain（PTD）と呼ばれるペプチドが，細胞外から細胞内へと

図❶ 細胞内治療ターゲットに対する薬物療法とPTDの応用技術



細胞内の特定オルガネラには数多くの疾患関連タンパク質が含まれており，難治性疾患を根治可能な高分子医薬の細胞内適用が望まれている。そこで，膜透過性が乏しい高分子医薬の細胞内送達をめざし，細胞内移行ペプチド（PTD）の応用が注目されている。

移行する活性を有することが見出され，これらを細胞内薬物導入キャリアとして応用しようとする試みに注目が集まっている。HIV-1由来のTatペプチドに代表されるPTDは，塩基性アミノ酸を多く含む配列を有することを共通点とし，タンパク質，核酸，ナノ粒子などの様々な高分子物質を，その活性を保持したまま細胞内へと送達できることが知られている。また，従来までの物質導入法であるカチオニックリポソーム法などのトランスフェクション試薬や，エレクトロポレーションなどの直接導入法などと比較して，一般に高効率で細胞内への物質導入が可能であるという利点を有していることから，そのキャリアとしての利用に大きな期待が寄せられている。

Ⅱ．Protein Transduction Domain (PTD)

PTDに関する研究は，1988年にGreenやFrankelらによってHIV-1の転写因子であるTatタンパク質が，細胞外から細胞内への移行活性を有していることが見出されたことに始まる。1990年代に入ると，Tatの細胞内移行活性は，タンパク質分子内の塩基性に富む十数個のアミノ酸配列によって担われていることが明らかにされた。さらにその後の研究により，このペプチド配列を結合させれば，Tat以外のタンパク質をも生細胞内に導入できることが発見され，このペプチド配列はprotein transduction domain（PTD）と呼ばれるようになった。現在では，Tatペプチドと同様に細胞内移行活性をもつ，ショウジョウバエ・ホメオドメインのAntennapedia由来ペプチド（Antp），HIV-1のmRNA核外輸送タンパク質Rev由来ペプチド（Rev），単純ヘルペスウイルスの構造タンパク質N末端由来のVP22ペプチド（VP22）などがPTD活性を有することが報告されており[1]（表❶），細胞内への薬物導入キャリアとしての応用に大きな期待が寄せられている。これらPTDに共通してみられる特徴は，

表❶　代表的なPTDとそのアミノ酸配列

PTDの代表例	由　来	配列(R,K,H：塩基性アミノ酸)
Tat	HIV-1	GRKKRRQRRRPPQ
Antp	*Drosophila*	RQIKIWFQNRRMKWKK
Rev	HIV-1	TQRARRNRRRRWRERQR
VP22	HSV	NAKTRRHERRRKLAIER

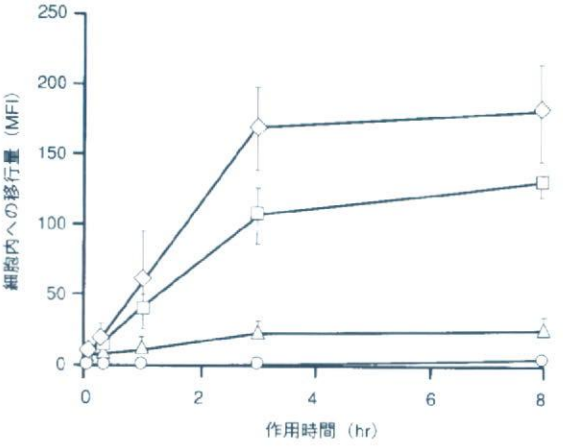
アルギニンやリジンといった塩基性アミノ酸が配列中に多数みられることであり，アルギニンのみを7～十数個，人工的に連結したポリアルギニン配列にもPTD活性があることが明らかにされるなど[2]，PTDの塩基性が細胞内移行に重要な役割を担っているものと考えられている。実際にわれわれの行った検討においても，PTDの細胞内移行活性にこのような塩基性アミノ酸の重要性を示すデータが得られており（**図❷**），そのアミノ酸配列よりもアミノ酸組成が重要である可能性が強く示唆されている。

図❷では，HeLa細胞に対し，FITCラベルしたペプチドを添加し，細胞内への取り込みをフローサイトメトリーにより評価した結果を示している。PTDの代表例であるTatペプチド，ならびにTatペプチド内のアミノ酸配列をランダムに組み換えたTatスクランブルペプチドでは，3時間以内に大多数のペプチドが取り込まれ，レセプター依存性エンドサイトーシスにより効率的に細胞内に取り込まれるRGDSペプチドよりも，さらに優れた細胞内移行活性を有することがわかる。とりわけ，Tatペプチドには劣るものの，Tatスクランブルペプチドが優れた細胞内導入効率を有するという事実は興味深く，本知見はPTDの細胞内への導入に，そのPTDの塩基性，すなわちアミノ酸組成が重要であるという先の可能性を裏づけるものである。

Ⅲ．PTDの応用例

これまでに細胞内で機能する活性タンパク質をPTDを用いて細胞内へと導入し，疾病治療へと応用しようとする試みは多数報告されている。**表❷**には，数あるPTDの応用研究の中から，*in vivo* への適用に成功した例を記載した[3)13)]。最も例が多いのは，癌に対する応用であり，細胞死を誘発するペプチドやカスパーゼといったタンパク質，あるいは癌免疫療法をめざした細胞内への抗原デリバリーが盛んに行われている。また，逆にBcl-xLをはじめとする細胞死抑制分子を用い，脳虚血再灌流障害の

図❷　HeLa細胞に対するTatペプチドの細胞内移行特性



FITC修飾した各種ペプチドの細胞内移行を，フローサイトメトリーにより解析した。その結果，Tatペプチド（◇），Tatスクランブルペプチド（□）は，レセプター依存性エンドサイトーシスで取り込まれるRGDSペプチド（△），ネガティブコントロールのFITCデキストラン（○）に比べ，有意に細胞内移行活性が優れていることがわかる。なお，各種ペプチドのアミノ酸配列は以下のとおりである。Tatペプチド：GRKKRRQRRRPPQ，Tatスクランブルペプチド：RPGRRQPRKQKRR，RGDSペプチド：RGDS。

軽減をめざす研究[12]では，現在でも多様な細胞死抑制因子を用いた報告がなされている。

PTDの利点として忘れてはならないのは，タンパク質やペプチドにとどまらず様々な高分子を細胞内へと導入することができることであろう。2000年にLewinらによって報告された手法は，PTDを基礎研究へも応用展開できることを示している。彼らは，Tatペプチドで標識した超常磁性酸化鉄ナノ粒子を投与し，造血幹細胞や神経前駆細胞をラベルすることに成功している[13]。酸化鉄ナノ粒子でラベル化された細胞は，生体内で正常に分化し，その分布はMRIによってイメージングが可能である。現在，本方法は様々な幹細胞の *in vivo* での動態を追跡する手法として応用されている。

PTDの応用技術は，細胞内への物質輸送だけにとどまらず，生体バリアを乗り越えるためのツールとして応用する試みも存在する。2000年にはRothbardらによりポリアルギニン：R7とシクロスポリンを結合させることで，皮膚における抗炎症効果を増強したという報告がなされた[8]。その作用増強メカニズムは明確ではないが，まず細胞内へと取り込まれたPTD融合体の一部が，組織内

表❷ PTDの *in vivo* への適用例（文献3～13より）

治療標的・応用	導入分子(Cargo)	PTD	文 献
腫瘍	PKCδ inhibitory peptide	Tat	3
腫瘍	Caspase-3 fusion protein	Tat	4
腫瘍（ワクチン）	OVA CTL peptide	Antp	5
腫瘍（DCワクチン）	OVA	Tat	6
炎症	IKKβ C-terminal peptide	Antp	7
炎症（皮膚）	Cyclosporin A	R7	8
PNP欠損症	Purine nucleoside phosphorylase	Tat	9
ポリグルタミン病	polyQ binding peptide 1（QBP-1）	Tat, VP22 etc.	10
神経細胞死	GDNF	Tat	11
脳虚血再灌流障害	Bcl-xL	Tat	12
細胞動態トレース	超常磁性ナノ粒子	Tat	13

部へと浸透しえたことによるものであると考えられている。本概念によって，現在では経口投与製剤開発において，小腸上皮細胞といった生体バリアを乗り越えるためのDDSツールとしてのPTDの応用にも注目が集まっている。

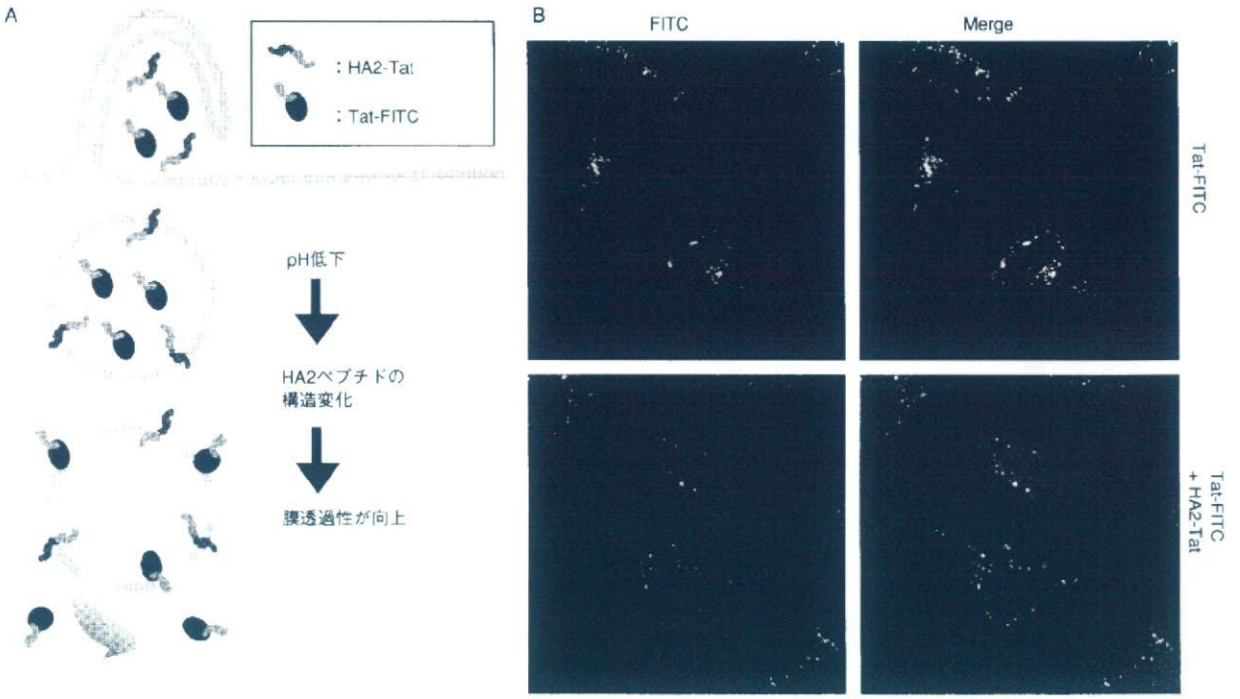
しかしながら，このような成功例が存在する一方で，PTDを利用した現存の応用研究のほとんどは *in vitro* での検討にとどまり，**表❷**に示したような *in vivo* への適用はごくわずかに限定されてしまっている。それは，PTDの応用範囲は細胞内に微量存在することにより機能を発揮する分子に限られていること，つまり細胞内に高濃度存在させなければ十分な効果を発揮しえない分子への応用は，現存するPTDの細胞内導入効率では不十分であることが一因と考えられている。そのような現状を打破するためには，PTDの細胞内移行メカニズムを詳細に理解し，その物質導入を改良しうる方法論の開発が必須であると考えられる。

著者らは，このような細胞内移行メカニズム解析の一環として，Tat-FITCの細胞内導入後の挙動を共焦点レーザー顕微鏡で観察した（**図❸**）。その結果，細胞内に取り込まれたTat-FITCは，大部分はエンドソーム内にトラップされ，そのほとんどが治療標的の存在する細胞質や標的オルガネラへと到達しえないということが明らかとなった。したがって，標的オルガネラへの効率的な薬物送達を達成し，PTDを *in vivo* への適用にかなうツールとして応用していくためには，①PTDの細胞内挙動を理解し，エンドソームからの効率的なエスケープを達成すること，②PTDの細胞内導入活性自身を強化することで，細胞内取り込みの絶対量を増大させること，といったPTDの有効性向上に関するアプローチを融合することが有効であるものと考えられる。以上の背景を踏まえ，次項からは，われわれが行ってきたPTDの有効性向上をめざした2つの基盤技術開発について紹介する。

IV. PTDの有効性向上 -HA2ペプチドによるエンドソームからのエスケープ-

HA2ペプチドは，インフルエンザウイルス表面に存在する感染に必須のタンパク質，HAタンパク質由来であり，fusogenic peptideとして知られている。インフルエンザウイルスが細胞内に侵入する際には，HAタンパク質はエンドソーム内の酵素によってHA1とHA2の2つのフラグメントに切断され，HA2フラグメントの働きによってエンドソーム膜の透過性が亢進し，ウイルス粒子の効率的な細胞質内への侵入が促進される。最近の研究によって，このHA2フラグメントのN末端ペプチド配列（influenza HA2 peptide）は，エンドソーム内低pH環境下で構造変化を生じ，それによって膜透過性を向上させることが明らかとされた[14]。著者らは，HA2とTatの融合体HA2-Tatを利用し，モデル薬物としてのTat-FITCと同じエンドソーム内へ取り込ませることで，これらの効率的な細胞質内への送達が可能になるものと考えた（**図❸**）。まず，Tat-FITCとHA2-Tatペプチドを同時にHeLa細胞に作用させ，その細胞内挙動を共焦点レーザー顕微鏡で観察した。Tat-FITC単独作用では，FITC由来の蛍光（緑）がドット状に観察されたのに対し，HA2-Tatペプチドを共存させると，その蛍光が核を含めた細胞全体に拡散し

図❸ Influenza HA2 ペプチドを用いた Tat-FITC のエンドソームからのエスケープ



緑：Tat-FITC，青：hoechst33342（核），赤：FM4-64（エンドソーム）

A. HA2 ペプチドは，低 pH 環境下で構造変化を起こし，エンドソーム膜の透過性を向上させる。
B. Tat-FITC 単独作用群（上段）では，そのほとんどがエンドソームにトラップされてしまうのに対し，HA2 ペプチドを共存させた群（下段）では，Tat-FITC が細胞全体に分散していることがわかる。（グラビア頁参照）

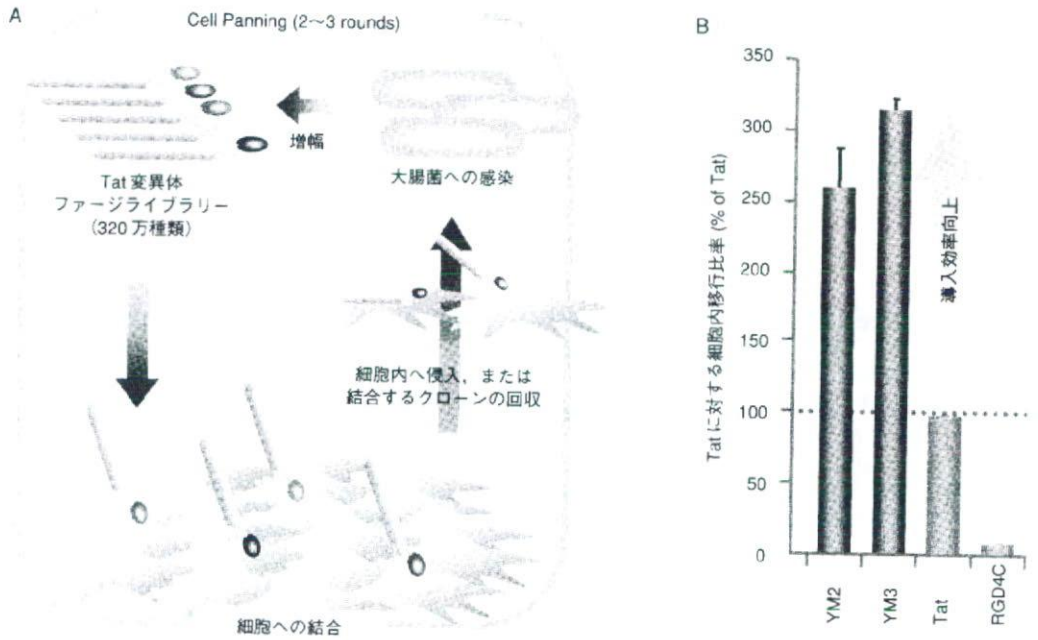
て観察された。本方法論は，細胞質内への積極的な薬物送達という観点から，細胞質や特定オルガネラを標的とした薬物治療の有効性を向上可能なアプローチとなるものと期待される。

V．PTD の有効性向上 - ファージ表面提示法を用いた新規 PTD の創製 -

PTD による物質導入の高効率化で最もシンプルなアプローチは，PTD 自身の細胞内導入活性を向上させることであろう。そのため，優れた PTD の開発をめざして，現在世界的に PTD の立体構造解析に基づいたアナログ作製や細胞への吸着性向上を目的としたカチオン性アミノ酸導入体の作製が，ペプチド合成法によりトライアンドエラーで進められている[15]。しかしながら，これらの方法は膨大な時間・労力を費やすばかりか，作製可能なペプチドの多様性（種類）にも限界があるなど，期待どおりの PTD はほとんど得られていない。以上の背景から著者らは，ファージ表面提示法を駆使することで，その細胞親和性・特異性や細胞内移行性といった機能をハイスループット解析できるテクノロジーの開発を試みている。

ファージ表面提示法は，数百万から数十億種類ものタンパク質ライブラリーをファージ表面へと再現する技術であり，その膨大な変異体の中から，活性増強，特異性の変化といった分子進化を遂げた機能性変異体をわずか 1 ～ 2 週間という短期間にスクリーニングする手法である[16]。著者らは，Tat 配列中のアルギニンがその細胞内移行に重要な役割を担っていることに着目し（図❷），アルギニン以外の 5 つのアミノ酸をランダム化した Tat ペプチドライブラリー（20^5 = 3,200,000 種類）を構築することで，野生型 Tat ペプチドよりも細胞内移行活性に優れた新規 PTD を得ようと試みた[17]。作製したファージライブラリーを培養細胞へと添加し，細胞へ結合あるいは細胞内へ侵入したファージを回収し再度大腸菌に感染させることによって，ファージを増幅した（図❹）。こ

図❹ ファージ表面提示法を駆使した新規PTDの創製技術



A. Tatペプチド配列中5つのアミノ酸をランダムに改変したTatライブラリーの多様性は320万種類以上にも及ぶ。これらの中から細胞に結合，または細胞内に取り込まれるクローンのみを選択・増幅するcell panningを2～3回繰り返すことによって，効率的に細胞内に移行するTat変異体をスクリーニングした。

B. 得られたTat変異体の中には，3倍以上の効率で細胞内に移行する新規PTDが含まれていた。 （グラビア頁参照）

のような一連のcell panningを2～3回繰り返したファージプールの中には，効率的に細胞内に取り込まれる新規PTDを表面提示したファージクローンが含まれているはずである。実際に得られたクローンの中には，図❹Bで示すような，野生型Tatよりも3倍以上効率的に細胞内に取り込まれるクローンが含まれていた。現在，本方法論のさらなるシステムアップをめざし，さらに細胞内移行活性に優れる，あるいは細胞特異性を有する新規PTDの創製などへと，本テクノロジーを応用展開してゆく予定である。

おわりに

PTDは発見されてから10年以上の歴史をもち，古くから多くの研究者がその応用を試みてきた。しかしながら，近年のPTD基礎研究の進展により，PTDの細胞内移行メカニズムが徐々に明らかになるにつれて，現存するPTDの有効性をさらに向上するアプローチも可能となりつつある。今回の総説では，エンドソームからのエスケープを促すこと，新規PTDを創製することの双方によって，PTDの有効性向上をめざすアプローチを紹介した。今後は，これら2つの異なるアプローチを融合することで，さらなる有効性の向上をめざす予定である。また将来的には，このような研究をシステムアップすることで，細胞特異性や標的指向化といったわれわれの望む機能を付与した新規PTDを人工的に作り出すことも可能となるかもしれない。本稿で紹介した技術開発が，今後の細胞内タンパク質を治療標的とした新規DDS製剤の誕生への一助となることを期待し，本稿の結語とする。

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学生時代より「ファージ表面提示法を駆使した機能性人工タンパク質の創製」をキーワードとし，サイトカインの改変，抗体，ペプチドの高機能化によるタンパク性医薬品の品質向上をめざした研究を行ってきた。また現在では，得られたアミノ酸改変体に関するX線結晶構造解析も同時に推進することによって，アミノ酸改変により得られた新機能とその構造との連関評価についても検討中である。

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

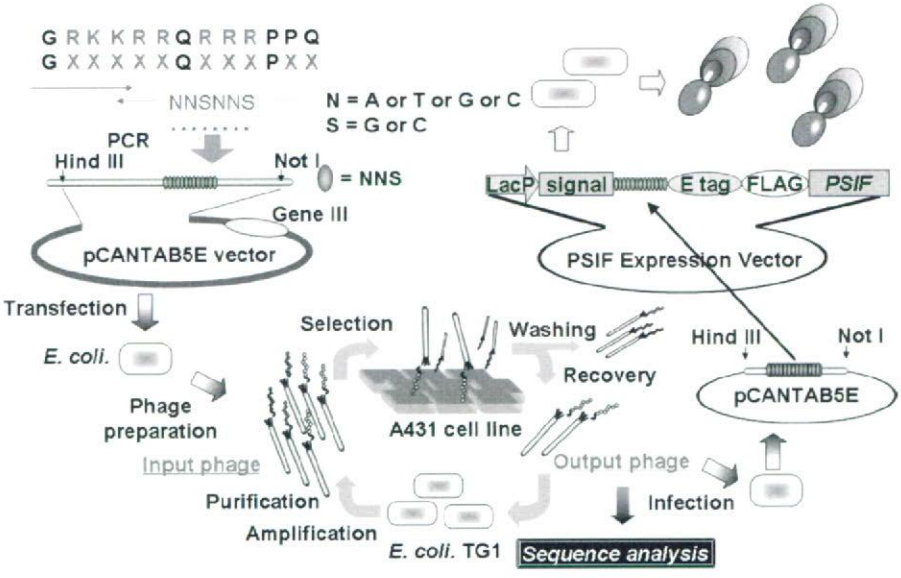

Fig. 1. Schema for Screening CPPs

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

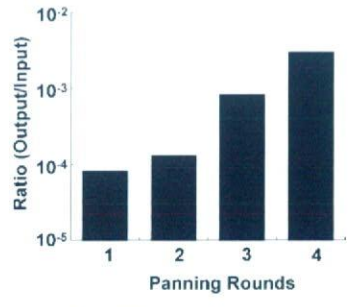

Fig. 2. Enrichment of Phage Clones by Biopanning

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.

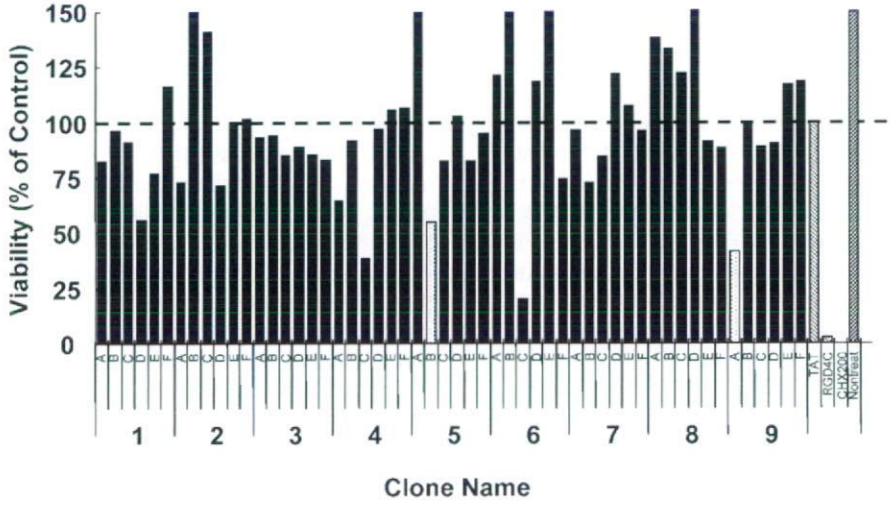

Fig. 3. Measurement of Penetrating Activity as an Index of Cytotoxicity to A431 Cells

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

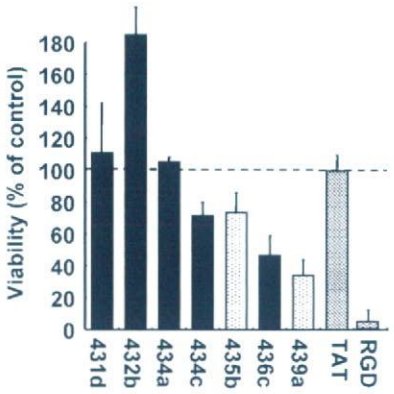

Fig. 4. Evaluation of Cell Penetrating Activity of Individual Clones

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%).

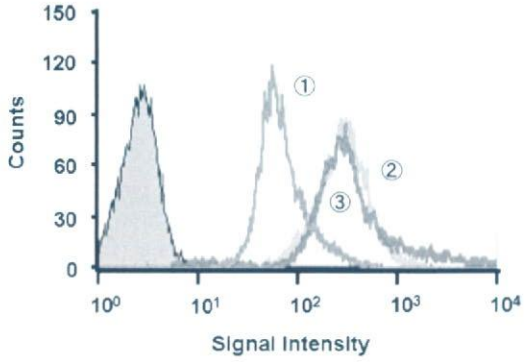

Fig. 5. Intracellular Penetrating Activity of FITC-Labeled CPPs on A431 Cells

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.

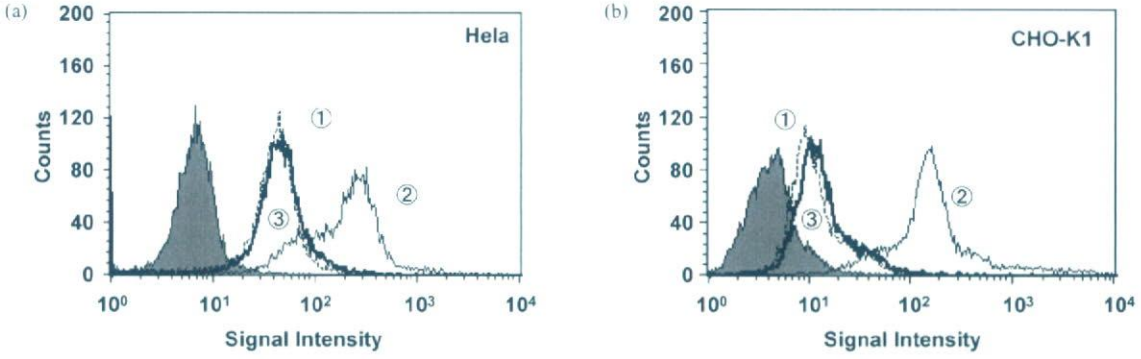

Fig. 6. Intracellular Penetrating Activity of FITC-Labeled CPPs on Human and Murine Cell Lines

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.

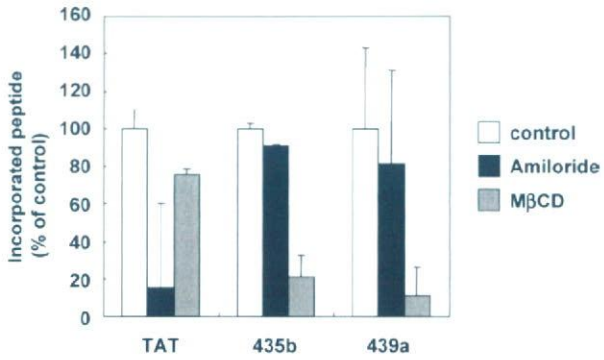

Fig. 7. Inhibitory Effects of Amiloride and MβCD for Peptide Incorporation on A431 Cells

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-

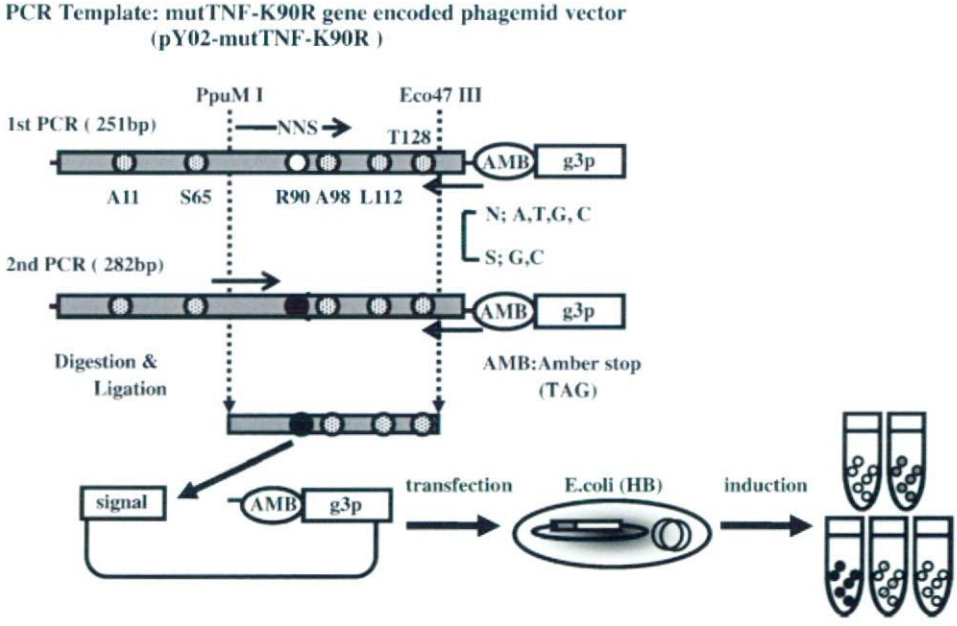


Fig. 1. Construction of mutTNF-R90X *E. coli* library.

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.

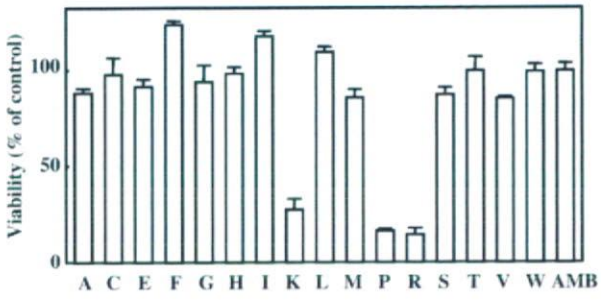


Fig. 3. Selection of clones by cytotoxicity assay using L-M cells. The cytotoxicity of mutTNF-R90X was assessed on L-M cells using culture supernatant of *E. coli* cells carrying the phagemid vector encoding mutTNF-R90X. L-M cells (3×10^4 cells/well) treated with actinomycin D were incubated with mutTNF-R90X for 24 h, and viabilities were assessed by methylene blue assay.

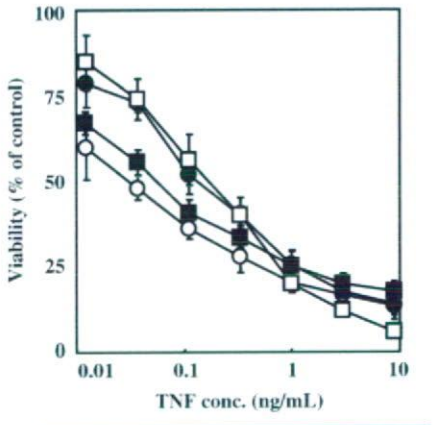
city to L-M cells. Other mutTNF-R90Xs have almost no affinity for anti-TNF-α antibody or show cytotoxicity. These results indicate that, as was expected, amino acid residue 90 should be lysine, arginine, or proline to retain its bioactivity and conformation.

3.2. Characterization of purified mutTNF-R90, P90, and K90

To assess their properties in detail, recombinant mutTNF-R90R, R90P, and R90K proteins were purified, and used in a binding analysis for TNF receptors, TNFR1 and TNFR2. As the result of gel filtration chromatography (GFC), the three mutTNF recombinant proteins exhibited the same retention time, indicating that they formed homotrimers (data not shown). Bioactivity of the three mutTNFs via mouse TNFR1 was confirmed using a cytotoxicity assay in L-M cells. As we reported previously, the cytotoxicity of mutTNF-R90P was almost the same as that of human wtTNF, while mutTNF-R90K was 3-fold higher and mutTNF-R90R was 5-fold higher (Fig. 4). Bioactivity of these mutTNFs via human TNFR1 was also assessed in HEp-2 cells. The cytotoxicity of mutTNF-R90P was the same as that of wtTNF. Interestingly, mutTNF-R90K and R90R exhibited 10-fold higher cytotoxicity than wtTNF (Fig. 5). Finally, bioactivity via human TNFR2 was assessed on GM-CSF production from PC60-hTNFR2 cells (human TNFR2 transfected PC60 cells). All three mutTNFs showed 2-fold higher bioactivity via TNFR2 than wtTNF (Fig. 6). These results indicate that mutTNF-R90R and R90K can stimulate TNFR1 more selectively than wtTNF.

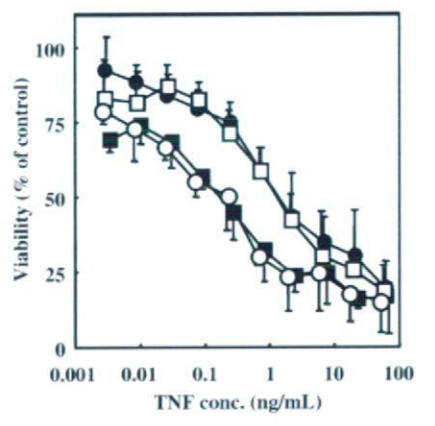
3.3. Kinetics of mutTNFs on TNFR1 and TNFR2 affinity

The increase in affinity for TNF receptors may be one of the factors that contribute to the bioactivity enhancement. Therefore, we measured the affinities of mutTNFs for TNFR1 and TNFR2 using surface plasmon resonance (BIAcore). By comparison of the dissociation constants (KD) of mutTNFs against TNFR1, the affinities of mutTNF-R90K and R90P were 2 to 3-fold higher than wtTNF (Table 1). The affinity of mutTNF-R90P, however, was half that of wtTNF. Thus, the



	EC50 (ng/mL)
□ wtTNF	0.17
■ mutTNF-R90K	0.05
○ mutTNF-R90R	0.03
● mutTNF-R90P	0.14

Fig. 4. Cytotoxicity of mutTNFs against L-M cells. The bioactivity of mutTNF via mouse TNFR1 was measured by the cytotoxicity assay against L-M cells in the presence of actinomycin D. Each value represents the mean ± SD. The EC_{50} shows the concentration of TNF required to inhibit L-M cell viability by 50%.



	EC50 (ng/mL)
□ wtTNF	1.30
■ mutTNF-R90K	0.17
○ mutTNF-R90R	0.15
● mutTNF-R90P	1.40

Fig. 5. Cytotoxicity of mutTNFs against HEp-2 cells. The bioactivity of mutTNF via human TNFR1 was measured by a cytotoxicity assay against HEp-2 cells in the presence of cycloheximide. HEp-2 cells (4×10^4 cells/well) were incubated with mutTNFs for 18 h, and their viabilities were assessed by methylene blue assay. Each value represents the mean ± SD. The EC_{50} shows the concentration of TNF required to inhibit HEp-2 cell viability by 50%.