GTPase activity, it is possible that thioredoxin may play a role in Rho-mediated cytoskeletal remodelling during cell migration.[29]

In summary, our data show that activation of nicotinic acetylcholine receptors (nAChRs) induces endothelial cell migration. Furthermore, growth factor (VEGF and bFGF)–induced endothelial cell migration involves nAChR activation. By transcriptional profiling we have identified convergent genomic responses of ECs to nicotine, VEGF, and bFGF. Identification of concordantly regulated genes may provide novel insights into molecular processes mediating EC migration and angiogenesis. Indeed, using this approach we found that TXNIP, by regulating thioredoxin activity, is centrally involved in nAChR-mediated EC migration. Our studies provide evidence for a cholinergic contribution to growth factor-induced EC migration. The nAChRs may play an important role in growth factor-induced angiogenesis, and thus may be a target for therapeutic modulation in disorders of pathological or insufficient angiogenesis.

Disclosures

Dr Cooke holds equity in Athenagen Inc, which has licensed Stanford University patents for the use of nAChR agonists and antagonists for disorders of angiogenesis. Dr Cooke is an inventor on these patents, and receives royalties from the licenses. A patent is being filed based upon the intellectual property described in this manuscript that may benefit J.P.C., M.K.C.N., E.C., and J.W.

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10 ヒト細胞治療薬の品質と安全性確保について

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研究テーマはバイオ医薬品及び細胞治療薬の品質・安全性確保技術の開発。趣味は読書。

Key words：細胞治療薬，薬事法，細胞・組織加工医薬品，品質と安全性

Abstract

再生医療・細胞治療の開発が急速に進んでいるが，臨床研究や高度先進医療としての開発に比較し薬事法の規制を受ける細胞治療薬の開発が遅れているといわれている。より再生医療・細胞治療を広く国民に提供して行くには，臨床研究等の成果を生かし薬事法の規制のもとに細胞組織加工医薬品等（細胞治療薬；医薬品や医療機器を含む）として開発することが望まれる。本稿では，細胞治療薬を適切に開発して行くために求められるウイルス等の感染因子に対する安全確保，製法の確立や恒常性の維持，品質管理のありかた等について概説した。

はじめに

発生学や幹細胞研究の飛躍的な進展に加え，種々の細胞への分化誘導や増幅法などの培養技術やバイオテクノロジー応用技術の進歩により，ヒトまたは動物の細胞や組織を培養，加工し，さまざまな疾患の治療に用いる細胞治療薬やそれを用いた医療技術の開発が進んでいる。さらに，これらの開発では治験や臨床研究といった異なるアプローチがとられており，医薬品・医療用具といった薬事法上の規制のかかる製品開発を目指す場合ばかりでなく，高度先進医療としての実用化を目指している場合もある。本稿では，特に薬事法の規制を受ける細胞治療薬（細胞・組織加工医薬品等）の指針で求められている開発に当たっての要点を概説する。さらには各国の規制状況との比較を行い，実用化において特に注意を払うべき点について考察したい。

細胞治療薬は，極めて複雑な構造を持ち，かつ生きているというダイナミックな特性を併せ持つことから，従来の医薬品に適用されていた品質管理や，非臨床試験や臨床試験の必要事項は必ずしも適用出来るわけではない。さらに，生きた細胞を投与するために，これまでのバイオ医薬品等のように高度な精製やウイルス不活化・除去工程を適用することが困難であり，安全性に関して特別な配慮が必要とされる。厚生労働省からは，表1にあげたような細胞治療薬に関するいくつかの指針や基準が出されている。特に，平成12年に出された医薬発第1314号通知の別添1[1]，及び別添2[2]は，ヒト細胞治療薬の規制の根幹をなす指針である。別添1は，細胞治療薬の製造に当たって，その採取行為から加工，製造における取り扱いや使用に当たっての基本的要件を示している。別添2は，ヒト由来細胞治療薬に焦点をあて，その品質・安全性・有効性確保のための要件をまとめたもので，承認申請のみならず治験前の確認申請で求められる資料についても明らかにされている。この確認申請の制度は，細胞治療薬については未知・未経験の要素が多いことから，その治験
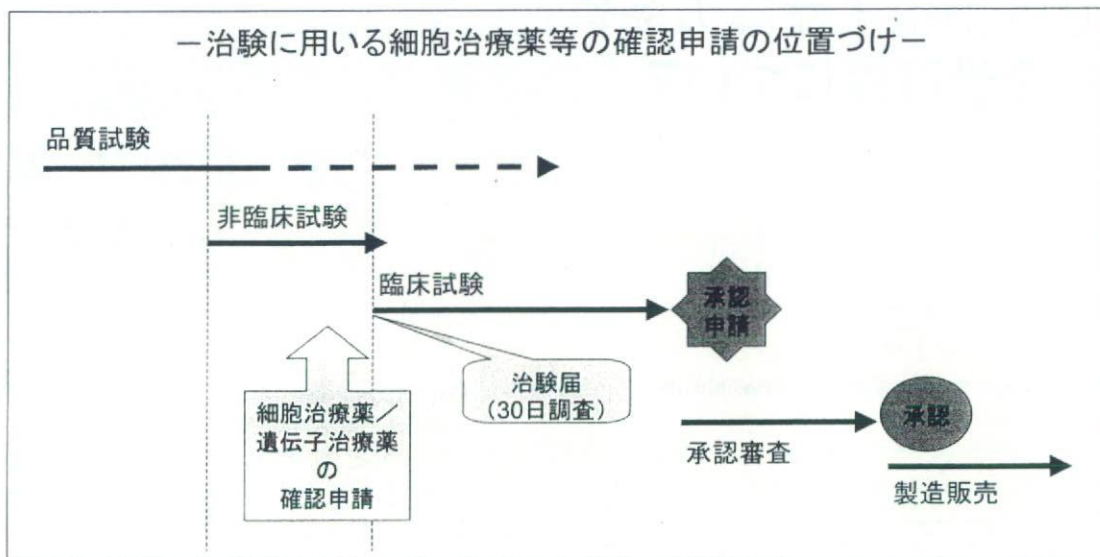
図1 薬事法に基づく先端医薬品の品質・安全性の確保

表1 我が国における細胞治療薬（再生医療）に関連する
指針や通知

細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方
（医薬発第1314号 別添1）
ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針
（医薬発第1314号 別添2）
生物由来製品及び特定生物由来製品の指定並びに生物由来原料基準の制
定等について
（医薬発第052001号）
生物由来製品に関する感染症定期報告制度について
（医薬発第051508号）

を開始する前に一定の品質・安全性を担保する
ことを目的として，厚生労働大臣にその確認を
求めるとされているものである（図1）。確認
申請は，治験の申請前に行われ，その段階で必
要とされるデータはあくまでも安全に治験を実
施するに足る品質・安全性が確保されているか
を確認するためのものである。このような確認
申請は，他には遺伝子治療薬にのみ適用されて
いる制度である。

　第1314号については現在見直し作業が進行中
であり，指針をヒト自己由来細胞製品とヒト同
種由来細胞製品に分ける予定である。それぞれ
の指針については，今後の議論を通じて変更さ
れる可能性があり，本稿ではこれまでの指針に

沿った概説を行うと共に，必要に応じて現時点
で示されている改正案についても触れていくこ
とにする。

1．ヒト由来細胞治療薬関連指針の概要

1）ヒト由来細胞を用いた細胞・組織加工医薬品等の定義

　細胞治療に用いる細胞・組織加工医薬品等と
は，ヒトあるいは動物由来の細胞・組織を加工
した医薬品又は医療用具と定義される。本稿で
は前述したように，細胞・組織加工医薬品等を
細胞治療薬と略す。前記した通知や指針は，細
胞治療に用いる医薬品や医療用具を企業が開発
しようとする場合を対象としており，細胞治療
の臨床研究については対象外とされている。

2）「指針」の対象とする範囲

　「指針」等の対象とする範囲として，輸血用
血液製剤，移植医療としての骨髄移植，臍帯血
移植，ヒト皮膚や骨等を直接利用する医療行為
は含まれていない。また細胞・組織の加工とし
ては，in vitroでの増殖，薬剤処理による細胞の
活性化あるいは生物学的特性の改変，遺伝子工

学的改変を指し，単なる遠心操作等の細胞・組織の分離や抗生物質処理及びガンマ線等の滅菌，冷凍，解凍は含まれない。欧米では，我が国で移植医療として分類される製品についても細胞治療薬として規制がかけられており，この点が大きな違いである。日米欧の細胞治療薬（細胞治療製品）の規制の違いについては，表2を参照されたい。

3）細胞治療薬等の品質や安全性面での問題点

「指針」の安全性面で最も重視されている点はウイルス等の感染症伝播をいかに防止するかである。細胞治療に用いる細胞は滅菌や高度な精製といった処理ができないため，原材料や製造に用いられる試薬や血清等へのウイルス等の混入を如何に防止するかが最重要課題となる。また，製品に感染因子が混入した場合，患者ばかりでなく患者の家族や医療従事者等へも感染が広がる危険性があり，公衆衛生の観点も含め十分な対策が求められる。この点は，欧米のガイドラインとも共通している点であり，細胞治療薬の基本的要件である。

しかし，ウイルス試験にも検出限界があり，また未知のウイルスの存在も考えられるため，ウイルスの潜在を前提とした対策が求められる。原材料となる細胞・組織に関する記録や最終製品の製造記録や試験及び検査記録の保存，可能であれば採取した細胞・組織の一部を保管することが求められている。これは，将来患者に当該製品が原因と推定されるような感染症が発症した場合の原因解明を可能とするための措置である。また，製品が生物由来原料基準に基づき，特定生物由来製品や生物由来製品の指定を受けた場合には，それぞれの指定に応じた上乗せ的な安全対策が必要となる。

2．指針等で求められる細胞治療薬の要件

1）基原または発見の経緯及び外国等における使用状況

細胞治療薬の開発の経緯やその特徴などについ

て明らかにすることが求められる。また，外国等での使用状況についても明らかにする必要がある。一方，ヒト由来細胞を用いた細胞治療薬の開発では，先行して実施された国内での臨床研究の技術移転をうけているケースも多くあり，技術移転を受けた臨床研究の実施状況についての情報も提供されなければならない。
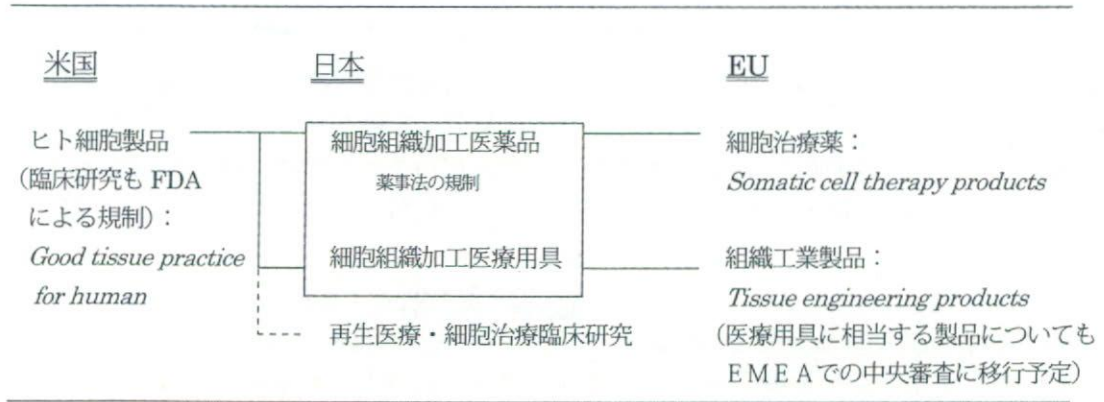
2）原材料となる細胞・組織の由来と選択基準

第1314号指針では，原材料として用いられる細胞・組織が自己由来であるか非自己であるかを明確にすることが求められるが，改正予定の指針では自己と同種とに分けられる予定である。細胞・組織の入手方法及びその生物学的特徴について説明し，細胞・組織を選択した理由を明らかにする必要がある。原材料となる細胞・組織の特性と適格性について，形態学的特徴，増殖特性，生化学的指標，免疫学的特徴，特徴となる細胞由来産生物質等，遺伝型や表現型から適切な指標を選択して解析し，明らかにすることが求められる。

特にHBV，HCV，HIV，HTLVや，必要に応じてパルボウイルスB19，サイトメガロウイルスやEBウイルスについて血清学的試験や核酸増幅法等の検査を行う必要がある。さらに，細菌や真菌等の試験が必要となる。また，問診や病歴等も考慮した上で，ドナーとしての適格性を評価する必要がある。この場合，ヒト同種由来細胞製品のみならず，ヒト自己由来細胞製品についても，製造工程での作業従事者の安全性，他の製品に対する交差汚染防止の観点から，ウイルス等の必要な試験の実施を考慮することが求められる。ウイルス等の検査においては，PCR等を用いても検出出来ないウインドウ期の存在があることから，適切な時期に再検査を行うことが推奨されている。

3）採取行為及び利用の妥当性

細胞・組織採取時のドナーに対する説明及び同意の内容を明らかにし，細胞の採取部位，採取方法が科学的及び倫理的に適切であることを

表2　細胞治療製品の規制の枠組み　一日米欧比較

米国	日本	EU
ヒト細胞製品 （臨床研究も FDA による規制）： *Good tissue practice* *for human*	細胞組織加工医薬品 薬事法の規制 細胞組織加工医療用具 再生医療・細胞治療臨床研究	細胞治療薬： *Somatic cell therapy products* 組織工業製品： *Tissue engineering products* （医療用具に相当する製品についても ＥＭＥＡでの中央審査に移行予定）

示す必要がある。また，ドナーに対して細胞・組織の利用目的，個人情報の保護，その他採取に関する事項について理解を得るよう文書を用いて充分に説明し，自由意志に基く同意を文書によって得ることが必要である。さらに，採取施設において細胞・組織の採取の倫理及び科学的観点からの充分な審議が行える倫理委員会を設置することが求められる。

　原材料となる細胞・組織について，安全性確保上必要な情報が確認できるようにドナーに関する記録が整備，保管されていることが必要である。これらの記録の保管は，製造記録とともに製品の最終有効期限より少なくとも10年間とされている。この期間については，遅発性感染症に関する新たな情報の蓄積によって今後再検討が必要とされている。また，治療の成否の検証や患者等が感染症を発症した場合等の原因究明のために，採取した細胞・組織の一部等の適当な試料を，適切な期間保存することが推奨されている。

4）製造方法
①原材料等
　細胞治療薬の製造に際しては，製品がロットを構成する場合には，原材料，最終製品，必要に応じて中間段階の製品についてロットごとに品質管理法を設定する必要がある。ロットを構

成しない場合は，各製品の使用目的や使用方法に適合する品質規格，出荷基準等を設定しなければならない。

①-1）細胞培養方法
　製造工程で細胞培養を行う場合は，培地の組成，培養条件，培養期間，収率等を具体的に記載することが求められている。使用する材料は，医薬品又は医薬品原料に匹敵する基準で品質管理されているものを用いる必要がある。全ての成分を含む培地成分に関しては，ロットごとの無菌性試験を実施するとともに，目的とする培養に適合していることを確認するための性能試験を実施することが必要である。

　血清は，必須でなければ使用しないことが望ましく，使用が避けられない場合には，血清からの感染因子の混入・伝播の防止策を設ける必要がある。特にウシ血清を用いる場合には，i) 血清の由来を明確にし，ii) 牛海綿状脳症の発症地域以外の血清を用いること，iii) ウイルス等の感染因子に関して適切な否定試験を行ったものを用いること，iv) 潜在的なウイルスのリスクを避けるための放射線処理等の安全対策を実施するとともに，使用した血清の一部を保管しておくことが求められる。大学等での臨床研究では多くの場合自己血清が使用されているが，技術移転を受けて開発されてきた製品では，多くの場合ウシ血清が用いられることが多い。　これは，ヒト血清では開発企

業がその製品の恒常性，一定の品質を担保することが困難であったためと推察される。もちろんウシ血清の使用は必須でなければ使用しないことが求められるが，やむを得ない場合にはその臨床上の有用性等も考慮して判断されることになる。ヨーロッパ医薬品庁（EMEA）の細胞治療薬の指針ではヒト血清を用いる場合には自己由来血清を用いるべきとされており，またウシ血清を用いる場合には，我が国の同様の要件が求められている[3]。

抗生物質については極力使用を避けるべきであるが，やむを得ず使用する場合には最終製品での残存性を極力低減化する使用方法を考えるべきである。他の培地成分や添加される試薬等についても，最終製品での残存性を考慮し，生体に悪影響を及ぼさないものを選択することが求められる。

フィーダー細胞として異種細胞を用いる場合には，「異種移植に関する指針」[4]等を参考に安全性を確保することが必要となる。その際，セルバンクシステムを確立し，マスターセルバンク(MCB)で徹底的な安全性評価を行うと共に，条件を超えて製造された細胞についても安全性を評価し，フィーダー細胞として製造に用いる期間における，安全性やその機能の担保を行っておくことが求められる。

②-2）非細胞成分と組み合わせる場合

細胞成分とともに最終製品の一部として用いられる原材料（シートやマトリックス，医療材料等）に関しては，品質・安全性に関する適切な情報を提供することが求められる。必要な試験については，「医療用具の製造（輸入）承認申請に必要な生物学的試験の基本的考え方について」（医薬審発第0213001号）[5]等を参照し，試験結果を示すと共に当該原材料を使用することの妥当性を示すことが必要となる。妥当性の提示に当たっては，文献からの知見，情報を合理的に活用することが可能である。

また目的とする細胞との相互作用について明らかにすることが求められる。特に，基材との相互作用により，臨床適応に必要な細胞の機能や増殖性，安定性に悪影響を及ぼすことがない

かを明らかにしておく必要がある。

③-3）細胞に遺伝子工学的改変を加える場合

細胞に遺伝子工学的改変を加える場合は，①目的遺伝子の由来，②入手方法，③クローニング法，④細胞バンク作成法や管理法，更新法，⑤目的遺伝子の構造，⑥導入遺伝子の性質，⑦目的遺伝子産物の構造，⑧遺伝子構成体の作製手順，原材料，性質，⑨遺伝子構成体の構造や特性，⑩ベクターや遺伝子構成体を作製するための細胞やウイルスのバンク化，バンクの管理法について明らかにすることが求められる。

②製造工程

原材料の細胞・組織等の受け入れから最終製品製造における加工の方法（製造工程）の概要を示すと共に，各工程での処理の内容，工程管理，品質管理について詳細な説明が求められる。

細胞の加工

細胞・組織の受け入れのために必要な試験を実施するとともに，受け入れ基準を設定しておく必要がある。採取した細胞塊や組織等について，必要且つ可能であればポビドンヨード液等を用いた除菌・不活化を実施することが求められる。当然このような操作は採取した細胞塊や組織の表面に付着した細菌や真菌，ウイルス等の不活化，除去の処理にのみ適用可能である。

細胞の培養を行う場合には培地の組成，培養条件，培養期間，収率等を明らかにする必要がある。

細胞のバンク化を行う場合には，医薬品製造に用いられる細胞基材に関するICH Q5Dガイドライン[6]を参照することが求められる。

さらに採取した細胞の取り違え防止策やクロスコンタミネーションの防止策を明らかにし，その妥当性を説明することが求められる。患者に感染症が発症した場合の原因究明の一助にするために，採取した細胞・組織の一部を適切な期間保存しておくことを考慮すべきである。

③加工した細胞の特性解析

工程評価の一環として，加工した細胞の変化を調べておくことが求められる。このために，形態学的特徴，増殖特性，生化学的指標，免疫

学的指標，細胞の特性に関連する細胞由来産生物質などの指標から，適切なものを選び解析することが必要である。また培養期間中に望ましくない特性の変化が起きていないことを確認するために，規定された培養期間を超えて培養された細胞について試験を行い，目的としない望ましくない細胞の変化が起きないことを確認しておくことが必要となる。

培養期間や加工の程度に応じて，細胞の形質転換の可能性について評価しておくことが必要となる。しかし非常に短期間の培養であれば核型分析の必要性は低いと考えられる。また，細胞治療薬の大きな懸念として造腫瘍性があるが，そのリスクが高い場合には，ヌードマウスを用いた造腫瘍性試験等の実施も考慮すべきである。しかし，増殖能の変化，サイトカインや増殖因子に対する応答性等，その他の適切な試験も考慮し，科学的に合理的な試験を実施することが望ましい。例えば，FDA も一律に細胞治療薬について造腫瘍性試験を求めてはおらず，そのリスクの高い場合にのみヌードマウスを用いた試験が必要になるとされている。

④製造方法の恒常性

細胞治療薬の製造方法の恒常性を示すために，製造工程を通じて，加工した製品の細胞生存率や製品の有効性や安全性の面から求められる表現型や遺伝型の適切な指標，機能特性，目的とする細胞の含有率等が本質的に損なわれないことを評価しておくことが必要である。このために，複数の検体（ロット）を用いた試験を実施することが必要である。どの程度のロットを用いるのかは，培養工程等，どのような加工を行うか，あるいは確認申請のステージか承認申請かどうかによっても異なる。

5）最終製品の品質管理

細胞治療薬の品質管理には，①最終製品等の規格及び試験方法の設定，②適用ロット毎の原材料の品質管理，③製造工程の妥当性の検証と一定性の維持，④各工程の中間製品の品質管理を適正に行い，品質管理全体からみたその妥当

性を明らかにする必要がある。

最終製品については，表 3 に示したような品質管理項目及び試験を参考として，適切な規格・試験方法を設定することが必要となるが，ロットを構成しない場合には，個別製品が品質管理の対象となる。ロットを構成する場合には，個別の製品ではなく各ロットが品質管理の対象となる。例えば，自己由来細胞製品であっても，製造後，複数のアンプルに分注して凍結し，繰り返し投与する製品では，凍結した全アンプルが一つのロットを構成することになる。

確認試験では，目的とする細胞の生化学的特徴，免疫学的特徴，目的細胞の産生する物質などの指標から，適切なものを選択して設定することが必要となる。

細胞の純度試験としては，目的細胞以外の異常増殖細胞の出現，形質転換細胞の有無や混入細胞の有無等の細胞の純度について試験を行い規格を設定することが求められる。

製造工程由来不純物試験として，原材料に存在するか，又は製造過程で非細胞・組織成分，培地成分，資材，試薬などに由来し，製品中に混入物，残留物，あるいは新たな生成物，分解物等として存在する可能性があるもので，かつ品質，安全性面からみて望ましくない物質等について，適切な試験を実施し，規格を設定することが必要となる。例えば，ウシ胎児血清由来タンパク質，増殖因子，あるいは抗生物質など，必要に応じて最終製品への存在許容量を設定しておく必要がある。

無菌試験及びマイコプラズマ否定試験については，適切な検体を用いてあらかじめ試験を行い，全例において無菌性を確認しておくことが必要である。また，無菌性試験の結果が患者への投与後になる場合も無菌性試験を実施し，万が一投与後に無菌性が否定された場合の対処法について設定しておくことが必要である。エンドトキシン試験では，規格値は必ずしも実測値によらず，日本薬局方で示されている最終製品の 1 回投与量をもとにした安全域を考慮して設定すればよい。製造工程中で使用する生物由来

原料などを考慮して，最終製品等について必要なウイルスの試験を実施する必要がある場合もある。また，製造工程中，潜在しているウイルスが増幅する可能性がある場合には，中間製品，最終製品等でウイルスの存在を試験する必要もある。

それぞれ臨床使用目的に応じた細胞の効能試験を実施する必要がある場合もある。例えば，幹細胞等を用いて特定の細胞への機能分化を期待する場合には，その分化能を試験し，規格を設定する必要があると考えられる。また，遺伝子改変細胞であれば改変によってもたらされた特定タンパク質の発現について規格を設定することが求められる。

細胞から分泌される特性の生理活性物質がその効能である場合には，力価試験としてその生理活性物質の発現量に関する規格・試験法を設定することが必要となる。

確認申請の段階では，全ての規格が設定出来ることはまれであり，その後の臨床開発を通じて適切な規格が設定されていくものと考えられる。従って，いくつかの試験項目については少数の検体で得られた試験結果に基づいて暫定基準を設けておくことで対応可能な場合がある。

6）安定性

製剤化した細胞治療薬や重要な中間工程製品について，保存や流通期間を考慮した安定性試験を実施する必要がある。試験では，細胞の生存率，力価等の適切な項目を選び，試験を実施することが求められる。その結果に基づいて，貯法や有効期間を設定することが必要である。また凍結保存を行う場合には，凍結操作による，生存率や増殖能，力価等への影響を確認することが必要となる。

7）細胞治療薬の非臨床安全性試験

細胞治療薬の非臨床安全性試験として，可能であれば，科学的合理性のある範囲で動物を用いた試験，あるいは in vitro での試験を実施することが求められる。

ヒト由来細胞治療薬の試験用検体は貴重で限りがあり，又，異種細胞であるヒト細胞を動物に投与して得られる結果の有用性については限りがあると考えられることから，動物細胞を用いた製品モデルを作製し適切な実験動物に適用する試験系が，科学的合理性がある場合も考えられる。またこのような試験によって，より有用な知見が得られると考えられる場合には，試験の実施を考慮することが望ましい。場合によっては動物細胞を用いる試験系も考慮し，以上のようなアプローチにより試験を行なった際には，その妥当性を明らかにすることが必要である。

8）細胞治療薬の効能または性能を裏付ける試験

一般的に種の壁があるため，動物を用いてヒト由来細胞治療薬の効能や性能を裏付ける試験を実施することにはその解釈も含めて困難が伴う。このために，モデル動物の相同細胞を用いた評価系やヌードマウスに当該ヒト細胞を投与するなどの様々な工夫が試みられている。しかし，モデル動物での反応性がヒトと同じであるとは限らず，モデル動物を用いた試験が必ずしも適切な評価系とは言えないことも多い。従って，技術的に可能かつ科学的に合理性のある範囲で，実験動物，細胞等を用いて，適切に設計された試験により，細胞治療薬の機能発現，作用持続性，医薬品・医療機器として期待される効果を検討することが望ましい。また，既に確立された適当な動物由来細胞・組織製品モデルや疾患モデル動物がある場合には，それを用いて治療効果を検討することが必要となる。また，必要に応じて，文献や知見等により合理的に細胞治療薬の効力又は性能が他の治療法に比較して勝れていることが期待できる場合には，臨床開発の初期段階として詳細な動物実験等は必要とされない可能性もある。

特に開発に当たって参照可能な臨床研究の成果があり，効能または性能を裏付ける情報が得られる場合には，モデル動物を用いた試験の必

Influences of the recombinant artificial cell adhesive proteins on the behavior of human umbilical vein endothelial cells in serum-free culture

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Abstract

To improve the safety of cellular therapy products, it is necessary to establish a serum-free cell culture method that can exclude animal-derived materials in order to avoid contamination with transmissible agents. It would be optimal if the proteins necessary to a serum-free culture could be provided as recombinant proteins. In this study, the influences of recombinant artificial cell adhesive proteins on the behavior of human umbilical vein endothelial cells (HUVECs) in serum-free culture were examined in comparison with the influence of plasma fibronectin (FN). The recombinant proteins used were Pronectin F (PF), Pronectin F PLUS (PFP), Pronectin L (PL), Retronectin (RN), and Attachin (AN). HUVECs adhered more efficiently on PF or PFP than on FN. No cells adhered on PL. Regarding the VEGF or bFGF-induced cell growth, the cells on PF and PFP proliferated at a similar rate to the cells on FN. RN and AN were less effective in supporting cell growth. Since cell adhesion on PF and PFP induced phosphorylation of focal adhesion kinase, they are thought to activate integrin-mediated intracellular signaling. The cells cultured on PF or PFP were able to produce prostaglandin I_2 or tissue-plasminogen activator in response to thrombin. However, thrombin caused detachment of the cells from PF but not from PFP or FN, meaning that the cells were able to adhere more tightly on PFP or FN than on PF. These data indicate that PFP could be applicable as a substitute for plasma FN.
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Keywords: Cell adhesive protein; Recombinant protein; Fibronectin; Endothelial cells

1. Introduction

The widespread development of cellular therapy products has advanced to the stage of non-clinical and clinical testing [1–3]. Regulatory documents for human somatic cell therapy including instructions for investigational new drug applications have been published [4–6]. To guarantee the safety of both product recipients and the public at large, it is crucial to prevent contamination of cellular therapy products by infectious agents [7].

Serum-free culture is one of the desired methods for manufacturing cellular therapy products when safety issues are a concern [8,9]. Although serum is a very effective additive

for a culture medium that can support cell adhesion, survival, growth, and functions, animal-derived materials such as serum may contain transmissible agents or human allergens [10–13]. Since serum is composed of proteins, sugars, lipids, vitamins, and other ingredients, its quality is affected by the genetic and environmental circumstances of the animal used, which means there are lot-to-lot variations in composition and potency. This variability could reduce the consistency of cellular therapy products, and a serum-free culture that could contribute to improving the consistency of the cell features is thus needed.

Protein factors often need to be added to serum-free culture to substitute for the functions of serum. The proteins used for this purpose must also pose no risk of infection. Because recombinant proteins can be produced without using animal-derived materials, and pose little risk of contaminating human pathogens, they are a useful biomaterial for culturing cells

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when safety issues are a concern. In addition, the functions of recombinant proteins can be improved by modifying their amino acid sequences, which is also an advantage. In this study, using a serum-free culture of human umbilical vein endothelial cells (HUVECs), the usefulness of recombinant artificial cell adhesive proteins as a plasma fibronectin substitute was evaluated.

The extracellular matrix glycoprotein fibronectin consists of two similar polypeptide chains, each with a molecular mass of approximately 250 kDa joined at their respective C termini by disulfide bonds [14,15]. The RGD (arginine–glycine–aspartic acid) recognition sequence located within the molecule was the first amino acid motif shown to mediate cell adhesion [16,17]. Intracellular signaling induced by cell adhesion on fibronectin plays a critical role in cytoskeletal reorganization, cell cycle progression, and cell survival [18,19].

This study defines the influences of five different kinds of recombinant artificial cell adhesive proteins on endothelial adhesion, proliferation, and antithrombotic function in serum-free culture for which plasma fibronectin is needed. The recombinant proteins used were Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, and Attachin (Table 1).

2. Materials and methods

2.1. Recombinant cell adhesive proteins

Pronectin F, Pronectin F PLUS, and Pronectin L were purchased from Sanyo Chemical Industry (Kyoto, Japan), Retronectin from TaKaRa (Shiga, Japan), and Attachin from Bio999 (Taipei, Taiwan). All of these proteins were produced via bacterial fermentation. Pronectin F is a genetically engineered protein containing repeating units of the RGD sequence interspersed with a β-silk peptide for structural stability [20]. It is

comprised of 980 amino acids. Based on its sequence, the molecular weight is estimated to be 72,728. The amino acid sequence is fMDPVVLQRRDWENPGVTQLNRLAA HPPFASDPMGAGS(GAGAGS)$_6$GAAVTGRGDSPASAAGY-[(GAGAGS)$_9$GAAVTGRGDSPASAAGY]$_{12}$-(GAGAGS)$_2$GA GAMDPGRYQLSAGRYHYQLVWCQK. Pronectin F PLUS is a positively charged water-soluble variant of Pronectin F that is produced by chemical modification [21,22]. Pronectin L is a protein polymer that exhibits IKVAV epitopes from the laminin alpha chain with a similar backbone as Pronectin F [21]. It is comprised of 1019 amino acids. The molecular weight is estimated to be 75,639. The amino acid sequence is fMDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGA GS(GAGAGS)$_6$GAAPGASIKVAVSAGPSAGY-[(GAGAGS)$_9$ GAAPGAIKVAVSAGPSAGY]$_{12}$-(GAGAGS)$_2$GAGAMDPG RYQLSAGRYHYQLVWCQK. Retronectin consists of a central cell-binding domain, a high affinity heparin-binding domain II, and a CS1 site within an alternatively spliced type III connecting segment region of human fibronectin [23]. It is comprised of 574 amino acids. The molecular weight is estimated to be 62,631. Attachin is an artificial fusion protein with the molecular weight of 30 kDa that has several functional domains, including a fibronectin-like cell attachment domain [24]. It has reportedly been used to promote the adhesion of several kinds of cell lines, including CHO-K1, MDBK, PK-15, L929, Vero, COS, U373, Swiss 3T3, and MRC-5 [25].

2.2. Cells and materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Sanko Junyaku (Tokyo, Japan) and maintained

Table 1
Fibronectin and recombinant cell adhesive proteins used in this study

Proteins	Structure	Molecular Weight
Fibronectin	heparin/fibrin, collagen, cell, heparin III CS fibrin — NH2–[...]–COOH / NH2–[...]–COOH, ★RGD	250K x 2
Pronectin F	Head-[(GAGAGS)$_9$GAAVTGRGDSPASAAGY]$_{12}$-Tail ★	73K
Pronectin F Plus	Positively charged, water-soluble variant of Pronectin F	73K
Pronectin L	Head-[(GAGAGS)$_9$GAAPGASIKVAVSAGPSAGY]$_{12}$-Tail ◆	76K
Retronectin	Chimeric protein of human fibronectin fragment NH2–[...]–COOH	63K
Attachin	A fusion protein constructed by molecular biotechnology	30K

Fibronectin.

▯ Type I module.

◯ Type II module.

▢ Type III module.

★ : cell attachment sequence derived from fibronectin.

◆ : cell attachment sequence derived from laminin.

in EGM-2 media (Cambrex, Walkersville, MD) on collagen-coated dishes (Asahi Techno Glass, Tokyo, Japan). EGM-2 media is modified MCDB 131 containing 2% fetal bovine serum, VEGF, bFGF, IGF-1, EGF, heparin, hydrocortisone and ascorbic acid. The cells were kept in a humidified, 5% CO_2 environment at 37 °C. Cells between passages 3 and 5 were used for all experiments.

The serum-free media used was human endothelial SFM (Invitrogen, Carlsbad, CA) [26]. Ten nanograms/ml of epidermal growth factor (EGF) (Invitrogen) and 20 ng/ml of basic fibroblast growth factor (bFGF) (Invitrogen) were added as supplements. Fibronectin is recommended for use as a cell attachment factor.

2.3. Cell adhesion assay

Recombinant cell adhesive proteins were diluted with phosphate buffered saline (PBS(−)), and plated on multiwell non-treated polystyrene plates (BD Falcon, Franklin Lakes, NJ). The plates were incubated for 2 h at room temperature, and then the protein solutions were removed and the wells washed with PBS(−). The concentration of the recombinant proteins used was 10 µg/ml (2 µg/cm^2), unless the description states otherwise. The amount of absorbed protein was quantified with a QuantiPro BCA Kit (Sigma, St. Louis, MO) using bovine serum albumin as a standard.

The HUVECs were harvested using trypsin and washed twice with PBS(−). The cells were then suspended in serum-free media, and added to each well of 96-well plates previously coated with recombinant cell adhesion proteins at a cell density of 1×10^4 cells/well. After incubating for 60 min under 5% CO_2 at 37 °C, the supernatant was removed, and the wells rinsed with PBS(−) to remove non-adherent cells. Following fixation of the adherent cells by 4% paraformaldehyde for 10 min, the paraformaldehyde was removed and the cells washed once with distilled water. A 0.5% (w/v) solution of crystal violet was then added to the wells. After staining for 25 min, the cells were rinsed five times with distilled water and the crystal violet that was absorbed on the adherent cells was solubilized with 0.5% SDS. The optical density at 595 nm was measured on an EL340 plate reader (BioTek Instruments, Winooski, VT).

2.4. Measurement of cell proliferation

HUVECs were harvested and suspended in serum free media, and plated to each well of the 96-well plates previously coated with recombinant cell adhesive proteins. VEGF (R&D Systems, Minneapolis, MN) or bFGF (Invitrogen, Carlsbad, CA) was added at concentrations of 1–100 ng/ml. After culturing for 2 days under 5% CO_2 at 37 °C, the cell number in each well was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Results are expressed as the mean value ± S.D. of triplicate determinations.

2.5. Phosphorylation of focal adhesion kinase

The HUVECs were collected by trypsin treatment (Invitrogen), resuspended in serum free medium, incubated for 2 h at 37 °C in suspension, and subsequently plated at 5×10^5 cells/dish on 60 mm diameter dishes pre-coated with cell adhesive proteins or bovine serum albumin (BSA) (10 µg/ml) [27]. After incubation for 2 h under 5% CO_2 at 37 °C, the cells were lysed in RIPA buffer (50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate), and the protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). 3.5 µg of the total cell lysates was resolved by SDS–PAGE, blotted onto Immobilon-P membranes (Millipore, Volketswil, Switzerland), and incubated in 1% BSA with anti-pY397 FAK antibody (Upstate Biotechnology Inc.,, Lake Placid, NY) followed by incubation with horseradish peroxidase-labeled secondary antibody (Cell Signaling Technology, Danvers, MA). The ECL system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and Luminoimage analyzer LAS 3000 (Fuji Film, Kanagawa, Japan) were used for detection. The membranes were stripped of bound antibody using a Re-Blot Plus Western Blot Recycling Kit (Chemicon, Temecula, CA), and the membrane was reprobed with anti-FAK antibody (Upstate Biotechnology). The labeled bands were quantified using the MultiGauge software program (Science Lab). The quantified value for phosphorylated FAK was normalized with that for total FAK in each sample, and the relative phosphorylation level was then calculated as a ratio against the cell lysate on fibronectin.

2.6. Secretion of Prostaglandin I_2 (PGI$_2$)

The HUVECs were harvested using trypsin, washed twice, and then suspended in serum-free media. The cells were placed on the 24-well plates coated with recombinant cell adhesive proteins at the density of 7×10^4/well. After culturing for 1 day, the cells were stimulated with 1 U/ml of thrombin or 30 ng/ml of VEGF. The cells were incubated for 1 h under 5% CO_2 at 37 °C, and then the supernatant was collected. The supernatant was centrifuged for 10 min at $2000 \times g$ in a microcentrifuge to remove any residual cells. The level of 6-keto Prostaglandin $F_1\alpha$, a major metabolite of Prostaglandin I_2, was determined using 6-keto Prostaglandin $F_1\alpha$ EIA Kit (Cayman Chemical, Ann Arbor, MI). Each culture condition was repeated in triplicate.

2.7. Secretion of tissue-plasminogen activator

The HUVECs were seeded as described above. After culturing for 1 day, the cells were stimulated with 0.01–1 U/ml of thrombin. Twenty-four hours later, the supernatant was collected and spun down for 10 min at $2000 \times g$ in a microcentrifuge. The level of tissue plasminogen activator was determined using the AssayMax Human Tissue-Type Plasminogen Activator ELISA Kit (Assay Pro, Brooklyn, NY). Each culture condition was repeated in triplicate. Phase contrast images were obtained before the supernatant was collected.

3. Results

3.1. Adhesion of HUVECs onto recombinant cell adhesive proteins

To examine the adhesion onto the recombinant cell adhesive proteins, HUVECs harvested and suspended in serum-free media were applied on plastic wells precoated with Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, Attachin, or fibronectin (Table 1). As shown in Fig. 1, Pronectin F, Pronectin F PLUS, Retronectin, or Attachin showed cell adhesion activity to a similar extent as fibronectin at the concentration of 10 μg/ml (2 μg/cm^2). Pronectin F and Pronectin F PLUS were superior to fibronectin when they were used at an amount of less than 1 μg/ml. The cells were observed under microscopy to have spread well on the recombinant proteins that they adhered to (Fig. 2). No cells had adhered onto Pronectin L or BSA.

In order to test if these differences are due to the difference in the absorbed amount of each protein, the protein absorbed on the plate was measured (Fig. 3). It was found that Pronectin F and Pronectin L had absorbed to the plate better than other proteins and that the absorbed amount of Pronectin F PLUS was lower than that of Pronectin F and Pronectin L. From the point of view of the efficiency of cell adhesion (Fig. 1) and the absorbed amount of protein (Fig. 3), the number of cells attached was not dependent on the amount of absorbed protein, suggesting that the observed effects were not due to differences in protein absorption but rather due to the character of each cell adhesion protein.

3.2. Influences on cell proliferation

Besides anchorage, cell adhesion to components of the extracellular matrix triggers signaling events affecting diverse
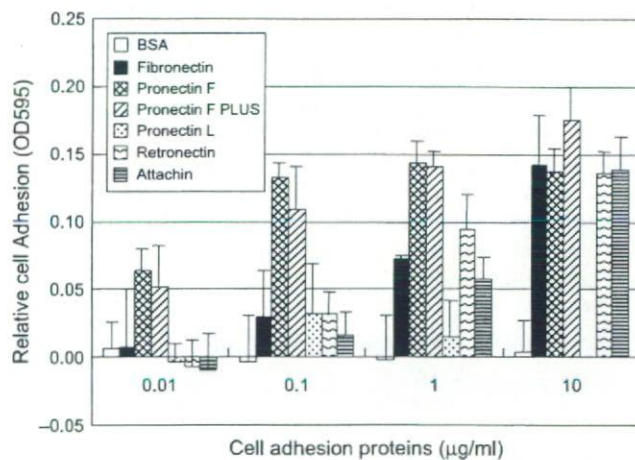

Fig. 1. Adhesion of HUVECs to recombinant cell adhesive proteins. HUVECs were plated into wells previously coated with Fibronectin, Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, Attachin, or BSA, and then incubated for 1 h at 37 °C. Attached cells were fixed and stained with crystal violet and quantified by absorbance reading. Results are expressed as mean value ± S.D. of triplicate determinations. The 0.1 OD corresponded to $0.61 \pm 0.085 \times 10^4$ cells.

cellular traits and activities, including survival, proliferation, and other functions. Therefore, the influences of the recombinant cell adhesion proteins on cell proliferation were examined. The HUVECs in serum-free media were seeded on the plates that were coated with recombinant cell adhesive proteins, and then cultured in the presence or absence of basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) at concentrations from 1 to 100 ng/ml. After culturing for 2 days, the cell numbers in each well were examined (Fig. 4). When HUVECs were cultured in the presence of bFGF, the cells on Pronectin F or Pronectin F PLUS had grown to the same level as that on fibronectin in each concentration of bFGF, showing that Pronectin F and Pronectin F PLUS have the same growth support potency as fibronectin (Fig. 4A). However, the cell numbers on Retronectin or Attachin reached 70% or 40% of that on fibronectin, respectively; showing that the growth support potency of Retronectin and Attachin was lower than fibronectin. When VEGF-stimulated cell proliferation was examined, Pronectin F and Pronectin F PLUS also showed similar growth-supporting potency to fibronectin (Fig. 4B). Retronectin and Attachin were less effective than fibronectin.

3.3. Phosphorylation of focal adhesion kinase

Cell adhesive proteins activate intracellular signaling via cell surface integrins. In regulating the cellular responses to integrin-mediated adhesion, focal adhesion kinase (FAK) has emerged as a key signaling molecule [28–30]. Integrin–ligand engagement promotes FAK tyrosine phosphorylation that promotes FAK signaling activity. The phosphorylation of FAK Tyr-397, the only apparent autophosphorylation site, is known to create a high-affinity binding site for SH2 domains of the Src-family kinases, including c-Src and Fyn [31].

HUVECs cultured in growing media were harvested by trypsin treatment, and incubated in serum-free media for 2 h at 37 °C in suspension. After this incubation, the cells were plated onto the dishes in which recombinant cell adhesive proteins were coated, and then incubated for 2 h under 5% CO_2 at 37 °C. Cell lysates were prepared using RIPA buffer, and the phosphorylation level of FAK on Tyr 397 was then examined by Western blotting. As shown in Fig. 5, FAK Tyr-397 is phosphorylated under an attached condition (lane 1), becomes dephosphorylated upon cell suspension (lane 2), and then becomes rephosphorylated after replating. The FAK phosphorylation level after replating on recombinant proteins differed in each sample (lanes 3–10). Phosphorylation level of cells on BSA (lane 3) or Pronectin L (lane 7), to which cells did not adhere, was still lower after replating. Pronectin F (lane 5) or Pronectin F PLUS (lane 6) induced FAK phosphorylation to the similar extent to Fibronectin (lane 4), suggesting that Pronectin F and Pronectin F PLUS are thought to activate intracellular signaling via integrin as well as fibronectin. Retronectin that has RGD domain of Fibronectin also induced FAK phosphorylation (lane 8).
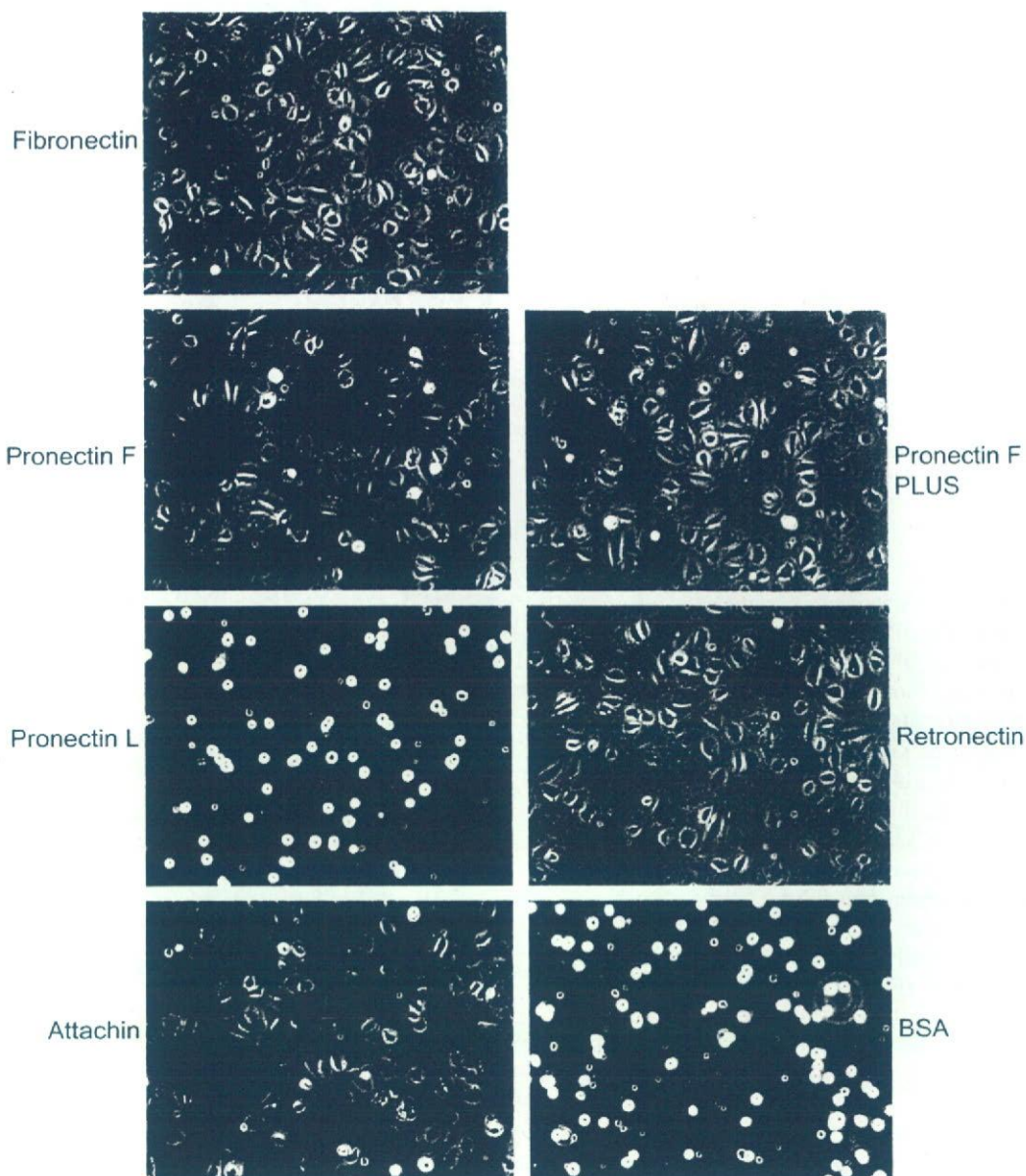
Fig. 2. The phase contrast images of cell attachment and spreading on recombinant cell adhesive proteins. HUVECs were seeded into wells previously coated with 10 μg/ml of Fibronectin, Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, Attachin, or BSA. After 1 h incubation at 37 °C, the cells were photographed with an inverted microscope.

3.4. Influences on antithrombotic functions of endothelial cells

In order to examine the influences of recombinant cell adhesive proteins on cellular functions, the production of prostaglandin I_2 (PGI_2) and tissue-plasminogen activator (t-PA), both of which play important roles in the antithrombotic feature of endothelial cells, was tested. Antithrombotic activity is the most important characteristic of endothelial cells that are used for artificial blood vessels or engineered vascularized tissues. PGI_2 inhibits platelet aggregation, thereby inhibiting the formation of thrombus. It is produced quickly after stimulation via the activation of phospholipase A_2. Since the half

life of PGI_2 is as short as 2 min, the concentrations of its metabolite 6-keto Prostaglandin $F_1\alpha$ were measured. t-PA is produced from endothelial cells via the induction of protein synthesis in response to the extracellular stimuli. It induces the processing of plasminogen to plasmin, and dissolves the fibrin clots.

HUVECs were plated on the recombinant cell adhesion protein-coated dishes and cultured for 1 day, then stimulated with VEGF or thrombin. As shown in Fig. 6A, PGI_2 was produced from VEGF- stimulated HUVECs on different kinds of cell adhesive proteins to the same level. In the serum-free media, the basal production of PGI_2 was higher, and the production after the stimulation was less than in the growing
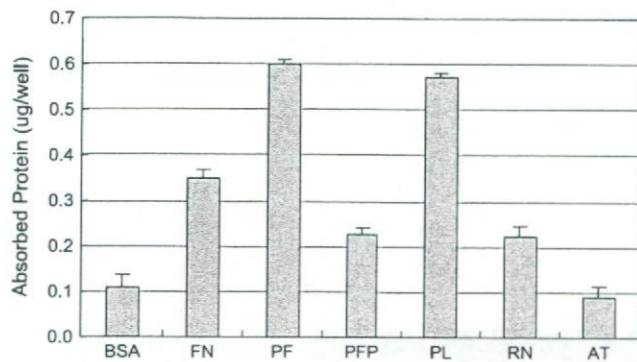
Fig. 3. Protein absorption on non-treated polystyrene plates. Ten µg/ml of bovine serum albumin (BSA), Fibronectin (FN), Pronectin F (PF), Pronectin F PLUS (PFP), Pronectin L (PL), Retronectin (RN) or Attachin (AN) was added to 96-well plates and incubated at room temperature for 2 h. Each well was washed with PBS(−) and the amount of absorbed protein was quantitated by QuantiPro BCA using bovine serum albumin as a standard. Each data point represents the mean ± S.D. of triplicate determinations.

media. Similar results were obtained when the cells were stimulated with thrombin (Fig. 6B).

Next, t-PA production from the thrombin-stimulated HUVECs was examined. As shown in Fig. 7, the t-PA concentration in the culture supernatant of HUVECs that adhered on Pronectin F PLUS was the same as that on Fibronectin. However, the concentration was lower in the supernatant on Pronectin F, Retronectin, or Attachin than on fibronectin. The basal production of t-PA in the serum-free media was less than in growing media; therefore, responsiveness to thrombin was readily observed in the serum-free culture.

During these experiments using thrombin, detachment of the thrombin-stimulated cells from Pronectin F, Retronectin, or Attachin was observed (Fig. 8). No detachment was seen in the cells that adhered on fibronectin or Pronectin F PLUS. This result might reflect the possibility that the adhesiveness of the cells to Pronectin F, Retronectin, and Attachin is weaker than to Pronectin F PLUS or fibronectin.

The following data support the results of Fig. 8, which are presented using microscopic images. Since HUVECs are adherent cells, detachment from the dishes leads to a loss of viability. Therefore, changes in the viable cell concentration after thrombin stimulation reflect cell detachment. As shown in Fig. 9, the production of t-PA and changes in the viable cell concentration after culturing in the presence of 0.01, 0.1, or 1 U/ml of thrombin were tested. At all three concentrations of thrombin used, cell detachment was observed only from Pronectin F. Although the t-PA concentration was increased in a dose-dependent manner by thrombin in the cells that adhered on Fibronectin or Pronectin F PLUS (Fig. 9A), there was less of an increase in t-PA production from the cells on Pronectin F when a low dose (0.01 or 0.1 U/ml) of thrombin was used. At those concentrations, the viable cell concentration on Pronectin F was decreased by thrombin stimulation, meaning that cell detachment occurred on Pronectin F (Fig. 9B). At a high concentration of thrombin (1 U/ml), the viable cell concentration decreased slightly even though cell detachment was
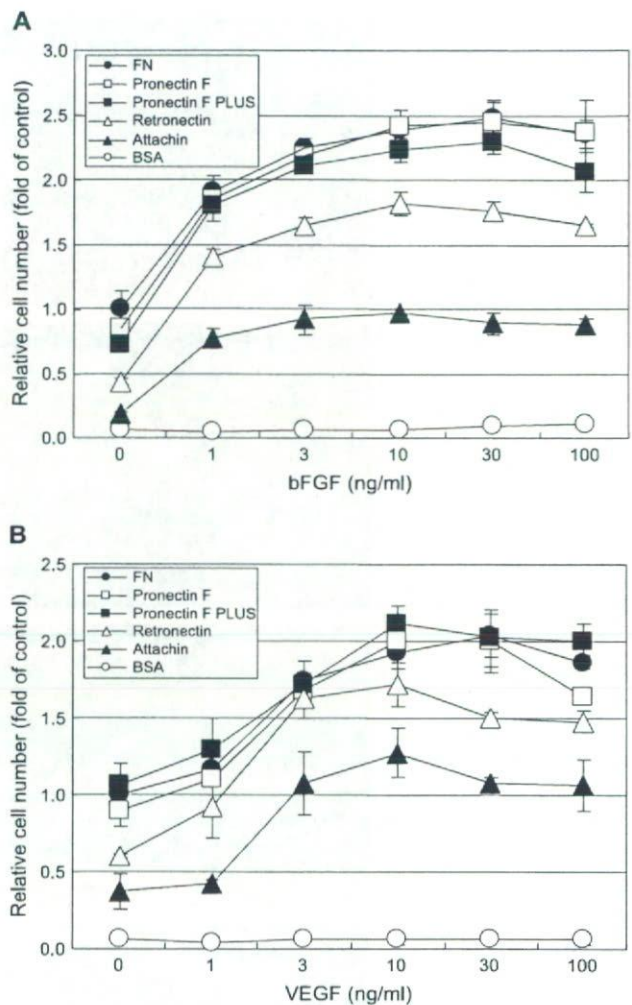

Fig. 4. Effects of recombinant cell adhesive proteins on the VEGF- or bFGF-induced cell proliferation. HUVECs were seeded into wells previously coated with 10 µg/ml of Fibronectin, Pronectin F, Pronectin F PLUS, Retronectin, Attachin, or BSA, and cultured for 2 days in the presence or absence of bFGF (A) or VEGF (B) at the concentrations indicated. The relative cell number was examined using a cell counting kit-8. The results are expressed as the mean value ± S.D. of triplicate determinations.

observed. This might be because the cell proliferative effect of thrombin canceled the decrease in cell viability. t-PA production corrected by the viable cell concentration is shown in Fig. 9C. By correcting the t-PA concentration using the viable cell concentration, the dose-dependency of thrombin in t-PA production was similar in Pronectin F to Pronectin F PLUS and fibronectin (Fig. 9C). From these results, in the cells on Pronectin F and Pronectin F PLUS, the signals from thrombin transduced similarly, but the strength of the cell adhesion differed.

4. Discussion

The goal of the present study was to establish a cell culture method that can improve the safety of cellular therapy products. Our focus is now on human endothelial cells, because
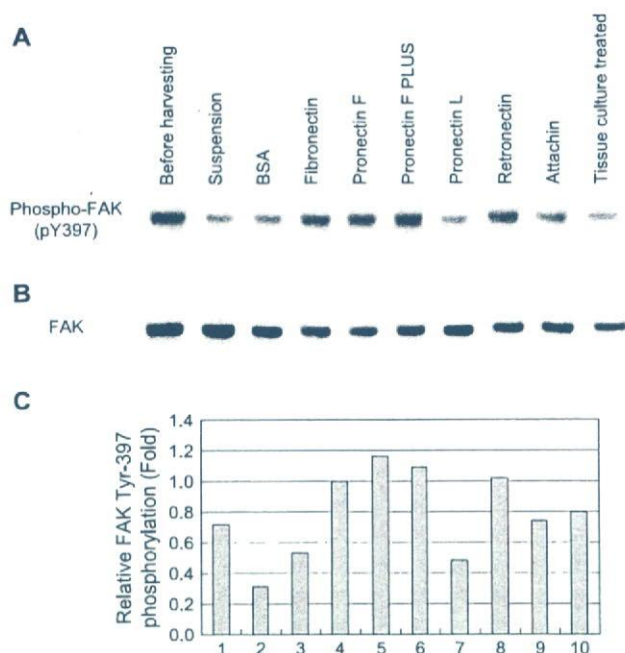
A

Phospho-FAK
(pY397)

B

FAK

C

Fig. 5. FAK activation in HUVECs adhered to recombinant cell adhesive proteins. HUVECs cultured in serum-containing EGM-2 media on collagen-coated plates were harvested and held in suspension for 2 h at 37 °C, then plated onto precoated plates with 10 μg/ml of Fibronectin, Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, or Attachin. After 2 h incubation at 37 °C, cell lysates were prepared using RIPA buffer. The cell extracts were analyzed for the phosphorylation (A) and protein levels of focal adhesion kinase (B) by Western blotting. The amount of proteins detected was analyzed using MultiGauge software (C). Lane 1: before harvesting; lane 2: after suspension; lane 3: BSA; lane 4: Fibronectin; lane 5: Pronectin F; lane 6: Pronectin F PLUS; lane 7: Pronectin L; lane 8: Retronectin; lane 9: Attachin; lane 10: tissue culture-treatment dish. Similar results were obtained in two independent experiments. Representative blots are shown.

A

B

Fig. 6. Effects of cell adhesive proteins on the VEGF- or thrombin- stimulated production of Prostaglandin I_2. HUVECs were seeded at 7×10^4 cells/well on 24-well plates previously coated with Fibronectin (FN), Pronectin F (PF), Pronectin F PLUS (PFP), Retronectin (RN), or Attachin (AN). After culturing for 1 day, the cells were stimulated with 30 ng/ml of VEGF (A) or 1 U/ml of thrombin (B). One hour later, the conditioned media were collected and 6-keto $PGF_1\alpha$ concentration was determined by enzyme immunoassay. Results are expressed as picograms of $PGF_1\alpha$/ml of the supernatant and represent the mean ± S.D. of triplicate determinations. 6-Keto $PGF_1\alpha$ production from HUVECs cultured in serum-containing growing media (EGM-2) plated on a collagen-coated dish was also examined (Col/EGM).

they are expected to be put into clinical use in the near future. Serum-free culture supplemented with recombinant proteins produced without using animal-derived materials would be optimal in order to avoid contamination by animal-derived materials and transmissible agents. This work was carried out as a step in demonstrating the feasibility of recombinant cell adhesion proteins in serum-free cultures of human endothelial cells, which might form the basis of future cellular therapies. HUVECs were used as a model of tissue-derived differentiated endothelial cells.

One of the uses of endothelial cells in cellular therapy is for the vascularization of engineered tissue. Vascularization of a tissue-engineered construct is mediated primarily through the ingrowth of surrounding vessels from the peri-implant tissue. However, this process is quite slow and is not sufficient to achieve appropriate vascularization of large defects [32]. The incorporation of endothelial cells or vascular-like structure in the tissue-engineered constructs before transplantation is a possible solution of this problem. Both human embryonic stem cell-derived endothelial cells and tissue-derived endothelial cells such as HUVECs have been shown to incorporate successfully into engineered skeletal muscle tissue [33–35].
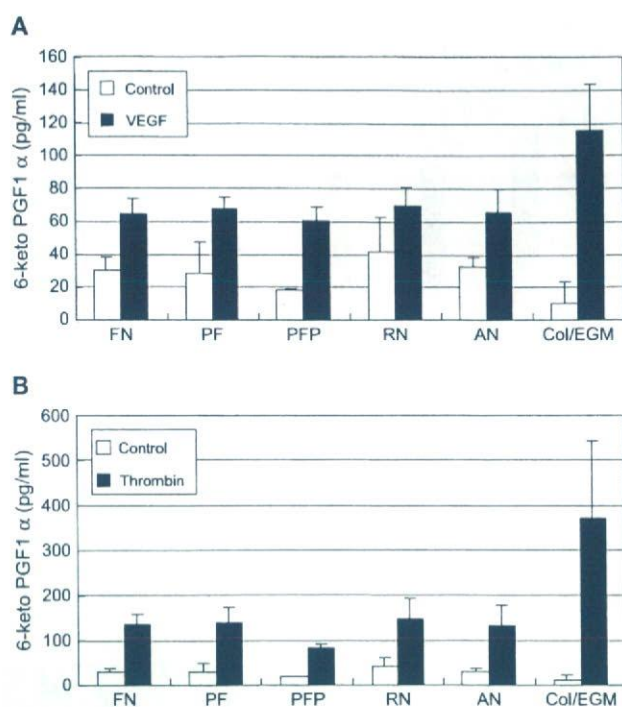
Tissue-derived human endothelial cells are also used for the endothelialization of artificial artery grafts [36]. Although serum-free media for human endothelial cells has been developed, being supplemented with a cell adhesion protein such as fibronectin is necessary [26]. Since plasma fibronectin is known to bind to several pathogens including viruses [37,38], use of a recombinant cell adhesion protein would be desired in order to avoid the contamination of infectious agents.

Another use of endothelial cells in cellular therapy is for therapeutic neovascularization, an important adaptation to rescue tissue from critical ischemia. In a rat model of myocardial infarction, transplantation of HUVECs has been shown to contribute to increased neovascularization [39]. Recently, much attention has been paid to outgrowth endothelial cells (OECs) obtained by *in vitro* culture of blood mononuclear cells [40–46]. OECs show a marked similarity to fully differentiated endothelial cells with respect to their cellular morphology, marker expression and potential to form capillary-like structures [43,44,47]. Because OECs can be obtained from blood mononuclear cells, they have the advantage that autologous endothelial cells could be obtained without invasive procedures. The potential use of OECs for therapeutic neovascularization has been shown in murine ischemia models
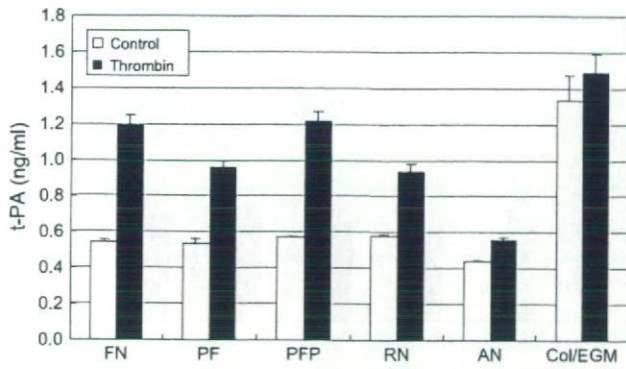
Fig. 7. Effects of cell adhesive proteins on the thrombin-stimulated production of tissue plasminogen activator. HUVECs were seeded at 7×10^4 cells/well on 24-well plates previously coated with Fibronectin (FN), Pronectin F (PF), Pronectin F PLUS (PFP), Retronectin (RN), or Attachin (AN). After culturing for 1 day, the cells were stimulated with 1 U/ml of thrombin. After 24 h incubation, the conditioned media were collected and t-PA concentration was determined by enzyme immunoassay. Results are expressed as nanograms of t-PA/ml of the supernatant and represent the mean $\pm$ S.D. of triplicate determinations. t-PA production from HUVECs cultured in serum-containing growing media (EGM-2) plated on collagen-coated dish was also examined (Col/EGM).

[47–50]. The culture conditions for OECs and for tissue-derived endothelial cells such as HUVECs are the same: endothelial basal media supplemented with fetal calf serum, VEGF, basic FGF, IGF-1, EGF and ascorbic acid. Fibronectin is the cell adhesion protein most commonly used to obtain OECs. Therefore, examining the usefulness of recombinant cell adhesion proteins as a substitute for plasma fibronectin in serum-free culture would lead to the establishment of a serum-free culture for OECs as well.

The cell culture method chosen has a direct influence on the quality and safety of cellular therapy products. The composition of media and serum are considered to affect the features of the cells, and animal-derived materials are known to carry the risk of infection or allergy. Thus, developing appropriate culture methods and ways of evaluating these methods are key to assuring the safety of cellular therapy products [11,12]. For example, when culturing human embryonic stem cells that are anticipated to be a useful source for cellular therapy products, non-human-type sialic acid (N-glycolyl neuraminic acid) derived from animal serum is incorporated into the sugar chain on the cell surface, which could induce an immune response
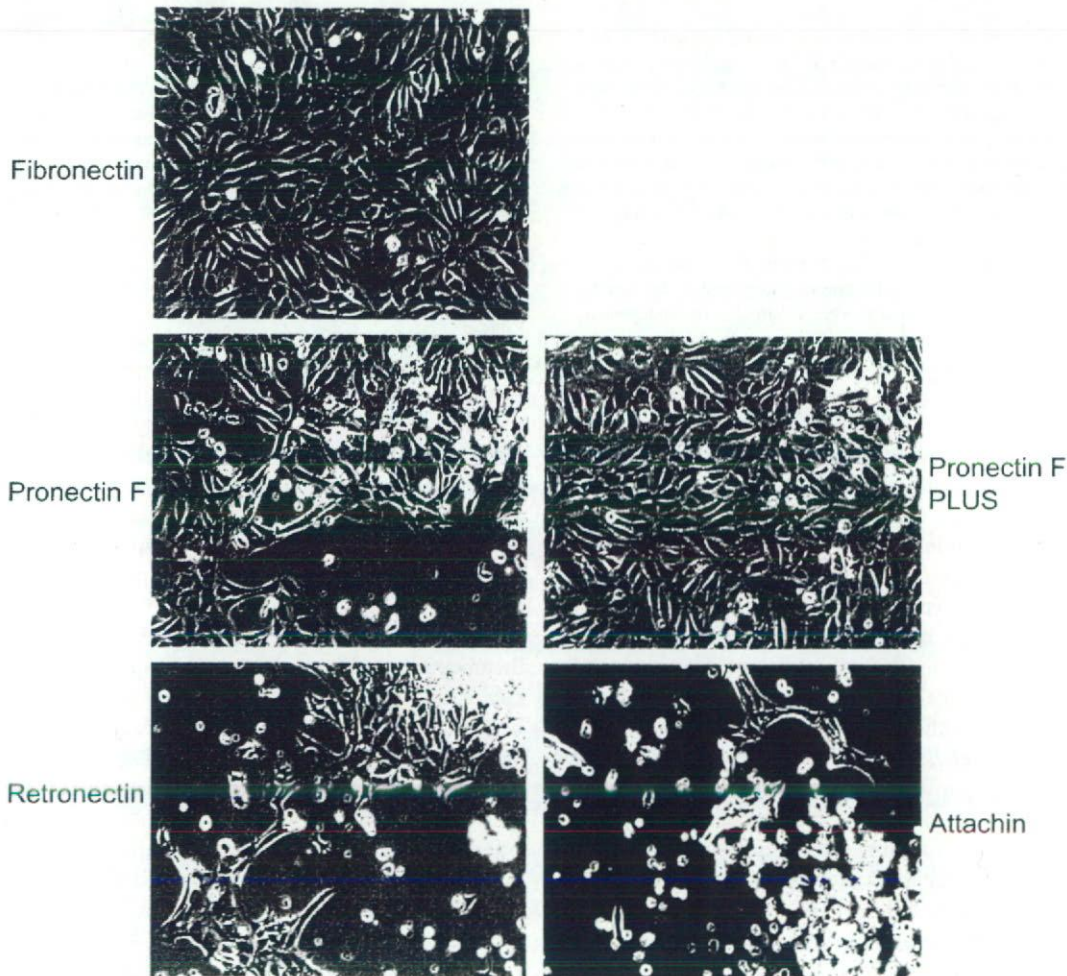

Fig. 8. Detachment of HUVECs cultured in the presence of thrombin. Cells were plated on the plates previously coated with Fibronectin, Pronectin F, Pronectin F PLUS, Retronectin, or Attachin, and cultured for 24 h in the presence of 1 U/ml of thrombin. The cells were photographed before harvesting the supernatant that is provided for the measurement of the t-PA concentration.

upon transplantation [51]. In addition, the transmission of unknown viruses from rodent feeder cells is also a concern [8]. Therefore, establishing cell culture methods that assure safety is an important goal in the development of cellular therapy products.

Because recombinant proteins can be produced without using animal or human-derived materials, providing the protein factors that are necessary to cell culture as recombinant proteins can contribute to improving the safety of cellular therapy products. Recombinant proteins also have an advantage in that they can provide consistent performance from lot to lot, and their function can be modified by molecular design. Artificial recombinant proteins have the possibility of being superior to naturally occurring proteins; for example, Retronectin, a deletion mutant of fibronectin that unfortunately showed less activity as an adhesive protein in this study, is much more effective in supporting gene transfer by retrovirus vectors than fibronectin [23]. If there is a concern in using artificial recombinant proteins, the residual artificial protein has the potential to show immunogenicity.

In our experiments to test cell adhesion, Pronectin F and Pronectin F PLUS were superior to fibronectin when they were used in lower concentrations (Fig. 1). This might be because both Pronectin F and Pronectin F PLUS have 13 repeats of the RGD sequence in one molecule, whereas fibronectin has only two. Pronectin F and Pronectin F PLUS are also structurally stable, and thus the coated substrates retain their performance for at least 2 years at room temperature, which is another advantage over fibronectin [52]. Pronectins were produced without using any animal-derived components (Dr. Kurokawa, Sanyo Kasei Kogyo, personal communication).

Pronectin F is not soluble in water since the silk-like protein sequence forms strong hydrogen bonds intermolecularly; thus, it must be dissolved in LiClO$_4$ [21]. Therefore, Pronectin F PLUS, a water-soluble variant of Pronectin F, was developed by chemical modification of the serine residues of Pronectin F [22]. As shown in Fig. 3, a higher amount of Pronectin F was absorbed on polystyrene dishes than Pronectin F PLUS. Since the surface of polystyrene plates is hydrophobic, hydrophobic Pronectin F might have been absorbed well. Pronectin L, which has a similar core sequence to that of Pronectin F, was also absorbed well on the dish, although no cells adhered on it.

In spite of their differences in absorption on the polystyrene dish, Pronectin F PLUS and Pronectin F showed similar efficiency with respect to the adhered cell number, growth support, induction of FAK phosphorylation, and production of PGI$_2$ and t-PA. Moreover, Pronectin F PLUS was more potent than Pronectin F in its support of cell adhesion under stimulation with thrombin (Fig. 9). The cells on Pronectin F PLUS were resistant to cell detachment due to the thrombin-induced cytoskeletal changes. This might be because that Pronectin F PLUS is positively charged by chemical modification. Since the cell surface possesses a negative charge, the adhesive intensity between the cells and Pronectin F PLUS was increased due to the static electrical interaction, resulting in the maintenance of cell adhesiveness after the cytoskeletal change
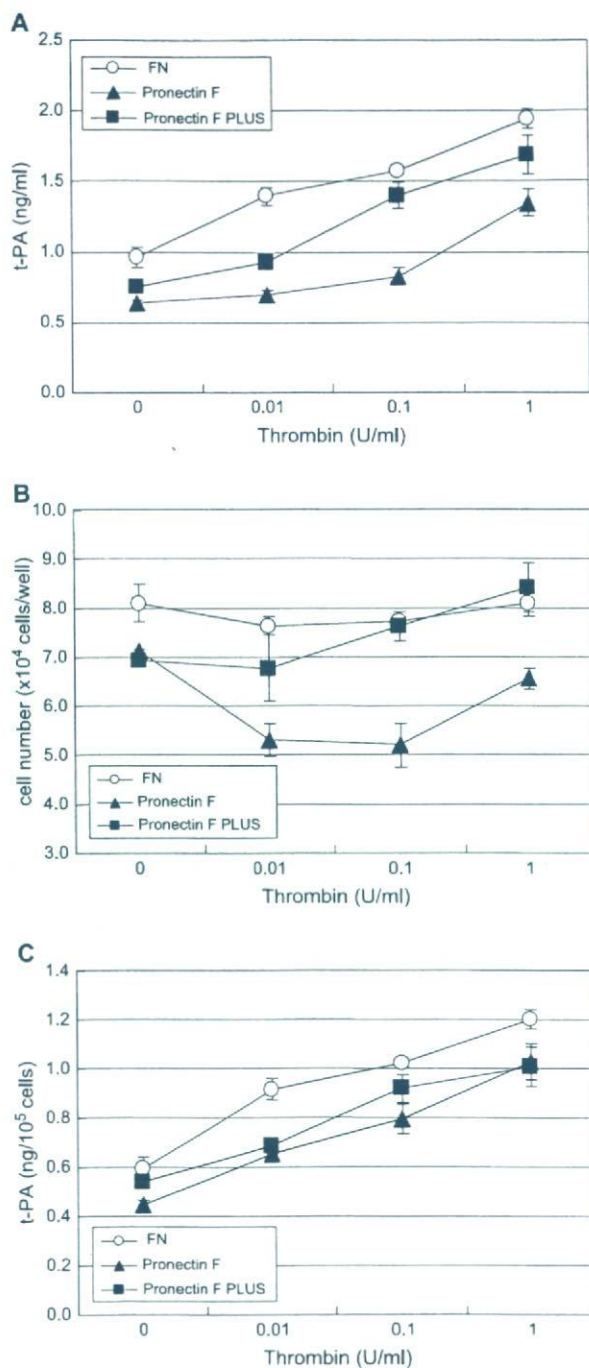

Fig. 9. Thrombin-induced production of t-PA and decrease in viability of HUVECs adhered on Pronectin F. HUVECs were seeded at 7×10^4 cells/well on 24-well plates previously coated with Fibronectin, Pronectin F or Pronectin F PLUS. After culturing for 1 day, the cells were stimulated with thrombin at the indicated concentrations. After 24 h incubation, the conditioned media were collected and t-PA concentration was determined by enzyme immunoassay (A). The cell numbers were determined using a Cell-Counting Kit-8 (B). Production of t-PA per 10^5 cells was calculated (C). Each data point represents the mean ± S.D. of triplicate determinations.

induced by thrombin. To our knowledge, this is the first observation to reveal the difference in biological properties between Pronectin F and Pronectin F PLUS. The retention of human endothelial cells on the lumen in the presence of physiological stimuli is an important prerequisite for artificial artery grafts to be successful, and, therefore, Pronectin F PLUS could also be suited for use in this type of graft.

In the present study, the usefulness of Pronectin F PLUS as a substitute for fibronectin was indicated; however, Pronectin F PLUS and Pronectin F were less effective than fibronectin in long-term cell cultures. We were able to culture HUVECs up to around 6 passages using the serum-free media with fibronectin. However, when Pronectin F or Pronectin F PLUS was used instead of fibronectin, the cell number did not increase beyond the second passage. Although Pronectin F PLUS worked better than Pronectin F, the efficiency of both was lower than that of fibronectin in the long-term maintenance of HUVECs. Since fibronectin has several functional domains that can bind to heparin, fibrin and collagen, signals other than the RGD-integrin interaction would be important in the long-term maintenance of the cells. The incorporation of domains other than that of RGD of fibronectin into Pronectin F PLUS would be necessary to improve its efficacy in long-term cell cultures.

For cell cultures, porcine trypsin is often used when cells are harvested. The use of trypsin may lead to the contamination of animal-derived materials or transmissible agents. In serum-free cultures of HUVECs, we were able to harvest cells for passage using a protease free of animal-derived materials such as TrypLE Select (Invitrogen, Carlsbad, CA, USA) or AccuMax (Chemicon, Billerica, MA, USA); therefore, trypsin is not necessarily used for the maintenance of HUVECs, which means that it is possible to eliminate the potential for contamination of animal-derived proteins.

In summary, Pronectin F PLUS was shown to have the closest activity to plasma fibronectin in the serum-free culture of HUVECs by examining VEGF- or bFGF-induced cell proliferation, FAK phosphorylation, and thrombin-stimulated production of PGI_2 or t-PA. The cell adherent activity of Pronectin F PLUS was superior to fibronectin at concentrations of less than 1 µg/ml. Although Pronectin F PLUS has the same amino acid sequence as Pronectin F, only HUVECs on Pronectin F PLUS were resistant to thrombin-induced cell detachment. Pronectin F PLUS is thus thought to be a promising substitute for plasma fibronectin in the serum-free culture of endothelial cells, although modification would be necessary to improve its efficiency in long-term cell cultures.

Acknowledgements

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Regulatory Aspects of Oncolytic Virus Products

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Abstract: Many types of oncolytic viruses, wild-type virus, attenuated viruses and genetically-modified viruses, have been developed as an innovative cancer therapy. The strategies, nature, and technologies of oncolytic virus products are different from the conventional gene therapy products or cancer therapy products. From the regulatory aspects to ensure the safety, efficacy and quality of oncolytic viruses, there are several major points during the development, manufacturing, characterization, non-clinical study and clinical study of oncolytic viruses. The major issues include 1) virus design (wild-type, attenuated, and genetically engineered strains), 2) poof of concept in development of oncolytic virus products, 3) selectivity of oncolytic virus replication and targeting to cancer cells, 4) relevant animal models in non-clinical studies, 5) clinical safety, 6) evaluation of virus shedding. Until now, the accumulation of the information about oncolytic viruses is not enough, it may require the unique approach to ensure the safety and the development of new technology to characterize oncolytic viruses.

Keywords: Gene therapy, cancer therapy, replicating virus.

INTRODUCTION

Oncolytic virus therapy has been developed as a new wave of cancer therapies. These therapies are dependent on the replication-selective nature of these viruses in tumor cells, which provides the marked breaths of cancer therapy. More than one century ago, evidence of oncolytic activity caused by replicating viruses was reported, and it was known the viruses could induce tumor lysis. Using these studies as a point of departure, rare but dramatic responses in cancer patients recovering from viral infections were reported. In the early development of oncolytic virus therapy, wild-type viruses with low pathogenicity to normal tissues, or attenuated viruses were selected for the treatment of cancer patients. However, some adverse events, such as the development of encephalitis in immune compromised patients, were reported [1-3]. Other works reported the oncolytic activity of wild-type or attenuated oncolytic viruses to be transient or limited to the site of injection [4-8]. Recently, attention has focused on overcoming the disadvantages of wild-type or attenuated oncolytic virus therapy, and many genetically modified viruses have been developed for cancer treatment. Progress in the development of genetically engineered oncolytic viruses has been based on recent advances in our understanding of the molecular biology of cancer and viruses, and on advances in genetic engineering technologies of virus genomes. Although many gene therapy clinical studies for the treatment of cancer have been conducted during the past decade using replication-incompetent virus vectors, these studies have not achieved satisfying results. Tumor-selective replicating viruses have been suggested to have an advantage over conventional gene therapy vectors for cancer therapy, and oncolytic viruses, especially genetically modified viruses, must be considered to be a special type of gene therapy product since their principle is directly associated with the transfer of the viral genome as the therapeutic gene [9]. In the present report, we review the development of oncolytic viruses as gene therapy products or attenuated virus products with specific reference to the associated regulatory issues.

Oncolytic virus therapy is based on several strategies, including tumor-selective replication, tumor-selective targeting, and/or the minimization of toxicity to normal cells. Many types of viruses have been utilized in oncolytic virus therapy; including adenovirus, herpes simplex virus (HSV), reovirus, Newcastle disease virus (NDV), vaccinia, measles virus, vesicular stomatitis virus (VSV) and Sendai virus [10-13]. During the development of oncolytic virus products, a number of major issues have arisen with respect to ensuring the quality, safety and efficacy of the products: 1) virus design (wild-type, attenuated and genetically engineered strains); 2) proof of concept in the development of oncolytic virus products; 3) the selectivity of oncolytic virus replication and targeting to cancer cells; 4) relevant animal models in non-clinical studies; 5) clinical safety; and 6) the evaluation of virus shedding. Since the strategies, nature and technologies of oncolytic virus products are different from those of conventional gene therapy products or cancer therapy products, we discuss the regulatory aspects of the development of oncolytic viruses in the present paper.

VIRUS DESIGN AND PRODUCT DEVELOPMENT

While many types of viruses are utilized for oncolytic virus therapies [10, 11, 14], selective replication in tumor cells is essential for the efficacy and safety of oncolytic viruses. Wild-type viruses and naturally occurring attenuated viruses are known to possess the ability to infect and kill transformed cells such as tumor cells. For example, VSV, NDV and reovirus have been used as oncolytic viruses with inherent tumor-selectivity [12, 15-18]. In the case of wild-type viruses or attenuated viruses, the mechanism underlying the virus-selectivity to tumor cells has been analyzed from various points of view. For instance, reovirus has an inherent preference for replicating cells with dysregulated growth factor-signaling cascades by Ras activation [17, 19]. Attenuated strains from HSV-1 have been reported to be potential anti-cancer therapeutics and have necessitated a thorough investigation into the molecular basis of host-cell permissiveness to HSV [20-22]. Since in the

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