①当該医薬品をヒトに適用する際の投与量および投与スケジュールを設定するための安全性情報を可能な限り得ること

②医薬品として期待される薬効以外の毒性が発現するおそれのある臓器・組織を可能な限り特定し，かつその毒性の種類，程度，可逆性や発現機序を検討しておくこと

である．また，

③臨床試験を含めた臨床使用時においてモニタリングすべき具体的な安全性評価項目を見出すこと

④承認・上市前にヒトでの知見を十分に得ることが事実上困難なケースが多い安全性（例えば，がん原性，生殖・発生毒性，遺伝毒性）に関する情報を得ること

も重要な目的である．したがって新医薬品の開発研究上，非臨床安全性試験の実施は，安全性薬理試験も含めて一般的に必要不可欠なものであり，それはバイオロジクスにおいても例外ではない．

また，開発途中や承認・上市後に製造方法を変更した場合や最終製品に重大な変更（例えば添加物に関する大きな変更）を加えた際にも，最終製品のcomparability（同等性/同質性）を確認するため，品質面での評価のみならず，場合によっては追加の安全性試験，薬物動態試験，薬力学試験や臨床試験が必要となることもある．

目的タンパク質の構造の多様性や不均一性，作用発現の動物種特異性，免疫原性，予期しない部位での作用発現の可能性など，バイオロジクスの物性面や作用面での特徴・特殊性からみて，従来の医薬品（特に化学合成医薬品）における非臨床安全性試験の種類・項目および試験方法をバイオロジクスにそのまま機械的に適用することは必ずしも妥当ではなく，従来とは異なる観点や方法で試験を実施すべき場合が多い．このため，細菌，酵母，昆虫，植物および哺乳動物細胞を含む種々の発現系を用い，特性解析がなされた細胞から製造される医薬品（動物工場/植物工場由来医薬品も含む．遺伝子治療用医薬品および細胞・組織利用医薬品は除く）について，別途検討すべき非臨床安全性試験の内容や考え方がICHガイドライン「バイオテクノロジー応用医薬品の非臨床における安全性評価」として厚生労働省から公表されている（章末参考文献3，表3-4脚注7）．これは，化学合成医薬品を主な適用対象とした他の非臨床安全性ICHガイドラインを補完する位置付けにある．

上記ガイドラインの基本的考え方によれば，あらゆる遺伝子組換え技術応用医薬品や細胞培養技術応用医薬品に対して適切とされる画一的な非臨床安全性試験のプロトコールなるものは存在し得ず，対象とする医薬品の特性や臨床上

の適用法などを考慮して医薬品毎にケース・バイ・ケースで合理的かつ柔軟に対応することが適切であるとされている．「ケース・バイ・ケース」といえども，あくまで当該医薬品の臨床上の安全性や有効性の適正な評価に役立つ知見を得ることを最終的な目標として，その時点で最も科学的に適切な試験を実施すべきであることは当然である．その上で，実施すべき試験の種類・項目および具体的な試験方法に関しては個々の医薬品毎に合理的な選択を行えばよい．逆に実施しないとした試験については，実施しなくてよいと判断した合理的な理由が必要である．

化学合成医薬品と同様，バイオロジクスの非臨床安全性試験においても，

①適切な動物種の選択

②用いる動物の例数，性別および週齢

③用いる動物の生理的状態

④投与量，投与経路，投与方法など動物への投与計画

⑤試験使用条件下での試料の安定性

などについて十分考慮しなければならない．

ガイドラインで適用対象としているバイオロジクスの多くには動物種特異性があるため，非臨床安全性試験において適切な動物種を選択することが特に重要である．*in vivo*での活性についてある特定の性質を予測したり，ヒトを含む複数の動物種の相対的な感受性を評価するためには，種々の動物由来の培養細胞系を利用することが適切なケースもある．①における「適切な動物種」とは，その動物種に目的産物の受容体が発現しており，用いる試料が薬理学的活性を示すような動物種のことである．例えば，モノクローナル抗体医薬品の場合における「適切な動物種」は，意図するエピトープ（抗原決定基）を発現し，かつヒトと類似した組織交差反応性を示すような動物種に相当する．ヒト疾患と類似していると考えられる実験動物モデル，例えば誘発性および自然発症性病態モデル動物，遺伝子ノックアウトモデル動物，トランスジェニック動物などを非臨床安全性試験に用いることにより有益な知見を得られる場合もあるが，その際にはこのような動物モデルを用いて安全性評価を行う科学的妥当性を事前に明確にしておく必要がある．それぞれの安全性評価では通常2種類の「適切な動物種」を使用した試験を計画すべきであるが，十分な周辺データおよび考察に基づいた正当かつ合理的な理由があれば1種類の「適切な動物種」を用いた試験のみでも許容され得る．

上記の動物種の選択とも関係するが，②の例数について，使用される動物の例数が少ない場合（ヒト以外の霊長類を用いた試験においてしばしばみられる），背景データなどの当該試験以外のデータの収集や総合的な考察をより綿

密に行う必要がある．観察の頻度を増やしたり観察期間を延長することによっても，例数が少ないことに起因する限界を部分的には補うことが可能である．

④の投与量，投与経路および投与回数は，臨床適用で予定される投与方法に可能な限り近い形とすべきである．投与量についてガイドラインでは「使用される動物種における医薬品の体内動態及び生物学的利用率並びに実験動物に安全かつ人道的に投与しうる投与量について考慮するべき」かつ「毒性用量及び無毒性用量（NOAEL）を含み，用量－反応関係に関する情報が得られるよう設定しなければならない」とされている．

ヒトに適用されるバイオロジクスの多くは動物で免疫原性を示すことから，動物における抗体産生がヒトでの抗体産生を直接意味するものではないものの，反復投与試験において当該医薬品の投与により産生する抗体を測定して（目的タンパク質に対する抗体の測定のみではなく，必要に応じて不純物などに対する抗体産生や添加剤共存による影響も含めた検討も行う），観察された抗体反応の特性（例えば，抗体力価，応答した動物数，中和抗体であるか否か）を明らかにし，その上で抗体発現が本薬の薬理作用および毒性所見に及ぼす影響について検討する必要がある．このため，用いた動物のほとんどで，新たに産生した抗体により当該医薬品の薬理作用または毒性作用が中和されるケースを除いては，抗体が検出されたことだけを理由として安易に本来必要である他の非臨床安全性試験を省略したり試験期間を短縮したりすることは不適切である．なお，反復投与毒性試験における投与期間について，ガイドラインでは一般的に1～3ヵ月，臨床で短期使用（例えば7日以内）および急性の致死的疾患に対する適応が検討されている薬剤では2週間，慢性疾患に対する適応が検討されている薬剤では6ヵ月前後が推奨されている．

さらに，最終製品（科学的妥当性があればそれと類似の剤型でも可）を用いての局所刺激性試験を実施する必要がある．ただし，単回または反復投与毒性試験に局所刺激性の評価を組み込むことにより，局所刺激性試験を独立して実施しなくてよい場合もある．同様に，安全性薬理試験のうち，摘出臓器を用いる試験や*in vitro*での試験以外の一部は，毒性試験に組み込んで実施することも可能である．また，反復投与毒性試験には可能な限りトキシコキネティクスを組み込むことが望ましい．

特に免疫毒性試験，生殖・発生毒性試験，遺伝毒性試験およびがん原性試験では，従前の化学合成医薬品で確立された試験方法をそのままバイオロジクスに適用して試験を実施しても無意味な結果しか得られない場合が多い．これらの試験の実施にあたっては，そもそもの試験目的を十分に考えた上で，品質面や薬理学的作用の面あるいは予定される適応症や投与期間等の臨床的側面など

個々の医薬品の特性に合わせた試験計画を個別に立案して試験を実施する必要がある．なお，科学的にみて合理的な理由があれば試験内容を簡略化したり，場合によっては試験を実施しなくても問題はない．

C 遺伝子治療用医薬品の品質・安全性確保

遺伝子治療用医薬品においては，

①ウイルスベクターの場合の複製（増殖）性ウイルスの検出方法，存在許容量と管理方法

②抗原性，特にウイルスベクターの場合に，目的遺伝子以外で発現するウイルスタンパク質による抗原性に対する留意と軽減方策

③標的細胞指向性の付与などによる目的外の細胞・組織への遺伝子導入の回避と投与量の軽減のための方策

④レトロウイルスベクターなどの染色体への遺伝子組み込みに伴う遺伝毒性，がん原性発現への慎重な対処

などが重要課題である（表3-5）．

D 細胞・組織利用医薬品の品質・安全性確保

細胞・組織利用医薬品・医療機器においては，

表3-5 バイオロジクスの品質・安全性確保の方策に関するガイドライン類（ICHガイドライン以外）

○生物学的製剤基準（1993年10月）[1]
○遺伝子治療用医薬品の品質及び安全性の確保に関する指針（1995年7月．一部改正2002年3月）[2,3]
○血漿分画製剤のウイルスに対する安全性確保に関するガイドライン（1999年8月）[4]
○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（2000年12月）[5]
○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（2000年12月）[6]
○異種移植の実施に伴う公衆衛生上の感染症問題に関する指針（2002年7月）[7]
○日局生物薬品のウイルス安全性確保の基本要件（第14改正日本薬局方第1追補・参考情報）（2002年12月）[8]
○医療用具の製造（輸入）承認申請に必要な生物学的安全性試験の基本的考え方（2003年2月）[9]
○生物由来原料基準（輸血用血液製剤総則，血漿分画製剤総則，人細胞組織製品原料基準，人尿由来原料基準，人由来原料基準，反芻動物由来原料基準，動物細胞組織製品原料基準，動物由来原料基準）（2003年5月）[10]
○BSEリスク評価の基本的な考え方（2003年8月）[11]

1 1993年10月1日付け厚生省告示第217号（その後，適宜一部改正）
2 http：//wwwhourei.mhlw.go.jp/~hourei/cgi-bin/t_docframe.cgi?MODE＝tsuchi&DMODE＝CONTENTS&SMODE＝NORMAL&KEYWORD＝&EFSNO＝3109
3 http：//wwwhourei.mhlw.go.jp/~hourei/cgi-bin/t_docframe.cgi?MODE＝tsuchi&DMODE＝CONTENTS&SMODE＝NORMAL&KEYWORD＝&EFSNO＝3638
4 http：//wwwhourei.mhlw.go.jp/hourei/doc/tsuchi/150702-a.pdf
5 http：//www.nihs.go.jp/mhlw/jouhou/cell/cell-a.pdf
6 http：//www.nihs.go.jp/mhlw/jouhou/cell/cell-b.pdf
7 http：//www.mhlw.go.jp/general/seido/kousei/i-kenkyu/index.html#isyoku
8 http：//jpdb.nihs.go.jp/jp14supp1/da1tuiho.pdf
9 http：//wwwhourei.mhlw.go.jp/~hourei/cgi-bin/t_docframe.cgi?MODE＝tsuchi&DMODE＝CONTENTS&SMODE＝NORMAL&KEYWORD＝&EFSNO＝3732
10 2003年5月20日付け厚生労働省告示第210号
11 http：//wwwhourei.mhlw.go.jp/hourei/doc/tsuchi/150806-c.pdf

①原材料となる細胞・組織から由来する感染症発生のリスク防止
②非自己細胞・組織の移植による望ましくない免疫反応や細胞分泌タンパク質による免疫原性
③移植細胞・組織のがん化の可能性
④移植細胞・組織が産生する目的外の生理活性物質が生体に及ぼす影響
⑤細胞の遺伝子改変，分化，増殖などに用いる試薬や培地成分による有害作用の回避

などに対する検討と対処が，製品特異的な安全性確保の方策として必要である（表3-5）．

E 動物工場/植物工場由来医薬品の品質・安全性確保

動物工場/植物工場由来医薬品においては，
①動物由来の異種間感染性物質の混入の可能性の排除
②製品（タンパク質や細胞・組織）による望ましくない免疫反応の回避に関する対策

が特に重要である（表3-5）．

F 感染性物質

バイオロジクスの安全性問題を物質面から考える際，大別して3つの観点がある．1つめは有効成分そのものに関わる安全性の問題，2つめは不純物などに関わる安全性の問題であり，これらについてはすでに論述した．3つめは汚染物質，特に感染性物質に関わる安全性の問題である．3者いずれも製品の安全性確保を図る上でゆるがせにできないポイントであるが，前2者が製品毎の個別対応の色彩が濃いのに対し，感染性物質に関わる問題はバイオロジクス全体に共通するものが多く，また，重篤な感染症の発生などの深刻な健康被害を招く可能性もあるのできわめて慎重な対応が必要である．

一般にヒトや動物を起源とする医薬品や添加剤を製造しようとする場合，あるいはその他製造過程において使用される細胞や組織，培地成分，クロマトグラフ用カラムの担体の成分，試薬などがヒトや動物などに由来する場合において留意すべき安全性上のきわめて重要な課題に，ウイルス，その他の微生物（細菌・真菌，マイコプラズマ）あるいはプリオンによる汚染の可能性がある．

このうち，細菌・真菌およびマイコプラズマによる汚染については，起源動物や原材料，あるいは医薬品製造基材（原薬の品質・安全性を確保する上で決定的に重要な位置付けにあると定めた原薬製造のための出発素材．表3-5にあげた生物由来原料基準で「原料又は材料」とされるもの）の段階をはじめ，製造工程の

適切な段階における適切な微生物学的検査や管理あるいは製品段階での無菌試験やマイコプラズマ否定試験などで対処することが一般的方策となっている．

反芻動物由来原料で問題となるプリオンについては，表3-5にあげた「BSEリスク評価の基本的な考え方」など，1996年の「牛海綿状脳症（BSE）に関する医薬品等の当面の安全性確保策について」（http：//www1.mhlw.go.jp/houdou/0804/98.html）以降の一連のBSE対策により原産国，使用部位，製造工程および製品の使用方法に基づく規制が行われており，これに従って対応することで安全性確保を図ることになる．

ウイルス安全性の確保については，バイオロジクス全般で基本的考え方は共通しているが，細部における問題とその対策や具体的アプローチは各製品の種類や製造方法毎に異なるところも多い．代表的バイオロジクスについては，すでにウイルス安全性に関するガイドライン類が各種整備されている（表3-4，表3-5）．これらのガイドラインをとおして，医薬品等のウイルスに対する総合的な安全性確保を図るために必要な基本的方策の共通認識とされることを要約すると，表3-6に示したとおりになる．これら表3-6 ①～⑨の方策を，段階的にかつ複数以上，相互補完的に活用していくことによって，医薬品等のウイルス面での安全性を確保，向上させることが重要である．

さらに，事前に予測あるいは検知できないウイルスなどによる健康被害の発生とその対応に備えて，原材料記録の保管管理，医薬品製造基材（血液，細胞・組織）の一部の貯留保管，ドナー記録・販売記録の保管管理，当該製品投

表3-6　ウイルスに対するバイオロジクスの総合的な安全性確保を図るために必要な基本的方策

①ウイルス汚染の可能性（汚染源）について熟知しておくこと
②原材料およびその起源たるヒトや動物の適格性に関して慎重に検討し，評価すること
③医薬品の製造基材と定めた段階のもの，すなわち原料または材料（例えば，原血漿，加工した細胞，セルバンク，プールした尿，細胞培養液，構造遺伝子，発現ベクターなど）において徹底的なウイルス試験とその結果の解析，評価を行い，ウイルス存在の有無および存在するウイルスの種類や性質について検討すること（なお，③は原材料やその起源たるヒトや動物における検討，評価と相互補完的に実施することが合理的な場合もある）
④ウイルスやウイルス様粒子が存在した場合，ヒトへの有害性がどの程度あるかを検討，確認すること
⑤ヒトに感染性や病原性を示すウイルスが存在しないような製造関連物質（培地成分，試薬，抗体を使用したアフィニティークロマトグラフ用担体など）を選択すること
⑥必要に応じて，製造工程の適当な段階において製品（例えば，細胞培養液を集めた未加工/未精製バルク，最終製品）の（外来性）ウイルス否定試験を実施するための適切な試験計画を策定すること
⑦製造工程による十分なウイルスクリアランスを達成するために，ウイルスの除去/不活化に効果的な方法を各種組み合わせて工程中に採用すること
⑧周到なウイルスクリアランス試験計画を立てること
⑨製造工程のもつウイルス不活化/除去能を評価する試験を⑧に基づいて実施し，評価すること

与に起因する可能性のある感染症発生の有無などの追跡調査，感染症の定期報告，当該製品が投与された患者の臨床記録・製品記録・製品およびドナーや患者由来の検査試料をしかるべき期間保存する措置，その他関連情報の積極的収集と情報提供なども，製品の種類や特殊性に応じて実施する必要がある．ただしこれらの多くは，血液製剤や細胞・組織利用医薬品など感染性物質混入のリスクが比較的高く保健衛生上の危害の発生または拡大を防止するための措置を講ずることが必要な製品（後述の厚生労働大臣により指定される「特定生物由来製品」）に対して求められるもので，細胞基材由来ペプチド・タンパク質性医薬品の多くにはあてはまらない．

ところで，製品のウイルス汚染ひいては健康被害発生を最も効果的に回避できるか否かの大きなポイントは，表3-6の②および③の段階，すなわち医薬品製造の上流の段階でウイルス汚染に関するチェックをいかに適切かつ厳密に行うかにかかっている．その具体的方策は，製品が（i）血液製剤，（ii）ヒト細胞・組織利用製品，（iii）ヒト尿由来製品，（iv）その他のヒト原料由来製品，（v）動物細胞・組織利用製品，（vi）その他の動物原料由来製品，（vii）反芻動物原料由来製品のいずれのカテゴリーに該当するかなどによってそれぞれ異なる．

生物由来原料基準で定められているとおり，例えばヒト細胞・組織利用製品については，細胞・組織採取から製品に至るまでの過程においてウイルス不活化/除去などの処理が一般的には困難なことから，ドナースクリーニングの段階で，製品の利用の目的に応じた適切な問診などの診断および検査を行い，ドナーとしての適格性を慎重に判断することとされている（表3-7）．

一方，ヒト尿由来製品では，原材料/医薬品製造基材にあたる一定処理後のプール尿においてB型肝炎ウイルス（HBV）抗原検査および核酸増幅検査

表3-7　ヒト細胞・組織利用医薬品におけるドナーの適格性

○B型肝炎ウイルス（HBV），C型肝炎ウイルス（HCV），ヒト免疫不全ウイルス（HIV），ヒトTリンパ球向性ウイルス（HTLV）およびヒトパルボウイルスB19感染症については，問診および検査（血清学的試験や核酸増幅検査（NAT）—例えばPCR法による—など）により否定する必要がある
○ヒトサイトメガロウイルス（CMV）およびEpstein Barrウイルス（EBV）感染については必要に応じて検査により否定することが求められる
○(i) 梅毒トレポネーマ，クラミジア，淋菌・結核菌の細菌による感染症，(ii) 敗血症およびその疑い，(iii) 悪性腫瘍，(iv) 重篤な代謝・内分泌疾患，(v) 膠原病，血液疾患，(vi) 肝疾患，(vii) 痴呆症（伝達性海綿状脳症およびその疑いのある者）については既往歴，問診などによる診断を行うとともに，輸血や移植医療を受けた経験の有無などから適格性を判断する
○免疫適合性などを考慮する
○ウインドウピリオド（病原体またはそれに対する抗体が検出できない感染初期の時期）の存在を考慮して可能な限り再検査を実施する
○なお，患者自己由来の細胞・組織を用いる場合は必ずしもドナースクリーニングを必要としない

（NAT）によるHBV，C型肝炎ウイルス（HCV），ヒト免疫不全ウイルス（HIV）検査が必要であるとされている．

細胞基材由来のペプチド・タンパク質性医薬品の場合は，細胞基材の由来となるヒト・動物レベルでの安全性にはあまり拘泥しない代わりに，セルバンクを医薬品製造基材と位置付け，この段階で徹底的なウイルス試験を行うことにより安全性を担保する．さらに，大量培養後の細胞でもしかるべきウイルス試験を念のため行うことにより，安全性の確保を徹底するという方策をとっている．

動物細胞・組織利用製品の場合は，

①細胞・組織採取の過程での病原微生物汚染の防止

②動物種毎の微生物学的特性を考慮したドナー動物の選択

③動物種に応じた適切な感染症に関する試験項目の設定

④適切な封じ込め設備などが整った施設におけるドナー動物の飼育管理

⑤生きた細胞または組織を用いる場合にあってはウイルス感染リスクの検証を行うこと

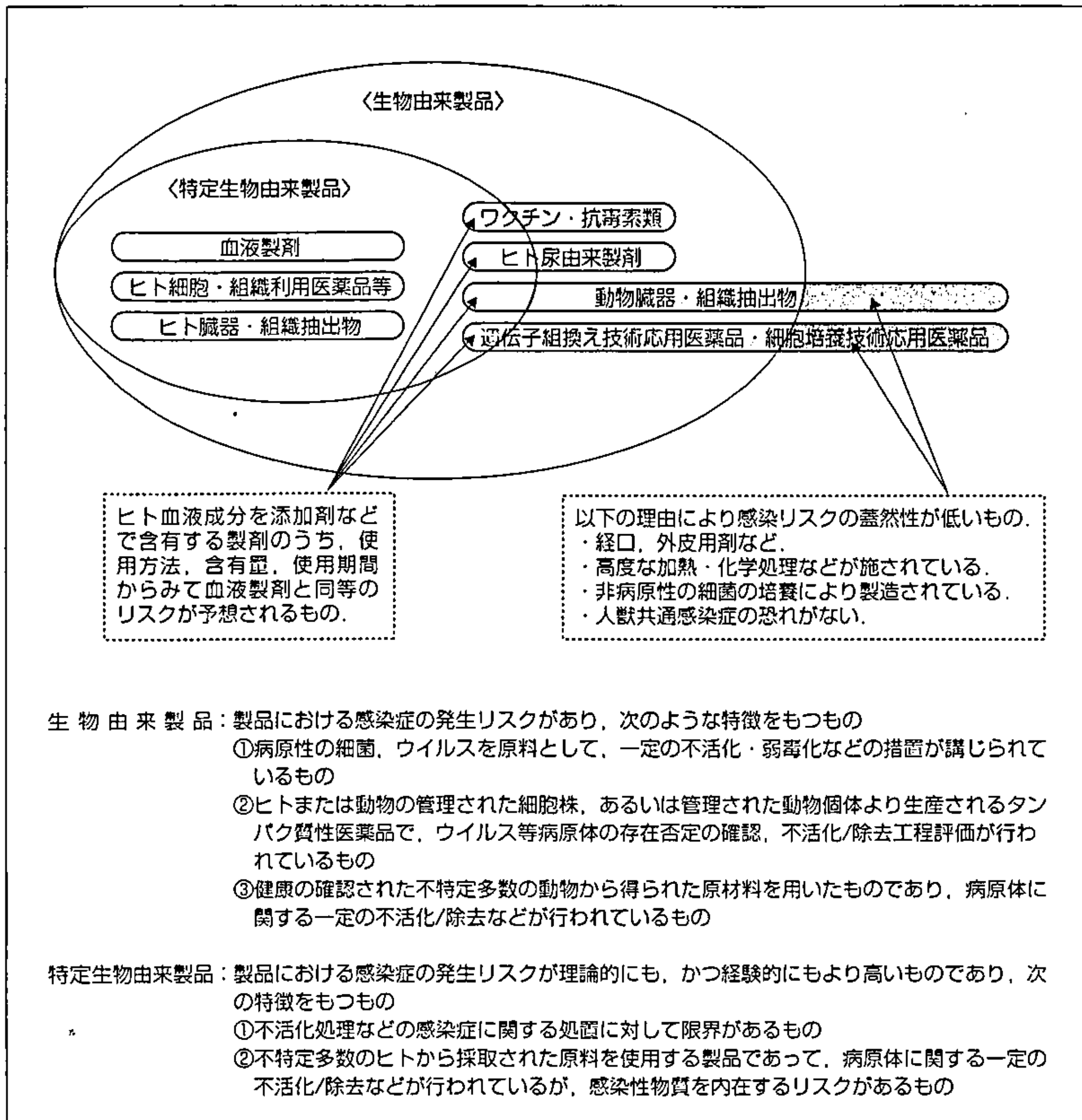
などが必要とされている．

動物原料由来製品の場合は，動物個体レベルで健康な動物あるいは食肉基準に適合した動物またはSpecific Pathogen-Free（SPF）動物を選択すること，さらに，動物レベルまたは原材料・医薬品製造基材レベルで動物種毎のウイルス学的特性（特にヒトへの感染性をもつウイルスの存在の可能性）に留意した検討を行うことが必要とされている．このウイルス試験の種類や程度は，製品の種類や以降の製造工程（不活化/除去工程など）での検討を勘案してケース・バイ・ケースで考えるのが合理的である．

医薬品・医療機器における感染リスクの評価に際して最も重要なことは，製造に用いられるヒト・動物由来原材料に感染性物質が混入するリスクの程度について合理的・客観的かつ可能な限り定量的な評価を行った上で，製品の臨床的有用性も勘案しながら，個々の製品の製造工程がもつ感染性物質のクリアランス能および投与経路に応じた患者の感染リスク（さらには発病リスク）を踏まえての現実的な議論を行うことである．

2002年の薬事法改正に伴い，新たに「生物由来製品」および「特定生物由来製品」という規制区分が設けられ，2003年7月から施行された（http：//wwwhourei.mhlw.go.jp/~hourei/cgi-bin/t_docframe.cgi?MODE＝hourei&DMODE＝CONTENTS&SMODE＝NORMAL&KEYWORD＝&EFSNO＝427）（図3-2）．「生物由来製品」とは，ヒト・その他の生物（植物以外）に由来する原材料を用いて製造される医薬品等のうち，製品による感染症伝播に関するリスク評価などの科学的見地に基づき「保健衛生上特別の注

図3-2　生物由来製品と特定生物由来製品



意を要するもの」として厚生労働大臣により個別に指定されるものであり，その中にはワクチン・抗毒素類，ヒトや動物の培養細胞由来の遺伝子組換え技術応用医薬品，動物成分抽出製剤などが含まれる．生物由来製品の中でも科学的見地もしくは行政的にみて感染症伝播に関するリスクについてさらに厳重な注意が必要，すなわち「保健衛生上の危害の発生又は拡大を防止するための措置を講ずることが必要なもの」は「特定生物由来製品」として指定される．2003年5月現在，既承認の医薬品等の中から，血液製剤，ヒト胎盤（プラセンタ）抽出物を含有する製剤，およびヒト血清アルブミンを添加剤として含み一個人（患者）に長期間適用されることが想定される製剤が特定生物由来製品として指定されている．この薬事法改正に伴って，特定生物由来製品を用いる際には

表3-8　バイオロジクス全体に共通する安全性確保上の要点（まとめ）

○原材料の採取段階も含めた製造工程の厳密な管理
○各バイオロジクスに特徴的な有効成分および目的物質由来不純物や製造工程由来不純物・汚染物質などの特性・品質解析や品質管理
○有効成分および不純物などに関わる安全性の確認
　・予期せぬ作用，抗原性・免疫原性・局所刺激性，新たに産生する抗体の影響などの確認
○感染性物質に関わる安全性の確保
　・細菌・真菌，マイコプラズマ，プリオン，ウイルス

医療機関において患者に十分な情報提供を行い患者の理解を得ることも義務付けられた．

本章で述べてきたバイオロジクス全体に共通する安全性確保上の要点を，表3-8にまとめる．

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Anti-tumor activity of chemokine is affected by both kinds of tumors and the activation state of the host's immune system: implications for chemokine-based cancer immunotherapy☆

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Abstract

In this study, we screened the anti-tumor activity of murine chemokines including CCL17, CCL19, CCL20, CCL21, CCL22, CCL27, XCL1, and CX3CL1 by inoculating murine B16BL6, CT26, or OV-HM tumor cells, all of which were transfected with chemokine-expressing fiber-mutant adenovirus vector, into immunocompetent mice. A tumor-suppressive effect was observed in mice inoculated with CCL19/B16BL6 and XCL1/B16BL6, and CCL22/OV-HM showed considerable retardation in tumor growth. In the cured mice inoculated with CCL22/OV-HM, a long-term specific immune protection against parental tumor was developed. However, we were unable to identify the chemokine that had a suppressive activity common to all three tumor models. Furthermore, an experiment using chemokine-transfected B16BL6 cells was also performed on mice sensitized with melanoma-associated antigen. A drastic enhancement of the frequency of complete rejection was observed in mice inoculated with CCL17-, CCL19-, CCL22-, and CCL27-transfected B16BL6. Altogether, our results suggest that the tumor-suppressive activity of chemokine-gene immunotherapy is greatly influenced by the kind of tumor and the activation state of the host's immune system.
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Keywords: Adenovirus vector; Chemokine; Transfection; Anti-tumor activity; Gene immunotherapy

Chemokine consists of a superfamily of small (8–14 kDa), secreted basic proteins that regulate relevant leukocyte-migration and -invasion into tissue by interacting with their specific receptors, which belong to the superfamily of seven-transmembrane domain G-protein-coupled receptors [1,2]. The function of chemokine, which is capable of attracting specific immune cells, is demonstrated in inflammatory disease sites as well as normal lymphoid tissues [2]. Because of these properties, chemokine is considered as the intriguing molecule for cancer immunotherapy, which is based on the premise of

☆ *Abbreviations:* Ad, adenovirus vector; AdRGD, RGD fiber-mutant Ad; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FBS, fetal bovine serum; MOI, multiplicity of infection; NK, natural killer; PBS, phosphate-buffered saline; $TCID_{50}$, tissue culture infectious $dose_{50}$.

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the eradication of tumor cells as a consequence of interaction with immune cells that have migrated and accumulated in tumor tissues [3]. To date, more than 40 chemokines have been identified, and several chemokines have been demonstrated as candidates for cancer treatment for use either as sole agents or with an adjuvant [4–8].

We hypothesized that efficient in vitro transfection of chemokine gene into tumor cells could render the tumor sufficient chemokine expression in vivo for screening anti-tumor activity. The chemokine, secreted from inoculated tumor cells, would induce the accumulation of immune cells in the tumor tissue. Consequently, the interaction between the immune cells and the tumor cells should initiate and/or demonstrate the anti-tumor immune response. Among the various methods of gene transduction, recombinant adenovirus vector (Ad) can provide high-level transduction efficacy to a variety of cell types [9,10]. However, some tumor cells exhibit a resistance to Ad-mediated gene transduction due to a decline in the expression of a coxsackie-adenovirus receptor, a primary Ad-receptor, on their surface. We previously demonstrated that, compared with conventional Ad, the fiber-mutant Ad harboring the RGD sequence in the HI loop of the fiber knob (AdRGD) could more efficiently transduce foreign genes into several kinds of tumor cells due to their directivity to αv-integrin positive in the majority of tumors [11–13]. Therefore, chemokine-expressing AdRGD would be useful not only for screening the anti-tumor activity of chemokines by in vitro transfection, but also for developing in vivo cancer gene immunotherapy.

In the present study, we first confirmed the vector performance of eight AdRGDs encoding each mouse chemokine, CCL17, CCL19, CCL20, CCL21, CCL22, CCL27, XCL1, or CX3CL1. The anti-tumor activity of these chemokines was investigated in mice by inoculating three kinds of murine tumor cells, B16BL6 melanoma, CT26 colon carcinoma, and OV-HM ovarian carcinoma cells, transfected with each chemokine-expressing AdRGD. In addition, we examined the growth and rejection ratio of chemokine gene-transduced B16BL6 cells in mice sensitized with melanoma-associated antigen (gp100).

Materials and methods

Cell lines and animals. Human lung carcinoma A549 cells were purchased from ATCC (Manassas, VA, USA). Murine melanoma B16BL6 cells (H-2^b) and human embryonic kidney 293 cells were obtained from JCRB cell bank (Tokyo, Japan). Murine colon carcinoma CT26 cells (H-2^d) were kindly provided by Dr. Nicholas P. Restifo (National Cancer Institute, Bethesda, MD, USA). Murine ovarian carcinoma OV-HM cells (H-$2^{b/k}$) were kindly provided by Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Osaka, Japan). A549 and 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. B16BL6 cells were cultured in a minimum essential medium supplemented with 7.5% FBS and antibiotics. CT26 and OV-HM cells were grown in an RPMI 1640 medium supplemented with 10% FBS and antibiotics. Murine pre-B lymphoma L1.2 cells and their stable transfectants of a specific chemokine receptor, L1.2/CCR4, L1.2/CCR6, L1.2/CCR7, L1.2/CCR10, L1.2/XCR1, and L1.2/CX3CR1 cells [14], were maintained in an RPMI 1640 medium supplemented with 10% FBS, 50 μM of 2-mercaptoethanol, and antibiotics. All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Female C57BL/6 (H-2^b), BALB/c (H-2^d), and B6C3F1 (H-$2^{b/k}$) mice, ages 7–8 weeks, were purchased from SLC (Hamamatsu, Japan). All of the animal experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Vectors. Replication-deficient AdRGD was based on the adenovirus serotype 5 backbone with deletions of E1/E3 region. The RGD sequence for αv-integrin-targeting was inserted into the HI loop of the fiber knob using a two-step method as previously described [11]. Mouse cDNAs of CCL17, CCL19, CCL20, CCL21, CCL22, and XCL1 were obtained from pExCell-mCCL17, pT7T3D-Pac-mCCL19, pFastBac1-mCCL20, pT7T3D-Pac-mCCL21, pBluescript SK(+)-mCCL22, and pExCell-mXCL1, respectively. The expression cassette containing each mouse chemokine cDNA under the control of the cytomegalovirus promoter was inserted into E1-deletion site for constructing AdRGD-CCL17, -CCL19, -CCL20, -CCL21, -CCL22, and -XCL1, respectively, by an improved in vitro ligation method as previously described [15,16]. Mouse CCL27-expressing AdRGD (AdRGD-CCL27), mouse CX3CL1-expressing AdRGD (AdRGD-CX3CL1), gp100-expressing AdRGD (AdRGD-gp100), β-galactosidase-expressing AdRGD (AdRGD-LacZ), luciferase-expressing AdRGD (AdRGD-Luc), and AdRGD-Null, which is identical to the AdRGD vectors without the gene expression cassette, were previously constructed [11,17–19]. AdRGD-LacZ, -Luc, and -Null were used as negative control vectors in the present study. All recombinant AdRGDs were propagated in 293 cells, purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80 °C. Titers (tissue culture infectious $dose_{50}$; $TCID_{50}$) of infective AdRGD particles were evaluated by the end-point dilution method using 293 cells.

RT-PCR analysis. A549 cells were transfected with each AdRGD at an MOI (multiplicity of infection; $TCID_{50}$/cell) of 50 for 2 h, and then the cells were washed twice with phosphate-buffered saline (PBS) and cultured for 24 h. The expression of mouse chemokine mRNA in these A549 cells was confirmed by an RT-PCR analysis as follows: total RNA was isolated from transduced A549 cells using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions, following which RT proceeded for 60 min at 42 °C in a 50-μl reaction mixture containing 5 μg total RNA treated with DNase I, 10 μl of 5× RT buffer, 5 mM $MgCl_2$, 1 mM dNTP mix, 1 μM random hexamers, 1 μM oligo(dT), and 100 U ReverTra Ace (Toyobo, Osaka, Japan). PCR amplification of each mouse chemokine and human β-actin transcripts was performed in 50 μl of a reaction mixture containing 1 μl of RT-material, 5 μl of 10× PCR buffer, 1.25 U *Taq* DNA polymerase (Toyobo), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.4 μM primers. The sequences of the specific primers used for PCR amplification and the expected PCR product sizes are defined in Table 1. After denaturation for 2 min at 95 °C, 30 (mouse chemokine) or 20 (human β-actin) cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C (human β-actin), 60 °C (mouse CCL17, CCL19, CCL20, CCL22, and CX3CL1), 62 °C (mouse CCL21 and XCL1), or 63 °C (mouse CCL27), and extension for 30 s at 72 °C were repeated and followed by completion for 4 min at 72 °C. The PCR product was electrophoresed on a 3% agarose gel, stained with ethidium bromide, and visualized under ultraviolet radiation. EZ Load (Bio-Rad, Tokyo, Japan) was used as a 100 bp molecular ruler.

In vitro chemotaxis assay. A549 cells were transfected with each AdRGD at an MOI of 50 for 2 h, and then the cells were washed twice with PBS and cultured in media containing 10% FBS. After 24 h

Table 1
Primer sequences used for PCR amplification

Gene	Primer sequence (5′ → 3′)	Product size (bp)
Mouse CCL17	(F) TGC TTC TGG GGA CTT TTC TG (R) CCT TGG GTT TTT CAC CAA TC	242
Mouse CCL19	(F) GAA AGC CTT CCG CTA CCT TC (R) TGC TGT TGC CTT TGT TCT TG	164
Mouse CCL20	(F) CGA CTG TTG CCT CTC GTA CA (R) CAC CCA GTT CTG CTT TGG AT	157
Mouse CCL21	(F) CTG AGC CTC CTT AGC CTG GT (R) TCC TCT TGA GGG CTG TGT CT	381
Mouse CCL22	(F) TAT GGT GCC AAT GTG GAA GA (R) GCA GGA TTT TGA GGT CCA GA	102
Mouse CCL27	(F) CTC CCG CTG TTA CTG TTG CT (R) AGT TTT GCT GTT GGG GGT TT	331
Mouse XCL1	(F) ATG GGT TGT GGA AGG TGT G (R) GGG AAC AGT TTC AGC CAT GT	250
Mouse CX3CL1	(F) GCA GTG ACC GGA TCA TCT CT (R) GGC ACC AGG ACG TAT GAG TT	701
Human β-actin	(F) CCT TCC TGG GCA TGG AGT CCT G (R) GGA GCA ATG ATC TTG ATC TTC	202

cultivation, cells were washed and incubated with an assay medium (phenol red-free RPMI 1640 containing 0.5% bovine serum albumin and 20 mM Hepes, pH 7.4) for another 24 h. The resulting conditioned medium was collected, and its chemoattractant activity was measured by an in vitro chemotaxis assay across a polycarbonate membrane with 5-μm pores (Chemotaxicell-24; Kurabo, Osaka, Japan) using L1.2 transfectants expressing the specific receptor for chemokines. Recombinant chemokines corresponding to each specific receptor (mouse: CCL19, CCL20, CCL22, CCL27, XCL1, and CX3CL1) were purchased from DakoCytomation (Kyoto, Japan) and used as a positive control for cell migration. Migration was allowed for 2 h at 37 °C in a 5% CO_2 atmosphere. The migrated cells were lysed and quantitated using a PicoGreen dsDNA quantitation reagent (Invitrogen, Tokyo, Japan), and the migration activity was expressed in term of the percentage of the input cells calculated by the following formula: (% of input cells) = (the number of migrated cells)/(the number of cells placed in Chemotaxicell-24; 1×10^6 cells) × 100.

Evaluation of growth of chemokine gene-transduced tumor cells in immunocompetent mice. B16BL6, CT26, and OV-HM cells were transfected with each AdRGD at an MOI of 400, 50, and 10, respectively. After 24 h cultivation, the cells were harvested and washed three times with PBS, and then 2×10^5 transduced B16BL6 cells, 2×10^5 transduced CT26 cells, and 1×10^6 transduced OV-HM cells were intradermally inoculated into the flank of C57BL/6 mice, BALB/c mice, and B6C3F1 mice, respectively. The major and minor axes of the tumor were measured using microcalipers, and the tumor volume was calculated by the following formula: (tumor volume; mm^3) = (major axis; mm) × (minor axis; $mm)^2$ × 0.5236 [20]. The mice were euthanized when one of the two measurements was greater than 15 mm. On day 60 after tumor inoculation, the tumor-free mice were judged as individuals that could achieve complete rejection. In some cases, the mice that could completely reject a primary tumor were rechallenged by intradermal injection into the flank with 1×10^6 parental or irrelevant tumor cells without chemokine gene-transduction at 3 months after the initial challenge.

Evaluation of growth and rejection ratio of chemokine gene-transduced B16BL6 cells in mice sensitized with melanoma-associated antigen. The immunization of mice with melanoma-associated antigen was performed by the administration of dendritic cells (DCs) transduced with the gp100 gene. The isolation, cultivation, and gene transduction procedures for C57BL/6 mouse bone marrow-derived DCs conformed to the methods previously described [21]. DCs transfected with Ad-RGD-gp100 at an MOI of 50 for 2 h were intradermally injected into the right flank of C57BL/6 mice at 5×10^5 cells/50 μl. At 1 week after the vaccination, 2×10^5 intact or transduced B16BL6 cells were inoculated into the left flank of the mice. The tumor growth and complete rejection were assessed as described above.

Results

Expression of chemokine mRNA and protein in cells transfected with AdRGD

In order to verify the vector performance of mouse chemokine gene-carried AdRGDs, we first examined

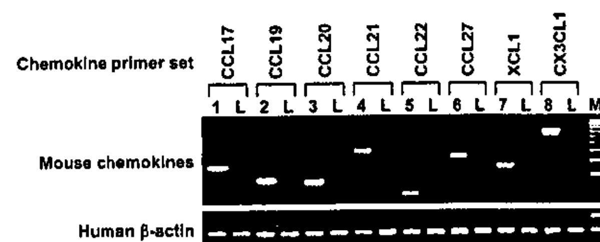

Fig. 1. RT-PCR analysis of chemokine mRNA expression in A549 cells transfected with each chemokine gene-carried AdRGD. PCR for mouse chemokine and human β-actin transcripts was performed on the same RT samples using each specific primer set (summarized in Table 1) to ensure the quality of the procedure. Lane L is negative control using AdRGD-LacZ-transfected A549 (LacZ/A549) cell-derived RT material. Lanes 1–8 represent CCL17/A549, CCL19/A549, CCL20/A549, CCL21/A549, CCL22/A549, CCL27/A549, XCL1/A549, and CX3CL1/A549, respectively. Lane M is a 100 bp molecular ruler.

mRNA expression in transfected cells by an RT-PCR analysis (Fig. 1). In this experiment, human lung carcinoma A549 cells were used instead of murine tumor cells to eliminate the influence of the expression of endogenous mouse chemokine. A549 cells transfected with AdRGD-CCL17, -CCL19, -CCL20, -CCL21, -CCL22, -CCL27, -XCL1, or -CX3CL1 expressed corresponding mouse chemokine mRNA, whereas no PCR products derived from the transcripts of the mouse chemokine gene were detected in AdRGD-LacZ-transfected A549 cells. Next, using in vitro chemotaxis assay, we investigated whether A549 cells transfected with each chemokine gene-carried AdRGD could secrete chemokine protein as a biologically active form into culture supernatants. As shown in Fig. 2, the culture supernatants of each chemokine gene-transduced A549 cell could induce greater migration of cells expressing the corresponding chemokine receptor than those of the intact A549 cells or the AdRGD-Luc-transfected A549 (Luc/A549) cells. The migration of parental L1.2 cells for chemokine receptor-transfectants was not observed in recombinant chemokine-added wells, and they were

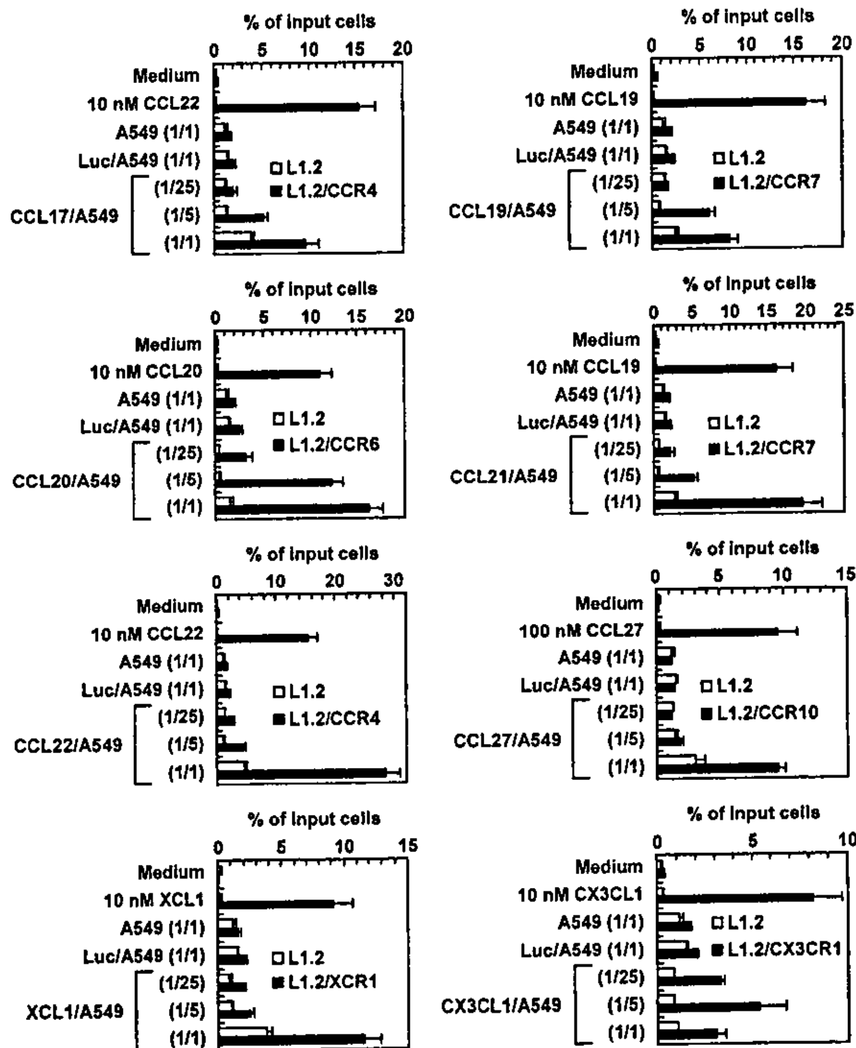

Fig. 2. Chemoattractant activity of culture supernatants of A549 cells transfected with each chemokine gene-carried AdRGD against the stable specific chemokine receptor-expressing cells. The culture supernatants of intact A549 cells, AdRGD-Luc-transfected A549 (Luc/A549) cells, and chemokine gene-transduced A549 cells were prepared and diluted with an assay medium. The fractional values with parentheses in each panel express the dilution factor. These samples and recombinant chemokines dissolved with the assay medium were added to a 24-well culture plate. Cells expressing specific receptors for CCL17 and CCL22 (L1.2/CCR4), CCL20 (L1.2/CCR6), CCL19 and CCL21 (L1.2/CCR7), CCL27 (L1.2/CCR10), XCL1 (L1.2/XCR1), or CX3CL1 (L1.2/CX3CR1) were suspended with the assay medium and placed in a Chemotaxicell-24 installed on each well at 1×10^6 cells. Likewise, parental L1.2 cells for these transfectants were prepared and added to Chemotaxicell-24. Cell migration was allowed for 2 h at 37 °C in a 5% CO_2 atmosphere. The cells that migrated to the lower well were lysed and quantitated using a PicoGreen dsDNA quantitation reagent. The data are expressed as means ± SE of the triplicate results.

maintained at low levels against the culture supernatants of intact A549, Luc/A549, and chemokine gene-transduced A549 cells. These results clearly demonstrated that all AdRGDs encoding each chemokine gene could deliver the concerned gene to target cells, and that transfected cells could secrete the chemokine protein which maintained original chemoattractant activity.

In vivo anti-tumor effect by transfection with chemokine-expressing AdRGD

B16BL6 and CT26 cells were each transfected with eight kinds of chemokine-expressing AdRGDs and AdRGD-Luc, as a control vector, at an MOI of 400 and 50, respectively. OV-HM cells were transfected with

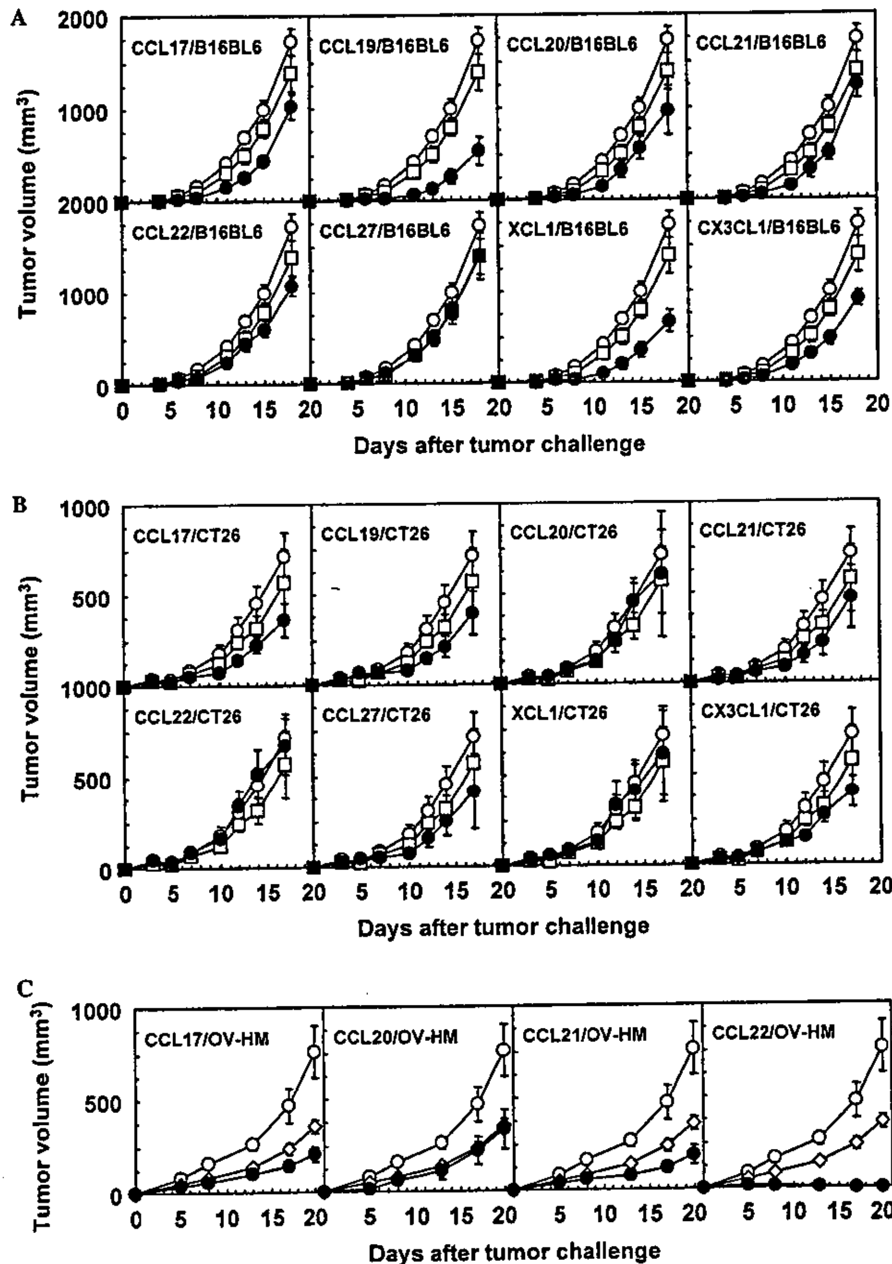

Fig. 3. In vivo growth of three kinds of murine tumor cells transduced with the chemokine gene. B16BL6 cells (A), CT26 cells (B), and OV-HM cells (C) were transfected with each chemokine-expressing AdRGD at an MOI of 400, 50, and 10, respectively, for 24 h. C57BL/6 mice, BALB/c mice, and B6C3F1 mice were intradermally injected in the flank with 2×10^5 transduced B16BL6 cells, 2×10^5 transduced CT26 cells, and 1×10^6 transduced OV-HM cells (●), respectively. Similarly, mice were inoculated with three kinds of intact tumor cells (○), AdRGD-Luc-transfected B16BL6 cells or CT26 cells (□), or AdRGD-Null-transfected OV-HM cells (◇), as control groups. The tumor volume was calculated after measuring the major and minor axes of the tumor at indicated points. Each point represents the mean ± SE of 6–10 mice. The data are representative of two independent experiments.

AdRGD-CCL17, -CCL20, -CCL21, -CCL22, or control AdRGD-Null at an MOI of 10. These transduced tumor cells were intradermally inoculated into H-2 haplotype-matched mice, and tumor growth was compared with that of intact tumors. As shown in Fig. 3, the tumorigenicity of B16BL6 and CT26 cells was hardly affected by transfection with the control vector, whereas OV-HM cells transfected with AdRGD-Null exhibited a slight delay of tumor growth as compared with intact OV-HM cells. Among 20 combinations of chemokine and tumor cells, an obvious tumor-suppressive effect was recognized in mice inoculated with CCL19/B16BL6, XCL1/B16BL6, or CCL22/OV-HM cells. In contrast, the in vivo growth of CCL27/B16BL6, CCL20/CT26, CCL22/CT26, XCL1/CT26, and CCL20/OV-HM cells was the same as that of the control vector-transfected cells, and only a slight delay of tumor growth was observed in five B16BL6 groups (CCL17, CCL20, CCL21, CCL22, and CX3CL1), five CT26 groups (CCL17, CCL19, CCL21, CCL27, and CX3CL1), and two OV-HM groups (CCL17 and CCL21). Importantly, CCL22/OV-HM cells not only demonstrated considerable retardation in tumor growth but were also completely rejected in 9 of 10 mice. In the rechallenge experiment, these cured mice were intradermally injected with 1×10^6 parental OV-HM cells or irrelevant B16BL6 cells at 3 months after the initial challenge. Five of six mice rechallenged with OV-HM cells remained tumor-free for more than 2 months, whereas rechallenging with B16BL6 cells perfectly developed palpable tumors in three additional mice within 2 weeks (data not shown). These results indicate the generation of long-term specific immunity against OV-HM tumor in mice that could once reject CCL22/OV-HM cells.

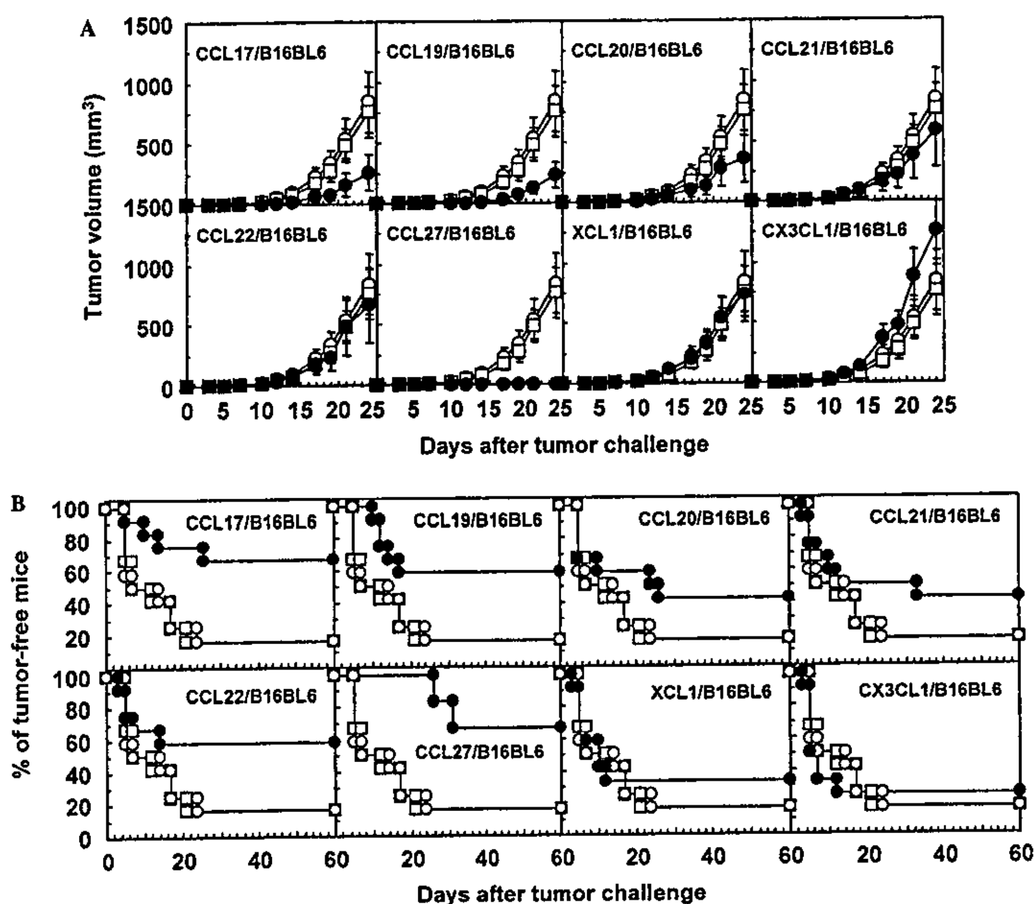

Fig. 4. Growth and rejection ratio of B16BL6 cells transduced with the chemokine gene in mice primed with melanoma-associated antigen. C57BL/6 mouse bone marrow-derived DCs were transfected with AdRGD-gp100 at an MOI of 50 for 2 h and they were intradermally injected into the right flank of syngeneic mice at 5×10^5 cells. At 1 week after the vaccination, these mice were intradermally inoculated in the left flank with 2×10^5 B16BL6 cells transfected with each chemokine-expressing AdRGD at an MOI of 400 for 24 h (●). Likewise, intact B16BL6 cells (○) or AdRGD-Luc-transfected B16BL6 cells (□) were inoculated in the gp100-primed mice, which were used as control groups. (A) The tumor volume was assessed three times per week. Each point represents the mean ± SE of results obtained from 12 mice. (B) Data are expressed in terms of the percentage of mice without visible tumor against the total mice tested in each group.

Growth and rejection ratio of chemokine gene-transfected B16BL6 cells in gp100-primed mice

For the purpose of examining the influence of chemokine against tumor growth in hosts specifically sensitized with tumor-associated antigen, B16BL6 cells transfected with chemokine-expressing AdRGD were inoculated into mice that were vaccinated with DCs presenting gp100, one of the identified melanoma-associated antigens. As shown in Fig. 4A, CCL17-, CCL19-, or CCL27-transfection was very effective for tumor growth suppression in gp100-primed mice, whereas AdRGD-Luc-transfected B16BL6 cells did not show any difference in tumor growth as compared with intact cells. A remarkable enhancement was observed in the complete rejection ratio at 2 months after tumor inoculation in the CCL22-transfected group as well as in the CCL17, CCL19, and CCL27 groups (Fig. 4B). Also, transfection with AdRGD-CCL20 or -CCL21 moderately improved the rejection ratio of B16BL6 cells in gp100-primed mice. XCL1 did not show a notable difference in both the growth and the rejection ratio of B16BL6 cells as compared with the control groups, and CX3CL1-transfected cells showed a tendency to promote tumor growth as compared with the intact B16BL6 cells.

Discussion

The application of chemokines to cancer immunotherapy has recently attracted great attention, because of their chemoattractant activity for a variety of immune cells as well as the angiostatic activity of some chemokines such as CXCL9 and CXCL10. In addition, it has been known that some tumor cells express a lower level of chemokines than normal cells [22]. Therefore, we may obtain novel cancer gene immunotherapy capable of demonstrating an excellent therapeutic effect, if a specific chemokine is adequately expressed at a local tumor site by gene transduction. The tumor-suppressive activity of several chemokines was observed in actuality in various experimental tumor models using the in vitro transfection method [8,23–27]. We also previously demonstrated that a CC family chemokine, CCL27, could suppress OV-HM tumor growth via transfection into the tumor cells due to the local recruitment of T cells and natural killer (NK) cell, whereas the transfection of CX3CL1 did not show a significant effect [19]. However, there are few reports comparing the antitumor activity of a specific chemokine between distinct tumor models.

Thus, we screened the potential anti-tumor activity of CCL17, CCL19, CCL20, CCL21, CCL22, CCL27, XCL1, and CX3CL1 in three murine tumor models by in vitro transfection. In order to efficiently transduce the chemokine gene into tumor cells, we constructed the AdRGDs carrying an expression cassette containing each murine chemokine cDNA by an improved in vitro ligation method. AdRGD can enhance gene transduction efficiency against a variety of tumor cells as compared with conventional Ad because of the expression of the RGD sequence, the αv-integrin-targeting peptide, at the HI-loop in their fiber knob [11–13]. Moreover, the improved in vitro ligation method enables speedy construction of a series of AdRGDs for screening by easy insertion of the expression cassette for the concerned gene into E1-deletion site [15,16]. With respect to the RT-PCR analysis and in vitro chemotaxis assay, transfection using our eight AdRGDs encoding the chemokine gene allowed tumor cells to express each corresponding chemokine mRNA and secrete a specific chemokine protein in a biologically active form (Figs. 1 and 2). Murine B16BL6 melanoma, murine CT26 colon carcinoma, and murine OV-HM ovarian carcinoma cells were transfected with chemokine-expressing AdRGDs at the MOI, which was suitable for adequately introducing a reporter gene into each tumor cell in preliminary examinations. To address the possibility of growth suppression depending on the cytotoxicity by AdRGD itself or secreted chemokine, we evaluated the viability of tumor cells transfected with each AdRGD at 48 h after transfection by MTT assay. The in vitro growth of the transfected cells was essentially identical to that of the intact cells with the exception of the OV-HM cells transduced with AdRGD-CCL19 or -XCL1 (data not shown). Therefore, CCL19 and XCL1 were excluded from the in vivo experiment using OV-HM cells.

Although a slight delay in tumor growth was observed in most of the combinations of tumor cells and chemokines, only CCL19/B16BL6, XCL1/B16BL6, and CCL22/OV-HM cells demonstrated a notable tumor-suppressive activity in immunocompetent mice as compared with the control vector-transfected cells (Fig. 3). In particular, CCL22-transfection was highly efficacious for the repression of OV-HM tumor growth, since complete rejection was observed in 9 of 10 mice. Furthermore, five of six cured mice could resist rechallenge with parental OV-HM cells, indicating the generation of a long-term tumor-specific immunity by rejection of CCL22/OV-HM cells. CCL22 exhibits a strong chemoattractant activity for a variety of immune cells including T cells, NK cells, and DCs. Guo et al. [28] also reported that the intratumoral injection of conventional Ad encoding human CCL22 resulted in a marked tumor regression in a murine 3LL lung carcinoma model with significant cytotoxic T lymphocyte (CTL) activity. However, CCL22-transfection did not show an anti-tumor effect in both B16BL6 and CT26 cells, and the chemokine that could demonstrate an obvious suppressive effect common to tumor cells of all three kinds was not found even if the results of CCL27/OV-HM and

CX3CL1/OV-HM cells, which were examined in our previous work [19], were included. In addition, some chemokines such as CCL17, CCL20, CCL21, and CX3CL1 failed to induce a notable suppressive effect against all three kinds of tumors although their chemoattractant activity for immune cells was reported. These complicated phenomena suggest that the anti-tumor effect via chemokine expression might be affected by several factors, for example, (1) the immunogenicity of the tumor cells, (2) the quantity and population ratio of the immune cells accumulated in tumor tissue, and (3) the activation state and deviation of the immune system in host.

We considered that not only the accumulation but also the activation of immune cells in tumor tissue is very important in cancer immunotherapy using chemokines, because several approaches that combined chemokines with cytokines or costimulatory molecules resulted in the synergic enhancement of anti-tumor activity as compared with the application of chemokine alone [29–32]. DCs, unique antigen-presenting cells capable of priming and stimulating naive T cells, not only play a critical role in establishing antigen-specific adaptive immune responses but also regulate the innate immune system [33–35]. Because of these properties, DCs loaded with tumor-associated antigen are ideal for generating a primary immune response against cancer as "nature's adjuvant" [33,36]. We previously reported that the vaccination of DCs transfected with gene coding gp100, one of the melanoma-associated antigens, by AdRGD could induce anti-B16BL6 tumor immunity based on increasing cytotoxic activities of NK cells and gp100-specific CTLs [21]. When chemokine-transfected B16BL6 cells were inoculated into mice vaccinated with gp100-expressing DCs, CCL17, CCL19, CCL22, and CCL27 could promote resistance to tumor formation (Fig. 4). Upon comparing the outcomes in Figs. 3A and 4, CCL19 demonstrated B16BL6 tumor-suppressive activity in both intact and gp100-primed mice, whereas the enhancement of the anti-tumor effect by CCL17, CCL22, or CCL27 was observed only in gp100-primed mice. Surprisingly, the anti-tumor activity of XCL1 detected in intact mice was lost in gp100-primed mice, and the CX3CL1/B16BL6 tumor grew more rapidly than the control tumor in gp100-primed mice. We speculated that the weak anti-B16BL6 tumor activity of XCL1 or CX3CL1 was masked by vaccine efficacy of gp100-expressing DCs, and that the angiogenic activity of CX3CL1 [37] might be emphasized in a tumor-specifically sensitized host.

Collectively, our data suggested that the tumor-suppressive activity of chemokine was greatly influenced by the kind of tumors and the activation state of the immune cells, and that a search for an effective chemokine for cancer immunotherapy should be performed in an experimental model that can reflect clinical status, including the immunogenicity of tumors, the state of the host's immune system, and the combination of other treatments, as much as possible.

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Antibody-targeted cell fusion

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Membrane fusion has many potential applications in biotechnology. Here we show that antibody-targeted cell fusion can be achieved by engineering a fusogenic viral membrane glycoprotein complex. Three different single-chain antibodies were displayed at the extracellular C terminus of the measles hemagglutinin (H) protein, and combinations of point mutations were introduced to ablate its ability to trigger fusion through the native viral receptors CD46 and SLAM. When coexpressed with the measles fusion (F) protein, using plasmid cotransfection or bicistronic adenoviral vectors, the retargeted H proteins could mediate antibody-targeted cell fusion of receptor-negative or receptor-positive index cells with receptor-positive target cells. Adenoviral expression vectors mediating human epidermal growth factor receptor (EGFR)-targeted cell fusion were potently cytotoxic against EGFR-positive tumor cell lines and showed superior antitumor potency against EGFR-positive tumor xenografts as compared with control adenoviruses expressing native (untargeted) or CD38-targeted H proteins.

Cell fusion is essential for fertilization and for the development of placenta, muscle and bone[1]. It provides a basis for stem cell plasticity[2] and is central to the pathogenesis of numerous viral infections[3–5]. Cell fusion is also a scientific tool, used in the production of monoclonal antibodies[6], the identification of oncogenes and tumor suppressor genes[7] and the elucidation of chromosomal functions[8]. Cell fusion has therapeutic applications in cancer gene therapy[9], virotherapy[10] and the generation of novel cancer vaccines[11,12]. We are therefore developing technology to control cell fusion, restricting and redirecting it to achieve target specificity.

Fusion of measles-infected cells is mediated by the viral H and F proteins, which together form a fusogenic membrane glycoprotein complex[3]. The H protein mediates attachment to either one of the viral receptors CD46[13] or SLAM[14] on the cell surface, and signals to the F protein to trigger cell fusion[15]. The steps required to retarget this cell fusion reaction are ablation of H protein–mediated CD46 and SLAM receptor recognition and introduction of a new binding specificity in the H glycoprotein, while preserving its ability to trigger conformational changes in the F protein that lead to membrane fusion. We have demonstrated that single-chain antibodies (scFvs) against carcinoembryonic antigen (CEA) and CD38 (a myeloma cell marker) could be displayed at the C terminus of hybrid H proteins where they triggered F protein–mediated fusion upon binding via the antibody to the targeted receptor[16,17]. However, fusion was also triggered via the natural measles receptors, CD46 and SLAM, which are widely expressed on normal tissues.

We sought to improve target specificity by introducing mutations in the H protein that would block its interactions with CD46 and SLAM. It is known that amino acids 451 and 481 in the H protein play an important role in the interaction with CD46[18]. In addition, by alanine scanning mutagenesis, we recently identified mutations at positions 529 and 533 that ablate fusion through SLAM[19]. We fused an anti-CD38 scFv to the C terminus of the H protein and mutated residues involved in binding to CD46 (451,481) and SLAM (529, 533) (Fig. 1a). Receptor-specific fusion support by the chimeric H protein expression plasmids was determined after F protein–plasmid cotransfection in cells expressing either CD46, SLAM or CD38 (Fig. 1b). Syncytial cytopathic effect was scored by counting syncytia. Paired mutations at positions 451 and 529, or 481 and 533, supported fusion via the targeted CD38 receptor but not via CD46 or SLAM. These data proved conclusively that antibody-targeted cell fusion can be achieved. However, the fusion support activity of the fully retargeted H chimeras on Chinese hamster ovary (CHO)-CD38 cells was considerably lower than that of the original nonablated chimeric protein, H-CD38.

To address the issue of suboptimal fusion support by fully retargeted chimeric H glycoproteins, we focused on residue 481, as amino acid substitutions at this position can have a strong effect on fusion triggering activity[20]. We therefore generated additional H protein chimeras mutated as before at residue 533 (R533A) but with different substitutions at position 481 (Y481M, Y481Q, Y481A) in place of Y481N. Interestingly, all of the new 481-substituted H protein chimeras retained the fully retargeted phenotype but showed higher fusion support activity than the original Y481N mutant on CHO-CD38 cells. In particular, the CD38-targeted chimera carrying mutation Y481A in addition to R533A (H_{AA}-CD38) was slightly more fusogenic on CHO-CD38 cells than was the nonablated chimera H-CD38 (Fig. 1b,c).

Because fusion support activity has been reported to depend on efficient transport and cell surface expression of the H protein[21], we determined total quantities of several chimeric H proteins both in whole cell lysates of transfected cells and at the cell surface (Fig. 1d,e). In CHO-CD38 cells, total cellular H protein expression from the

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