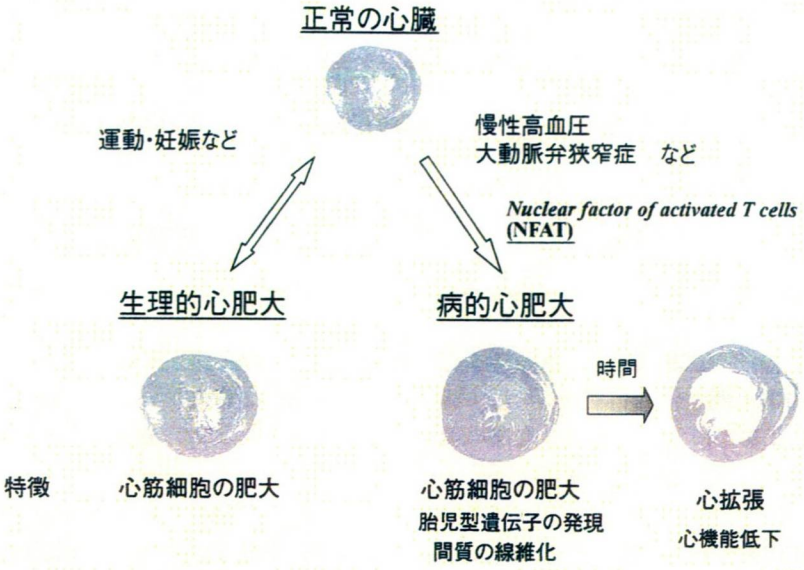

図1　心臓の形態変化（心肥大）と心不全との関係

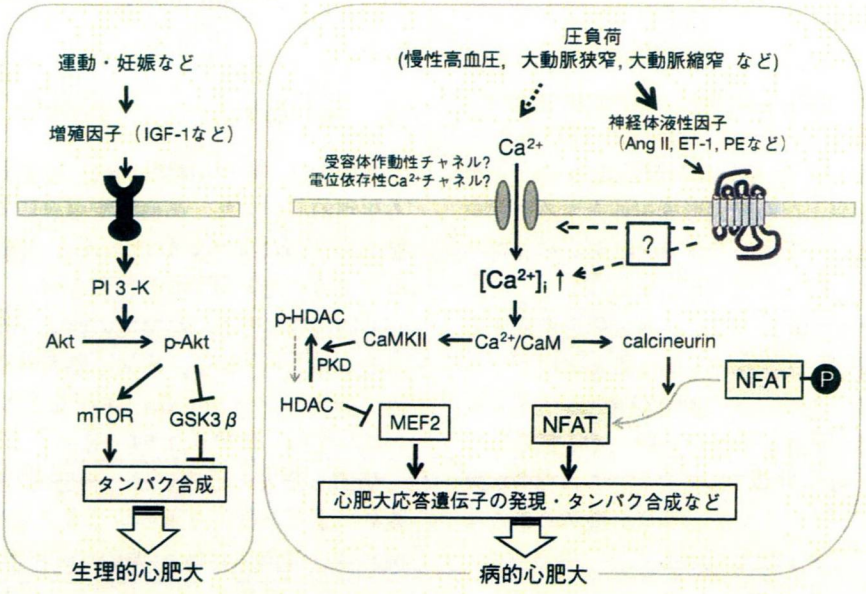

図2　生理的心肥大と病的心肥大の発症メカニズムの違い

では活性化されず，病的心肥大でのみ活性化されることから，心不全の初期誘導因子として一躍脚光を浴びるようになった．その後，NFATと並行して心肥大を誘導する転写因子 myocyte-enhancer factor（MEF）の活性を負に制御するヒストン脱アセチル化酵素（HDAC）がCaMKIIによって不活性化されることも明らかとなり(2, 3)，病的心肥大における Ca^{2+} シグナリングの重要性が確立されてきた．しかしながら，NFATやHDACの活性化に結びつく Ca^{2+} 供給源については未だに明らかにされていない．そこで我々は，心筋細胞の肥大形成に関わる Ca^{2+} シグナリングを評価する実験系を構築した（図3）．Ca^{2+} シグナリングは，一般的に，①刺激による細胞内 Ca^{2+} 濃度上昇（amplitude）が大きくなるか，②間歇的な Ca^{2+} 振動の頻度（frequency）が増加することで活性化されると考えられている．CaMKIIが自身の活性化のために大きなamplitudeを必要とするのに対し，NFATはamplitudeが小さくても Ca^{2+} 振動の頻度増加を自身の活性とし

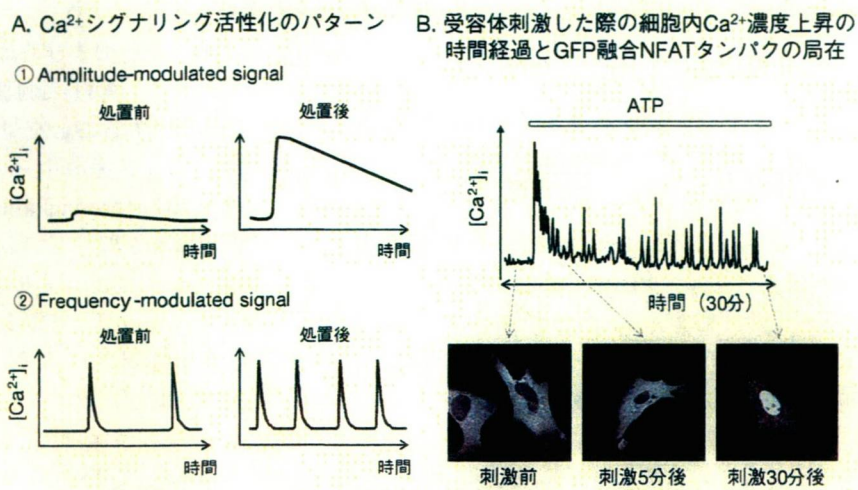

図3 心筋細胞の受容体刺激により活性化される Ca^{2+}シグナリングの解析方法

て検出することができる．我々は，アゴニスト刺激により持続的に生じる間歇的な Ca^{2+}濃度上昇（Ca^{2+}振動）とNFAT活性化を測定することで，心肥大形成に関わる Ca^{2+}シグナリング活性化の機序解析を行った．

3. 心筋細胞のTRPCチャネル

心臓の興奮収縮活動は，電位依存性L型 Ca^{2+}チャネルやリアノジン受容体チャネルを通過する Ca^{2+}によって引き起こされる．しかし，これらの Ca^{2+}透過チャネルが心肥大シグナルの活性化にも貢献しているとは考えにくい．そこで，有力な候補として最近注目を集めているのがtransient receptor potential（TRP）チャネルである(4)．このうち，TRPCファミリーは，イノシトール代謝回転と連関した受容体を刺激することによって活性化される「受容体作動性カチオンチャネル（RACC）」の分子実体であると考えられている．動物のTRPCチャネルは7種類（TRPC1-TRPC7）存在している．TRPC3，TRPC6，TRPC7はジアシルグリセロール（DAG）によって直接活性化されるというユニークな特徴をもつ．特に，TRPC6チャネルは機械的伸展刺激によりDAGによる活性化が増強されることが最近明らかにされている（井上らの頁参照）．ラットの心筋細胞にはTRPC2以外の全てのチャネルが発現しているものの，各々のチャネルの生理的役割についてはまだ明らかではない．

4. 心肥大を誘導するDAG感受性TRPCチャネル

非興奮性細胞におけるNFAT活性化や生理機能の発現は，主にイノシトール-1，4，5-三リン酸（IP_3）受容体を介した Ca^{2+}放出によって制御されることが良く知られている．しかし，心室筋細胞における IP_3受容体の発現量がリアノジン受容体の発現量の約1/100程度であることや(5)，実際の細胞の興奮によって生じる周期的な Ca^{2+}スパイクはリアノジン受容体を介した Ca^{2+}放出（Ca^{2+}-induced Ca^{2+} release：CICR）によるものであることから，心筋の IP_3受容体が非興奮性細胞と同じような役割を担うとは考えにくい．我々は，ホスホリパーゼ-C（PLC）活性化により IP_3とともに生成されるDAGがAng II刺激によるNFAT活性化に重要であることを見出した(6)．このメカニズムには，TRPC3/TRPC6を介した陽イオン流入による膜電位のシフト（脱分極）とそれに伴う心筋細胞の自動発火頻度の増加（すなわち電位依存性 Ca^{2+}チャネルの活性化を介した Ca^{2+}流入量の増加）が関与していた．さらに，心筋細胞にTRPC3またはTRPC6に対するsiRNAを処置することで，Ang II刺激により誘発される肥大応答がほぼ完全に抑制された．

TRPCチャネルの下流で働く電位依存性 Ca^{2+}チャネルの実体については，まだ明確にできていない．しかし最近，T型 Ca^{2+}チャネルの遺伝子欠損マウスが圧負荷で誘発される心肥大を抑制することが報告された(7)．我々も，Ang II刺激で誘発される持続的な Ca^{2+}スパイクとNFATの活性化がL型 Ca^{2+}チャネル阻害薬（ニトレンジピン）のみならずT型 Ca^{2+}チャネル阻害薬（ミベフラジル）でも抑制されることを最近見出している．ニトレンジピンはT型 Ca^{2+}チャネルも抑制しうることから(8)，ラット新生児の心筋細胞においてもT型 Ca^{2+}チャネルがNFAT活性化や心肥大

を誘導する Ca^{2+} 流入経路として働いているのかもしれない．

5. TRPC チャネル発現増加と心不全

圧負荷や虚血によって生じるイオンチャネルの量的・質的変化（リモデリング）もまた不整脈や心機能低下を引き起こす原因になると考えられている．TRPC チャネルもまた，肥大心や不全心で発現量が増加することが明らかにされている．例えば，Kuwahara らは，マウスへの圧負荷処置や心不全患者で TRPC6 の発現が増加していることを見出している(9)．さらに，TRPC6 の発現増加と肥大マーカー分子（ANP）の発現上昇とが相関することから，TRPC6 が単に心肥大を仲介しているだけでなく，心疾患時に見られた発現上昇がポジティブフィードバック的に働いている可能性もあると指摘している．Bush らは，ラットへの圧負荷処置で TRPC3 の発現が増加すること，また β アドレナリン受容体を刺激しても TRPC3 の発現が増加することを示した(10)．さらに，恒常的活性型のカルシニューリンを発現させたマウスでも TRPC3 の発現が増加していていることを示した．一方，ヒトの心不全末期の患者では，Kuwahara らが報告した TRPC6 ではなく TRPC5 の発現上昇がみられたと報告している．Ohba らはラット腹部大動脈の狭窄による圧負荷をかけた際に，TRPC1 の発現が増加することを示している(11)．このように，動物種や実験モデルによって発現誘導される TRPC サブタイプに違いはあるものの，TRPC チャネルを介したシグナリングが心肥大の発症・進展に関与するという点では一致した見解が得られている．

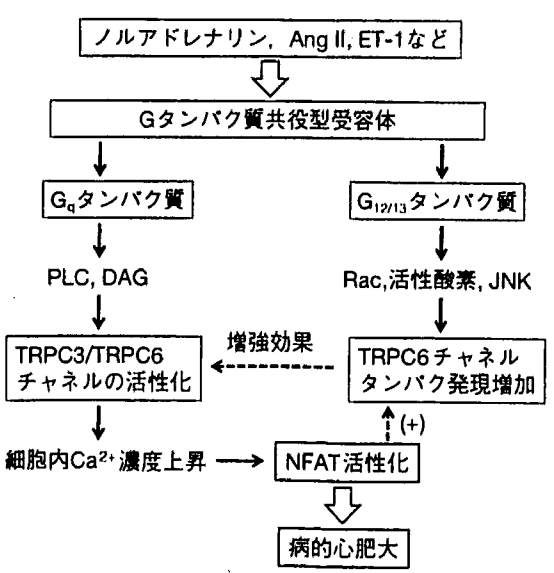

図4　G タンパク質を介した心肥大応答における TRPC チャネルの役割（ラット新生児心筋細胞の場合）

我々は，ラット心筋細胞および心線維芽細胞に Ang II 刺激やエンドセリン（ET）-1 刺激を行うことで TRPC6 の発現量が増加することを見出した(12)．このメカニズムには，G_{12} ファミリー（$G_{12/13}$）タンパク質を介した c-Jun NH_2-terminal kinase（JNK）および AP-1 転写活性の増加が関与していた．心筋細胞の $G_{12/13}$ タンパク質の α サブユニット（$G\alpha_{12/13}$）の機能を阻害しておくと，Ang II や ET-1 刺激により誘発される心肥大応答が抑制される(13, 14)．従って，Ang II や ET-1 刺激は，G_q タンパク質を介して TRPC3/TRPC6 チャネルを活性化するとともに，$G_{12/13}$ タンパク質を介して TRPC6 の発現量を増加させ，協調的に Ca^{2+}-NFAT シグナリングを活性化している可能性が考えられる（図4）．

6. 受容体 -TRPC チャネルタンパクの複合体形成と心肥大

G_q タンパク質と共役しうる全ての受容体が心肥大を誘発するわけではない．例えば，ATP やカルバコール刺激では，IP_3 が産生され，extracellular signal-regulated kinase（ERK）が活性化されるにも関わらず，心肥大応答は引き起こされない(15)．心筋細胞に ATP 刺激を行うと，刺激直後から basal Ca^{2+} 濃度の上昇を伴った強い Ca^{2+} 振動が引き起こされ，30 分後には消失した．これに比べ，Ang II 刺激や ET-1 刺激では，刺激した 5-10 分後から強い Ca^{2+} 振動が起こりはじめ，その Ca^{2+} 振動は 30 分から 1 時間後も持続していた．すなわち，ATP 刺激による Ca^{2+} 応答は Ang II や ET-1 刺激のそれと比べて持続しないことがわかった．そこで免疫沈降を行ったところ，AT1 受容体，ET_A 受容体は TRPC6 と共沈するのに対し，$P2Y_2$ 受容体は TRPC6 と共沈しないことが明らかとなった．これらの結果は，G_q タンパク質共役型受容体と TRPC3/TRPC6 チャネルとの複合体形成が，G_q タンパク質を介した心肥大応答に重要であることを示唆している．

7. TRPC チャネルを標的とした心不全治療の可能性

心筋に TRPC チャネルを過剰発現させると心肥大が誘発されることは示されているものの，TRPC チャネルの機能阻害が実際に病的心肥大を抑制するかどうかについてはまだ報告されていない．ただ，細胞レベルでは，2-APB，SK&F96365，BTP2 などの TRPC チャネル阻害作用をもつ化合物が心肥大を軽減させるこ

とがすでに報告されている(10, 16). 電位非依存性のTRPCチャネルは, いわゆる電位依存性L型Ca^{2+}チャネルとは役割が異なるため, 心臓の興奮収縮活動に直接関わってこない. すなわち, TRPCチャネル選択的阻害薬は心機能を低下させずに心肥大を抑制することが期待できる. しかしその一方で, TRPCチャネルが心臓以外の多くの組織に発現していることや, 細胞の恒常性維持に関わっていることも明らかにされており, 普通のノックアウトマウスでの解析は困難が予想される.

そこで我々は, 京都大学・森研究室との共同研究により, in vivoの心肥大に対するTRPC3選択的阻害薬(Ethyl-1-(4-(2, 3, 3-trichloroacrylamide) phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate (Pyr3))の効果を調べた. Pyr3は, ラット心筋細胞において, Ang II刺激や機械的伸展刺激によるNFAT活性化や心肥大応答を濃度依存的に抑制した. そこで, 血圧・心拍数に影響を与えない用量のPyr3をマウスに投与したところ, vehicle投与マウスと比べて, 大動脈狭窄による圧負荷で誘発される心肥大(心重量の増大と心筋細胞面積の増加)や心不全マーカー遺伝子の発現増加が有意に抑制されていた(17). また, 6週間の圧負荷によって生じる心収縮機能の低下が, Pyr3投与によって有意に改善されることも明らかとなった. この研究は, TRPCチャネルを阻害する化合物が治療薬になりうることを個体レベルで証明した最初の知見である.

Pyr3以外だけでなく, ホスホジエステラーゼ(PDE)阻害薬によるDAG感受性TRPCチャネルの抑制効果にも着目している. DAG感受性TRPCチャネルタンパク質は, プロテインキナーゼGやプロテインキナーゼCによってリン酸化を受けることでチャネル活性が抑制される(18). すなわち, ホスホジエステラーゼ(PDE)阻害薬や心房性ナトリウム利尿ペプチドなどは, リン酸化依存的にTRPC3やTRPC6チャネル活性を抑制すると考えられる. 実際, 我々は, PDE-V阻害薬を心筋細胞に処置するとTRPC6のリン酸化が増加することを見出している. この結果は, TRPC6チャネルの抑制が心肥大に効果を示す可能性を示している. 最近, シルデナフィル(バイアグラ®)の心肥大抑制効果について個体レベルでの報告がなされ, PDE-V阻害薬の臨床応用への期待が高まってきている(19).

さらに我々は, $G\alpha_{12/13}$タンパク質のαサブユニットが圧負荷による心臓の線維化(コラーゲンの蓄積)誘導に関わることを最近報告した(20). 線維化は心臓の弛緩機能を悪化させることから, 拡張不全の原因として注目されている. 線維化とTRPCチャネルとの関連については全くわかっていない. 今後, 心臓の$G\alpha_{12/13}$タンパク質を介したTRPCチャネル発現増加の意義を明らかにしていくことで新たな展開が見つかるかもしれない.

8. おわりに

NFAT活性を指標にして心肥大形成に関わるCa^{2+}シグナル解析を行った結果, DAG感受性TRPCチャネル(TRPC3/TRPC6)という新しい分子を見出すことができた. TRPCチャネルと心肥大との関連が示されつつある中, 我々はTRPCチャネルが心不全治療薬の新たな標的分子となりうる可能性を個体レベルで証明するところまでようやく辿り着いた. 心臓のTRPチャネル研究はまだ始まったばかりであり, 今後は, TRPCチャネルの生理的役割(特に興奮収縮活動における役割)についても明らかにしていかなければならない.

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Yoji Sato
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◎再生医療や細胞治療のツールとして使用することを目的に，細胞または組織を加工した製品を“細胞・組織加工医薬品/医療機器”(細胞組織製品)という．わが国では先ごろ，初の細胞組織製品として重症熱傷治療用培養皮膚製品が薬事法上の承認を受け，また世界に先がけてわが国で開発されたヒト人工多能性幹細胞(iPS 細胞)も再生医療・細胞治療への応用に熱い期待が集まっており，国内外で細胞組織製品の熾烈な開発競争が繰り広げられている．しかし，細胞組織製品の本格的な実用化・産業化に至るためには，その安全性評価方法の理解・確立が必須である．本稿ではヒト細胞組織製品の安全性の評価・確保について，最近の厚生労働省の関連指針を軸に概説する．

Key word：細胞・組織加工医薬品，細胞・組織加工医療機器，確認申請，品質管理，ヒト幹細胞臨床研究指針

再生医療(患者の組織の再生・修繕または置換を目的とする治療)および細胞治療(加工した細胞を患者の生理的機能の修復・修正または調節を目的として使用する治療)に使用するために，細胞あるいは組織を加工した製品を“細胞・組織加工医薬品”または“細胞・組織加工医療機器”という．本稿では，これらをまとめて“細胞組織製品”とよぶ．わが国では平成 19 年(2007)に重症熱傷治療用培養皮膚製品が，国内初の細胞組織製品として薬事法上の承認を受けた．近い将来にはさらに多くの細胞組織製品が実用化されると見込まれている．

しかし，本格的な細胞組織製品の実用化・産業化を達成するためには，検討すべき課題はまだ多い．なかでも重要な課題は，細胞組織製品の安全性をどう確保したらよいのか，ということである．本稿ではヒト細胞組織製品の安全性の評価・確保のポイントについて，厚生労働省の関連指針を軸に解説する．

ヒト細胞組織製品の品質・安全性ガイドライン

わが国でのヒト細胞組織製品の開発の多くは医師の裁量による臨床研究(あくまで研究が主目的)として行われてきたが，商品化をめざした活動としてのヒト細胞組織製品の開発も近年盛んに行われている．逆に，ヒト細胞組織製品の品質・安全性ガイドラインに関しては商品としてのヒト細胞組織製品に対するものが先行しており，大学などの研究機関での臨床研究を対象にした『ヒト幹細胞を用いる臨床研究に関する指針』(以下“ヒト幹細胞臨床研究指針”，厚生労働省，平成 18 年)においても，臨床研究で用いられるヒト細胞組織製品の品質・安全性に関しては商品としてのヒト細胞組織製品に対する品質・安全性ガイドラインを準用する形となっている．

商品としてのヒト細胞組織製品の製造・販売には薬事法に基づく承認が必要となる．使用目的による分類からすれば，“医薬品”あるいは“医療機器”のいずれかに分類されることになるが，どちらに分類されるかによって安全性確保の基準に自動的に差が出るわけではない．いずれにせよ細胞

組織製品は取扱い方法の分類からすれば“特定生物由来製品”とみなされる可能性が高いと想定され，そうなれば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じる必要がある．とくに細胞組織製品は臨床使用経験が少ないために知見の蓄積も乏しく，リスク予測が難しいため，わが国では治験でヒトに使用する前に製品の安全性と品質の“確認”を厚生労働大臣に求めなければならない(医薬発 906 号通知，平成 11 年)．この手続きは“確認申請”とよばれている．つまり開発者は新規細胞組織製品について治験実施に適うだけの安全性と品質をあらかじめ示さなければならない．

現在わが国には，細胞組織製品の品質・安全性確保についての主幹となるガイドラインとして，①『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』(以下，“基本的考え方”；医薬発 1314 号通知別添 1，平成 12 年)，②『ヒト(自己)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』(以下“ヒト自己指針”，薬食発第 0208003 号，平成 20 年)，および③『ヒト(同種)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』(以下“ヒト同種指針”，薬食発第 0912007 号，平成 20 年)が出されている．②および③は“医薬発 1314 号通知別添 2”(平成 12 年)の改訂版として位置づけされている．なお，これらの指針は輸血用血液製剤，移植医療としての骨髄移植，臍帯血移植，ヒト皮膚や骨などを直接利用する医療行為を対象としていない点で欧米の指針とは異なるので，注意を要する．

上記“基本的考え方”は，細胞・組織を取り扱う際の基本的要件を示すとともに，細胞組織製品の品質・安全性，ならびに細胞・組織の取扱いに関する科学的および倫理的妥当性を確保することを目的とし，細胞組織製品の承認後のみならず，治験時においても適用される．“基本的考え方”のなかで細胞組織製品の安全性に関してもっとも強調されているのは，細菌，真菌，ウイルスなどの汚染の危険性への対策である．

“ヒト自己指針”および“ヒト同種指針”はそれぞれ，ヒト(自己)由来およびヒト(同種)由来の細胞組織製品の品質および安全性の確保のための基本的な技術要件についてまとめたもので，製造販売承認申請時のみならず，治験開始前の確認申請で求められる資料について記されている．ヒト(自己)由来製品とヒト(同種)由来製品との間の根本的な差異は，自己由来の細胞・組織を用いる場合にはその細胞・組織を介する感染症伝播のリスクおよび免疫学的な問題が理論上ないことである．しかし，自己由来であっても製造工程におけるクロスコンタミネーションの問題や製造従事者，医療従事者などの安全上の問題は，同種由来の場合と同様に存在する．また，培養工程においてウイルスの増殖するリスクを考慮することが必要な場合もある．さらに自己由来の場合，個別製品の製造となるので，それらの品質のばらつきを最小限にとどめる工夫が必要な反面，製品レベルでの各種試験の実施に試験検体の量的制約がある．それらに留意した合理的な品質確保の方策(製造工程のより厳密な恒常性維持・管理など)を採用する必要がある．なお，自己由来であっても遺伝子改変細胞の場合には相応の留意が必要である．

“ヒト自己指針”および“ヒト同種指針”の安全性確保策

“ヒト自己指針”および“ヒト同種指針”のコンセプトとして細胞組織製品の品質・安全性確保の方針は，原材料となる細胞・組織の適格性をはじめ，原材料の品質管理・培養方法を含めた製造方法の恒常性の確保，工程評価を含めた妥当性の検証，中間製品の品質管理，最終製品の規格設定および品質管理によって担保することになっている．しかし，細胞組織製品の原料となる細胞・組織は複雑な構造と，“生きている”という動的な特性をもつため，従来の医薬品などに適用されてきた品質管理の必要事項がかならずしも適用できるとは限らない．したがって，細胞組織製品の安全性に関しては特別な配慮が必要となってくる．

1. 感染因子

原材料から最終製品までの製造工程を通じ，安全性に関してもっとも強調されているのは，感染因子の汚染の危険性への対策である．細胞組織製品では，従来の医薬品のような高度な精製やウイルスの不活化・除去を製造時に行うことは非常に

難しい．このため，原材料および製造工程での感染因子の混入をいかに防ぐかが課題となる．

ヒト同種由来製品の場合はドナーからの感染リスクの評価が最重要であり，各種感染症に対する試験や既往歴に関する問診・調査を入念に行う必要がある．ウイルス感染の初期には検査で検出不可能なウィンドウ期があることから，適切な時期に再検査することが推奨される．また，製造工程でのウイルス増殖リスクがある場合にも製造工程中の適切な段階で再検査が必要となる場合がある．ヒト自己由来製品の場合，製造時のクロスコンタミネーションや製造従事者らへの伝播の可能性について，とくにB型肝炎ウイルス，C型肝炎ウイルス，ヒト免疫不全ウイルスおよび成人T細胞白血病ウイルス感染には留意する．

2. 最終製品の品質管理

最終製品の品質管理では必要で適切な規格および試験方法を設定し，その根拠を明らかにする必要がある．“ヒト自己指針”および“ヒト同種指針”では一般的な品質管理項目および試験として，①細胞数・生存率，②確認試験，③細胞の純度試験，④細胞由来の目的外生理活性物質に関する試験，⑤製造工程由来不純物試験，⑥無菌試験およびマイコプラズマ否定試験，⑦エンドトキシン試験，⑧ウイルス試験，⑨効能試験，⑩力価試験，⑪力学的適合性試験が例示されている．また，最終製品および重要な中間製品については細胞の生存率・力価などに基づく安定性試験を実施し，妥当な貯法や有効期限を設定することが求められる．

3. 非臨床安全性試験

製品の特性および適用法から評価が必要と考えられる安全性関連事項について，技術的に可能であれば，科学的合理性のある範囲で，適切な動物を用いた試験または *in vitro* での試験を実施する．なお，非細胞・組織成分および製造工程由来の不純物などについては可能なかぎり，動物を用いた試験ではなく理化学的分析法による評価が求められている．ヒト由来の試験用検体は貴重であり，また，ヒト由来の製品を実験動物などで試験してかならずしも意義ある結果が得られるとは限らない．逆に，動物由来の製品モデルを作成し，適切な実験動物に適用する試験系により試験を行うことで，より有用な知見が得られると考えられるような場合には，むしろ，そのような試験系を用いることに科学的合理性がある場合がある．場合によっては細胞を用いる試験系も考慮し，このようなアプローチにより試験を行った際には，その試験系の妥当性について明らかにする必要がある．

ヒト細胞組織製品の安全性において感染症伝播とともに懸念されることとして，製品の造腫瘍性があげられるが，最終製品ごとの造腫瘍性試験を一律に課すのは合理的ではない．たとえば，自己由来細胞でも文献上の知見や類似品の使用経験などから造腫瘍性が考えにくいものについては，規格の培養期間を超えて培養した細胞について目的外の形質転換を起こしていないことを明らかにすることでよい場合もあると考えられる．一方，多分化能を有する幹細胞は体細胞と比較して腫瘍化の可能性が高いとも思われるので，それに配慮した試験を計画する必要があると考えられる．

4. 効力または性能を裏づける試験

現在の科学で技術的に可能かつ科学的合理性のある範囲で，実験動物または細胞などを用い，適切に設計された試験により，細胞・組織加工医薬品などの機能発現，作用持続性および医薬品・医療機器として期待される効果を検討することが求められる．ただし確認申請段階では，当該製品の効力または性能による治療が他の治療法と比較したときはるかに優れて期待できることが国内外の文献または知見などにより合理的に明らかにされれば，かならずしも詳細な実験的検討は必要とされない．

5. 体内動態

製品を構成する細胞・組織および導入遺伝子の発現産物について，技術的に可能かつ科学的合理性がある範囲で，実験動物での吸収および分布などの体内動態に関する試験などにより，患者などに適用された製品中の細胞・組織の生存期間，効果持続期間を推測し，目的とする効果が十分得られることを明らかにする．当該細胞・組織が特定の部位(組織など)に到達して作用する場合には，その局在性を明らかにする．

6. 臨床試験

確認申請の段階における安全性については，臨

床上の有用性を勘案して評価されるものである．細胞組織製品について予定されている国内の治験計画について，①対象疾患，②対象とする被験者および被験者から除外すべき患者の考え方，③細胞組織製品の適用を含め，被験者に対して行われる治療内容，④既存の治療法との比較を踏まえた臨床試験実施の妥当性，⑤現在得られている情報から想定されるリスクおよびベネフィットを含め，被験者への説明事項の案，を踏まえて評価する．

なお，臨床試験は適切な試験デザインおよびエンドポイントを設定して実施する必要があり，目的とする細胞・組織の由来，対象疾患および適用方法などを踏まえて適切に計画する．

おわりに

安全な再生医療・細胞治療に国民が広くアクセスできるためには，これらの治療法のメインツールであるヒト細胞組織製品が治験を通じて薬事法上の承認を得ることが望ましい．大学などで実施される臨床研究は医師法下の医療行為の一環であり，薬事法の規制対象ではなく，そこで用いられるヒト細胞組織製品の品質・安全性には実施施設によるばらつきが出てしまうことがこれまで懸念されていた．前述の“ヒト幹細胞臨床研究指針”では，研究に用いるヒト細胞組織製品について商品化をめざした製品の治験に準じる品質管理を求めており，今後は臨床研究で用いられる製品でも一定の品質・安全性が確保されていくと予想される．

ただし現状では，ヒト細胞組織製品の臨床研究のデータが医薬品などの申請資料として利用できずに改めてデータを取得し直すケースがまだ多く，細胞組織製品の実用化のうえでの大きな時間的・経済的な障害として問題となっている．この問題を解決し，臨床研究の段階から将来の医薬品としての開発を見越したうえでのデータの蓄積を行えるような，シームレスな体制を確立することが今後の課題となると考えられる．

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The Journal of Immunology

Importance of Neonatal FcR in Regulating the Serum Half-Life of Therapeutic Proteins Containing the Fc Domain of Human IgG1: A Comparative Study of the Affinity of Monoclonal Antibodies and Fc-Fusion Proteins to Human Neonatal FcR

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Importance of Neonatal FcR in Regulating the Serum Half-Life of Therapeutic Proteins Containing the Fc Domain of Human IgG1: A Comparative Study of the Affinity of Monoclonal Antibodies and Fc-Fusion Proteins to Human Neonatal FcR

Takuo Suzuki,* Akiko Ishii-Watabe,* Minoru Tada,* Tetsu Kobayashi,* Toshie Kanayasu-Toyoda,* Toru Kawanishi,† and Teruhide Yamaguchi*

The neonatal FcR (FcRn) binds to the Fc domain of IgG at acidic pH in the endosome and protects IgG from degradation, thereby contributing to the long serum half-life of IgG. To date, more than 20 mAb products and 5 Fc-fusion protein products have received marketing authorization approval in the United States, the European Union, or Japan. Many of these therapeutic proteins have the Fc domain of human IgG1; however, the serum half-lives differ in each protein. To elucidate the role of FcRn in the pharmacokinetics of Fc domain-containing therapeutic proteins, we evaluated the affinity of the clinically used human, humanized, chimeric, or mouse mAbs and Fc-fusion proteins to recombinant human FcRn by surface plasmon resonance analysis. The affinities of these therapeutic proteins to FcRn were found to be closely correlated with the serum half-lives reported from clinical studies, suggesting the important role of FcRn in regulating their serum half-lives. The relatively short serum half-life of Fc-fusion proteins was thought to arise from the low affinity to FcRn. The existence of some mAbs having high affinity to FcRn and a short serum half-life, however, suggested the involvement of other critical factor(s) in determining the serum half-life of such Abs. We further investigated the reason for the relatively low affinity of Fc-fusion proteins to FcRn and suggested the possibility that the receptor domain of Fc-fusion protein influences the structural environment of the FcRn binding region but not of the FcγRI binding region of the Fc domain. *The Journal of Immunology*, **2010, 184: 1968–1976.**

In healthy humans, IgG1 exhibits a long serum half-life of ~21 d (1). This prolonged half-life of IgG can be explained by the interaction with neonatal FcR (FcRn). FcRn is a heterodimer of the MHC class I-like H chain and the β_2-microglobulin (β_2m) L chain (2). Although this receptor was originally studied as a transporter of IgG from mother to fetus, subsequent studies have shown that this receptor also plays a critical role in regulating IgG homeostasis (3, 4). FcRn binds to the Fc domain of IgG at pH 6.0–6.5 but not, or weakly, at pH 7.0–7.5 (5). Therefore, FcRn protects IgG from degradation by binding to IgG in endosome and releases IgG into plasma (6). As indicated by previous studies in which amino acid substitutions in the Fc domain of IgG for modifying the affinity to FcRn can alter the serum half-life of the IgG, the affinity to FcRn is thought to play a critical role in determining the serum half-life of IgG (7–12).

Recently, therapeutic use of mAb products has become more important for various diseases, including cancer as well as autoimmune and infectious diseases (6, 13, 14). In addition to the mAbs, the Fc-fusion proteins (e.g., etanercept, alefacept, and abatacept) have been developed and have received considerable attention. These Fc-fusion proteins consist of an extracellular domain of membrane receptor linked to the Fc portion of human IgG1. They work like Abs by binding to ligands for the receptors. The receptor portions of etanercept and alefacept are, respectively, the extracellular ligand-binding portion of the human 75-kDa TNFR and the extracellular CD2-binding portion of the human leukocyte function Ag 3. Abatacept consists of the extracellular domain of human CTLA-4 linked to the modified Fc portion of human IgG1.

Most of the mAb products and Fc-fusion protein products have the Fc domain of human IgG1 (6, 14). Accumulating evidence regarding their clinical use has revealed that their serum half-lives are variable, ranging from 4 to 23 d, regardless of the presence of the Fc domain of human IgG1 (6). Although many factors such as m.w., posttranslational modifications including glycosylation, electrical properties, interactions with FcRs or target molecules, and features of the target molecules may influence their serum half-life, the reasons for the variability of half-life have not been elucidated. Among such factors, FcRn might play a critical role in regulating half-life; however, comparative studies between the affinities of these therapeutic proteins to FcRn and their half-lives in humans have not been reported. Therefore, although some Fc domain-containing therapeutic proteins exhibit shorter half-lives in humans, it remains unclear whether the shorter half-lives are due to the lower affinity to FcRn or other factors.

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Abbreviations used in this paper: β_2m, β_2-microglobulin; FcRn, neonatal FcR; HER2, human epidermal growth factor receptor 2; ND, not detected; R^2, coefficient of determination; SPR, surface plasmon resonance.

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In this study, we examined the affinity of clinically used mAbs and Fc-fusion proteins to recombinant human FcRn by surface plasmon resonance (SPR) analysis. The analytes used were human Ab (adalimumab), humanized Abs (daclizumab, omalizumab, palivizumab, and trastuzumab), chimeric Abs (infliximab and rituximab), mouse Ab (muromonab-CD3), and Fc-fusion proteins (etanercept, alefacept, and abatacept). We found that the affinities of the therapeutic proteins tested to FcRn were closely correlated with their serum half-lives, with a few exceptions. Because Fc-fusion proteins, which have relatively short half-lives (4–13 d), were shown to have lower affinity to FcRn than mAbs, we further investigated the reason for this difference by examining the affinity of the proteins to FcγRI or the affinity of papain-digested proteins to FcRn in SPR analyses. Our results suggested the possibility that the receptor portions of Fc-fusion proteins make a difference in the higher-order structure of the FcRn-binding region of Fc (i.e., CH2-CH3 interface) or interfere with binding between the Fc domain and FcRn by steric hindrance.

Materials and Methods

Therapeutic proteins and reagents

Abatacept (Bristol-Myers Squibb, Princeton, NJ), adalimumab (Abbott, Baar, Switzerland), alefacept (Biogen Idec, Cambridge, MA), daclizumab (Hoffmann-La Roshe, Nutley, NJ), etanercept (Takeda Pharmaceutical, Osaka, Japan), infliximab (Tanabe Phamaceutical, Osaka, Japan), muromonab-CD-3 (Jansen Pharmaceutical, Tokyo, Japan), omalizumab (Novartis Pharma Schweiz, Bern, Switzerland), palivizumab (Abbott Japan, Osaka, Japan), rituximab (Zenyaku Kogyo, Tokyo, Japan), and trastuzumab (Chugai Pharmaceutical, Tokyo, Japan) were purchased via reagent distributors. Recombinant human TNF-α was purchased from Wako (Osaka, Japan).

Purification of human FcRn

Stably transfected CHO cells expressing both the soluble portion of the hFcRn H chain (residues 1–267 of mature protein) and β_2m were provided by P. J. Bjorkman (California Institute of Technology, Pasadena, CA). Expression and purification of hFcRn were performed according to the method previously reported by West and Bjorkman (15), with slight modifications. Briefly, the CHO cells expressing soluble hFcRn and β_2m were cultured in α-MEM containing 5% dialyzed FBS, 100 μM methionine sulfoximine, and penicillin/streptomycin. Cell culture supernatant was collected every 2–3 d and was filtered with a 0.45-μm filter, and sodium azide was then added to 0.05%. The harvested supernatant was acidified to pH 5.8 and then applied to a human IgG column. After washing the column with 50 mM Bis-Tris (pH 5.8), hFcRn complexed with β_2m was eluted with 40 mM Bis-Tris/20 mM Tris (pH 8.1). The eluted fractions containing hFcRn were applied to a Uno-Q1 column, and hFcRn was eluted with pH gradient using 40 mM Bis-Tris/20 mM Tris (pH 8.1) and 40 mM Bis-Tris/20 mM Tris (pH 5.8).

SDS-PAGE and Western blotting

Each fraction of protein eluted from the Uno-Q1column was diluted in 1× SDS loading buffer and was separated in 15% polyacrylamide gel (Bio craft, Tokyo, Japan). After the electrophoresis, the gels were stained with Imperial protein stain (Pierce, Rockford, IL). For Western blotting, proteins separated by SDS-PAGE were electroblotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were immunoreacted with rabbit anti-hFcRn H chain peptide (Leu^{135}-Gly^{148}) Ab produced by Medical and Biological Laboratories (Nagoya, Japan) and then with HRP-conjugated secondary Abs (Cell Signaling Technology, Danvers, MA). The bands of hFcRn were detected using ECL Plus Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

SPR analyses

Analysis of affinity between FcRn and Fc domain-containing therapeutic proteins. The purified recombinant hFcRn was diluted with 10 mM sodium acetate (pH 5.0 or 4.5) and was immobilized onto a CM5 biosensor chip (Biacore, Uppsala, Sweden) using an amine coupling kit (Biacore) at relatively low densities (mainly 300–350 resonance units) to avoid mass transport limitation. The reference cell was treated with *N*-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and ethanol amine using an amine coupling kit without injecting the FcRn. Fc domain-containing proteins were diluted with the running buffer (50 mM sodium phosphate/150 mM NaCl [pH 6.0]) and injected at 25°C. The running buffer was allowed to flow at a rate of 20 μl/min. The injections were performed using the KINJECT mode (volume, 40 μl; dissociation time, 150 s). For regeneration, the regeneration buffer (100 mM Tris/200 mM NaCl [pH 8.0]) was injected for 4 min. Kinetic constants were calculated from the sensorgrams using the bivalent analyte model of BIAevaluation software 4.1.

To obtain the consistent results, we would indicate two points. First, it is necessary to set the bulk refractive index to zero to avoid wrong fitting, because the binding is rapidly reached to the near-equilibrium state. Second, it is necessary to set the injection point correctly. For example, if the sensorgrams of infliximab shown in Fig. 2 were analyzed with the injection point shifted to 0.5 s earlier, the values of k_{a1}, k_{d1}, and K_D were 1.95E+05 $M^{-1}s^{-1}$, 0.136 s^{-1}, and 697 nM, respectively. When the injection points of the sensorgrams are unclear, it may be better to use the average values of data resulting from two or more different injection points.

Analysis of affinity between FcγRI and Fc domain-containing therapeutic proteins. Recombinant human FcγRI, which consists of human FcγRI (Gln^{16}-Pro^{288}) and His-tag, was purchased from R&D Systems (Minneapolis, MN). Fc domain-containing proteins were immobilized to a CM5 biosensor chip in 10 mM sodium acetate (pH 5.0) using an amine coupling kit. Kinetic analyses of FcγRI binding were performed according to Ellsworth et al. (16) with some modifications. The running buffer, HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% Surfactant P20 [pH 7.4]) (Biacore), was allowed to flow at 20 μl/min. The injections of FcγRI were performed using the KINJECT mode (volume, 40 μl; dissociation time, 150 s). To regenerate the immobilized proteins, the regeneration buffer (10 mM glycine-HCl [pH 1.8]) was injected for 15 s. Kinetic constants were derived from the sensorgrams using the 1:1 binding model of BIAevaluation software 4.1.

Papain digestion

The papain (Wako) was activated in the buffer (50 mM sodium phosphate/150 mM NaCl [pH 6.0], 1 mM cysteine, 4 mM EDTA, and 1 mg/ml papain) at 37°C for 15 min. Next, 1 mg/ml Ab or Fc-fusion protein was digested with 0.1 mg/ml activated papain in 50 mM sodium phosphate (pH 6.0), 150 mM NaCl, 0.1 mM cysteine, and 4 mM EDTA at 37°C for 24 h.

Results

Purification of soluble human FcRn

FcRn binds to the Fc domain at acidic pH and then releases it at neutral pH. Recombinant soluble hFcRn expressed from CHO cells was purified using a human IgG column by binding at pH 5.8 and releasing at pH 8.1. The fraction purified by the IgG column was electrophoresed at lane 10 of SDS-PAGE gel (Fig. 1*B*). This fraction was then purified using an anion-exchange column with a pH gradient elution. The elution diagram is shown in Fig. 1*A*. Three main peaks were observed. The proteins in these peaks were electrophoresed (Fig. 1*B*) and subjected to Western blot analysis using anti-hFcRn H chain peptide Ab (Fig. 1*C*). Several bands were observed at ~32 kDa in these fractions, and these bands were immunoreactive to anti-hFcRn H chain peptide Ab. These results indicated that the purified FcRn had several isoforms, possibly because of the difference in posttranslational modification, including glycosylation or proteolysis. As shown in Fig. 1*C*, the signals of the higher m.w. bands of hFcRn tend to be weak. There is a possibility that the sugar chain at Asn^{125} of hFcRn interfered with the reactivity of the hFcRn to the anti-hFcRn H chain peptide Ab used. We analyzed the affinity of therapeutic mAbs and Fc fusion proteins to FcRn by SPR using the peak I, II, or III fractions eluted from the anion-exchange column. The K_D values were higher when peak I was used as a ligand in SPR analyses than when peaks II or III were used (data not shown). Because the m.w. of the proteins in peak I was smaller than that in peak II/III and the protein content of peak I varied depending on the lot of the cell culture supernatant, peak I seemed to consist of immature FcRn. The K_D values calculated from the experimental data using peaks II and III were comparable (data not shown). We, therefore, used the main peak (i.e., peak III) in the following experiments.

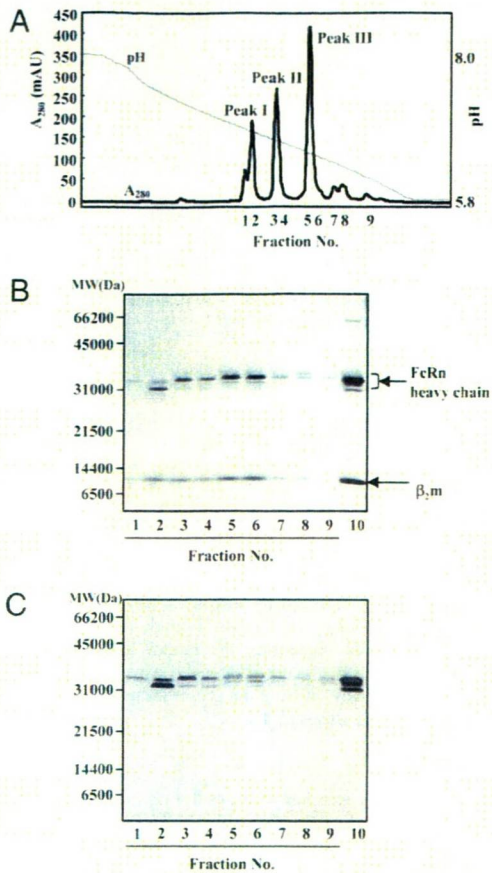
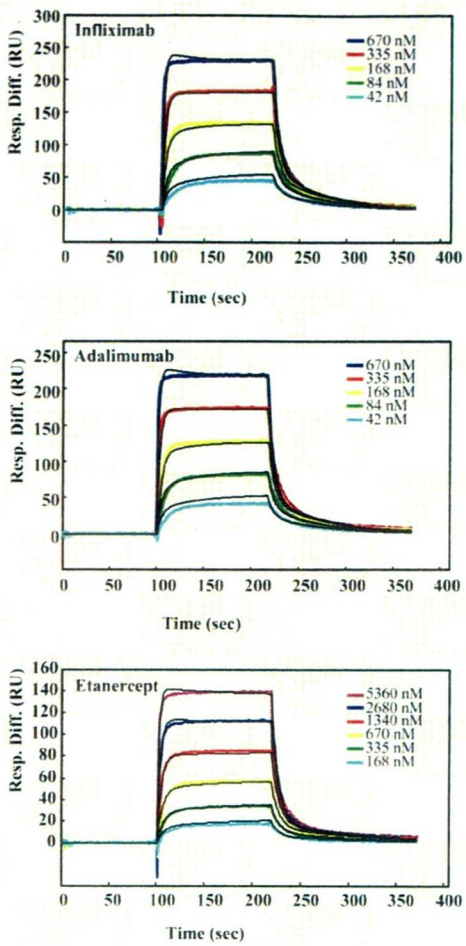


FIGURE 1. Purification and electrophoretic characterization of recombinant human FcRn. *A*, The elution diagram of the anion-exchange chromatography used for the purification of recombinant human FcRn. *B*, SDS-PAGE of the proteins in the fractions indicated in *A*. The protein applied to the anion-exchange column was electrophoresed in *lane 10*. The gel was stained with Imperial protein stain. *C*, Western blot analysis of eluate from the anion-exchange column by anti-hFcRn H chain Ab.

SPR analyses of the affinity between FcRn and Fc domain-containing proteins

Purified FcRn was immobilized onto a CM5 biosensor chip at relatively low densities as described in *Materials and Methods*. Five or six concentrations of Fc domain-containing therapeutic proteins were then injected. Because injection at higher concentrations caused nonspecific binding to flow cells, we analyzed the affinity of therapeutic proteins using sensorgrams obtained at the concentrations at which nonspecific binding was not observed. For example, infliximab was injected at concentrations of 670, 335, 168, 84, and 42 nM, and we analyzed the affinity to FcRn with the bivalent analyte model (Fig. 2). The colored lines were observed sensorgrams, and the black lines were fitting lines generated by the BIAevaluation software. The K_D value (= k_{d1}/k_{a1}) calculated from these sensorgrams was 727 nM. The affinities of adalimumab and etanercept to FcRn were 672 and 3612 nM, respectively (Fig. 2).

The affinities of the 11 kinds of Fc domain-containing proteins to FcRn were measured (Fig. 3). Adalimumab, daclizumab, infliximab, palivizumab, and rituximab were injected at concentrations of 42–670 nM. The concentrations of abatacept, alefacept, and etanercept used were 168–5360 nM, and those of muromonab-CD3, omalizumab, and trastuzumab were 84–1340 nM. Under this condition, the tested therapeutic proteins, except for muromonab-CD3, bound to FcRn. The K_D values measured in our experiments and the serum half-lives in humans reported in the literature are shown in Fig. 3*A*.



	Infliximab	Adalimumab	Etanercept
$k_{a}1$ (1/Ms)	2.09E+05	2.41E+05	3.71E+04
$k_{d}1$ (1/s)	0.152	0.162	0.134
$k_{a}2$ (1/RUs)	4.56E-05	3.98E-05	3.27E-05
$k_{d}2$ (1/s)	9.78E-03	8.80E-03	5.44E-03
Rmax (RU)	3.76E+02	3.47E+02	1.93E+02
Chi2	1.80E+01	1.74E+01	5.37E+00
$K_D=k_{d}1/k_{a}1$	**727 nM**	**672 nM**	**3612 nM**

FIGURE 2. Representative sensorgrams of SPR analyses. Infliximab (*upper panel*) or adalimumab (*middle panel*) was injected at concentrations of 42–670 nM and etanercept (*lower panel*) at concentrations of 168–5360 nM. The colored lines are the observed sensorgrams, and the black lines are fitting lines generated by the bivalent analyte model of BIAevaluation software. The association of KINJECT was started at ~100 s, and the dissociation of KINJECT was at ~220 s. The table describes the kinetic values calculated from the sensorgrams of infliximab, adalimumab, and etanercept.

The K_D values and the average values of the serum half-lives are plotted in Fig. 3*B*. The K_D values were closely correlated to the half-lives (contribution ratio = 0.8675) when the results were analyzed after excluding the data for infliximab, rituximab, and trastuzumab (Fig. 3*C*). Concerning infliximab, rituximab, and trastuzumab, which have relatively short half-lives and comparable affinity to other long half-life Abs to FcRn, other critical factor(s) seemed to be involved in regulating their half-lives (see *Discussion*). Although it was impossible to plot the data for mouse mAb muromonab-CD3,

A

Structure	Nonproprietary name	Binding target	Affinity to FcRn K_D (nM)	Half-life (days) cited from the literature	
Human antibody	Adalimumab	TNFα	672	14.7-19.3	Weisman et al., 2003
Humanized antibody	Daclizumab	CD25	846	20	Vincenti et al., 1998
	Omalizumab	IgE	1237	20	Casale et al., 1997
	Palivizumab	RSV F protein	750	19-27	Subramanian et al., 1998
	Trastuzumab	HER2	773	2.7-10	Tokuda et al., 1999
Chimeric antibody	Infliximab	TNFα	727	9.5	Cornillie et al., 2001
	Rituximab	CD20	508	9.4	Maloney et al., 1997
Mouse antibody	Muromonab-CD3	CD3	ND	0.75	Hooks et al., 1991
Fc-fusion protein	Abatacept	CD80/CD86	2633	13.1	prescribing information
	Alefacept	CD2	2506	11.3	prescribing information
	Etanercept	TNFα	3612	4	Lee et al., 2003

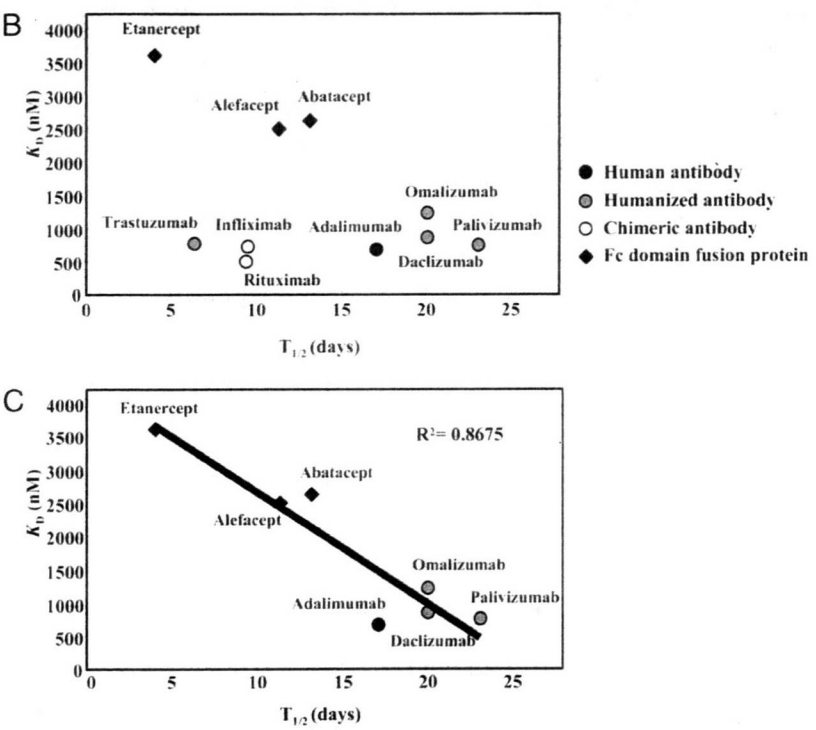


FIGURE 3. K_D values of binding between Fc domain-containing therapeutic proteins and hFcRn and the correlation with their serum half-lives. *A*, The K_D values obtained in our study and the half-lives in humans cited from the literature. The half-life values were obtained from the article reviewed by Lobo et al. (6) [adalimumab (17), daclizumab (18), etanercept (19), infliximab (20), muromonab-CD3 (21), omalizumab (22), palivizumab (23), rituximab (24), and trastuzumab (25)] or from the manufacturer prescribing information. *B*, The graphical presentation of the K_D values and serum half-lives described in *A*. The means of half-lives are plotted on the *x*-axis, and the values of affinity to FcRn are on the *y*-axis. Filled rhombi, Fc domain fusion proteins; closed circle, human Ab; gray circles, humanized Abs; open circle, chimeric Abs. *C*, Regression line of the plots of seven therapeutic proteins. ND, not detected; R^2, coefficient of determination.

which exhibited no significant binding to human FcRn, the half-life of this Ab in humans is the shortest (0.75 d) among the therapeutic proteins examined in this study (21). These results also show the importance of the binding affinity to FcRn in determining the serum half-life. The correlation described above was also observed when other fractions of hFcRn described in Fig. 1 (peaks I and II) were used in SPR analyses (data not shown).

The affinity between FcγRI and Fc domain-containing proteins

Because the affinities of Fc fusion proteins (etanercept, alefacept, and abatacept) to FcRn were lower than those of mAbs, the FcRn-binding region (CH2-CH3 domain interface) of Fc-fusion proteins seems to be structurally different from that of mAbs. We also analyzed the affinity of these proteins to FcγRI to test whether the structural environment around the FcγRI-binding region (hinge proximal region of CH2) is different between Fc-fusion proteins and Abs. Because the regeneration procedure in the SPR assay inactivated FcγRI but not Fc domain-containing therapeutic proteins, therapeutic proteins were immobilized to CM5 biosensor chips, and FcγRI was used as an analyte. The sensorgrams of Fc-fusion proteins (abatacept, alefacept, and etanercept) and mAbs (adalimumab and infliximab) are shown in Fig. 4*A*. The data were analyzed with a 1:1 binding model. The K_D values of the two Fc fusion proteins (alefacept and etanercept) and Abs (adalimumab and infliximab) were comparable (Fig. 4*B*). The K_D values obtained in this study were similar to the data reported for IgG [reviewed by van de Winkel and Anderson (26)]. In contrast, abatacept had a lower affinity to FcγRI. In abatacept, a series of selected mutations those can alter the binding affinity to FcγR were introduced to reduce Fc-mediated cytotoxic effects (Fig. 5) (28, 29). Therefore, the data in Fig. 4 show that the change in the affinity of Fc domain to FcγRI, which is caused by amino acid substitutions, was detected in our experiments. These results suggest that the region interacting with FcγRI (i.e., the hinge proximal region of CH2) was not structurally different between Fc fusion proteins, except for abatacept, and Abs examined.

The affinity between FcRn and Fc domains generated by papain treatment

In Fig. 5, the amino acids sequences of abatacept, alefacept, etanercept, adalimumab, infliximab, and omalizuamb are aligned. The differences in the primary structure of the Fc regions were Glu^{376} and Met^{378} of etanercept, which are attributed to the IgG1 allotype, and Ser^{162}, Ser^{165}, and Ser^{174} of abatacept, which are due

A

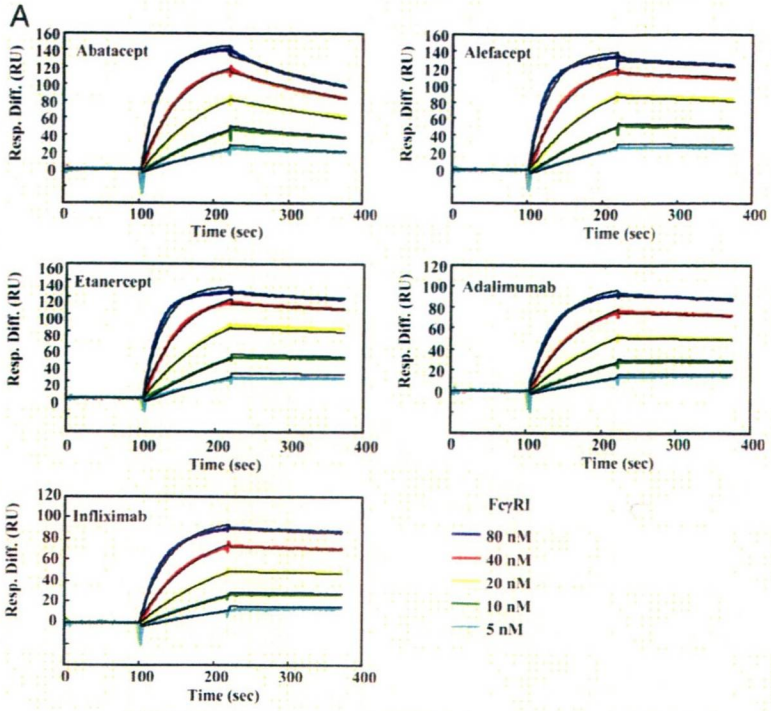


FIGURE 4. The affinity of Fc-fusion proteins and Abs to FcγRI. The Fc-fusion proteins (abatacept, alefacept, and etanercept) and mAbs (adalimumab and infliximab) were immobilized onto CM5 biosensor chips. Recombinant protein of the extracellular domain of FcγRI was injected at concentrations of 5–80 nM and analyzed with a 1:1 binding model (*A*). The colored lines are the observed sensorgrams, and the black lines are fitting lines. Association phase, ~100–220 s; dissociation phase, ~220 s. *B*, The K_D values calculated from the sensorgrams shown in *A*.

B

	Affinity to FcγRI K_D (nM)
Abatacept	4.82
Alefacept	0.857
Etanercept	0.818
Adalimumab	0.779
Infliximab	0.763

to the engineering for decreasing affinity to FcγR and improving protein production (28). To test the possibility that this limited structural difference or posttranscriptional modifications such as glycosylation can give rise to the difference in binding affinity to FcRn, we digested the Fc-fusion proteins or mAbs with papain and analyzed the affinity of their Fc domains to FcRn. The electrophoretic pattern of etanercept and adalimumab digested with papain is shown in Fig. 6*A*. Both etanercept and adalimumab were digested sufficiently for 24 h at 37°C under the conditions described in *Materials and Methods*, whereas digestion was not sufficient after incubating for 2 h. Therefore, the therapeutic proteins digested with papain for 24 h were used for the SPR analyses. The sensorgrams of etanercept (670 nM) and adalimumab (670 nM) were much different without incubation with papain, but they became almost identical after papain digestion (Fig. 6*B*). We measured the affinities to FcRn of five therapeutic proteins (etanercept, alefacept, adalimumab, infliximab, and omalizumab) digested with papain (Fig. 6*C*). Etanercept and alefacept are Fc-fusion proteins with low affinity to FcRn, and omalizumab is an Ab showing lower affinity to FcRn than other Abs. Because it was possible that the proteins were cleaved, in part, into smaller fragments than the Fc domain, the estimated K_D values may have been larger than the actual values. However, it was very clear that the affinities of etanercept, alefacept, infliximab, and omalizumab were increased by papain treatment (Fig. 6*C*).

The affinity of Fc-fusion protein and Abs became comparable after papain digestion, showing that the differences in amino acid sequences or posttranslational modification of the Fc domain did not contribute to the difference in the binding affinity of these proteins to FcRn. It therefore seems likely that the receptor domain of the Fc-fusion protein makes a difference in the higher-order structure of the FcRn-binding region of Fc (i.e., CH2-CH3 interface) or interferes with the binding between Fc domain and FcRn by steric hindrance. Moreover, such a difference or interference seems to be involved in determining the affinity to FcRn for some kinds of Abs, because the K_D values of infliximab and omalizumab were also increased significantly by papain treatment.

The affinity between FcRn and therapeutic proteins binding with target molecules

On the basis of the results suggesting the possibility that another region besides the Fc domain influences the affinity of Fc domain-containing proteins to FcRn, we assumed that binding with the target molecule would also change the affinity to FcRn. Because adalimumab, infliximab, and etanercept bind to the same target molecule, TNF-α, we analyzed the effects of binding with TNF-α on the affinity of these therapeutic proteins to FcRn. First, 0–2680 nM TNF-α was added to 335 nM infliximab and incubated for at least 1 h. The resulting mixture was then injected into the flow cell, and the affinities to FcRn were analyzed. By adding TNF-α, the shape of the sensorgram was drastically altered (Fig. 7*A*). The Abs (adalimumab and infliximab) can maximally bind to two TNF-α trimers, whereas etanercept binds to one TNF-α trimer. When the relative concentrations of TNF-α are low, three molecules of the Ab can bind to each TNF-α trimer, and cross-linked TNF/Ab complexes are formed (30). To evaluate the affinity

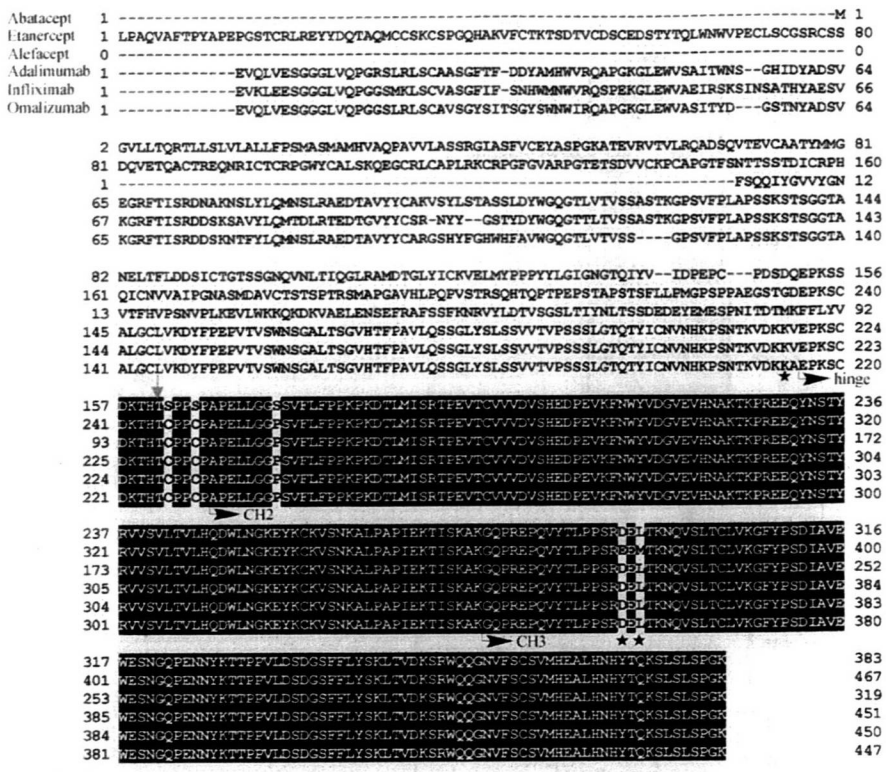


FIGURE 5. The amino acid sequences of abatacept, alefacept, etanercept, and H chains of adalimumab and infliximab. The amino acids marked with a star are different among allotypes of IgG1. The gray arrow is the cleavage site of IgG1 with papain (27). The amino acid sequences were obtained from the following links: abatacept, http://whqlibdoc.who.int/druginfo/18_2_2004_INN91.pdf; alefacept, http://whqlibdoc.who.int/druginfo/DRUG_INFO_14_4_2000_INN-84.pdf; etanercept, http://whqlibdoc.who.int/druginfo/DRUG_INFO_13_2_1999_INN-81.pdf; adalimumab, www.info.pmda.go.jp/shinyaku/g080405/10015900_22000AMX01598_A100_1.pdf; infliximab, www.info.pmda.go.jp/shinyaku/g020102/40031500_21400AMY00013_Q100_2.pdf; and omalizumab, www.drugbank.ca/drugs/DB00043.

between FcRn and TNF-α–binding proteins, excess TNF-α was added to adalimumab, infliximab, and etanercept (8-fold molar excess to 42–670 nM Abs and 4-fold to 168–2680 nM etanercept) to avoid forming nonuniform complexes. The sensorgrams were fitted by the bivalent analyte model (Fig. 7*B*). Although the fitted lines did not completely match the observed sensorgrams, the K_D values of infliximab, adalimumab, and etanercept to FcRn were calculated to be 2057, 1321, and 4286 nM, respectively (Fig. 7*C*). The affinity of infliximab–TNF-α complex or adalimumab–TNF-α complex was lower than that of infliximab or adalimumab, respectively (Fig. 7*C*). These results suggest that at least for these anti–TNF-α Abs, binding with target molecules decreases the affinity to FcRn. They may also suggest that the anti–TNF-α Abs complexed with TNF-α will be degraded more rapidly than anti–TNF-α Abs free from TNF-α in vivo.

Discussion

To our knowledge, this is the first article to elucidate the affinities of clinically used Fc domain-containing therapeutic proteins to FcRn in a comparative study. Because the affinities of these therapeutic proteins to FcRn were found to be highly correlated with the serum half-lives in humans, with the exception of infliximab, rituximab, and trastuzumab, the importance of FcRn in regulating the serum half-life of Fc domain-containing therapeutic proteins was suggested. The key observation was that the Fc-fusion proteins showed lower affinity to FcRn than Abs. These data provided us with one of the answers to the question of why the Fc-fusion proteins containing the Fc domain of human IgG1 exhibit a shorter half-life than human IgG1.

In the current study, we used the bivalent analyte model of BIAevaluation software. Most studies analyzing Fc-FcRn interactions have used the bivalent analyte model (15, 31) or the heterogeneous ligand model (7, 15, 31). Although the sensorgrams in our experiments were able to be fitted by both models, they were better fitted by the bivalent analyte model. Considering that two molecules of hFcRn bind to each IgG, resulting in a 2:1 binding stoichiometry (15), the bivalent analyte model seems to be suitable. It has been reported that the dual bivalent analyte model better fits the data of the FcRn-Fc interaction (32), although there are cases in which the bivalent analyte model does not work well. In the article about the dual bivalent analyte model, it was speculated that high-affinity and low-affinity types of FcRn existed on the surface of the BIAcore chip and that the low-affinity type receptor was probably an experimental artifact (32). Possibly because the content of the low-affinity type of FcRn on the chip is comparatively low in our immobilizing condition, the sensorgrams in our experiments might have been well-fitted by the bivalent analyte model.

Among the therapeutic proteins tested in this study, the Fc fusion proteins showed relatively lower affinities to FcRn (Figs. 2, 3), although the affinities to FcγRI are comparable to those of Abs (Fig. 4). Although the Fc domain binds to FcRn via the CH2-CH3 domain interface (33), the primary structures of the Fc domains of tested therapeutic proteins were almost the same, and cleavage of the Fc domains from Fab or the receptor region gave similar K_D values to FcRn (Fig. 6). These results suggest that the receptor regions of Fc-fusion protein alter the conformation of the FcRn-binding region (CH2-CH3 domain interface), not of the FcγRI-binding region (hinge proximal region of CH2 domain), or cause steric hindrance on the CH2-CH3 domain interface. The influence of regions besides the Fc domain on FcRn-binding regions would also be the case for Abs, as shown in Fig. 7.

Our results presented in this study can provide valuable information regarding the molecular design of novel Fc domain-containing therapeutic proteins and demonstrate the usefulness of FcRn-binding analysis in the characterization of Fc domain-containing therapeutic proteins. In addition to the Fc fusion proteins used in this study, rilonacept, a Fc-fusion protein consisting of ligand-binding domains of the extracellular portions of the human IL-1 receptor component (IL-1RI) and IL-1 receptor accessory protein linked to the Fc portion of human IgG1, and romiplostim,

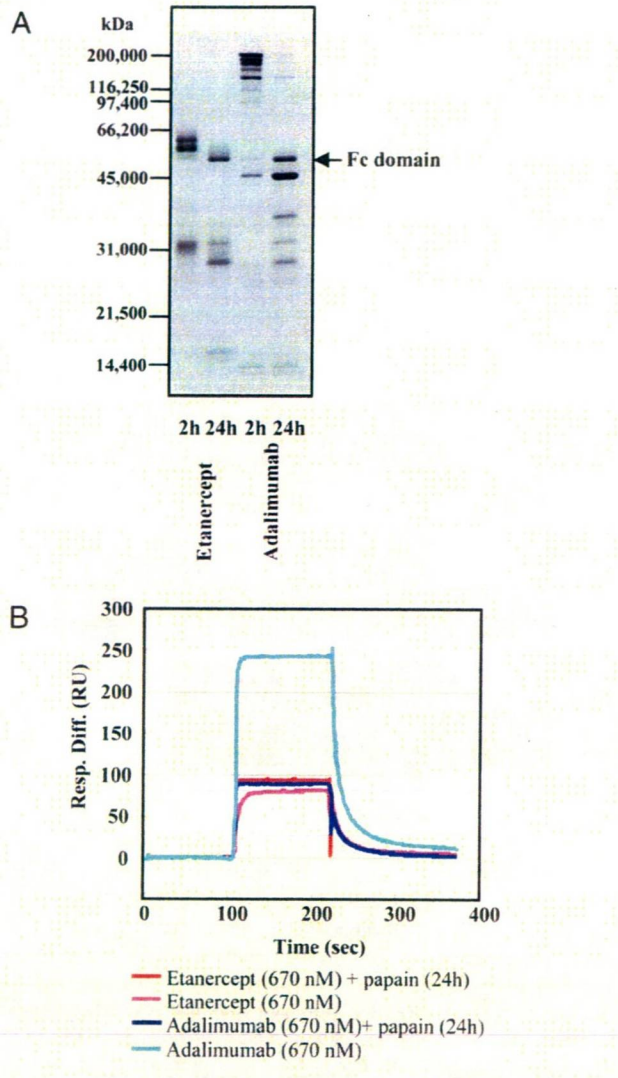
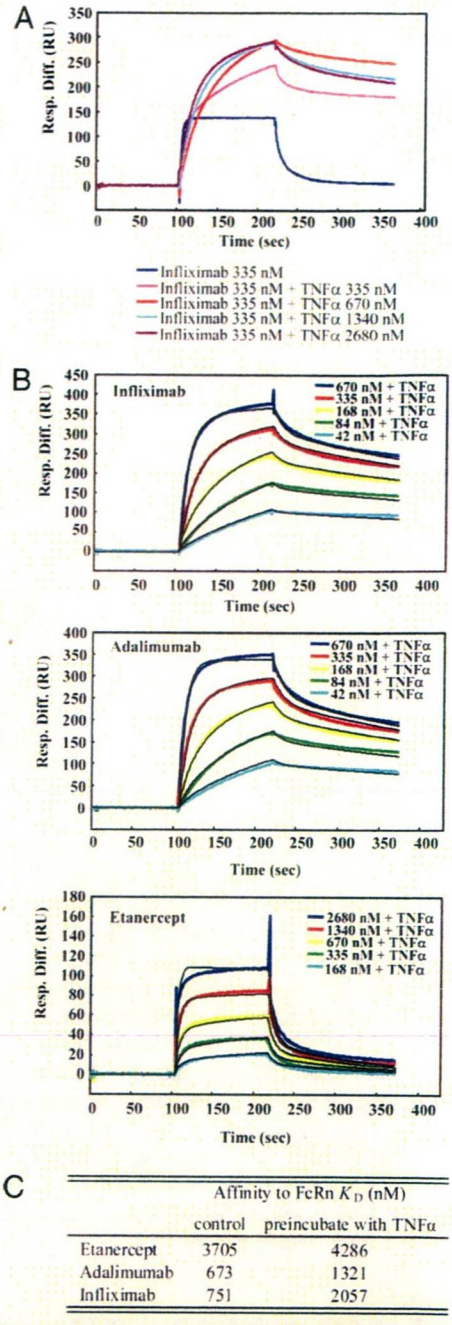


FIGURE 6. Effects of papain digestion on the affinities of Fc domain-containing therapeutic proteins to FcRn. *A*, The nonreduced SDS-PAGE of etanercept and adalimumab digested with papain for 2 and 24 h. *B*, The comparison between the sensorgrams of etanercept and adalimumab with or without papain digestion. *C*, Comparison of the affinity to FcRn among etanercept, alefacept, adalimumab, infliximab, and omalizumab, which were digested or not digested with papain. The K_D values were calculated from the sensorgrams at the range of concentrations described as follows. The concentrations of papain-digested etanercept, papain-digested alefacept, adalimumab, papain-digested adalimumab, infliximab, papain-digested infliximab, and papain-digested omalizumab were 42–670 nM; those of etanercept and alefacept were 168–5360 nM, and those of omalizumab were 42–1340 nM. Each bar shows the average K_D value + SD, which was calculated from three independent experiments. $^{**}p < 0.01$. NS, no significant difference according to Student *t* test.



C

	Affinity to FcRn K_D (nM)	
	control	preincubate with TNFα
Etanercept	3705	4286
Adalimumab	673	1321
Infliximab	751	2057

FIGURE 7. Effects of binding with the target molecules on the affinities of Fc domain-containing therapeutic proteins to FcRn. *A*, The sensorgrams of infliximab (335 nM) preincubated with TNF-α (0–2680 nM). *B*, The sensorgrams of infliximab (*upper panel*), adalimumab (*middle panel*), and etanercept (*lower panel*) preincubated with TNF-α (8-fold molar excess to 42–670 nM Abs and 4-fold to 168–2680 nM etanercept). The sensorgrams were fitted by the bivalent analyte model. *C*, The K_D values calculated from the sensorgrams shown in *B*. The values of infliximab, adalimumab, and etanercept derived from the same series of experiments are also shown as controls.

a Fc-peptide fusion protein consisting of human IgG1 Fc domain linked at the C terminus to a peptide containing two thrombopoietin receptor-binding domains, were approved recently (34, 35). The

development of Fc-fusion proteins will receive further attention. Although the Fc domains are used with the intent of prolonging the half-lives of receptor proteins, the half-lives tend not to be fully prolonged to the level of IgG1. It remains unclear whether the receptor regions of Fc-fusion proteins alter the conformation of the CH2-CH3 domain interface or the regions cause steric hindrance on the binding site of FcRn; however, the molecular design of Fc-fusion proteins having a higher affinity to FcRn might be possible in either case.

Reflecting the increasing interest in the development of mAbs and related products, the newly revised guideline for such products was adopted by the European Medicines Agency in 2008 (www.emea.europa.eu/pdfs/human/bwp/15765307enfin.pdf). In the guidelines, it is mentioned that FcRn-binding activity should be provided, as appropriate, in product characterization. Because regions other than the Fc domain might affect the affinity of the protein to FcRn (Figs. 6, 7), the affinity to FcRn should be evaluated as an important quality attribute related to the pharmacokinetic profile, even if the protein has a native Fc domain of IgG1, especially in cases of Fc-fusion proteins. Meanwhile, because it was demonstrated that oxidation of two labile methionines, Met^{252} and Met^{428}, in human IgG1 attenuates binding of the Ab to FcRn (36), alteration of the affinity to FcRn during the production process or storage will reflect structural changes of the protein, including Met oxidation, that will lead to shortening the serum half-life. In addition to IgG, albumin is also known to bind to FcRn in a pH-dependent manner and is protected from degradation (37, 38). The albumin-fusion proteins (e.g., albumin-IFN) or drugs having an albumin-binding moiety are being developed. FcRn-binding characteristics would also be important as a quality attribute of such products, which is related to the pharmacokinetic profile.

As mentioned above, the existence of several Abs having a short half-life and high affinity to FcRn suggested the involvement of other critical factor(s) in regulating the serum half-life of Abs such as trastuzumab, rituximab, or infliximab. Trastuzumab is a humanized Ab directed against human epidermal growth factor receptor 2 (HER2), which is expressed in some types of breast cancer cells. It has been reported that trastuzumab is taken up by HER2-expressing cells via HER2-mediated endocytosis (39, 40). Rituximab, a chimeric Ab directed against CD20, is also internalized in an Ag-mediated manner (41). Because the ligand-dependent internalization is followed by degradation of Abs, this property seems to be an important reason for the short half-life of trastuzumab and rituximab. It has been reported that, in general, the half-life of monoclonal IgG Abs increases depending on the degree of humanization in the order of murine < chimeric < humanized < human (6, 41, 42). Because infliximab and rituximab are chimeric Abs, the involvement of common factors influencing the half-life of chimeric Abs such as the presence of human anti-chimeric Ab would be another reason for the shorter half-life.

As shown in Fig. 7, the affinities of infliximab–TNF-α complex and adalimumab–TNF-α complex seemed to be lower than those of infliximab and adalimumab. If the affinity of therapeutic proteins/target molecules complexes to FcRn is lower than that of the free therapeutic proteins, the complexes will be degraded faster. Therefore, the half-lives of such therapeutic proteins seem to be shortened in the case that the target molecules are abundant in the bodies of patients. In contrast, if the affinity to FcRn of therapeutic proteins/target molecule complexes is higher than that of the free drugs, the complexes of drug and target molecules will have longer half-lives than free drugs. Because there are many factors affecting the elimination of Abs [reviewed by Tabrizi et al. (41)], further studies are necessary to elucidate the critical factors impacting the half-lives of Fc domain-containing proteins, in addition to the affinity to FcRn. Binding characteristics of the Fc domain-containing proteins or their complex with target molecules to FcγRs would be one of the important issues to be examined in regard to the impact on their elimination.

In conclusion, we showed the importance of the affinity to FcRn in determining the serum half-life of Fc domain-containing therapeutic proteins. Further investigation regarding the molecular structures that regulate the affinity of the engineered protein to FcRn will accelerate the development of therapeutic proteins with a desired half-life.

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