免疫反応でインヒビター力価が上昇したときに第VIII因子製剤の持続投与を中止した．その後は，インヒビター力価は7週間で5,700 BUまで増加し，続いて漸減した．一方，40歳時の中和療法後は，インヒビター力価が上昇した後，さらに10日間，第VIII因子製剤の投与を継続した．インヒビター力価は，製剤投与中に67 BUを示した後は，漸減し，13週後には8 BUとなった．2度の中和療法での条件の相違は，用いた遺伝子組換え第VIII因子製剤の製造メーカーとインヒビター力価の上昇後に製剤投与を継続したかどうかである．同じインヒビター症例でも，第VIII因子に対するインヒビター力価の上昇率と最高値を示した時期が異なることは興味深い．

症例1の40歳時の脳出血エピソードでは，持続投与中にインヒビターの上昇が起きた後でも，かろうじて0.1 U/ml程度の血漿第VIII因子活性を認めていたため持続投与を継続した．インヒビターはその反応動態の違いからtype Iとtype IIに分けられる．インヒビター抗体と凝固因子を反応させた時，インヒビターの濃度依存性に凝固因子活性を低下させ，抗体過剰の条件では残存因子活性を完全に失活させるものをtype Iインヒビターという[9]．それに対して，インヒビター量が多くなるにもかかわらず，凝固因子活性の失活程度が弱くなり，抗体過剰状態でも低値の活性が共存する場合をtype IIインヒビターという[10]．本症例において，既往免疫反応後にも関わらず，低値の血漿残存第VIII因子活性を認めていたのは，本症例の再上昇時のインヒビターが，type IIの性質であったことが考えられる．第VIII因子製剤持続投与を継続して，第7日から第14日まで血漿第VIII因子活性を低値ながらも維持したことは，硬膜外血腫の治療上有意義であったと推察している．持続投与開始第8日に，第VIII因子インヒビターの上昇に対し再度中和を試みて第VIII因子製剤のボーラス投与を行なったが，血漿第VIII因子活性は上昇しなかった．type IIインヒビターの力価測定では希釈直線性を認めず，高倍率希釈検体でのインヒビター力価は比較的高くなる[11]．この場合，便宜上，正常血漿中の第VIII因子活性を50%失活させる最小希釈検体の成績を採用している．したがって，本症例の既往免疫反応後のインヒビター中和量の計算において，インヒビター力価は見かけ上低かった可能性が考えられる．以上より，本症例では既往免疫反応前後でインヒビターの性質がtype Iからtype IIに変化したことが推測される．すなわち，type IIインヒビターの場合には理論計算式通りに*in vivo*での中和が得られないことを考慮する必要がある．一方では，インヒビター力価が高くても，type IIインヒビターの場合には，製剤の投与により低値の活性が維持できる可能性も示唆している．

症例2の第IX因子インヒビター症例では外傷性足背部皮下血腫に対して，まずPCCによるバイパス療法を行ったが，十分な止血が得られず，再出血後の治療に難渋した．第IX因子を含有するPCCの連日投与にも関わらず，第IX因子インヒビターは2.1 BU/mlと比較的低力価であったため，第IX因子製剤によるインヒビター中和と持続輸注療法に切り替えた．理論的に算出したインヒビター中和量の第IX因子製剤投与によって，期待した血漿第IX因子活性が得られ，第IX因子ハイレスポンダーインヒビター症例に対しても同療法の効果が確認できた．

症例2は，PCC中の第IX因子に対する免疫反応は示さず，第IX因子精製製剤の投与後にインヒビター力価が192 BUまで上昇したことが特徴的である．PCCは過去に繰り返し投与されており，患者はPCC中の第IX因子には，ある程度の免疫寛容を獲得していたと考えられる．また，短期間に比較的大量の第IX因子精製製剤に暴露されたことが，免疫反応につながったと推測できる．

以上，ハイレスポンダーのインヒビター症例の止血療法として，凝固因子製剤によるインヒ

ビターの中和と持続輸注療法について検討した．10 BU/ml 程度のインヒビターは，理論上の抗体中和必要量の凝固因子製剤投与により中和され，ほぼ期待通りの血漿凝固因子活性が得られた．ただしその効果は既往免疫反応が起きるまでの数日間であることに留意し，最低1週間は凝固因子活性を連日モニターする必要がある．血友病ハイレスポンダーインヒビターの重篤な出血もしくは止血コントロールが不良の出血に対し，本法は効果的であるが，短期間に確実に血中凝固因子活性を上昇させたい場合に限定される止血療法と考えられる．

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Inhibitor Neutralization and Continuous Infusion of Clotting Factor Concentrates for Hemophilia Patients with High-responding Inhibitor

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Key words: hemophilia, high responder, inhibitor, continuous infusion, intracranial hemorrhage

We report successfully controlled severe bleeding in two hemophiliacs with a high responding titer. This was accomplished by neutralizing the inhibitors with a bolus infusion of a sufficient amount of the relevant blood coagulation factor concentrate, which was followed by a continuous infusion of the concentrate to maintain the titers of the factors in plasma at required levels.

The first patient, a 38-year-old man with severe hemophilia A, suffered from an intracer-

ebellar hemorrhage. The inhibitor titer on his admission was 2.1 Bethesda units (BU)/ml. An initial bolus dose of 5,000 U of recombinant factor VIII (FVIII) concentrate was administered, followed by a continuous infusion of the concentrate at 3.8 U/kg/hr. Plasma FVIII : C level was maintained at 0.9-1.44 U/ml over a period of 4 days, resulting in the reduction of the size of hematoma. At the age of 40, he suffered from a parietal lobe intracerebral hemorrhage. Despite the use of prothrombin complex concentrate (PCC) as a bypassing agent, the brain CT revealed a new hemorrhage in the subdural space. The inhibitor titer at this episode was 10 BU/ml. An initial bolus dose of 12,000 U of recombinant FVIII concentrate, followed by a continuous infusion of the concentrate at 4-6 U/kg/hr raised the plasma FVIII : C level to 0.54-2.04 U/ml over 5 days and resulted in the reduction the size of hematoma.

The second patient was a 4-year-old boy with severe hemophilia B. He was hurt on the right foot, having developed a large subcutaneous hematoma. The bypassing therapy with the prothrombin complex concentrate over a period of 6 days did not reduce the hematoma size. The inhibitor titer at the time of his admission was 2.1 BU/ml. He was given an initial bolus dose of 2,000 U of factor IX (FIX) concentrate, followed by a continuous infusion of the concentrate at 13 U/kg/hr that yielded the FIX : C level at 1.3 U/ml, and reduction in the size of the hematoma.

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日本血栓止血学会　血友病標準化検討部会編

血友病在宅自己注射療法の基本ガイドライン

(2003年版)

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I. はじめに

血友病の在宅自己注射療法（家庭療法）は，凝固因子製剤による早期止血や定期注射による血友病性関節症などの慢性障害を防止することを目的に1983年に認可され，現在では全国に広く普及している．しかし，家庭療法の導入基準はもとより，患者教育，遵守事項，管理項目に関して施設間に差違がある上，患者が備えるべき関連知識や注射技術の顕著な格差も生じている．また，認可当初に比較して，医療施設側の家庭療法に対する定期的な管理や評価が不十分になっている可能性も指摘されている．さらに1996年からは，インヒビター保有患者のバイパス止血療法に関しても家庭療法が認められており，バイパス止血療法の特殊性に留意した指針が求められている．日本血栓止血学会血友病標準化検討部会では，これらの問題点を再度整理し，標準化することで家庭療法からの脱落や逸脱を防ぎ，さらに安全で効果的な血友病止血治療を推進させるために，家庭療法の基本ガイドラインを作成した．本ガイドラインでは，臨床実地に即した対応ができるように，主に医療施設側の管理上の項目を箇条書きにして示した．なお，本ガイドラインは必要に応じて随時変更し，本誌上で更改される．

II. 家庭療法に関する従来のマニュアル，ガイドラインおよび教育資料

これまで血友病家庭療法の教育には，東京医科大学臨床検査医学科および荻窪病院小児科が中心となって編集し，バクスター株式会社[1]あるいは日本赤十字社[2]が発行するホームインフュージョン教育マニュアル（図1）が広く使用されてきた．また，1993年には厚生省健康政策局と日本医師会が監修し，在宅自己注射法マニュアル等作成委員会が編集した在宅自己注射（血友病・下垂体小人症）ガイドライン[3]とマニュアル[4]が発行されている（図2）．これらのマニュアルやガイドラインは，いずれも現在入手が困難となっており，また内容や体裁も古くなり改訂が望まれている．

家庭療法が開始された1983年頃には，図3に示すような血友病教育プログラム，患者・家族テキストと，そのテキストに応じた自己評価問題とその解答[5]をセットにした冊子も発行されていた．これは米国のNational Hemophilia Foundation（NHF）とThe World Federation of Hemophilia（WFH）のプログラムを参考に荻窪病院小児科で作成された．内容はかなり詳細で，当時慎重に家庭療法が導入されていたことが伺える．この冊子は現在入手が不可能であるが，広島県へモフィリア友の会のウェブサイト（http://www.aaieba.gr.jp/）には，血友病に関する様々な基礎知識あるいは補充療法・家庭療法に関する知識を問う問題とその解答が掲載されている．

III. 家庭療法の現状と問題

現在，家庭療法を行っている患者やその家族の中には，初期の教育を医師や看護師から受けていない例や，血友病の病態，臨床症状とその対応，遺伝形式，自己注射の手技等，教育内容の一部しか教育されていない例が見受けられる．このため，製剤の注射量が極端に少ない例，逆に多過ぎる例，出血部位や出血の程度に関わらず注射量や注射回数が固定されている例，出血してから輸注するまでの時間が長い例，必要な連続注射を行っていない例など，家庭療法の実際が不適切と思われる事例がある．中には家庭療法を管理していく上で必要な輸注記録をつける習慣すらない例も少なくない．

一方，医療施設側の家庭療法上の管理や記録の保存が，認可当初に比較して不十分になっている可能性も指摘されている．家庭療法は一度開始すると，患者の生涯に渡り継続することが原則になる．その間には患者の成長や身体的ならびに環境の変化が予測される．また住居の移

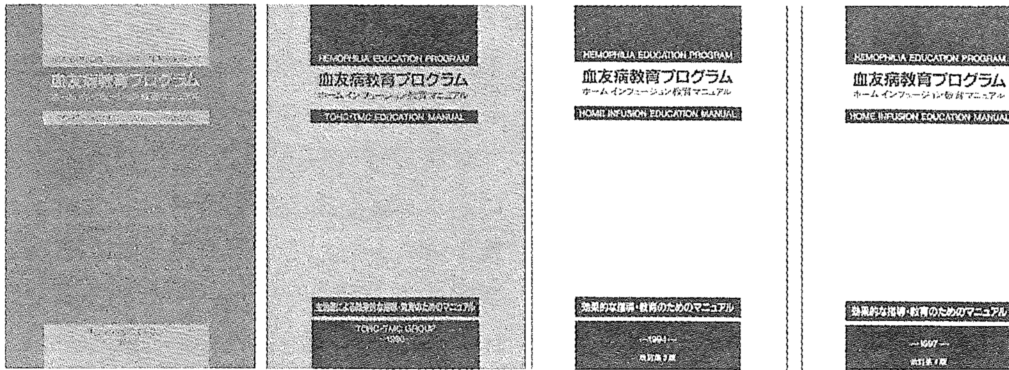

図 1　血友病教育プログラム（ホームインフュージョン教育マニュアル）

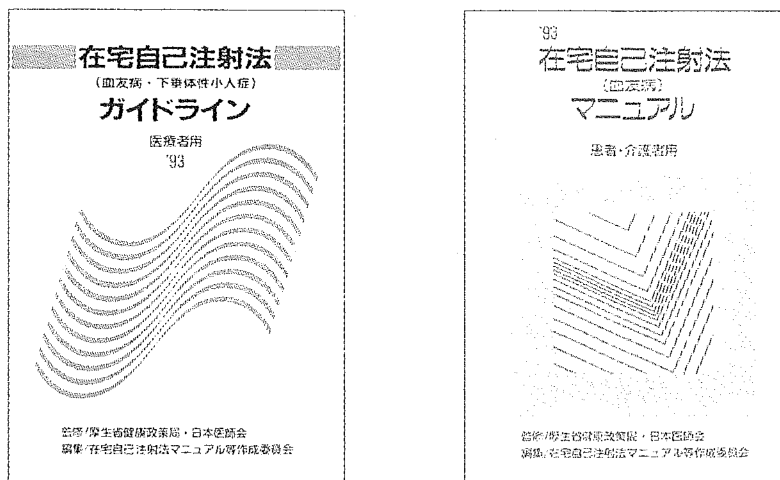

図 2　在宅自己注射法（血友病，下垂体性小人症）ガイドライン
と在宅自己注射法（血友病）マニュアル

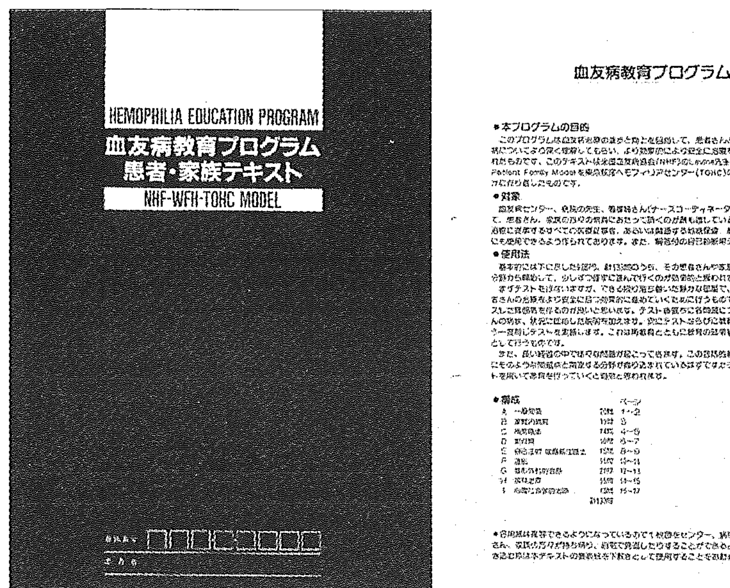

図 3　血友病教育プログラム　患者・家族テキスト　NFH-WFH-TOHC MODEL

動に伴い医療施設を移る可能性もある．血友病医療における医療者と患者の関係は長期に渡り，時に両者間には，ある種の「馴れ」が生まれることもある．その結果，ともすると医療者は，患者の家庭療法を客観的に評価し記録することを省略しがちになる．長期に渡る，定期的な教育水準や注射技術の確認や再評価に関しては，当初は十分な配慮がなされておらず，基準なども設けられていなかった．

製剤の安全性や取り扱い上の利便性が向上したことも相まって，幼少時から本療法を開始した若年層の血友病患者には，血友病性関節症等の慢性障害は着実に少なくなっている．しかしながら，現在の家庭療法は，以上のように不確

表 1 家庭療法の目的，意義

1. 出血時の早期補充療法あるいは定期補充療法を医療施設以外で効率よく行うことにより，出血の苦痛を予防・軽減させる
2. 出血による後遺症および慢性障害の発生を予防・軽減させる
3. 出血時に通院する際の身体的，時間的，経済的負担を減らす
4. 出血症状に伴う学校生活や社会生活の質の低下を軽減させる
5. 活動内容や行動範囲を広げ，社会適応をはかり，心身両面での自立を促す

表 2 凝固因子補充療法の方法（インヒビター保有症例を除く）

1. 出血時の補充療法：
   - Ⅰ. 軽度から中等度の出血に対して；可及的早期に止血をはかり，慢性障害（関節症，偽腫瘍など）の発生を回避する
   - Ⅱ. 重度の出血（頭蓋内出血，消化管出血，外傷）に対して；早期に補充療法を開始することで生命予後を改善する
2. 予備的補充療法：運動会や遠足などの身体活動の事前に凝固因子製剤を注射し，出血を回避する方法
3. 定期補充療法：
   - Ⅰ. 一次定期注射；重症型の血友病患者を対象に2歳未満あるいは最初の関節出血後（2回目の関節出血以前）に定期的（例えば週に2あるいは3回）に凝固因子製剤の注射を開始し，これを長期間行うことにより関節症を未然に防止しようとする方法
   - Ⅱ. 二次定期注射
     - a. 2歳以上あるいは2回以上関節出血を来した後から開始し，定期的（例えば週に2あるいは3回）に凝固因子製剤の注射を長期間行う方法
     - b. 頻回の出血あるいは慢性滑膜炎などに対し，定期的（例えば週に2あるいは3回）に凝固因子製剤の注射を短期間（数週間から数年）行う方法

表 3 家庭療法の適応基準

1. 本療法を患者ならびに家族が望んでいる
2. 本療法の目的，意義，遵守事項を患者と家族が十分に理解している
3. 本療法が患者の身体的，精神的苦痛を軽減し，生活の質を高めることが予想される
4. 医師・医療スタッフと患者や家族との間に安定した信頼関係が築かれている
5. 患者や家族が心理的に安定している
6. 患者は当該製剤による重篤な副作用の既往がない

表 4 患者や家族の遵守事項

1. 定期的（最低3か月毎）に受診すること
2. 家庭治療に関して主治医の評価と指導を受けること
3. 治療経過や製剤の家庭内在庫状況を記録し，病院に定期的に提出すること
4. 製剤は規定の方法で管理し，奨められた輸注量，輸注方法を守ること
5. 製剤は，兄弟を含む患者の間で流用しないこと
6. 針や注射器などの医療廃棄物を適切に処理すること
7. 出血症状が強いときや判断に迷うときには主治医に連絡すること

表5 家庭療法の教育項目（下線のは必須項目）

1．基本的知識
- I．血液凝固と血友病に関する基本事項
  - a．止血の仕組みの基本；一次止血，二次止血，血管内皮細胞，血小板，凝固因子
  - b．血友病の病態；第VIII因子，第IX因子，APTT，X連鎖劣性遺伝，重症度，保因者，有病率
- II．出血症状とその対応
  - a．急性出血部位と症状；関節内，筋肉内，腎・尿路，鼻，皮下，腸腰筋，消化管，外傷，頭蓋内，咽頭・喉頭・頚部
  - b．慢性障害と症状；関節症，偽腫瘍
- III．補充療法
  - a．注射量と上昇期待値；半減期，回収率
  - b．注射方法と注射量；初回注射・連続注射，定期注射，出血部位と程度に対する補充量，副作用と対応
- IV．インヒビターとその治療
  - a．インヒビターの基本；発生率と有病率，ベセスダ単位，ハイレスポンダー・ローレスポンダー，免疫寛容療法，バイパス止血療法，中和療法
  - b．バイパス止血療法の実際（インヒビター保有患者においては必須）

2．注射の実技と製剤の管理
- I．重篤出血時の対応方法
- II．副作用出現時の対応方法
- III．製剤の保管，管理方法
- IV．製剤の溶解方法
- V．静脈注射の実際
- VI．効果の判定と繰り返し注射の方法
- VII．止血管理の記録方法
- VIII．廃棄物の取り扱い方法
- IX．主治医あるいは病院との連絡方法
- X．製剤の一回処方量
- XI．家庭内での製剤の適正な在庫量
- XII．定期受診の必要性

表6 家庭療法の認可

1．適応規準（表3）を満たしている
2．各施設で規定の教育プログラムを受け，教育目標（表5）を達成している
3．担当医師が適当と認めている
4．遵守事項（表4）を守ることに同意できる

表7 輸注記録表の記載項目

1．出血エピソード
2．輸注までの時間
3．製剤名，輸注量，製剤のロット番号（シール貼付）
4．輸注毎および全体の止血の評価
5．副作用
6．併用薬
7．製剤の処方量と在庫量（出納表）
8．気付いたことやメモ
9．主治医への質問事項
10．主治医からのコメント，指導

実，不十分な問題点を抱えたまま行われているのも事実である．

IV．基本ガイドライン

表1から**表9**に示す基本ガイドラインは，これまで使われてきたマニュアルやガイドラインの内容を大きく刷新するものではない．また，各施設で用いられているマニュアルや教育資料，輸注記録表を統一させることを目的とはしていない（**表1**）．むしろ，最小限必要な項目

表 8　家庭療法の継続・管理：

（以下の項目を定期的に確認してカルテに記載する）
1. 定められた期間内に定期的に受診している
2. 輸注記録表の回収，評価，保存がなされている
3. 製剤の処方頻度，一回処方量が適切である
4. 製剤の出納管理が適切になされている
5. 家庭療法の更新：5年毎あるいは患者の発達段階（小学校入学時，中学校入学時など，あるいは父母などによる注射から自己注射に移行する場合）に応じて随時，適応規準・遵守事項・教育規準を確認し，必要に応じた再教育を行う

表 9　家庭療法実施医療機関のありかた

1. 血友病包括医療体制が整備されている（基幹病院）
   a. 血友病患者を積極的に診療している医師，看護師がそれぞれ1名以上常勤している
   b. 家庭療法の教育プログラムをもち，管理体制が整っている
   c. 各種の凝固因子製剤が院内薬剤部に常備され，外来処方が可能
   d. 各科（内科，小児科，整形外科，リハビリテーション科，歯科，脳外科など）の医療連携体制があり，血友病の合併症にも対応できる
   e. 入院施設がある
   f. 心理・社会的な相談に対応できる
   g. 患者団体と連携できる
   h. 遺伝相談に応じることができる
   i. 患者や他の医療施設からの連絡の受け入れ態勢が確立している：休日・夜間の連絡方法，主治医など
2. 上記の体制が未整備な医療機関においては，基幹病院との医療連携を維持している（二次医療施設）
3. 血友病医療の最新情報を入手し実践することに努めている

表 10　バイパス製剤による家庭療法に共通する留意事項

1. 当該製剤による重篤な即時性の副作用の既往がないこと
2. 原則として軽度あるいは中等度の出血を対象とする
3. 出血症状が出てから可及的早期（2時間以内が望ましい）に注射を開始する
4. 重度の出血（頭蓋内出血，消化管出血，外傷など）に対しては早期に注射を行った上で，基幹病院に連絡し指示を受ける

を列挙することで，標準化を進めていくことを第一義的に構成した．血友病診療施設においては，本ガイドラインの各項目を確認し，用いられている資料に適宜追加補足されることを期待したい．

凝固因子補充療法の方法（**表2**）は，出血時の補充療法，定期補充療法，予備的補充療法に大別して記載した．現在わが国における家庭療法は，出血時の補充療法や予備的補充療法が主体である．今後は，一次定期注射も含めた効果的な定期補充療法が普及し，血友病患者が生涯にわたり合併症の危険から解放されることが望ましい．日本小児血液学会の血友病委員会（委員長：吉岡　章，奈良県立医科大学小児科教授）では，定期補充療法に関する前方視的研究の計画を進めている（担当：瀧　正志，聖マリアンナ医科大学小児科助教授）．今後は日本血栓止血学会の血友病標準化検討部会が相互協力し，定期補充療法の普及に努めたい．

家庭療法の教育（**表5**）に関しては，各施設で各種の資料や冊子が用いられていると想像される．本項目に関しては，教育内容の詳細はあえて省き，必要項目のキーワードの列挙に留めた．詳細に関しては，ある程度の自由度をもって，各施設で適宜既存の資料などを用いて教育されることを期待するものである．基本的知識は多項目に渡るため，患者の年齢によっては，すべての理解を求めるのは難しいこともある．

表 11 プロプレックスSTによる家庭療法のプロトコール

1. 1回注射量：50 ～100単位/kg
2. 注射間隔：最低6時間
3. 注射終了：2回注射後にも止血が不十分な時には病院に連絡する
4. 自己評価のポイント：注射6時間後の止血の評価，注射終了24時間後の止血の評価

表 12 ファイバによる家庭療法のプロトコール

1. 1回注射量：50 ～100単位/kg
2. 1日最大注射量：200単位/kgまで
3. 注射間隔：最低8時間
4. 注射終了：2回注射後にも止血が不十分な時には病院に連絡する
5. 自己評価のポイント：注射8時間後の止血の評価，注射終了24時間後の止血の評価

表 13 オートプレックスによる家庭療法のプロトコール

1. 1回注射量：25 ～100単位/kg
2. 注射間隔：最低6時間
3. 注射終了：2回注射後にも止血が不十分な時には病院に連絡する
4. 自己評価のポイント：注射6時間後の止血の評価，注射終了24時間後の止血の評価

また，2.の「注射の実技と製剤の管理」を習得した上で，家庭療法をまず導入し，追って1.の「基本的知識」の教育を深めていくことも考えられる．そこで，基本的知識の中の必要最低限の項目をアンダーラインで示した．注射の実技と製剤の管理はすべて必須項目とした．

輸注記録表も施設ごとに様々な様式が用いられているが，本ガイドラインでは，記録表に記録すべき必要最低限の項目を列挙した（**表7**）．近年，各製剤の外箱には，ロット番号が示されたシールが3枚添付されている．輸注記録表のロット番号欄に書き写す代わりに，これらのシールを貼り付けることが可能である．新しい項目としては，処方された製剤量と家庭内の在庫量を記入する項目を設けた．これは既存の輸注記録表には，おそらく取り上げられることの少なかった項目である．患者や家族が，貴重な凝固因子製剤を自己管理する意識を高める上で必要であり，また，医療者側の薬剤の出納記録としても重要になる．

家庭療法の継続と管理（**表8**）は新規の事項である．長い年月に渡って，家庭療法の質を維持するためには，医療者側が主体的に管理基準を遵守し，評価していく必要がある．患者の受診頻度は，一般的なものとして，「最低3カ月毎」（**表4**）としたが，個々の患者の状況や実績に応じて適宜定められるものであろう．輸注記録表は定期受診時などに患者が持参する．そこで主治医は，個々の記録を遡って患者と共に確認し，評価やコメントを加えたうえで，その記録を患者と医師でそれぞれ保存することが望ましい．製剤の処方量は，製剤使用頻度，来院頻度，冷蔵庫の保存スペースなどにより，患者毎にある程度定まってくる．1カ月分の平均使用量を基準とし，多くとも定期受診の間に使用する量を限度とする．製剤の出納に関して，出庫量はカルテに記載した処方量で把握はできるが，家庭での在庫量はわかりにくい．したがって，出納管理は患者の輸注記録表などで行うことが望ましい．

家庭療法の長期継続管理（**表8-5**）に関しては，5年を目安に，あるいは患者の発達段階に応じて見直す機会を設けることが望ましい．小学校入学時や中学校入学時など，あるいは父母などによる注射から自己注射に移行する時期などが見直す機会としては相応しい．それらの際には，改めて，適応基準（**表3**），遵守事項（**表4**），知識や技術（**表5**）の確認を行う．問

表 14 ノボセブンによる家庭療法のプロトコール

1. 注射量 ：90〜120 μg/kg
2. 注射間隔：2〜3時間ごと
3. 注射回数：1回の出血につき原則として3回まで
4. 注射終了：止血効果がみられたら，さらに1回追加注射をする．2回連続して止血効果が感じられないときや3回注射しても止血が不十分な時にはいったん中止し，病院に連絡する．
5. 自己評価のポイント：注射2〜3時間ごとの止血の評価，注射終了24時間後の止血の評価

題点があれば十分に説明と指導を行い，時には再教育も必要になる．近年，重症型の血友病の場合は家庭療法の導入を低年齢から開始することがある．この場合，家庭注射の教育は患者本人よりも母親あるいは父親を対象に行われることになる．患者の成長にともない，家庭療法は親から患者本人へと受け継がれていくことになるが，この時期に医療者による本人への教育がなされない場合は，教育項目の一部が抜け落ちる可能性がある．家庭療法の定期的な更新が必要な理由はここにもあり，その時の患者の年齢，理解度に合わせた教育が必要とされる．

最後に，家庭療法を実施する医療機関のありかたにつき，大枠を示した（**表 9**）．家庭療法実施医療機関としては，血友病患者を積極的に診療している医師と看護師がそれぞれ1名以上常勤しており，整形外科，リハビリテーション科，口腔外科を含む他科との医療連携により，血友病の合併症を包括的に診療できる体制の整った，入院施設のある総合病院（基幹病院）であることが望ましい．基幹病院では凝固因子製剤を常備し，外来処方ができ，家庭療法に関する教育プログラムを含めた管理体制が整っていることが必要である．また，患者の心理的・社会的な相談や遺伝相談に対し，医療ソーシャルワーカーや医師が対応できる体制をもつことが望ましい．患者団体との良好な連携を維持することも求められる．

基幹病院で教育を受けて家庭療法が導入された患者は，基本的にその病院で治療管理を継続することになる．しかし，自宅が基幹病院の遠隔地にある場合などには，協力的な近医（協力医）に家庭療法の一部の機能を委託することがある．また，血友病の患者を初めに診断し治療を継続している診療所や病院（二次医療施設）では，家庭療法を導入する体制が整備されていない場合には，いったん基幹病院に教育などを委任し，その後の日常管理を二次医療施設で継続することもできる．これらの患者が，休日や夜間に診療が必要な際や緊急時には，直接基幹病院を受診するか，その担当医の指示を受けられる体制にしておくことが肝要である．また協力医や二次医療施設の担当医も，血友病診療上の疑問点や問題が生じた際には，基幹病院に遅滞なく連絡・相談できる関係を維持する必要がある．

V. インヒビター保有患者の家庭バイパス療法

インヒビター保有患者の止血管理は，未だすべての場合に対応できる方法はなく，血友病医療のなかでも難題とされる．家庭療法が認められた1983年の時点では，即時性の副作用が比較的少なく，用量依存性の止血効果が期待できる補充療法を，患者と家族に委嘱することが目的であった．インヒビター保有患者のバイパス療法に関しては，即時型のアレルギー反応や血栓性の副作用の可能性があり，止血効果の確実性に問題があったために，多くの施設では家庭療法には当面そぐわないと考えていた．ところが，ある種のバイパス製剤の止血効果が安定して期待できるインヒビター保有患者の中では，バイパス製剤の家庭療法の施行を求める声が高まってきた．それらを受けて，1996年にはバイパス製剤を用いた家庭療法も認可され，イン

ヒビター保有患者の止血管理も現在では病院を離れて行うことのできる環境にある．

現在わが国で使用可能なバイパス製剤は，プロプレックスST，ファイバ，オートプレックス，ノボセブンの4製剤である．これらの家庭療法導入に際しては，前述した家庭療法の基本ガイドラインを遵守した上で，上記の潜在的な問題点を十分に配慮する必要がある．**表10**には，すべてのバイパス製剤に共通する注意事項を掲げた．家庭でのバイパス療法では，止血効果が不十分であった時の判断と，基幹病院への連絡体制はさらに重要になる．止血効果を認めないまま，漫然とバイパス製剤を反復注射することは，出血症状が増悪する上，副作用を惹起する可能性があり，高価な製剤の空費を避けるうえでも好ましくない．各製剤の用法・用量を考慮した家庭療法での使用プロトコールを，**表11**から**表14**に示した．基幹病院の担当医には，これらを基準にした上で，各インヒビター保有患者に合わせた注射法を考案することが望まれる．

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Five Novel and Four Recurrent Point Mutations in the Antithrombin Gene Causing Venous Thrombosis

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Abstract

We analyzed the antithrombin (AT) gene in 9 unrelated Japanese patients with thrombotic disease. All 7 exons, the splice junctions, and the 5′-flanking region of the AT gene were amplified by polymerase chain reaction and sequenced directly. Nine different point mutations, all in the heterozygous state, were identified. Five novel (M-32T, M89K, L146H, Q159X, and L409P) and 2 previously reported (R132X and R359X) point mutations were identified in patients with type 1 deficiency. Two different missense mutations, R393C and R393H, located in the protease reactive site were detected in patients with type 2 deficiency. No other sequence abnormalities in the AT gene were detected by direct sequencing. None of the mutations was present in 100 alleles from 50 unrelated Japanese control subjects. Although type 1 deficiency was diagnosed in patient 7 on the basis of approximately 50% AT antigen and activity levels, the data indicated that the novel L409P mutation is a type 2 pleiotropic effects (PE) deficiency because its location in the C-terminal portion of the reactive site is similar to the locations of reported PE type mutations, and it is highly conserved among other serpins. *Int J Hematol.* 2003;78:79-83.
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Key words: Antithrombin; AT deficiency; Point mutation; Direct sequence; Venous thrombosis

1. Introduction

Antithrombin (AT) is the primary inhibitor of thrombin and other activated serine proteases in the blood coagulation system. The gene for AT is 13.5 kilobases (kb) in length and is located on human chromosome 1 at q23-25. It contains 7 exons (1, 2, 3a, 3b, 4, 5, and 6) and 6 introns [1]. AT is synthesized by hepatocytes as a 464–amino acid precursor. Before secretion into the blood, the 32–amino acid signal peptide is cleaved to yield the mature protein. Plasma AT is a single-chain glycoprotein with a molecular weight of 58.2 kd and disulfide bonds between residues 8-128, 21-95, and 247-430 [2,3].

AT belongs to a large group of structurally related proteins known as serine protease inhibitors (serpins) [4]. The reactive sites of AT are Arg393 (P1) and Ser394 (P1′). When AT interacts with a target protease, such as thrombin or factor Xa, the reactive sites are split off and form 1:1 inactive complexes linked by acyl bonds between the Ser residue (S1) of the protease active center and the P1 residue in AT [2].

Individuals with inherited AT deficiency have an increased incidence of thrombotic events. In 1965, Egeberg [5] first described a large pedigree that comprised 3 generations, each with persons with thrombotic disease associated with approximately half-normal levels of AT. The prevalence of such deficiency has been estimated at 1 in 2000, and the frequency among hospitalized patients with recurrent or extensive thrombosis has been estimated at 2% to 3% [6]. However, it was recently reported that AT deficiency has a prevalence of 1 in 630 in the general population and 3% to 5% in patients with thrombotic disease [7]. Classification of this hereditary deficiency is based on molecular defects [8,9], and 2 types are recognized. Type 1 is characterized by reductions in levels of both AT activity and antigen to approximately 50% of normal. Type 2 deficiency includes cases in which AT activity is reduced, and approximately 50% of the AT antigen is of a variant form. Type 2 deficiencies are subdivided into 3 types: the heparin-binding site (HBS) type,

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reactive site (RS) type, and multiple or pleiotropic effects (PE) type. These classifications are based on mutation sites and functional abnormalities. The PE-type deficiency has been reported in association with multiple defects in secretion, heparin binding, and thrombin inhibition. Reported PE-type mutations were located in exon 6, which encodes the C-terminal portion of the reactive site [10].

In the present study, we analyzed the AT gene in 9 unrelated Japanese patients with thrombotic disease and identified 5 novel and 4 previously described mutations.

2. Materials and Methods

2.1. Patients

Nine unrelated Japanese individuals with AT deficiency were included in the present study. Informed consent was obtained from all patients and family members involved in the study.

Patient 1 was a 46-year-old man with deep vein thrombosis (DVT) of the right lower extremity diagnosed when he was 38 years old. His father suffered cerebral infarction (CI) at age 40. Patient 2 was a 31-year-old woman with DVT of the left lower extremity diagnosed when she was 22 years old. At age 31, she showed severe emesis gravidarum and was given warfarin anticoagulant therapy at delivery. No thromboembolic episodes were noted in her family. Patient 3 was a 21-year-old woman who suffered sudden CI after the delivery of her first child. No episode of thrombosis was noted in family members. Patient 4 was a 36-year-old woman with DVT and pulmonary embolism. She had recurrent thrombotic episodes from age 14. Her father suffered CI at age 40 and developed flaccid paralysis of the left upper and lower extremities. Patient 5 was a 65-year-old man with a history of DVT. The first occurrence was at age 50. A second case of DVT that developed 4 years later was complicated by pulmonary embolism. No episode of thrombosis was noted in family members. Patient 6 was a 22-year-old man with DVT and pulmonary embolism diagnosed when the patient was 17 years of age. Pulmonary embolism resulted in the death of his father at age 48. Patient 7 was a 53-year-old man. At the age of 20 years, he suffered a hematogenous disorder in the lower extremities, and hypertension was diagnosed. At age 51, intestinal excision was performed for necrotic peritonitis caused by small-intestine thrombosis. At this time, the patient had already developed DVT. There was no family history of thrombosis. Patient 8 was a 51-year-old man with DVT of the left lower extremity diagnosed when he was 47 years of age. Any thrombus tendency in the family was unknown. Patient 9 was a 60-year-old woman who developed arterial thrombosis of the right lower extremity at the age of 54 years. She had DVT after an appendectomy at age 20. There were no thrombotic episodes in family members.

All 9 patients underwent warfarin anticoagulant therapy.

2.2. AT Assays

AT activity levels were determined by amidolytic heparin cofactor assay with chromogenic substrate (S-2238; Chromogenix Instrumentation Laboratory, Milan, Italy) for anti-IIa activity. AT antigen levels were determined by latex photometric immunology assay (LPIA; DIA-Iatoron, Tokyo, Japan).

2.3. Polymerase Chain Reaction

Genomic DNA was isolated from leukocytes by standard methods with protease K and phenol/chloroform extraction. After DNA extraction, all 7 exons, the splice junctions, and the 5′-flanking region of the AT gene were amplified by polymerase chain reaction (PCR) with *Taq* polymerase (*Takara Taq*; TaKaRa Shuzo, Shiga, Japan). All oligonucleotide primers used in this study were described previously [11]. Amplification was carried out in 100-μL reactions comprising 100 to 500 ng of genomic DNA, 100 pmol of each primer, 200 μM each of deoxynucleoside triphosphate, 1.25 mM $MgCl_2$, 50 mM KCl, 10 mM tris(hydroxymethyl)aminomethane HCl (pH 8.3), and 2 units of *Taq* DNA polymerase. Reactions were denatured at 95°C for 5 minutes and then amplified with 30 to 40 cycles consisting of denaturation at 94°C to 96°C for 30 seconds, annealing at 55°C to 65°C for 30 seconds, and extension at 72°C to 74°C for 30 seconds to 1 minute. For the last cycle, the extension time was increased to 10 minutes. After the reaction, the amplified products were electrophoresed on 3% NuSieve 3:1 agarose gels (BioWhittaker Molecular Applications, Rockland, ME, USA) for 1 hour 30 minutes at 120 V and visualized with ethidium bromide under UV transillumination.

2.4. DNA Sequence Analysis

PCR products were separated by electrophoresis on agarose gels and purified with the QIAquick gel extraction kit (Qiagen, Chatsworth, CA, USA). Purified templates were sequenced by the dideoxynucleotide chain termination method with a Thermo Sequenase premixed cycle sequencing kit (Amersham Life Science, Buckinghamshire, UK) according to the manufacturer's protocol and were analyzed with a Hitachi SQ5500 automated sequencer (Hitachi, Tokyo, Japan). Both nucleotide positions in the AT gene and amino acid residues in AT were numbered according to the method of Olds et al [1].

2.5. Restriction Fragment Length Polymorphism Analysis

We performed restriction fragment length polymorphism (RFLP) analysis to confirm the presence of each mutation or polymorphism. PCR products were digested with restriction enzymes (*Nla* III, *Dde* I, *Taq* I, *Bfa* I, *Hae* III, *Msp* I, and *Msc* I; New England Biolabs, Beverly, MA, USA) per the conditions suggested by the manufacturer. The digested bands were examined by electrophoresis, ethidium bromide staining, and UV transillumination.

2.6. Structural Analysis of AT Protein

A 3-dimensional model of AT was obtained from the Protein Data Bank (code 2ANT), and the native form was mod-

Table 1.
Results of Phenotype and Genotype Analyses*

Patient	Sex	Age†	Thrombotic Event	Antithrombin Activity, %	Antithrombin Antigen, %	Nucleotide Change	Exon	Amino Acid Change	Restriction Enzyme
1	M	38	DVT	53	55	72T>C	1	M-32T‡	*Nla* III
2	F	31	DVT, PE	35	39	2730T>A	2	M89K‡	*Dde* I
3	F	21	CI	62	76	5389C>T	3a	R132X	*Taq* I
4	F	14	DVT, PE	53	58	5433T>A	3a	L146H‡	
5	M	50	DVT, PE	59	49	5471C>T	3a	Q159X‡	*Bfa* I
6	M	17	DVT, PE	51	57	9819C>T	5	R359X	*Hae* III
7	M	20	DVT	44	50	13344T>C	6	L409P‡	*Msp* I
8	M	47	DVT	58	78	13295C>T	6	R393C	*Hae* III
9	F	20	DVT	59	92	13296G>A	6	R393H	*Msc* I

*DVT indicates deep vein thrombosis; PE, pulmonary embolism; CI, cerebral infarction.
†Age at onset of thrombosis.
‡Novel mutation.

ified as a template. Protein structure was analyzed with the molecular visualization program RasMol v2.5.

3. Results

3.1. Phenotype Analysis

AT activity, antigen levels, and thrombotic symptoms of the 9 study patients are shown in Table 1. Six patients (patients 1, 2, 4, 5, 6, and 7) showed reduced levels of both AT activity and antigen, and these patients were given the diagnosis of type 1 deficiency. Two patients (patients 8 and 9) showed a low level of AT activity in comparison with the level of antigen and were given the diagnosis of type 2 deficiency. Patient 3 had an AT activity level that was lower than the AT antigen level, but the AT deficiency type was not clear.

DVT had occurred in all except patient 3. Four patients (patients 2, 4, 5, and 6) were given the diagnosis of pulmonary embolism. In 6 patients (patients 2, 3, 4, 6, 7, and 9), the onset of thrombosis occurred at a young age, and the thrombosis was recurrent. In all patients except patient 7, venous thrombosis was not a result of underlying disease, such as hypertension, diabetes mellitus, or hyperlipidemia, or of lupus anticoagulant.

3.2. Genotype Analysis

Nine different point mutations, all in the heterozygous state, were found in our patients (Table 1). These mutations were not found by RFLP analysis or direct sequencing in DNA from 50 healthy subjects. Other sequence abnormalities were not detected by direct sequencing in the coding regions, splice junctions, or 5′-flanking region of the AT gene.

We found that the 9 point mutations included 5 novel and 4 previously reported mutations.

Five novel, 4 missense and 1 nonsense, distinct mutations were identified in the heterozygous state. In patient 1, the novel M-32T mutation was found in exon 1, which encodes the signal peptide for AT. In patient 7, the L409P mutation was detected in exon 6, which encodes the C-terminal end of the reactive site.

Four previously reported mutations also were found in the heterozygous state. Two distinct missense mutations in the P1 of the reactive site were detected, and 2 nonsense mutations were present elsewhere in the gene.

The G-to-A transition found in patient 9 created an *Msc* I restriction site (Figure 1). We performed *Msc* I RFLP analysis of patient 9's family; however, the abnormal digestion pattern seen in the proband was not found in other family members.

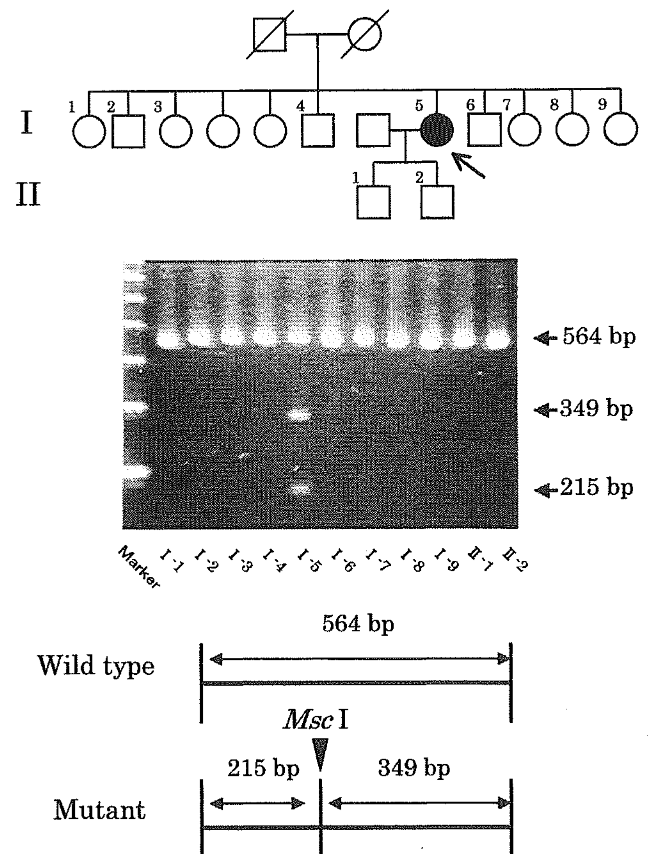

Figure 1. *Msc* I restriction digestion of exon 6 from patient 9 and family members. The G-to-A transition creates an *Msc* I restriction site, and digestion yields 349–base pair (bp) and 215-bp bands, which are present only in the proband. Arrow indicates the proband.

4. Discussion

Inherited AT deficiency has been recognized as a cause of thrombotic disease. The 9 patients in this series all developed thrombosis, DVT, pulmonary embolism, or CI. Injury, appendectomy, parturition, or surgery was a trigger for the thrombotic event in some of these patients.

A novel M-32T mutation was detected within the initiation codon (ATG) of the signal peptide in patient 1. Although approximately 100 distinct mutations in the coding region of the AT gene have been reported in type 1 deficiency, only 3 mutations, Y-16S, L-10P, and C-4X, occur within the signal peptide [12,13]. The signal peptide plays an important role in translation. Fitches et al [13] studied the central hydrophobic domain of the AT signal peptide and found that the L-10P mutant does not undergo posttranslational glycosylation and is not translocated to microsomes. The M-32T mutation in the initiation codon would likely have a significant effect on translation. It is possible that translation of the M-32T mutant does not begin at the original start position.

Novel M89L and L146H mutations were identified in exons 2 and 3a in patients 2 and 4, respectively. The amino acid substitutions that cause type 2 HBS deficiency occur in polar basic residues (arginine and lysine) in the N-terminal end, which encodes exon 2 and a part of exon 3a. These basic residues are located in helices A and D. Methionine and leucine are nonpolar neutral amino acids, and methionine 89 and leucine 146 are located on helix B and the sheet of strand 2A (s2A), respectively. AT antigen and activity levels decreased in parallel in these 2 cases. Therefore, type 1 deficiency was diagnosed in patients 2 and 4.

Twelve nonsense mutations in the AT gene, all of which cause type 1 deficiency, have been described [12]. We detected a novel Q159X mutation in patient 5. The R132X and R359X mutations identified in patients 3 and 6 were described previously by Perry et al [14] and Chowdhury et al [15], respectively. It was difficult to ascertain the deficiency type in patient 3 on the basis of phenotype alone because there was discrepancy between her AT antigen and activity levels. However, type 1 deficiency was diagnosed on the basis of the AT mutation detected.

Although patient 7 was classified as having type 1 deficiency on the basis of the approximately 50% AT antigen and activity levels, the novel L409P mutation detected in this patient is associated with type 2 PE deficiency.

It was recently suggested that deficiencies resulting from mutations in the distal hinge region should be called PE-type deficiencies [16]. The distal hinge region is formed by strand 1 of sheet C (s1C) and the turn connecting this strand to strand 4 of sheet B (s4B). Previously reported PE-type mutations were found on strands 1C, 4B, and 5B with RasMol software (Figure 2). Leucine 409 also is located on strand 4B near the amino acid residues associated with other PE-type deficiencies.

The mutated amino acid residues associated with PE-type deficiencies are highly homologous among serpins. Leucine 409 (P16′ position) of AT was also highly conserved with the corresponding position (P16′) in other serpins (Figure 3).

Eleven distinct PE-type cases have been reported in the AT mutation database [12], including the PE cases with approximately 50% AT antigen and activity levels reported herein. Other PE-type deficiencies, including A404T (AT Oslo 5) [10], P407L (AT Utah) [17], and R425T [12], have approximately 50% the normal level of AT antigen in plasma.

These findings suggest that the L409P case (patient 7) might be classified as a PE type because this mutation is located in the C-terminal portion of the reactive site, a location similar to that of other reported PE-type mutations, and is highly conserved in other serpins.

The missense mutations identified in patients 8 and 9 are both located in the P1 residue and are classified as type 2 RS deficiencies. It was reported that the 393C variant, which is similar to the mutation in patient 8, is linked by disulfide bonds to albumin [18-20]. Five Cys variants identified from the residue except for the P1 residue have been reported. AT Rouen (R24C) [21], Toyama (R47C) [22], and Rosny (P402C) [10] were reported as type 2 deficiencies, and R57C [15] and T166C [23] were reported as type 1 deficiencies. However, there are no data indicating that the Cys variant is linked by disulfide bonds to albumin. It appears that the center loop of the reactive site possesses a molecular structure that reacts easily with other proteins.

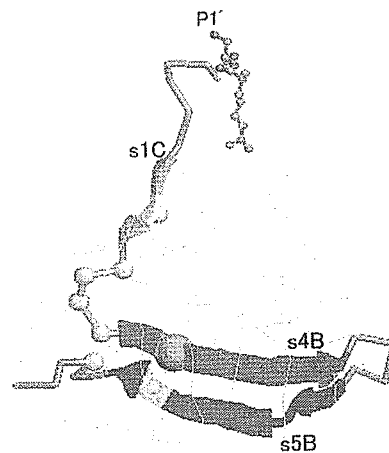

Figure 2. Schematic showing selected regions of the native form of intact antithrombin in the 3-dimensional structure (2ANT). The peptide main chain is indicated as the backbone. The distal hinge region is formed by strand s1C and the s1C-s4B turn. The reported pleiotropic effects–type mutations are marked in yellow. Strands 1C (purple), 4B (blue), and 5B (violet) are indicated. The position of Leu409 is indicated in red. The reactive sites (P1-P1′) are shown as balls and sticks.

	P1	P1′								P9′					P14′		P16′			
Residue No.	393	394								402					407		409			412
AT	R	S	L	N	P	N	R	V	T	F	K	A	N	R	P	F	L	V	F	I
HC II	L	S	T	Q	V	R	-	-	-	F	T	V	D	R	P	F	L	V	L	I
PCI	R	S	A	R	L	N	S	Q	R	V	-	F	N	R	P	F	L	M	F	I
α_1AT	M	S	I	P	P	E	-	-	-	V	K	F	N	K	P	F	V	F	L	M
α_1ACT	L	S	A	L	V	E	T	R	T	V	R	F	N	R	P	F	L	M	I	I
α_2AP	H	S	L	S	S	-	-	-	-	F	S	V	N	R	P	F	L	V	F	I
tPAI	R	M	A	P	E	E	-	-	-	I	I	H	D	R	P	F	L	M	V	V
C1I	R	T	L	L	V	F	E	-	-	V	Q	Q	-	-	P	F	L	M	V	L
Ovalbumin	A	S	V	S	E	E	-	-	-	F	R	A	D	H	P	F	L	F	C	I

Residue No.	413												425				429
AT	R	E	V	P	L	N	T	I	I	F	M	G	R	V	A	N	P
HC II	Y	E	H	R	T	S	C	L	L	F	M	G	R	V	A	N	P
PCI	V	-	-	D	-	H	H	I	L	F	L	G	K	V	H	R	P
α_1AT	I	E	Q	H	T	K	S	P	L	F	M	G	K	V	V	N	P
α_1ACT	V	P	T	D	T	Q	H	I	F	F	M	S	K	V	T	N	P
α_2AP	F	E	D	T	T	G	L	P	L	F	V	G	S	V	R	N	P
tPAI	R	H	N	P	T	G	T	V	L	F	M	G	Q	V	H	E	P
C1I	V	P	Q	Q	H	K	P	V	-	F	M	G	R	V	Y	D	P
Ovalbumin	K	H	I	A	T	H	A	V	L	F	F	G	R	C	V	S	P

Figure 3. Amino acid alignment of the C-terminal regions of serpins numbered according to antithrombin (AT). AT amino acid substitutions are boxed. HC indicates heparin cofactor II; PCI, protein C inhibitor; α_1AT, α_1-antitrypsin; α_1ACT, α_1-antichymotrypsin; α_2AP, α_2-antiplasmin; tPAI, tissue plasminogen activator inhibitor; C1I, C1 inhibitor; ovalbumin, chicken ovalbumin.

We have described 9 mutations in the AT gene isolated from unrelated Japanese patients and the relation between the locations of these mutations and structural abnormalities in the AT molecule.

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RESEARCH ARTICLE

Efficient gene transfer of a simian immunodeficiency viral vector into cardiomyocytes derived from primate embryonic stem cells

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Abstract

Background Embryonic stem (ES) cells continually proliferate and can generate large numbers of differentiated cells. Genetic manipulation of transplantable cells derived from primate ES cells offers considerable potential for development research and regenerative cell therapy. However, protocols for efficient gene transfer into primate ES-cell-derived cells have not yet been established.

Methods Spontaneously contracting areas were derived from cynomolgus monkey ES cells. Features of cardiomyocytes in the area were analyzed according to gene expression (RT-PCR), morphology (immunostaining and electron microscopy), and function (intracellular calcium transience). Beating cells were transduced using a simian immunodeficiency virus (SIV) vector expressing enhanced green fluorescence protein (EGFP), then transplanted into ischemic rat myocardium.

Results Beating cells derived from monkey ES cells displayed gene expression, ultrastructural and functional properties of early-stage cardiomyocytes. Highly efficient (97% cardiac phenotype) and stable transduction of these ES-cell-derived cardiomyocytes was achieved using SIV vector without altering contractile function. In addition, transduced cardiomyocytes survived in the myocardium of a rat myocardial infarction model.

Conclusions A lentiviral vector system based on SIV represents a useful vehicle for genetic modification of cardiomyocytes derived from primate ES cells, and can extend the application of primate ES cells to gene therapy. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords embryonic stem cell; cardiomyocyte; simian immunodeficiency virus; cell transplantation; myocardial infarction

Introduction

The generation of various differentiated cells from pluripotent embryonic stem (ES) cells provides a renewable resource not only for studying the mechanism of early development *in vitro*, but also for cell transplantation therapy. Among many specialized cells in adults, cardiomyocytes are terminally differentiated and have no or only limited regenerative capacity after injury such as myocardial infarction [1]. Thus, the transplantation of functional cardiomyocytes into the damaged myocardium would have therapeutic potential. Recent studies have demonstrated that human ES cells can differentiate into cardiomyocytes with structural and functional properties *in vitro* [2–5]. Although human ES cells hold promise for clinical applications, an alternative model system based on ES cells derived from

experimental animals may be necessary for pre-clinical studies, including allogenic transplantation. We established cynomolgus monkey (*Macaca fascicularis*) ES cell lines [6] that are similar to human ES cells but distinct from murine ES cells in terms of morphology, expression of surface markers, feeder- and leukemia inhibitory factor-dependence and other factors. These features indicate that cynomolgus ES cells represent a suitable pre-clinical model for cell transplantation therapy.

Gene transfer into transplantable cells has potential to enhance the effects of cell replacement therapy. Although murine ES cells can be transduced by electroporation or mouse stem cell virus (MSCV)-based retroviral vectors [7–9], primate ES cells are not efficiently transduced by these methods [10]. Lentiviral vectors can transduce both dividing and non-dividing cells and long-term expression of the transgene is stable in a wide range of target cells [11–13]. We have described the highly efficient transfer of a gene into cynomolgus monkey undifferentiated ES cells using a lentivirus vector based on simian immunodeficiency virus (SIV) [14].

The present study examines the differentiation of cynomolgus ES cells into functional cardiomyocytes and determines the efficiency and stability of gene transduction into these cardiomyocytes using an SIV-based lentiviral vector encoding the enhanced green fluorescence protein (EGFP) gene. We also evaluate the survival of transplanted cardiomyocytes derived from cynomolgus ES cells in the injured myocardium of a rat myocardial infarction model.

Materials and methods

Cell preparations

The cynomolgus monkey ES cell line CMK6 [6] was cultured in DMEM/F12 (Sigma, St. Louis, USA) on a mouse embryonic fibroblast feeder layer that was mitotically inactivated with mitomycin C (Kyowa, Tokyo, Japan). The medium was supplemented with 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine (Invitrogen, Carlsbad, USA), 1 mM sodium pyruvate (Invitrogen) and 15% fetal bovine serum (FBS, ICN Biomedicals, Inc., Ohio, USA). To induce differentiation, ES cells were dispersed into small clumps using collagenase IV (Wako, Osaka, Japan), transferred to plastic Petri dishes and suspension-cultured for 10 days. During this period, the cells aggregated to form embryoid bodies (EBs), which were then plated on plastic plates, and the appearance of spontaneous contractions was observed under a microscope. Rat neonatal cardiomyocytes were prepared from cardiac ventricles of 1-day-old Sprague-Dawley rats as described previously [15]. The cells were grown in DMEM (Sigma) supplemented with 10% FBS (ICN Biomedicals, Inc.), and 1% penicillin/streptomycin solution (Invitrogen). All experiments were carried out in full compliance with the institutional animal care and use committee of the Jichi Medical School.

RT-PCR

Total RNA from undifferentiated ES cells, contracting EBs, and heart tissue of an adult cynomolgus monkey that was killed for unrelated reasons was extracted using a RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA synthesized from 1 μg total RNA using SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) was amplified by PCR using the following primers selective for human cardiac genes (oligonucleotide sequences are given in brackets in the order of anti-sense, sense primer followed by the annealing temperature, cycles used for PCR and length of the amplified fragment): cardiac troponin T (cTnT, 5′-GGCAGCGGAAGAGGATGCTGAA and 5′-GAGGCACCAAGTTGGGCATGAACGA; 60 °C; 35 cycles; 150 bp), atrial myosin light chain (MLC-2A, 5′-ACAGAGTTTATTGAGGTGCCCC and 5′-AAGGTGAAGTG-TCCCAGAGG; 61 °C; 35 cycles; 381 bp), ventricular myosin light chain (MLC-2V, 5′-TATTGGAACATGGCCTC-TGGAT and 5′-GGTGCTGAAGGCTGATTACGTT; 61 °C; 35 cycles; 382 bp), α-myosin heavy chain (αMHC, 5′-GTCATTGCTGAAACCGAGAATG and 5′-GCAAAGTACTG-GATGACACGCT; 61 °C; 40 cycles; 413 bp), octamer-binding protein 4 (Oct-4, 5′-GAGAACAATGAGAACCTTC-AGGAGA and 5′-TTCTGGCGCCGGTTACAGAACCA; 55 °C; 35 cycles; 219 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5′-ATGCCAGTGAGCTTCCCGTT and 5′-CATCACCATCTTCCAGGAGC; 58 °C; 30 cycles; 473 bp).

Electron microscopy

For transmission electron microscopy, tissues were fixed in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, at 4 °C for 24 h, postfixed in 1% OsO_4 in PBS for 1 h, dehydrated in a graded ethanol series and embedded in Epon 812. Thin (60–90 nm) sections were stained with uranyl acetate and lead citrate, and observed using a JEM-2000EX transmission electron microscope operating at 80 kV.

Intracellular calcium transience

Intracellular calcium transience of the EBs and rat neonatal cardiomyocytes was measured as described previously [16]. Briefly, cells were loaded with fura-2-AM (Dojin Biochemicals, Kumamoto, Japan), washed and transferred to the chamber of a fluorescence spectrophotometer (CAF-100; Japan Spectroscopic Co., Tokyo, Japan). Fura-2 fluorescence was measured using a dual wavelength system. Fluorescence was monitored at 500 nm, with excitation at 340 and 380 nm in the ratio mode. After achieving a stable fluorescence signal, the cells were stimulated with 100 nM angiotensin II (Sigma) or endothelin-1 (Peptide Institute Inc., Osaka, Japan). The cells pretreated with [Sar^1,Ile^8]-angiotensin II (non-selective antagonist; Peptide Institute Inc.) or CV-11 974

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(angiotensin II type 1 receptor antagonist; kind gift from Takeda Chemical Industries, Ltd., Osaka, Japan) were also stimulated with angiotensin II.

Immunohistochemical staining

Contracting areas in EBs were mechanically dissected using a sterile micropipette. The samples were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) containing 8% sucrose at 4 °C for 4 h, washed with PBS containing 10, 20, and 30% sucrose in that order, embedded in OCT compound (Miles Laboratory, IN, USA), frozen in liquid nitrogen and cut into thin (8–10 μm) sections. We incubated the sections with monoclonal antibodies against human cardiac troponin I (cTnI) and cardiac myosin (both from Biogenesis, England, UK; diluted 1 : 200) for 1 h at room temperature. These anti-human antibodies cross-react against cynomolgus monkey cardiomyocytes but not against rat cardiomyocytes. Texas red labelled anti mouse IgG (Vector, Burlingame, CA, USA) was the secondary antibody. Samples were fixed in 4% paraformaldehyde in PBS for 20 min and immersed in 4′,6-diamidino-2-phenylindole (DAPI, 500 ng/ml; Sigma) containing Tris buffer (pH 7.4) for 10 min at room temperature to stain nuclei.

Vector construction and transduction

The SIV vector expressing the EGFP gene (Clontech, CA, USA) was produced by transient transfection into 293T cells as described [17]. Briefly, the envelope plasmid (pVSV-G; Clontech) encoding the vesicular stomatitis virus G (VSV-G) protein, the packaging plasmid (pCAGGS/Sagm-gtr), and the vector plasmid (pBS/CG2-Rc/s-CMV-ΔU) expressing the EGFP gene under the control of the cytomegalovirus (CMV) promoter were transfected into 293T cells and supernatants were harvested 48 h later. The SIV vector was concentrated by centrifugation of the supernatants at 42 500 *g* for 90 min and the titer assessed by fluorescence activated cell sorting (FACS) using 293T cells as targets was 1.87×10^8 TU/ml.

Spontaneously contracting EBs were transduced with SIV vector expressing the EGFP gene at a multiplicity of infection (MOI) of 100. Cells were washed with PBS 10 h later and incubated in fresh medium for 7–14 days. Contracting EBs were micro-dissected with a sterile micropipette and prepared for immunohistological analysis. Other dissected cells were enzymatically dispersed in trypsin/EDTA solution (0.05% trypsin, 0.53 mM EDTA; Invitrogen) for 7 min at 37 °C and resuspended in DMEM/F12 for use in cell transplantation experiments. To determine the transduction efficiency with the SIV vector, some dissociated cells were centrifuged with Cytospin and stained with anti-cTnI antibody. We then calculated the ratio (%) of EGFP-positive cells among cTnI-immunoreactive cells.

Cell transplantation in a rat myocardial infarction model

Immunodeficient (F344/N *rnu/rnu*) nude rats (male, initial body weight 140–180 g) were used ($n = 4$) to avoid graft-versus-host disease. Left thoracotomy proceeded under general anesthesia, then the pericardium was opened and the left descending coronary artery was ligated. Cynomolgus ES-cell-derived cardiomyocytes expressing EGFP (1×10^5 cells/50 μl) were implanted in the injured myocardium 30 min after myocardial infarction induction. Two weeks later, rats were killed and hearts were extracted for immunohistological study. All experiments in this study were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

Statistical analysis

Results are presented as mean ± SD. For comparisons between multiple groups, we determined the significance of differences between group means by ANOVA using the least significant difference for multiple comparisons. Differences at values of $p < 0.05$ were considered to be statistically significant.

Results

Differentiation of cynomolgus ES cells into cardiomyocytes

Rhythmically contracting areas appeared between 3 and 10 days after plating EBs on plastic plates, and were maintained for 3–4 weeks. Figure 1A shows that the ratio of EBs containing beating areas as a function of time after plating was 8.7% (of 751 EBs) at 14 days.

We investigated the expression of cardiac-specific genes in spontaneously contracting EBs. The RT-PCR results revealed that these cells expressed cTnT, MLC-2A, MLC-2V, and αMHC (Figure 1B). In contrast, Oct-4, a marker of undifferentiated ES cells, was expressed in undifferentiated ES cells, but not in contracting EBs or cynomolgus heart tissues. Light microscopy showed that the contracting areas were composed mainly of round or rod-shaped mononuclear cells. Myofibers were detected in the high power light microscopy image stained with toluidine blue (Figure 2A). Transmission electron microscopy revealed that these cells had the mature sarcomeric organization and desmosome structure of cardiomyocytes (Figures 2B–2D). To further elucidate whether these cells have functional features as cardiomyocytes, we measured the effect of angiotensin II or endothelin-1 on intracellular calcium transience. Intracellular calcium transience was obviously stimulated with angiotensin II or endothelin-1. The angiotensin II stimulated effect was completely inhibited by pretreatment with [Sar^1,Ile^8]-angiotensin II

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