It has been reported that the level of e-XDP, which are the fibrin degradation products by leukocyte elastase released from activated leukocytes [31], is useful for the diagnosis and prognosis of disseminated intravascular coagulation [32–34] and the diagnosis of symptomatic DVT [35]. However, no previous studies have measured the e-XDP level before and after orthopaedic surgery to evaluate its usefulness for diagnosis of postoperative VTE. In this study, the e-XDP level at postoperative day 1 was significantly higher in the thrombus group than that in the no-thrombus group. The diagnostic sensitivity, specificity and likelihood ratio of the e-XDP level at postoperative day 1 using a cut-off point of 8.2 U/ml were 75%, 59% and 1.84, respectively, for predicting postoperative asymptomatic VTE. Therefore, the e-XDP level at postoperative day 1 is estimated to be a blood marker for early prediction of postoperative asymptomatic VTE. Furthermore, by multiple logistic regression analysis, the e-XDP level at postoperative day 1 differed significantly among the other factors and there were no significant differences in age, sex, volume of intraoperative haemorrhage, operation time or other presurgical factors, except for the minimal blood pressure, or blood markers between the thrombus and no-thrombus groups. Therefore, this study has established that the e-XDP level at postoperative day 1 and the D-dimer level at postoperative day 4 are associated with asymptomatic VTE after TKA and are independent markers for predicting the postoperative occurrence of new asymptomatic VTE. Recent studies have demonstrated that leukocyte elastase-mediated fibrinolysis is activated to varying degrees depending on the amount of systemic inflammation, such as a major surgical procedure, and sepsis as an alternative pathway to the plasminogen activator-plasmin system [32–36]. We consider that leukocyte elastase-mediated fibrinolysis may have been mainly activated in the early phase as an alternative pathway, and then plasmin may have been mainly activated in the late phase as the plasminogen activator-plasmin system in patients who developed asymptomatic VTE after TKA. If leukocyte elastase causes asymptomatic VTE and subsequently symptomatic and fatal PE, inactivation of leukocyte elastase may prevent the development of symptomatic and fatal PE.

The postoperative occurrence of new asymptomatic VTE could not be predicted from the preoperative SFMC, D-dimer or e-XDP levels. Dunn et al. [37], who performed venography after TKA or THA, and Bounameaux et al. [30], who employed venography after TKA, compared the preoperative D-dimer levels between patients who did and did not develop DVT postoperatively and reported no significant differences. Our results are consistent with these previous reports, and we could not establish that preoperative blood coagulation markers are associated with VTE after TKA.

In this study, the early diagnosis of new asymptomatic VTE that can be predicted by the D-dimer level at postoperative day 4 and the e-XDP level at postoperative day 1 after TKA may prevent PE development after TKA. Therefore, in daily clinical practice, we consider that measurements of e-XDP at postoperative day 1 and D-dimer at postoperative day 4 are necessary for early detection of asymptomatic VTE in patients who have low risk factors because they are independent markers. However, we need to further verify these measurements in larger studies to determine the adequate cut-off points, sensitivities and specificities of e-XDP at postoperative day 1 and D-dimer at postoperative day 4 for preventing symptomatic and fatal PE in the perisurgical period. One limitation of our study is that we do not know whether early detection of asymptomatic VTE prevents symptomatic and fatal PE. Therefore, we have continued to follow up the patients in daily clinics after completing this study, and no patients have suffered from symptomatic and fatal PE to date. Another limitation of our study is that MDCT was performed 4 days preoperatively and postoperatively, and the results therefore reflect the state of asymptomatic VTE at these time points. We believe that almost all asymptomatic VTEs may disappear spontaneously within 2 or 3 days postoperatively and that an asymptomatic VTE that does not disappear spontaneously within 2 or 3 days postoperatively can cause symptomatic and fatal PE in low-risk patients after TKA. This is because the incidence of PE was reported to be high at days 3 or 4 postoperatively after TKA [15]. Furthermore, that is the earliest point at which the patients had less pain and could comfortably undergo MDCT during the postoperative period. However, since MDCT was not performed from the operative day to postoperative day 3, it can be assumed that not all asymptomatic VTE were detected in the perisurgical period. Therefore, the incidence of postsurgical asymptomatic VTE may be underestimated, and the roles of e-XDP and D-dimer levels in predicting asymptomatic VTE in the perisurgical period must be further verified in larger studies.

Conflict of interest

The authors state that they have no conflict of interest.

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第72回日本血液学会学術集会
シンポジウム4
Recent Advance in Thrombosis, Hemostatis, and Vascular Biology: From a Clinical Aspect

血友病に対する遺伝子細胞治療—最近の進歩

三室 淳，坂田洋一

Key words: Hemophilia, Gene therapy, Adeno-associated virus vector, Lentivirus vector

1. はじめに

現在行われている血友病の治療は，出血時に出血時に凝固因子製剤を投与するオンデマンド治療が大部分で，定期補充療法は一部の症例に行われているにすぎない。凝固因子の定期補充療法は出血リスクを減らすが週2ないし3回の凝固因子製剤の静脈注射を行う必要がある。凝固因子を体内で産生し治癒にも繋がる血友病遺伝子治療も精力的に研究され，臨床治験もいくつか行われてきた。ほとんどの臨床治験は十分な効果が得られていなかったが，最近始められた血友病B遺伝子治療では有効であるとの報告がされていることから，遺伝子治療は血友病の次世代治療法として期待されている。

しかし，遺伝子治療が完全に安全であるということではない。種々の疾患に対する次世代治療法として遺伝子治療が研究されてきた。レトロウイルスベクターをもちいγc遺伝子を導入する重症免疫不全症X-SCIDの臨床治験は最も成功した遺伝子治療であるが，T細胞白血病様の病態がおこるという重大な副作用も生じた[1~3]。この事象は血友病遺伝子治療には当てはまらないと考えられているが，遺伝子治療に附随する安全性の問題は常に検証されなくてはならないことである[4]。

2. 血友病遺伝子治療のクリニカルエンドポイント

血友病は重症（欠乏する凝固因子活性1%未満），中等症（欠乏する凝固因子活性1~5%未満），軽症（欠乏する凝固因子活性5%以上）に分けられ，重症では日常生活においても出血が起こるが，中等症では重症出血の頻度は低く，軽症例では日常生活ではほとんど出血しない。これらの臨床経験を踏まえて，血友病遺伝子治療の対象は重症例であり，エンドポイントは欠乏する凝固因子活性を1%以上に，理想的には5%以上に上昇させることである。このように，血友病治療として恒常的に発現させる目標凝固因子レベルの治療域が広く遺伝子治療の良い適応といえる。

自治医科大学分子病態研究部

3. 血友病遺伝子細胞治療の戦略

血友病はX染色体上の第VIII因子遺伝子あるいは第IX因子遺伝子の異常によりこれらの凝固因子欠乏が起こるため，現在の血友病の遺伝子治療研究は正常凝固因子遺伝子を導入し正常凝固因子を産生させることに主眼がおかれている。もちろん将来的には遺伝子異常の是正のような究極のストラテジーも期待出来る。

正常凝固因子を生体内で発現させる方法は，凝固因子遺伝子を搭載したベクターを直接生体に投与する体内法と，凝固因子を発現する細胞を移植する体外法に大別できる（図1）。

1）体外法におけるベクターの選択

ベクターはウイルスベクターと非ウイルスベクターに大別できる。ウイルスベクターは，遺伝子導入効率は優れるが免疫系との確執がある。すなわち，ヒトは病原体として認識するウイルスを排除するように，また次の感染に対処するように反応する。一方，ウイルスは宿主に感染し自己増殖する。このためウイルスベクターは細胞へ感染し遺伝子導入をおこなう効率が高いが免疫系を活性化する。一方，非ウイルスベクターは免疫系との反応は少ないと考えられているが遺伝子導入効率は低く，現在の遺伝子治療研究はウイルスベクターを用いる方法が主流である。

生体に直接投与可能なベクターには，(1) 遺伝子導入効率が良い (2) 染色体に組み込まれない (3) 非分裂細胞にも遺伝子導入可能である (4) 病原性がない，など

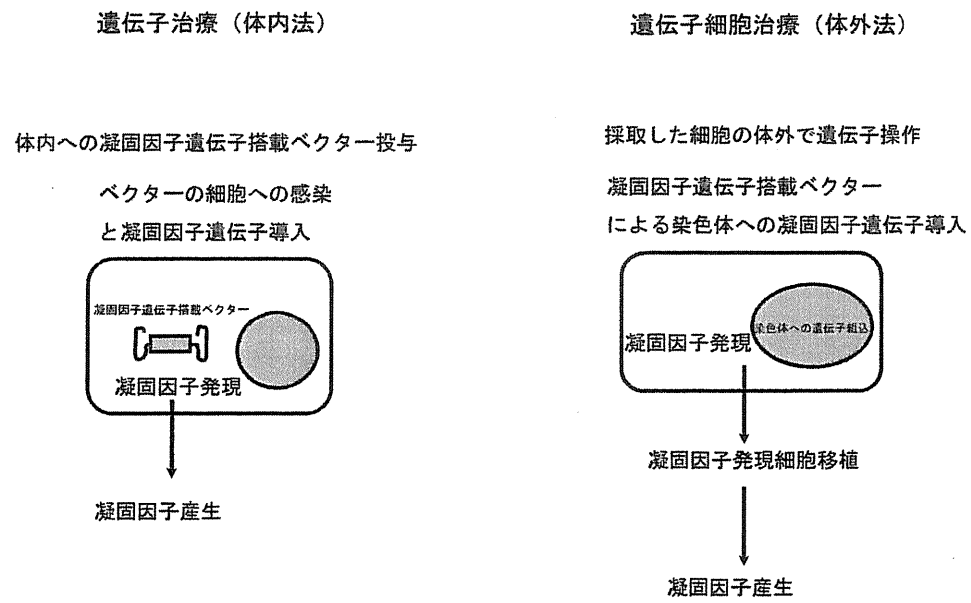

図 1　遺伝子・細胞治療
治療遺伝子搭載ベクターを直接体内に投与する体内法と，遺伝子操作を加えた細胞を移植する体外法を模式的に示す。

の性質が要求される。遺伝子治療研究の初期に用いられていたアデノウイルスベクターは病原性，血液障害性，さらに免疫系への影響が強く，レトロウイルスベクターとレンチウイルスベクターは染色体への組込が必至である。これらのベクターと異なり，アデノ随伴ウイルス（adeno-associated virus, AAV）ベクター（図 2）は上記条件を満たすことから遺伝子治療研究と臨床治験に用いられている。パーキンソン病遺伝子治療を始めとするAAV ベクターを用いた臨床治験が行われ，成果が得られている。ベクター開発が進み，血友病遺伝子治療研究では実験動物レベルで凝固因子の正常域からそれ以上までの発現も可能となり，最近の血友病 B 遺伝子治療臨床治験でも成果が得られつつある。

(1) アデノ随伴ウイルス（Adeno-associated virus, AAV）ベクター

非病原性のパルボウイルスの AAV に由来する AAV ベクターは，両端に Inverted terminal repeat（ITR）配列を残すのみで，他の大部分のウイルス由来遺伝子が除去されプロモーター・治療遺伝子・poly A 付加配列に置換されている（図 1）。自己複製能はなく，染色体への組込がほとんど起こらないため安全性が高く，さらに非分裂細胞にも遺伝子導入可能で導入遺伝子の長期発現が期待できるなど，遺伝子治療のベクターとしての適性を備えている。また，AAV 血清型（serotype）により細胞・臓器特異性が異なり，標的臓器に適した血清型 AAV ベクターを選択することが出来る。AAV serotype 2 (AAV2) ベクターは，血友病 B 遺伝子治療の臨床治験にも用いられてきたが，肝臓を遺伝子導入のターゲットとするには AAV5 ベクターや AAV8 ベクターが適していると考えられる。しかし，搭載可能な遺伝子サイズには依然として全長で約 5 kb の制限がある。FVIII 遺伝子を分割して AAV ベクターに搭載する dual vector system では同一細胞内で FVIII 重鎖と FVIII 軽鎖が発現し結合しなくてはならず，効率が悪い。極短い塩基長のミニマムプロモーターと BDDFVIII cDNA を用いた single vector system などの試みがなされ（図 2），肝臓への遺伝子導入効率が高い AAV8 ベクターと発現効率の高いミニマムプロモーターを用いることで高いレベルの第 VIII 因子発現が期待できる[5,6]。

(2) レンチウイルスベクター

従来用いられてきた MLV レトロウイルスベクターと比較し，分裂細胞，非分裂細胞のどちらにも効率の良い遺伝子導入が可能である。染色体への組み込みがおこり，分裂細胞へ治療遺伝子が組み込まれれば安定した治療遺伝子の発現が期待できる。レンチウイルスベクターとして human immunodeficiency virus（HIV）由来のベクターが詳細に検討され，第三世代化されほとんどのウイルス由来の遺伝子は除かれ LTR も改変され自己不活化（SIN vector）されている。central poly purine tract や WPRE などを組み込むことで遺伝子導入効率や発現効率が著しく改善し，さらにインシュレーターを組み込むことで安全性を高めている。第三世代化 HIV ベク

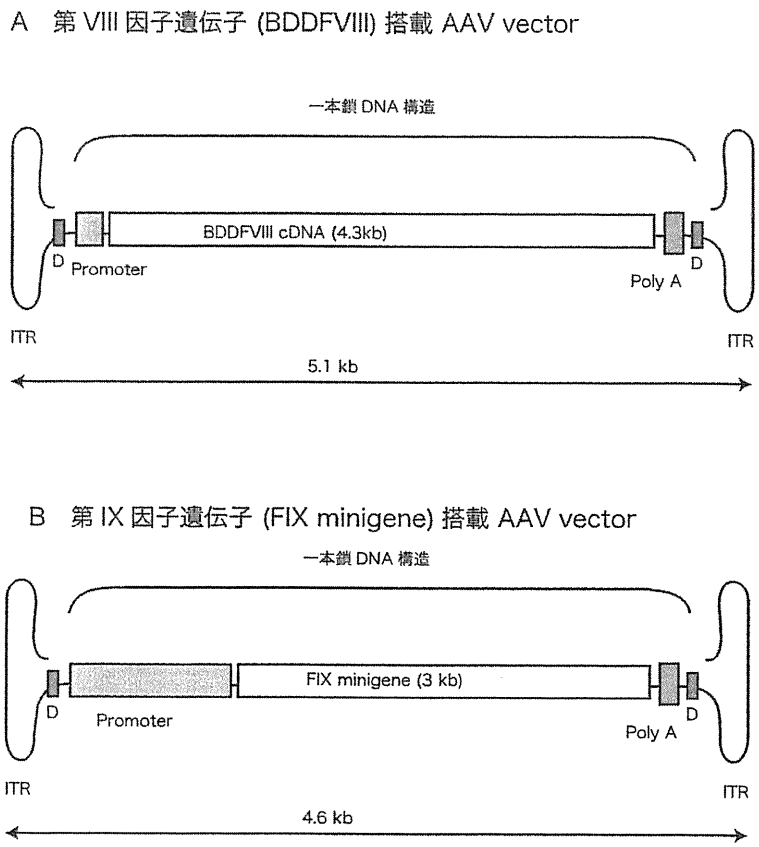

図 2 AAV ベクター

AAV は本来一本鎖 DNA ウイルスで，一般的に AAV ベクターといえば一本鎖 AAV ベクター single strand AAV vector を指す。inverted terminal repeat（ITR）配列に挟まれるように promoter–治療遺伝子–polyA 付加配列と並んでいる。AAV は複製の過程で二量体構造をとるが，AAV Rep の作用により一本鎖 DNA となり Capsid 内にパッケージされ AAV ベクターとなる。

ベクターが感染した細胞内で一本鎖 DNA として放出されるが，ベクター同士が結合あるいは相補鎖が合成されることで二本鎖となり効率のよい遺伝子発現が得られる。

A: 第 VIII 因子遺伝子搭載 AAV ベクター

AAV ベクターは搭載遺伝子長が約 5 kb に限られ，BDDFVIII cDNA は 4.3 kb あるため，0.3 kb までの極短い塩基長プロモーターしか用いることが出来ない。

B: 第 IX 因子遺伝子搭載 AAV ベクター

FIX 遺伝子搭載 AAV ベクターは FIX 遺伝子（イントロン配列を含む minigene）が約 3 kb であるため 1 kb 以上のプロモーターを用いることが出来る。

ターがサラセミア遺伝子治療臨床治験に用いられ治療効果が得られている[7~9]。しかし，遺伝子治療による HMGA2 遺伝子の活性化も起こっている。これ自体は治療に結びつくものであり白血病を引き起こすことに繋がっていないが，いわゆる挿入変異に関連する事象と考えられる。HIV ベクター以外では simian immunodeficiency virus（SIV）由来のベクターなどが検討されている。我々も SIV ベクターを利用して，CD34 陽性細胞への効率の良い遺伝子導入[10]と高い第 VIII 因子発現を得ることが出来た（図 3）。この方法を発展させ，血小板へ第 VIII 因子あるいは活性型第 VII 因子を，血小板プロモーターを用いて特異的に発現させることで血友病 A

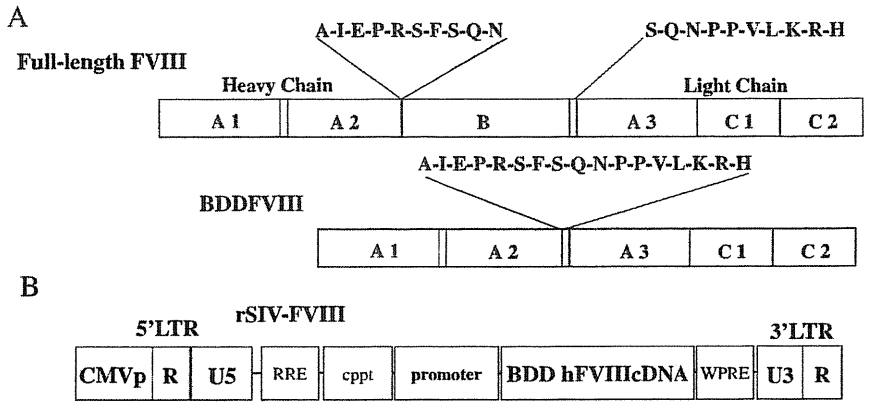

図３ 第 VIII 因子遺伝子搭載 SIV ベクター
A: 完全長 FVIII と B ドメイン欠如型 FVIII (B domain deleted FVIII, BDDFVIII) の構造を模式的に示す。B ドメインは細胞で産生される時に部分的に切除され，活性化反応で完全に除去され，FVIII 活性発現には直接は関係しない。FVIII cDNA には遺伝子発現を抑制する配列が B ドメインをコードする塩基配列にあるため，BDDFVIII を発現させる BDDFVIII cDNA が血友病 A 遺伝子治療研究で主として用いられている
B: 第 VIII 因子遺伝子を搭載する第三世代化された自己不活型 SIV ベクターを模式的に示す。LTR は修飾され自己不活型となり，遺伝子発現に重要な central poly purine tract (cppt) と遺伝子発現増強のための WPRE 配列を搭載している。BDDFVIII 遺伝子を搭載するように示してあるが，B ドメインを部分的に欠如する第 VIII 因子遺伝子も搭載可能である。

マウスの出血傾向を改善させることができ[11,12]，自己骨髄移植を応用した血友病 A 遺伝子治療の可能性を示し得た。また，同じベクターを用いて脂肪細胞への第 VIII 因子遺伝子導入と第 VIII 因子の発現にも成功している[13]。SIV ベクターは，まもなく眼科領域での臨床治験に用いられることになっており，標的細胞によっては良いベクターといえる。

(3) アデノウイルスベクター

アデノウイルスベクターは，免疫原性が高いこと，肝臓障害や造血障害が発生することが問題とされてきた。アデノウイルス由来の遺伝子の大部分を除去し，ベクター投与に伴う免疫反応や細胞障害性を低減した第 2 世代ベクター (Gutless adenovirus vector) を用い，血友病 A モデルマウスと血友病 A イヌに対してイヌ第 VIII 因子遺伝子を導入し有効性が示されているがヒトへ傷害性のない投与量までは減量できていない[14]。ポリエチレングリコール付加などの化学修飾で生体反応を軽減されたアデノウイルスベクターの開発が試みられているとされていたがその後の報告はない。

2) 体外法における細胞とベクターの選択

免疫学的排除を考えると，自己細胞移植が選択される。細胞治療においても細胞に遺伝子操作を加える必要がある。ウイルスベクターも非ウイルスベクターも用いることができるが，細胞治療においては染色体へ治療遺伝子を組み込む必要がある。遺伝子導入に伴う変異の影響を最小限にすることと，導入遺伝子のサイレンシングは解決されなくてはならない問題である。移植後の細胞寿命も考慮すると幹細胞の移植が，また移植後の増殖を懸念すれば幹細胞で遺伝子導入し終末分化させて移植する方法が考えられる。ES 細胞や iPS 細胞も研究されているが，腫瘍形成性の問題が解決される必要がある。ただし，遺伝子導入した自己 iPS 細胞由来の血小板や赤血球などは無核で腫瘍形成性の問題がなく，将来的には細胞治療の標的となりえる可能性を秘めている。

4. 血友病 A 遺伝子治療

1) 治療遺伝子における最近の進歩

(1) 改変第 VIII 因子遺伝子

第 VIII 因子 (FVIII) 遺伝子 (cDNA) は翻訳領域でも 7.1 kb あり，ウイルスベクターへの搭載も困難であること，また B domain をコードする領域を除くと FVIII の発現も増加することから，B domain をコードする領域をほぼ完全に除いた B domain deleted FVIII cDNA (BDDFVIII cDNA) が一般的に用いられてきた。B domain の様々な領域を残した FVIII の発現を検討した結果，B domain を部分的に残すと，B domain を完全

に除いたBDDFVIII cDNAよりもFVIIIの発現が上昇するとの報告があるが[15]，これに対しては異論もある[16,17]。発現されるFVIII分子のBドメインの配列によりインヒビター発生も差異があるとされるが，ヒトでインヒビターが出来やすいのはBドメインではないので疑問もある。いずれにしても，遺伝子導入に際し，完全長のFVIII cDNAを用いるよりもB domainをコードする塩基配列を部分的ないし完全に除去するとFVIII発現が上昇するのは間違いない。また，BDDFVIIIは組換第VIII因子製剤として既に臨床で用いられており，治療効果あるいはインヒビター発生率ともnative FVIIIと差がないことが報告されていることから治療遺伝子としてBDDFVIII cDNAを用いることには問題はないと考えられる。さらにコドンの最適化や発現抑制に働く可能性のある配列を変更することで遺伝子発現が数倍に上昇することが報告され[17]，今後は最適化された塩基配列のFVIII遺伝子が用いられる可能性がある。

(2) 改変第VII因子遺伝子

活性化第VII因子は，高濃度では第IX因子（FIX），FVIIIに依存せずに第X因子を活性化することができるため，インヒビターのある血友病患者の出血時の治療に使用されている。この理論に基づき，改変第VII因子遺伝子を用いて活性化型第VII因子を発現させる試みが血友病Bモデルの第IX因子欠損マウスで行われた。同じく，AAVベクターでイヌ活性化第VII因子を発現させ，血友病Aイヌおよび血友病Bイヌでも試みられ，止血効果がえられている[18]。

2) ベクターの進歩

非ウイルスベクターとしてhyaluronanとasialoorsomucoidでコートされたnano-capsuleにFVIII遺伝子を内包したベクターを血友病Aマウスに投与しtransposonを用いてFVIII発現が得られた報告があり，今後の展開が期待されるがtransposonを用いることによる染色体へのランダムな遺伝子組込の懸念は依然として残る[19]。

3) 遺伝子導入の標的臓器の最新の知見

第VIII因子の細胞内輸送にはERGIC-53（シャペロン分子）などが関わっており，第VIII因子の細胞からの分泌が全ての細胞でも効率よく行われるかは明らかでない。BDD FVIIIの細胞内輸送が完全長FVIIIと同じか否かも明らかではない[13]。第VIII因子遺伝子導入が高効率に行われても必ずしもFVIIIが血流へスムースに移行するとは限らず，FVIII遺伝子導入の標的細胞・臓器は慎重に選択する必要がある。肝臓，血液細胞，脂肪細胞，骨格筋，血管内皮細胞などが標的細胞・臓器として考えられている。

4) 血友病A遺伝子治療の臨床治験の最新の知見

現在までに3つの臨床治験（TKTトライアル，Chironトライアル，アデノウイルスベクターをもちいたトライアル）が行われている[14]。一時的な効果が認められたが，いずれも1年以上に及ぶ長期間の第VIII因子の上昇は得られていない。これ以後血友病A遺伝子治療臨床治験は行われていないのが現状である。遺伝子導入方法，標的組織・臓器，ベクター，プロモーターの選択と改良が必要と考えられ，今後の展開は，AAVベクターを用いる血友病B遺伝子治療臨床治験の成果次第と思われるが，血友病B遺伝子治療と同じストラテジーが用いることが出来るかは確実でない。

5. 血友病B遺伝子治療

血友病A遺伝子治療に比較し，血友病B遺伝子治療研究は進んでおり，臨床治験も進行中である。FIX遺伝子搭載AAV2ベクターを用いた血友病B遺伝子治療臨床治験（Avigen trial）は，血友病遺伝子治療で最も期待されていた。最初は骨格筋へFIX遺伝子導入が行われたが十分な成果が得られなかった。続いて同じベクターを用いて肝臓へのFIX遺伝子導入の臨床治験が行われたが，肝臓へのFIX遺伝子導入にともないT細胞性免疫反応によるAAV2ベクター感染肝細胞の排除という傷害事象が発生したため，臨床治験は中断されていた[14]。

1) ベクターの進歩

AAVベクターの血清型とプロモーターなどがみなおされた結果，肝臓へのFIX遺伝子導入にはAAV8ベクターが優れ，免疫反応を考慮するとAAV5ベクターも選択肢に入ると思われ，実際にAAV8ベクターが血友病B遺伝子治療臨床治験に用いられ始めた。

新規血清型AAV8ベクターに加えAAVベクター構築の進歩もある。AAVそのものは一本鎖DNAウイルスであり，AAVベクター感染細胞内でベクター搭載遺伝子を発現するためには二本鎖となる必要があるとされる（二本鎖DNAになる機序は議論がある）。近年開発された自己相補型AAVベクター self complementary AAV (scAAV) vector（二本鎖AAVベクター double strand AAV vector，ds AAV vectorとも呼ばれる）は，AAVベクターに搭載される遺伝子の後半部分の塩基配列が前半部分の相補的配列になっているため，ベクター内ですでに二本鎖DNA構造をとり，AAVベクターが感染した細胞内で相補鎖の合成などのステップを取る必要がなく効率よく遺伝子発現を行えると考えられている[20]。

2) 非ヒト霊長類を用いた血友病B遺伝子治療研究

これまで血友病遺伝子治療研究は血友病マウスや血友病イヌを用いて前臨床研究を進めてきた。しかし，これらの実験動物とヒトとの種差は大きく，これらを用いて得られた研究成果をヒトへ外挿できないことも多い。そ

の意味で，非ヒト霊長類を用いた血友病遺伝子治療研究を行うことは意義が大きい。我々も一部をヒト型にしたカニクイザル FIX 遺伝子を搭載した AAV8 ベクターをカニクイザルへ投与し前臨床研究を進めてきた[21]。詳細な成果は示せないが，肝臓へ効率の良い遺伝子導入を行いうる AAV8 ベクターも，カニクイザル肝臓への遺伝子導入はマウス肝臓への遺伝子導入効率著しく異なることが分かった。また，血中に存在する抗 AAV 抗体の影響も大きいことも明らかとなった[22]。このようにヒトでの血友病遺伝子治療の前臨床研究を非ヒト霊長類で行うことは重要であると思われる。

3) AAV ベクターによる遺伝子導入に対する既感染の影響

ヒトはウイルスから自己を守ため抗体を産生し，またウイルス感染細胞を排除する。野生型 AAV の既感染があると中和抗体が形成され，中和抗体が低力価でも存在すると AAV ベクターの遺伝子導入は著しく阻害されることがサルを用いた研究から明らかとなっている。特に，AAV8 ベクターのように血中に投与し肝臓へ遺伝子導入するタイプのベクターでは，短時間でも血中の中和抗体と反応すると遺伝子導入効率が著しく低下する。AAV のうち AAV2 に対するヒトの中和抗体陽性率は世界的にも高いものがある[23]。また，各血清型 AAV のキャプシドのアミノ酸配列は相同性が高く，ある血清型の AAV に対する抗体は，他の血清型 AAV に対しても阻害活性を有する可能性があり，一般人と血友病患者の抗 AAV 抗体価測定など，今後の検討が必要である。野生型 AAV 既感染はベクター感染細胞の免疫学的排除にも関わるとされる。scAAV8 ベクターを用いた FIX 遺伝子導入は血友病 B マウスやサルでの発現実験でも優れた FIX 発現能力を示したことを受け[24, 25]，臨床治験が開始され 2 例以上で臨床効果がえられている[26]。しかし，AAV8 中和抗体陽性の患者では有効性が得られなかったと報告されている。

6. 終わりに

血友病は遺伝子治療のよいモデルとされ，精力的に研究され臨床治験も行われてきた。現在進行している血友病 B 遺伝子治療では成果が得られつつあり，今後の展開が待たれる。血友病遺伝子治療における導入遺伝子由来の凝固因子に対するインヒビター産生の問題と，ベクター感染細胞に対する免疫反応など安全性の問題は今後さらに検討されるべきものと思われる。

著者の COI (conflicts of interest) 開示：本論文発表内容に関連して特に申告なし

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Regular Article

Local regulation of neutrophil elastase activity by endogenous α1-antitrypsin in lipopolysaccharide-primed hematological cells

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ABSTRACT

Neutrophil elastase released from activated neutrophils contributes in combating bacterial infection. While chronic inflammation results in anemia and decreased bone marrow activities, little is known about the effect of neutrophil elastase on hematological cell growth in severe inflammatory states. Here, we demonstrated that α1-antitrypsin, a physiological inhibitor of neutrophil elastase, functions as a regulator for cell growth by neutralizing neutrophil elastase activity in lipopolysaccharide-primed hematological cells. HL-60 cells were resistant to neutrophil elastase, as they also expressed α1-antitrypsin. The growth of HL-60 cells transduced with a LentiLox-short hairpin α1-antitrypsin vector was significantly suppressed by neutrophil elastase or lipopolysaccharide. When $CD34^+$ progenitor cells were differentiated towards a granulocytic lineage, they concomitantly expressed neutrophil elastase and α1-antitrypsin and prevented neutrophil elastase-induced growth inhibition. These results suggest that granulocytes might protect themselves from neutrophil elastase-induced cellular damage by efficiently neutralizing its activity through the simultaneous secretion of endogenous α1-antitrypsin.

Introduction

Neutrophil elastase is implicated in antimicrobial defense by degrading engulfed microorganisms [1–4]. Neutrophil elastase is a potent protease as it cleaves almost all connective tissue components as well as soluble proteins [5–7]. At the site of inflammation, neutrophil elastase released from azurophilic granules of the activated leukocyte is thought to mediate tissue destruction through its proteolytic cleavage of cell surface glycoproteins, extracellular matrix and junctional complexes [5,8]. The activity of neutrophil elastase is counteracted by endogenous inhibitors [9–11]. The serine protease inhibitor, α1-antitrypsin possesses potent anti-neutrophil elastase activities [12]. Abnormalities of α1-antitrypsin have been associated with liver damage arising from pathologic polymerization of the variant α1-antitrypsin, and with the development of pulmonary emphysema or panniculitis due to inflammatory stimuli leading to the unregulated activity of neutrophil elastase [13–16].

Neutrophil elastase is also known to provide feedback to granulopoiesis through direct proteolytic action on granulocyte-colony stimulating factor (G-CSF) [17]. Patients with mutations in the gene encoding neutrophil elastase (ELANE) display severe congenital neutropenia due to abnormal traffic of neutrophil elastase and induction of the unfolded protein response [18–20]. In addition, neutrophil elastase induces apoptosis of hematopoietic progenitor cells, which is prevented by a secretory proteinase inhibitor [17,21]. Chronic infection or inflammation results in anemia and decreased bone marrow activities [22,23], and hematopoietic efficacy declines with hematopoietic cell apoptosis and altered cytokine production [24,25]. However, little is known regarding the effect of neutrophil elastase on hematological cell growth or regulation by α1-antitrypsin in severe inflammatory states such as sepsis.

In this study, we investigated the possibility that the growth of hematological cells may be affected by the enzymatic activity of neutrophil elastase and that is regulated by endogenous alpha1-antitrypsin under the stimulation of lipopolysaccharide.

Materials and methods

Cell lines and cell cultures

The human leukemia cell lines HL-60 and K562 were obtained from the American Type Culture Collection (ATCC, Manassas VA, USA) and MEG-01 was purchased from the European Collection of Cell Cultures (ECACC, Down, UK). They were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated FBS (Gibco). Cells were adapted to serum free AIM-V Medium (Gibco) as needed. Human embryonic kidney 293 T cells

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were also purchased from the ATCC and grown in DMEM/F-12 medium (Gibco) supplemented with 10% heat-inactivated FBS. Human cord blood cells were isolated from healthy volunteers with informed consents according to the Declaration of Helsinki.

In vitro differentiation of human cord blood derived CD34$^+$ cells

Human cord blood-derived CD34$^+$ progenitor cells from four independent donors were isolated with a CD34 MicroBead Kit (Miltenyi Biotech, Auburn, CA, USA), and cultured with StemPro-34 SFM (Invitrogen, San Diego, CA, USA). These cells were differentiated into three lineages of hematological cells using appropriate cytokines: granulocytic (10 ng/mL GM-CSF, 10 ng/mL IL-3, and 50 ng/mL SCF), erythrocytic (3 U/mL EPO, 10 ng/mL IL-3 and 50 ng/mL SCF) and megakaryocytic (50 ng/mL TPO, 10 ng/mL IL-3, and 10 ng/mL IL-6) [26].

Measurement of neutrophil elastase activity

The enzymatic activity of neutrophil elastase was determined by an amidolytic reaction to a specific substrate, methoxysuc-AAPV-p-nitroanilide (Sigma-Aldrich, St. Louis, MO, USA) and measuring its absorbance at 405 nm with a Bechmark Plus Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA), as previously described [27].

Detection of human neutrophil elastase mRNA and related factor mRNA

RNA samples were subjected to reverse transcription-polymerase chain reaction (RT-PCR) using the following primer pairs: neutrophil elastase forward (5'-GTAAACTTGCTCAACGACATC-3') and reverse (5'-CTCACGAGAGTGCAGACGTT-3'); α1-antitrypsin forward (5'-CAGATCAACGATTACGTGGAGA-3') and reverse (5'-GCTTCATCATAGGGACCTTCAC-3'); and GAPDH forward (5'-AAGGTGAAGGTCGGAGTC-3') and reverse (5'-GAAGATGGTGATGGGATTTC-3'). The RT-PCR was performed using a GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA, USA) as previously described. In brief, reverse transcription was performed at 42 °C for 30 minutes followed by PCR. The initial denaturation step was at 95 °C for 5 minutes followed by 30 cycles of denaturation at 95 °C for 15 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 7 seconds.

Construction of lentiviral α1-antitrypsin shRNAi vectors

To knock down α1-antitrypsin, shRNAi vectors were constructed. As a control, a scramble sequence was inserted into the vector used. The piGENE hU6 vector (iGENE Therapeutics, Tokyo, Japan) was digested with the restriction enzymes EcoRI (Toyobo, Osaka, Japan) and HindIII (Fermentas Life Sciences, Ontario, Canada). The fragment incorporating the hU6 promoter and a BfuAI site was extracted with a MinElute Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and inserted into the pBC SK + vector (Stratagene, La Jolla, CA, USA), linearized with *Eco*RI and *Hind*III. After digestion with *Xba*I and *Xho*I (Toyobo), they were cut and inserted into the pLentiLox 3.7 vector (pLL3.7 vector; ATCC) previously treated with *Xba*I and *Xho*I. Short interfering RNAs (siRNAs) targeted towards human α1-antitrypsin were based on sequences within the NCBI database. Sense and antisense oligonucleotides of siRNA were designed by Hokkaido System Science (Sapporo, Japan). The sequences oligonucleotides used in this study were: short hairpin sense (5'-CACCGTTTGGGTATGTTTAGCATACGTGTGCTGTCCGTATGTTAAACATGCCTAAACTTTTT-3') and antisense (5'-GCATAAAAAGTTTAGGCATGTTTAACATACGGACAGCACACGTATGCTAAACATACCCAAAC-3'); scramble sense (5'-CACCGATCATAGATAGCACAGGTACGTGTGCTGTCCGTACTTGTGCTATCTGTGGTCTTTTT-3') and antisense (5'-GCATAAAAAGACCACAGATAGCACAAGTACGGACAGCACACGTACCTGTGCTATCTATGATC-3'). These oligonucleotides were ligated into pLL3.7 at the *Bfu*AI site. The pLL3.7 shRNAi plasmid was co-transfected with three plasmids containing PV, REV and VSV-G into 293 T cells. Viral RNA was purified using a QIAamp Viral RNA Mini kit (QIAGEN), and the number of viral particles was determined with a TaqMan PCR Core reagent kit (Applied Biosystems) and an ABI 7700 System (Applied Biosystems). The primers used for quantitative RT-PCR analysis were: forward (5'-GCTTTCATTTTCTCCTCCTT-3') and reverse (5'-GGCCACAACTCCTCATAA-3') along with a FAM-labeled probe (5'-ATCCTGGTTGCTGTCT-3').

Stimulation of hematological cells with neutrophil elastase or lipopolysaccharide (LPS)

Cultured hematological cells were treated with various concentrations of neutrophil elastase (Elastin Products, Owensville, MO, USA), α1-antitrypsin (Sigma-Aldrich) and sivelestat sodium hydrate (ONO Pharma, Osaka, Japan). For LPS stimulation, 1×10^6 cells were incubated with 10 ng/mL LPS (Sigma-Aldrich) at 37 °C for 45 minutes. After washing, cells were re-suspended in the medium with 100 nM N-formyl-Met-Leu-Phe (fMLP; Sigma-Aldrich) for another 45 minutes at 37 °C, as described previously [28].

Western blot analysis

Cells were lysed with a lysis buffer (10 mM Tris-HCl pH 7.6, 1% NP40, 0.15 M NaCl, 1 mM EDTA, 10 μg/mL aprotinin), and each sample consisted of 1×10^6 cells. After centrifugation, 10 μg of sample was applied to SDS-PAGE then electrically transferred to PVDF membranes. After blocking with 20 mM Tris-HCl, (pH 7.6) and 150 mM NaCl containing 2% bovine serum albumin (BSA) at 25 °C for 1 hour, the membrane was incubated with mouse anti-human α1-antitrypsin antibody (1:3000; Ikagaku, Kyoto, Japan) followed by goat anti-mouse IgG horseradish peroxidase-conjugated antibody (1:10000; Invitrogen) in 20 mM Tris-HCl, (pH 7.6), 150 mM NaCl containing 0.1% Tween20 for detection by an ECL Western Blot Detection system (GE Healthcare, Buckinghamshire, UK).

Enzyme-linked immunosorbent assays (ELISAs)

The levels of neutrophil elastase antigen were measured using a Human Elastase ELISA kit from Hycult biotechnology (Uden, Netherlands). The α1-antitrypsin antigen levels were measured using an AssayMax Human α1-Antitripsin Elisa Kit from Assaypro (St. Charles, MO, USA). Concentrations of transforming growth factor-β1 (TGF-β1) were determined by a Quantikine Human TGF-β1 Immunoassay kit from R&D Systems (Minneapolis, MN, USA). The manufacturer's recommended protocols were followed for each kit.

Immunohistochemistry

Cytospin slides containing 1×10^5 cells were prepared with a Cytospin3 (Shandon, Cheshire, UK). After fixation with ethanol, they were incubated with phosphate-buffered saline (PBS) containing 1% BSA and 1% non-immune goat serum for 10 minutes at 25 °C. After blocking, slides were stained for 2 hours at 4 °C using a rabbit anti-human neutrophil elastase antibody (1:200; Calbiochem, Darmstadt, Germany) or mouse anti-human α1-antitrypsin antibody (1:200; Ikagaku) diluted with PBS containing 1% BSA as primary reagents. After washing with PBS, they were incubated with Alexa Fluor-488 labeled goat anti-rabbit IgG antibody (1:500; Invitrogen) or Alexa Fluor-555 conjugated rabbit anti-mouse IgG antibody (1:500; Invitrogen) for 1 hour at 25 °C. Nuclei were stained with DAPI (Roche, Basel, Switzerland).

Terminal dUTP nick-end labeling (TUNEL) Assay

Cytospin slides with 1×10^5 cells were fixed with ethanol, and the TUNEL assay was performed using the DeadEnd Colorimetric

Apoptosis Detection System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Quantification of TUNEL positive cells was determined by mean percentage of apoptotic cells from the total number of cells counted in five fields per slide.

Statistical analysis

The SPSS statistical software package (SPSS, Chicago, IL, USA) was used for all statistical analyses of data. Variables not normally distributed were analyzed with the two-sided Mann-Whitney *U* test. A difference with $p<0.03$ was considered statistically significant.

Results

Effect of LPS on secretion of neutrophil elastase and proliferation in hematological cells

We stimulated three different hematological cell types, HL-60, K562 and MEG-01 cells with LPS. The antigen levels of neutrophil elastase were increased only in HL-60 cells (Fig. 1 A). However, the enzymatic activity of neutrophil elastase was markedly decreased until four hours after the stimulation (Fig. 1 B). LPS did not affect proliferation of any cell type (Fig. 1 C). Then, we cultured 1×10^5 cells/mL of HL-60, K562 and MEG-01 for 24 hours in the absence or presence of the supernatants derived from HL-60 cells that had been stimulated with 10 ng/mL LPS for one hour. Interestingly, the proliferation of K562 and MEG-01 cells was markedly inhibited in the presence of culture medium derived from LPS-stimulated HL-60 cells, although their supernatants did not suppress proliferation of naïve HL-60 cells (Fig. 1 D). These inhibitory effects on K562 and MEG-01 cells were canceled by a human neutrophil elastase specific inhibitor, sivelestat sodium (Fig. 1 D).

Effect of neutrophil elastase on proliferation of hematological cells

To determine whether neutrophil elastase could inhibit proliferation of hematological cells, HL-60, K562 and MEG-01 cells were cultured in the presence of neutrophil elastase. Proliferation of K562 and MEG-01 was significantly inhibited by treating with 1 nM neutrophil elastase for 72 hours (Fig. 2 A), and their proliferation was suppressed in a dose-dependent manner (Fig. 2 B). In contrast, neutrophil elastase had no effect on the proliferation of HL-60 cells. Both K562 and MEG-01 underwent apoptosis in a dose-dependent manner with neutrophil elastase (Fig. 2 C and D). There was no significant increase in the number of apoptotic cells when HL-60 cells were cultured in the presence of 5 nM neutrophil elastase.

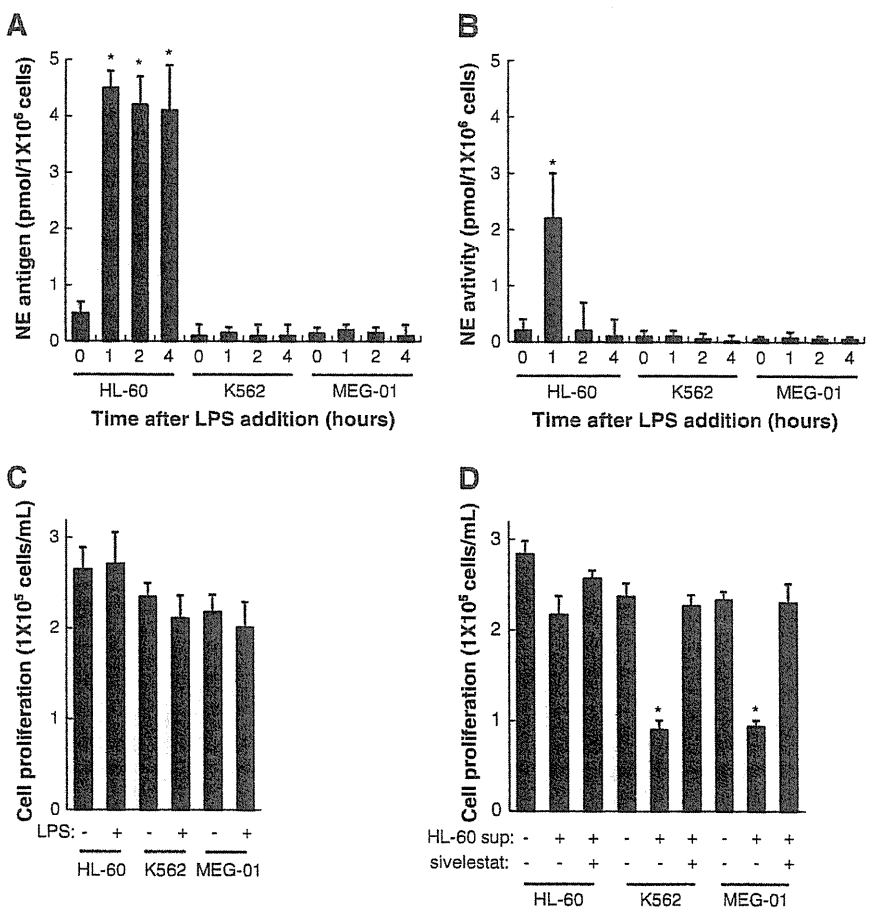

Fig. 1. Effects of LPS on secretion of neutrophil elastase and proliferation in hematological cells. A - B. 1×10^5 cells of three different hematological cell types HL-60, K562 and MEG-01 were stimulated with 10 ng/mL LPS for 0, 1, 2 and 4 hours. Neutrophil elastase antigen levels (A) and activity (B) were measured as described in the Materials and methods. C. 1×10^5 cells/mL HL-60, K562 and MEG-01 cells were cultured in the absence or presence of 10 ng/mL LPS. Cell numbers for each cell were counted at 24 hours by trypan-blue viable staining. D. 1×10^5 cells/mL of naïve HL-60, K562 and MEG-01 were cultured in the absence or presence of the supernatants derived from HL-60 cells that had been stimulated with 10 ng/mL LPS for 1 hour. HL-60, K562 and MEG-01 cells were also cultured in the presence of LPS-stimulated HL-60 supernatants treated with 100 nM sivelestat sodium. Cell numbers for each cell were counted at 24 hours. Values are mean ± SD; $^*p<0.03$ compared with controls. (A), (B) and (C) $n=5$, (D) $n=3$. NE, neutrophil elastase.

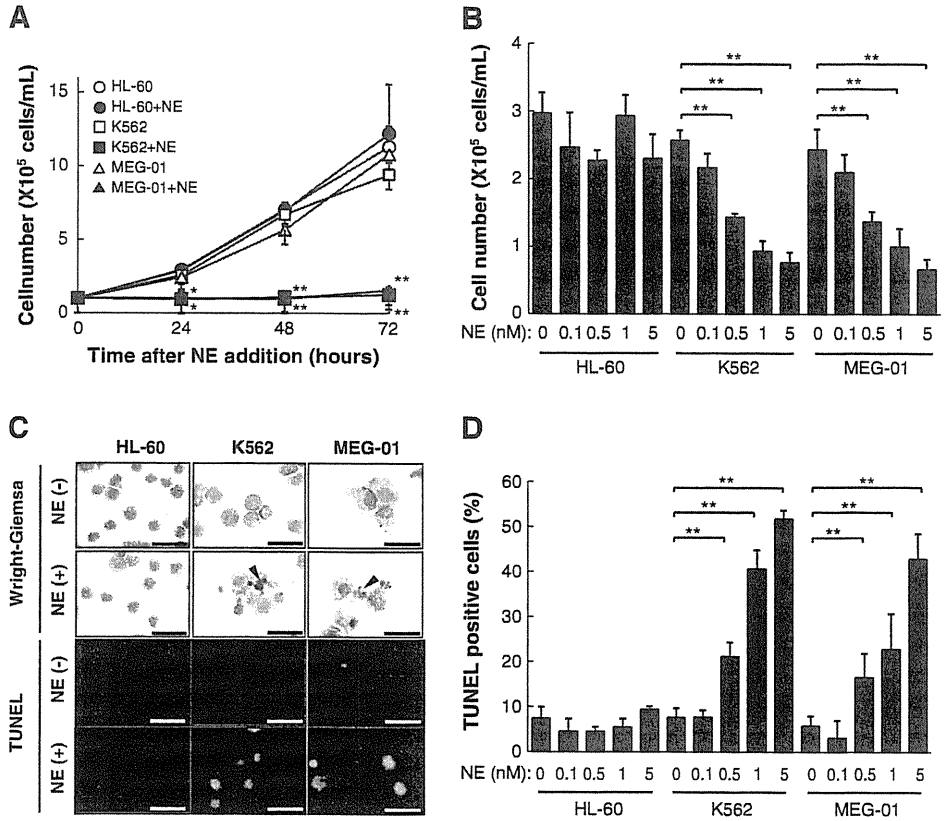

Fig. 2. Effects of neutrophil elastase on proliferation and induction of apoptosis in hematological cells. A. HL-60, K562 and MEG-01 cells were cultured in the absence or presence of 1 nM neutrophil elastase for 72 hours. Cell numbers for each cell were counted every 24 hours. B. HL-60, K562 and MEG-01 cells were cultured in the presence of 0–5 nM neutrophil elastase for 24 hours, and viable cell numbers were determined. C. Morphological changes in HL-60, K562 and MEG-01 cells were analyzed using Wright-Giemsa staining 24 hours post-stimulation with 1 nM neutrophil elastase (upper panels). For TUNEL labeling, apoptotic cells demonstrated a greater intensity of fluorescence (lower panels). Representative data from three independent experiments are shown. Arrow heads demonstrate apoptotic cells. Scale bars indicate 50 μm. D. The proportion of TUNEL-positive cells was calculated for each cell line treated with neutrophil elastase. Values are mean ± SD; $^{*}p<0.03$ and $^{**}p<0.01$ compared with controls. (A), and (B) $n=5$, (D) $n=3$. NE, neutrophil elastase.

HL-60 neutralizes neutrophil elastase activity by concomitant secretion of α1-antitrypsin

To clarify how HL-60 cells were not subject to apoptotic induction by neutrophil elastase, we measured residual antigen and enzymatic activity after its addition. We detected both antigen and enzymatic activity of neutrophil elastase in K562 and MEG-01 culture supernatants at levels similar to which they were added (Fig. 3 A and B). In HL-60 cultures, antigen levels of neutrophil elastase increased in a dose-dependent manner (Fig. 3 A), however enzymatic activity was not increased even after the addition of 0.3 or 1 nM neutrophil elastase (Fig. 3 B). Apoptotic effects on K562 and MEG-01 cultures were no longer evident when 1 nM neutrophil elastase was present together with its specific inhibitor, sivelestat sodium, at a concentration of 100 nM (Table 1), suggesting that cell type-specific apoptotic effects of neutrophil elastase might be based on its enzymatic activity. Thus, we focused on a natural inhibitor of neutrophil elastase, α1-antitrypsin. As shown in Fig. 3 C, HL-60 cells expressed α1-antitrypsin mRNAs, although the amount of mRNA did not increase after stimulation with neutrophil elastase. The α1-antitrypsin proteins were observed in the cytoplasmic granules of HL-60 cells but their appearance diminished following stimulation with neutrophil elastase (Fig. 3 D). Levels of α1-antitrypsin antigen in the culture medium of HL-60 cells significantly increased following stimulus with neutrophil elastase (Fig. 3 E). In contrast, α1-antitrypsin levels in HL-60 cell lysates were very low (Fig. 3 F).

Silencing of endogenous α1-antitrypsin expression with a shRNA lentiviral vector induces inhibition of HL-60 proliferation following the stimulation with neutrophil elastase

To confirm that the absence of α1-antitrypsin suppresses HL-60 growth when neutrophil elastase is present in the culture, we used siRNAs to knock down endogenous α1-antitrypsin expression. We examined the efficiency of the lentiviral shRNA for α1-antitrypsin with eGFP expression in HL-60 cells. As shown in Fig. 3 A, HL-60 cells transduced with the LentiLox-short hairpin or scramble α1-antitrypsin demonstrated expression of eGFP. Expression of α1-antitrypsin protein was significantly suppressed by transduction with LentiLox-short hairpin α1-antitrypsin (Fig. 4 B and C), demonstrating that silencing of endogenous α1-antitrypsin by a shRNA lentiviral vector system is effective in HL-60 cells. The proliferation of HL-60 cells transduced with the LentiLox-scramble α1-antitrypsin was not affected by neutrophil elastase (Fig. 4 D). However, α1-antitrypsin deficient HL-60 cells transduced with LentiLox-short hairpin sequences (Fig. 4 E) exhibited significant growth retardation after stimulation with neutrophil elastase (Fig. 4 D).

Effect of LPS on α1-antitrypsin deficient HL-60 cells transduced with a shRNA lentiviral vector

When HL-60 cells were stimulated with LPS, it was found that all cells secreted neutrophil elastase antigen regardless of whether they

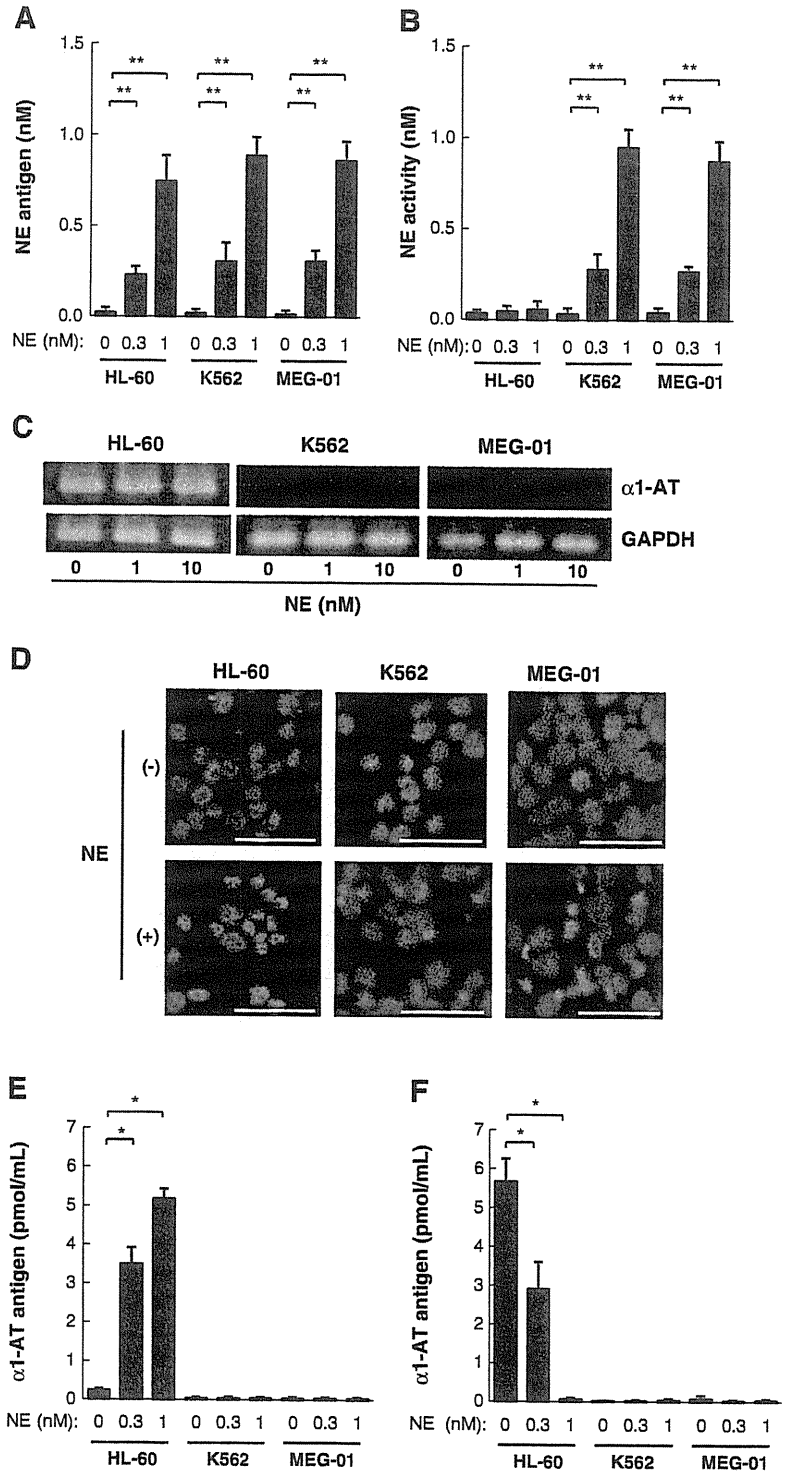

Fig. 3. HL-60 cells neutralize neutrophil elastase activity by secretion of α1-antitrypsin. A - B. Level of neutrophil elastase antigens (A) and enzyme activity (B) in a culture medium containing 1×10^5 cells/mL of HL-60, K562 and MEG-01 cells was measured 24 hours after the addition of neutrophil elastase (0, 0.3 and 1 nM), as described in the Materials and methods. C. HL-60, K562 and MEG-01 cells were cultured in the presence of 0–10 nM neutrophil elastase for 24 hours, and then α1-antitrypsin and GAPDH mRNA of each cell were analyzed by RT-PCR. D. HL-60, K562 and MEG-01 cells were cultured in the absence (upper panels) or presence (lower panels) of 1 nM neutrophil elastase for 24 hours, and then stained with anti-human α1-antitrypsin IgGs (red) and DAPI (blue) Scale bars indicate 50 μm. E-F. Levels of α1-antitrypsin antigens in culture medium (E) and cell lysate (F) of 1×10^5 cells/mL HL-60, K562 and MEG-01 cells were measured 24 hours after the addition of neutrophil elastase (0, 0.3 and 1 nM), as described in the Materials and methods. Values are the mean ± SD ($n=3$, in each group). $^{**}p<0.01$ compared with controls. NE, neutrophil elastase; α1-AT, α1-antitrypsin.

were transduced with LentiLox-scrambled or LentiLox-short hairpin α1-antitrypsin (Fig. 5 A). However, the neutrophil elastase activity in α1-antitrypsin deficient HL-60 cells was higher when compared to HL-60 controls or cells transduced with LentiLox-scrambled α1-antitrypsin (Fig. 5 B). Knock-down of α1-antitrypsin with the shRNA lentiviral vector efficiently suppressed α1-antitrypsin secretion

Table 1
Effect of sivelestat sodium on neutrophil elastase-induced apoptosis in hematological cells.

NE (nM)	sivelestat sodium (nM)	TUNEL positive cells (/1X103 cells)		
		HL-60	K562	MEG-01
0	0	4.7 ± 1.8	5.6 ± 1.9	3.0 ± 0.8
1	0	4.7 ± 0.6	47.2 ± 4.2**	39.8 ± 4.8**
1	10	4.2 ± 1.5	48.8 ± 5.4**	38.4 ± 2.5**
1	50	5.4 ± 2.3	34.7 ± 6.7**	28.0 ± 5.0**
1	100	4.4 ± 0.3	9.0 ± 2.2	7.7 ± 3.0

HL-60, K562 and MEG-01 cells were stimulated with 1 nM human neutrophil elastase in the presence of 0–100 nM of sivelestat sodium hydrate for 24 hours. TUNEL positive cells were calculated in each of 1×10^3 cells. Values are mean ± SD; n = 4; **$p<0.01$ compared with controls. NE, neutrophil elastase.

following LPS-stimulus (Fig. 5 C). As expected, the growth of HL-60 cells transduced with LentiLox-short hairpin α1-antitrypsin was significantly suppressed when treated with LPS compared to cells transduced with LentiLox-scramble α1-antitrypsin (Fig. 5 D). Additionally, when HL-60 cells were stimulated with LPS in the presence of sivelestat sodium, levels of α1-antitrypsin in the culture medium did not increase (data not shown).

Effect of neutrophil elastase on in vitro differentiated hematological cells derived from CD34$^+$ progenitor cells

Human cord blood-derived CD34$^+$ progenitor cells were treated with differentiation protocols to produce cells of granulocytic, erythrocytic and megakaryocytic lineages as previously described [29]. We found that

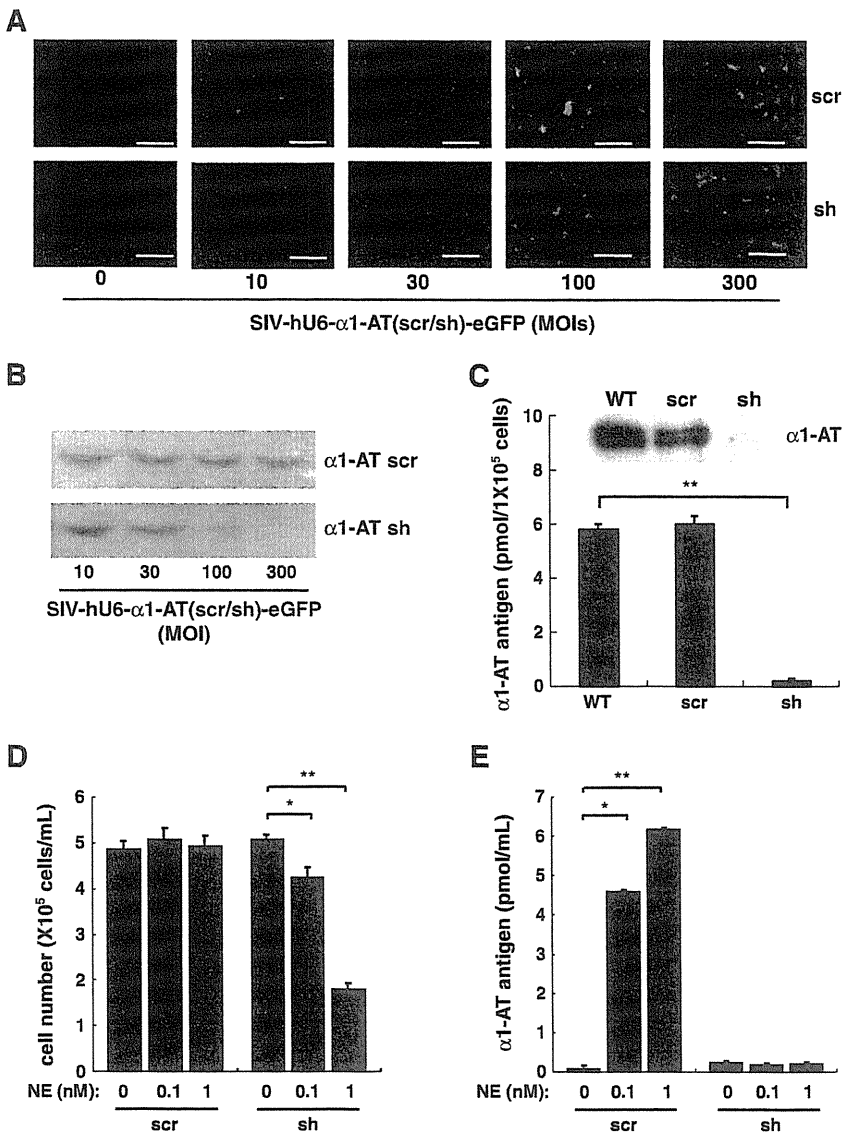

Fig. 4. Silencing of endogenous α1-antitrypsin expression with a shRNA lentiviral vector in HL-60 cells. A. HL-60 cells were transduced with LentiLox-scramble α1-antitrypsin (upper panels) or LentiLox-short hairpin α1-antitrypsin (lower panels) at multiplicities of infection (MOIs) of 0–300. Representative data from three independent experiments are shown. Scale bars show 50 μm. B. Cell lysates of HL-60 cells transduced with LentiLox vectors were analyzed with SDS-PAGE followed by Western blotting with anti-human α1-antitrypsin IgGs. Representative data from three independent experiments are shown. C. Amounts of α1-antitrypsin antigen were measured by ELISA in the cell lysates of 1×10^5 HL-60 cells transduced without (WT, $n=5$) or with LentiLoxs (scr, $n=5$; sh, $n=5$). D - E. HL-60 cells transduced with LentiLox-short hairpin α1-antitrypsin or LentiLox-scramble α1-antitrypsin were cultured in the absence ($n=5$) or presence of 0.1 nM ($n=5$) and 1 nM ($n=5$) neutrophil elastase for 72 hours and viable cell numbers (D) and antigen levels of α1-antitrypsin (E) were measured. Values are mean ± SD; *$p<0.03$ and **$p<0.01$ compared with controls. NE, neutrophil elastase; α1-AT, α1-antitrypsin; WT, non-transduced; scr, LentiLox-scramble α1-antitrypsin transduced; sh, LentiLox-short hairpin α1-antitrypsin transduced HL-60 cells.

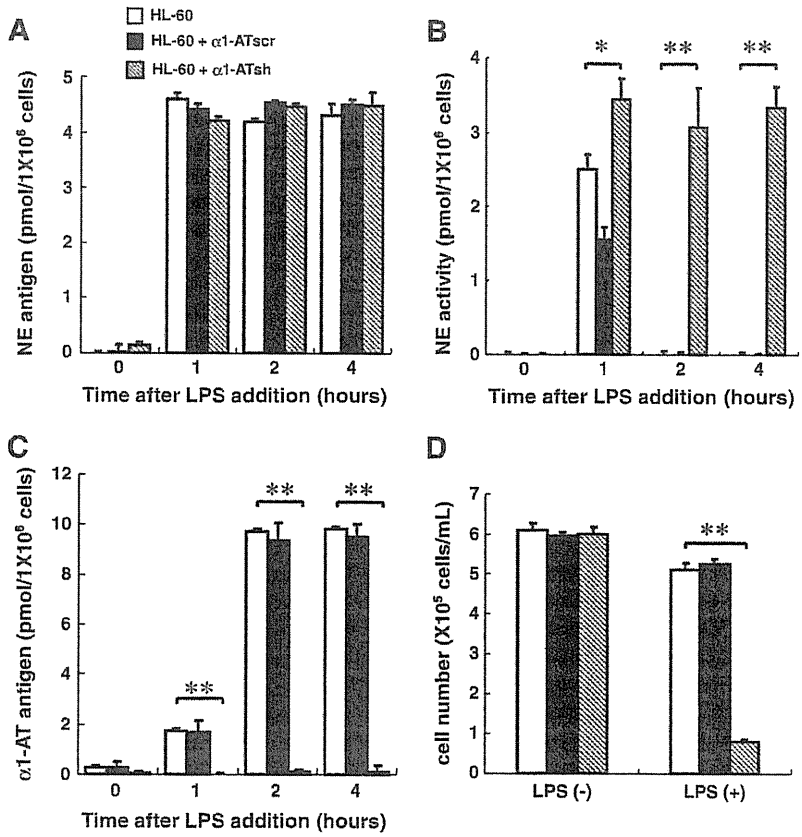

Fig. 5. Effects of LPS on α1-antitrypsin deficient HL-60 cells transduced with shRNA lentiviral vectors. A - C. 1×10^5 HL-60 cells ($n=4$, bars), LentiLox-scramble transduced HL-60 cells ($n=4$, shaded bars), LentiLox-short hairpin α1-antitrypsin transduced HL-60 cells ($n=4$, hatched bars) were stimulated with 10 ng/mL LPS for 0, 1, 2 and 4 hours. Neutrophil elastase antigen levels (A), activity (B) and α1-antitrypsin antigen levels (C) were measured as described in the Materials and methods. D. 1×10^5 HL-60 cells ($n=4$, bars), LentiLox-scramble transduced HL-60 cells ($n=4$, shaded bars), LentiLox-short hairpin α1-antitrypsin transduced HL-60 cells ($n=4$, hatched bars) were stimulated with 10 ng/mL LPS. After 48 hours, cell numbers of viable HL-60 cells were counted. Values are mean ± SD; $^*p<0.03$ and $^{**}p<0.01$ compared with controls. NE, neutrophil elastase; α1-AT, α1-antitrypsin; LPS, lipopolysaccharide.

progenitor cells could differentiate into three lineages, with the lineage of these cells determined by morphological assessment using a phase-contrast microscope, and detection of lineage-specific surface markers observed until 16 days after the induction of differentiation (Fig. 6 A). Interestingly, the only differentiated cells that concomitantly expressed neutrophil elastase with α1-antitrypsin mRNA were a granulocytic lineage, suggesting that this enzyme-inhibitor system may be up-regulated during granuloid cell differentiation. Proliferation of erythrocytic and megakaryocytic lineage cells was significantly suppressed by 1 nM neutrophil elastase as compared to granulocytic lineage cells (Fig. 6 D). Residual neutrophil elastase activity was found in erythrocytic as well as megakaryocytic cells with very little activity apparent in granulocytic cells (Fig. 6 E). However, only granulocytic lineage cells possessed α1-antitrypsin antigen in their culture supernatants after stimulation with neutrophil elastase (Fig. 6 F). We found that granulocytic lineage cells secreted significant amounts of active leukocyte elastase shortly after stimulation with LPS when compared to erythrocytic and megakaryocytic lineages (Fig. 6 B and C). When each lineage of cells was cultured with the supernatant derived from LPS-stimulated granulocytic cells, both erythrocytic and megakaryocytic lineage cells were significantly suppressed in their proliferation (Fig. 6 G).

Discussion

Neutrophil elastase is known to contribute towards combating bacterial infection [1–3]. Paradoxically, neutrophil elastase damages host tissues such as the intestine, kidney and lung during inflammation [10,30,31]. In our study, active neutrophil elastase was secreted not from K562 or MEG-01 but from HL-60 cells after stimulation of LPS (Fig. 1). In addition, the proliferation of K562 and MEG-01 was markedly inhibited after addition of culture medium derived from LPS-treated HL-60 cells, and the effects were canceled by sivelestat sodium. Several researchers have shown that neutrophil elastase induces endothelial and epithelial apoptosis [10,32]. We showed that neutrophil elastase significantly inhibited proliferation of K562 as well as MEG-01 cells by inducing apoptosis (Fig. 2). Interestingly, neutrophil elastase did not affect cell growth of HL-60 cells. Thus, the damage of hematological cells may involve a disturbance in the balance between neutrophil elastase activity and its inhibitor in response to LPS.

It has been reported that neutrophils respond to surface stimulation by secreting neutrophil elastase and α1-antitrypsin, and that there might be an inherent mechanism for damping the local effects of neutrophil elastase [33,34]. Apoptotic effects of neutrophil elastase on K562 and MEG-01 cultures were absent when the enzymatic activity of neutrophil elastase was neutralized by sivelestat sodium (Table 1) [31,35]. Only HL-60 cells exhibited resistance to apoptotic induction as they stored α1-antitrypsin, which was secreted rapidly after stimulation by neutrophil elastase (Fig. 3). These results suggest that cell type-specific effects of neutrophil elastase on hematological cell growth might be dependent on the neutralizing activity of neutrophil elastase by endogenous α1-antitrypsin.

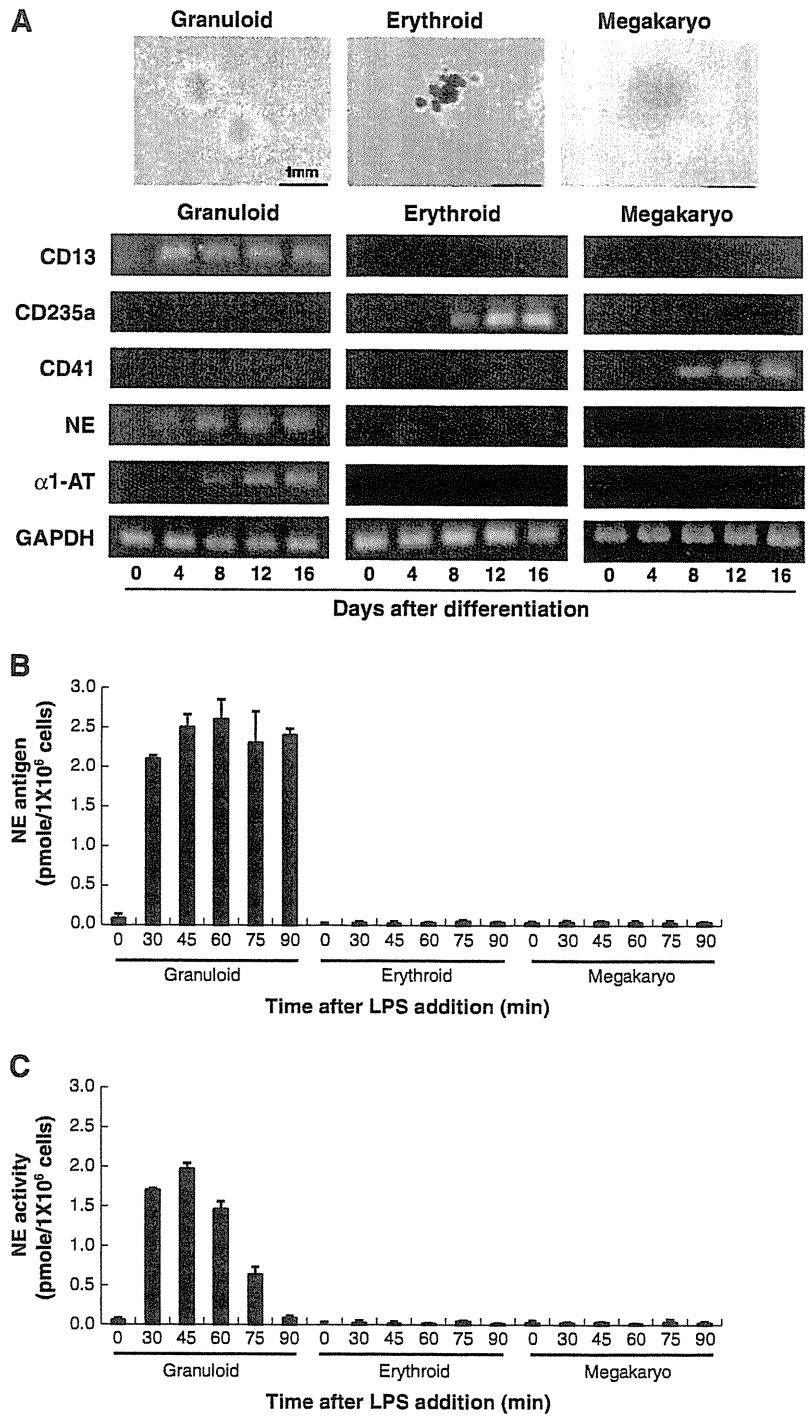

Fig. 6. *In vitro* differentiation of cord blood-derived $CD34^+$progenitor cells towards three lineages of hematological cells and their responses against LPS or neutrophil elastase. A. Human cord blood-derived $CD34^+$ progenitor cells were differentiated *in vitro* into granulocytic cells (left panels), erythrocytic cells (middle panels) and megakaryocytic cells (right panels) as described in the Materials and Methods. Each cell type was harvested at the indicated time points, and subjected to RT-PCR analysis for CD13, CD235a, CD41, neutrophil elastase (NE), α1-antitrypsin (α1-AT) and GAPDH mRNA expression. Representative data from three independent experiments are shown. B - C. 1×10^5 differentiated granulocytic, erythrocytic and megakaryocytic cells were stimulated with 10 ng/mL LPS for 0–90 minutes. Neutrophil elatastase antigen levels (B) and activity (C) were measured as described in Materials and methods. D. Differentiated granulocytic (circles), erythrocytic (filled circles) and megakaryocytic cells (triangles) were cultured in the presence of 1 nM neutrophil elastase for 72 hours. Cell numbers were determined every 24 hours. E - F. 1×10^5 differentiated cells were cultured with 0, 0.3 and 1 nM neutrophil elastase for 24 hours and neutrophil elastase acitvity (E) and α1-antitrypsin antigen levels (F) were measured. 1×10^5 differentiated granulocytic cells were also cultured for 24 hours in the presence of 1 nM neutrophil elastase that had been treated with 100 nM sivelestat sodium, and neutrophil elastase activity was measured (E). G. Differentiated granulocytic (circles), erythrocytic (filled circles) and megakaryocytic cells (triangles) were cultured for 72 hours in the presence of granulocytic cell-derived culture medium that had been stimulated with 10 ng/mL LPS for 45 minutes. Viable cell numbers were determined every 24 hours. The values given are the mean ± SD; $n = 4$; $^*p<0.03$ and $^{**}p<0.01$ compared with controls.

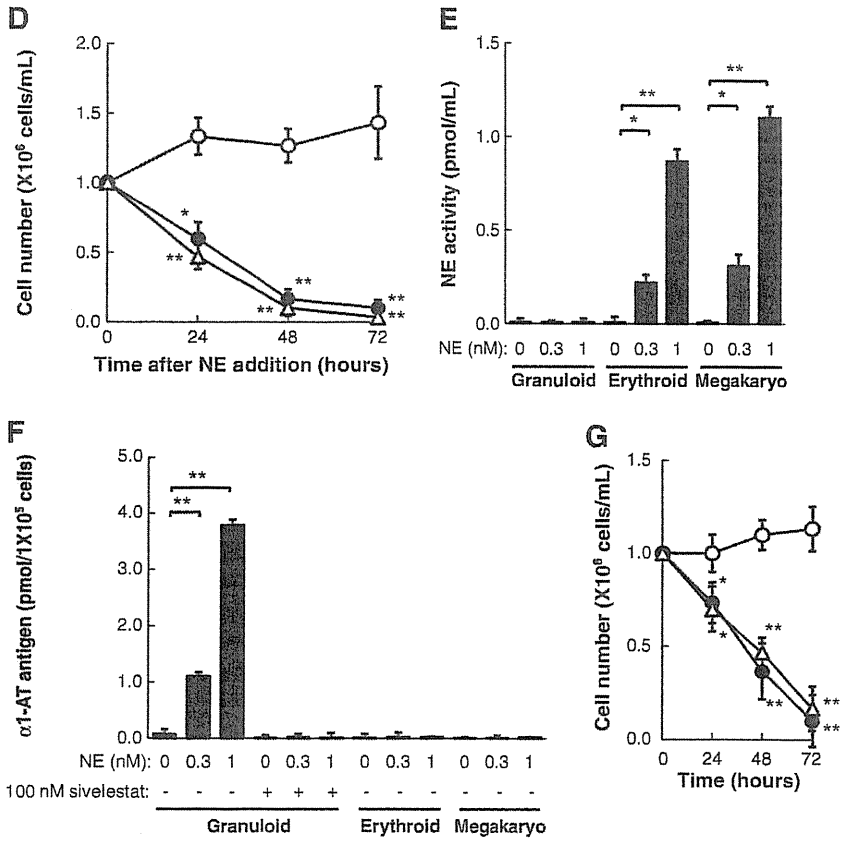

Fig. 6 (*continued*).

Leukocyte are known to release neutrophil elastase as well as α1-antitrypisn in response to LPS [28,36]. In our study, silencing of α1-antitrypsin expression with a short hairpin RNA lentiviral vector inhibited growth of HL-60 cells in the presence of neutrophil elastase (Fig. 4). In addition, HL-60 cells transduced with LentiLox-scrambled and -short hairpin α1-antitrypsin sequences secreted significant amounts of neutrophil elastase proteins shortly after stimulation with LPS (Fig. 5). The enzymatic activity of neutrophil elastase was only found in HL-60 cultures that had been transduced with LentiLox-short hairpin α1-antitrypsin which resulted in a significant reduction in cell growth. Moreover, K562 and MEG-01 cells did not express neutrophil elastase after LPS stimulation, and their proliferation was not significantly suppressed by LPS (Fig. 1). Taken together, our data suggests that LPS might stimulate secretion of neutrophil elastase, resulting in induction of neutrophil elastase-mediated apoptosis if its enzymatic activity is not efficiently neutralized by released α1-antitrypsin.

Although the cell surface receptor involved in neutrophil elastase has not been clearly identified, several candidates are suggested, such as protease-activated receptor (PAR)-2 and Toll-like receptor (TLR)-4 [8,37,38]. Additionally, the apoptosis induced by neutrophil elastase might be mediated through PAR-1 [39]. TLR-4 appears to be the principle receptor for LPS and mediates the activation of nuclear factor κB as well as the synthesis of proinflammatory cytokines such as interleukin-8 [8,40]. Tsujimoto *et al* showed that neutrophil elastase might be associated with expression of TLR-4 on monocytes and macrophages in the septic state [41]. In our study, TLR-4 was expressed in K562, MEG-01 and HL-60 cells [42], however, none of these hematological cells expressed PAR-1 or PAR-2 (data not shown), suggesting that the neutrophil elastase-induced apoptosis may be mediated through a signaling pathway similar to that for TLR-4.

Our *in vitro* differentiation models of hematopoiesis using $CD34^+$ progenitor cells showed that only granulocytic lineage cells expressed neutrophil elastase (Fig. 6), which is consistent with previous studies where levels of neutrophil elastase expression reach a maximum in the promyelocyte and are maintained until differentiation into a neutrophil [43,44]. Importantly, α1-antitrypsin was concomitantly expressed with neutrophil elastase during differentiation into a granulocytic lineage [34,45]. Additionally, α1-antitrypsin was secreted after stimulation by neutrophil elastase, and neutralized it to prevent growth inhibition. When we added inactive neutrophil elastase pretreated with sivelestat sodium to granulocytic lineage cells, we could not detect any α1-antitrypsin antigen (Fig. 6), suggesting that the enzymatic activity of neutrophil elastase might be crucial in secreting α1-antitrypsin. El Ouriaghli and coworkers reported that neutrophil elastase inhibits proliferation and induces apoptosis in $CD34^+$ cells along with degradation of G-CSF [17]. Although our *in vitro* hematopoietic differentiation system was free from G-CSF, concurrently secreted α1-antitrypsin may regulate neutrophil elastase activity. Granulocytic lineage cells released neutrophil elastase following stimulation with LPS, and active neutrophil elastase was neutralized for 90 minutes (Fig. 6). LPS stimulation did not affect erythrocytic or megakaryocytic lineage cells, however, the supernatant derived from granulocytic lineage cells 45 minutes after the addition of LPS significantly inhibited their proliferation as they could not inactivate neutrophil elastase. Erythropoiesis and megakaryopoiesis may be inhibited by neutrophil elastase due to the absence of α1-antitrypsin in these lineages in the inflammatory state. Thus, α1-antitrypsin released from granulocytes might play an important role for the maintenance of hematopoiesis in microenvironments such as the bone marrow niche where α1-antitrypsin might not be sufficiently supplied from plasma [36,46].

In conclusion, we demonstrated that hematological cells might be affected by neutrophil elastase which is regulated by endogenous α1-antitrypsin under the stimulation of lipopolysaccharide. We suggest that granulocytes could protect themselves from neutrophil elastase-induced cellular damage by efficiently neutralizing its activity with concomitant secretion of endogenous α1-antitrypsin. Extensive clinical studies would be required for understanding the precise mechanism of controlling neutrophil elastase activity by endogenous α1-antitrypsin in septic patients.

Authorship contribution

M. Dokai and S. Madoiwa designed and performed the research, analyzed the data, and wrote the paper; A. Yasumoto, Y. Kashiwakura, A. Ishiwata, A. Sakata and N. Makino performed experiments; S. Madoiwa, T. Ohmori, J. Mimuro, and Y. Sakata analyzed data and revised the paper.

Disclosure of conflicts of interest

The authors state that they have no conflict of interest to declare.

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Recovery of neurogenic amines in phenylketonuria mice after liver-targeted gene therapy

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Phenylketonuria (PKU) is a common genetic disorder arising from a deficiency of phenylalanine hydroxylase. If left untreated, the accumulation of phenylalanine leads to brain damage and neuropsychological dysfunction. One of the abnormalities found in hyperphenylalaninemic patients and a mouse model of PKU is an aminergic deficit in the brain. We previously showed correction of hyperphenylalaninemia and concomitant behavioral recovery in PKU mice after liver-targeted gene transfer with a viral vector. Here, we addressed whether such a functional recovery was substantiated by an improved amine metabolism in the brain. After gene transfer, brain dopamine, norepinephrine, and serotonin levels in the PKU mice were significantly elevated to normal or near-normal levels, along with systemic improvement of phenylalanine catabolism. The results of biochemical analyses validated the efficacy of PKU gene therapy in the central nervous system. *NeuroReport* 23:30–34 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Phenylketonuria (PKU; OMIM 261600) is a common inherited metabolic disorder, mostly arising from a deficiency of phenylalanine hydroxylase (PAH) [1]. PAH is exclusively responsible for converting phenylalanine into tyrosine, and its deficiency results in a systemic accumulation of phenylalanine in the body. Although the mechanisms involved are not fully understood, excessive amounts of phenylalanine are toxic to the developing brain and have a negative impact on neuropsychological function in adults. Therefore, the present treatment for PKU mandates strict restrictions of dietary protein in infancy and childhood to limit phenylalanine intake, and a similar diet is recommended for life. One possible mechanism responsible for the neurological dysfunction is an aminergic deficit, as earlier studies showed drastic decreases in neurotransmitters such as dopamine, norepinephrine, and serotonin (5-hydroxytryptamine, 5-HT) in the brains of untreated PKU patients [2,3]. A similar aminergic deficit was found in a mouse model of PKU (the *Pah*^{enu2} strain) [4–7]. We and other investigators have explored the feasibility of somatic gene therapy for PKU, and have shown that recombinant adeno-associated virus (AAV) vectors can achieve long-term corrections of hyperphenylalaninemia (HPA) in *Pah*^{enu2} mice [8–10]. We also demonstrated a behavioral recovery in the treated animals, indicating that some brain functions benefited from this approach [8]. In the present study, we addressed whether liver-targeted gene therapy for PKU would reinstate the metabolism of neurogenic amines, thereby improving homeostasis and the function of the central nervous system.

Materials and methods

Animals

All the animal experiments were carried out in accordance with the institutional guidelines under protocols approved by the Institutional Animal Care and Use Committee at Jichi Medical University (Shimotsuke, Japan). PAH-deficient C57BL/6-*Pah*^{enu2} mice (PKU mice, −/−) were homozygous for the same *Pah*^{enu2} mutation as that described in the original BTBR-*Pah*^{enu2} strain [4,5], but had been bred and backcrossed on the C57BL/6J background. Genotyping for the presence of the *Pah*^{enu2} mutation was performed by PCR analysis of tail biopsy DNA [9]. All the mice were maintained on standard mouse chow (CE-2 from Clea, Tokyo, Japan). Blood was collected from the tail veins on a filter paper for newborn mass screening (No. 545 from Advantec Toyo, Tokyo, Japan), and blood phenylalanine concentrations were determined by an enzymatic fluorometric assay using an Enzaplate PKU-R kit (GE Healthcare, Tokyo, Japan) and a Fluoroskan Ascent FL plate reader (Labsystems, Helsinki, Finland) [8,9]. *In-vivo* phenylalanine oxidation was evaluated by a noninvasive breath test using [1-^{13}C]L-phenylalanine [9,11].

In-vivo gene transfer

The construction and preparation of the AAV8-pseudotyped self-complementary AAV vector for PKU (scAAV8/

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