(2) サルへの移植実験の結果：現在，２頭目のサルの移植実験を終了し，移植後のデータを収集・解析中である．これまでに判明した実験結果を表にまとめた（**表１**）．移植後の末梢血単核球を調べたところ，約 2%が遺伝子標識された移植細胞由来であった．そのうち，単独移植群由来の細胞はほとんど検出されず，共移植すると生着がよいことが示された．

表１　２頭目の実験結果

	共移植群	単独群
移植部位	右側 上下肢	左側 上下肢
移植細胞とマーキングベクター	$CD34^+$細胞 LNL6	$CD34^+$細胞 G1Na
採取時 $CD34^+$細胞数	4.50×10^6	4.50×10^6
移植時 $CD34^+$細胞数	2.18×10^6	1.80×10^6
移植時 間質細胞数	4.97×10^6	なし
$CD34^+$細胞の 移植前の 遺伝子導入効率 （定量的 PCR）	340 %	290 %
移植後 28 日目 末梢血の 遺伝子標識 レベル （PCR）	2 %	ほとんど 検出されず

D. 考察

本年度は，本研究事業で２頭目のサルの移植実験を実施した．１頭目では，共移植群の $CD34^+$細胞を G1Na ベクターで標識し，単独移植群の $CD34^+$細胞を LNL6 ベクターで標識した．今回の２頭目は，ベクターによるバイアスを除外するために，使用ベクターをスイッチした．すなわち，共移植群の $CD34^+$細胞を LNL6 ベクターで標識し，単独移植群の $CD34^+$細胞を G1Na ベクターで標識した．また，今回の実験では，レシピエントのサルに対する移植前処置法を，全身放射線照射からブスルファン投与に変更した．全身放射線照射は，腸炎を引き起こし，長期間の下痢に悩まされ，１頭目のサルはそれが原因で死亡している．その点を踏まえて，今回はブスルファン投与に切り替えたが，放射線照射の場合と同等の骨髄抑制をもたらしながら，下痢などなく，全身状態は放射線照射後に比べてはるかに良好だった．移植後の末梢血単核球を調べたところ，約 2%が遺伝子標識された移植細胞由来であった．そのうち，単独移植群由来の細胞はほとんど検出されず，共移植すると生着がよいことが，２頭目のサルでも示された．

本研究事業では，平成 17 年度と 19 年度にサルを各１頭ずつ用いて実験を行ったが，いずれのサルでも，造血幹細胞を MSC と共に直接骨髄内へ移植すると，造血細胞の生着が促進されることを示すデータが得られた．しかし，サル実験は多くの費用と人手がかかり，結論を引き出すのに必要な数の実験を行うのは容易でない．そこで平成 18 年度は，別の大型動物実験として実施の比較的容易な，ヒツジ胎仔への移植実験系を用いて，MSC 共移植による造血細胞の生着促進効果を検討した．MSC を共移植した１頭のヒツジのみでサル造血細胞の生着が認められ,ヒツジの系でも共移植の効果が示唆された．

結局，サルとヒツジを用いた実験から，MSC を造血細胞とともに骨髄内へ直接移植すると，造血細胞の生着を高められることが分かったことになる．MSC の共移植による造血細胞の

生着促進効果を大型動物で検証した例はあまりなく，貴重な成果が得られたと言える．

造血幹細胞移植を受ける患者では，術前の化学療法や放射線照射による前処置によって骨髄微小環境が重度に破壊されるために，生着不全・不良を来すことがある．生着促進のためには骨髄微小環境の再建技術が望まれている．また，臍帯血を用いる移植では，臍帯血中の造血幹細胞の量が少ないため，少ない造血幹細胞を無駄なく効率よく生着させる技術が望まれている．これらは，MSC を造血細胞と共に移植することによって実現できるかもしれない．

E. 結論

サルおよびヒツジを用いた実験から，MSC を造血細胞とともに骨髄内へ直接移植すると，造血細胞の生着を高められることが示された．研究は当初の計画通り進んだと言える．造血幹細胞移植後の造血回復に関しては，造血幹細胞の体外増幅やサイトカイン投与による造血回復の促進など，主にドナー細胞の観点から研究が進められている．これに対して，レシピエントの骨髄微小環境の観点から造血幹細胞の生着促進を図る本研究はユニークといえ，今後もいっそうの進展を図りたい．

F. 健康危険情報（該当しない）

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む。）

なし．

厚生労働科学研究費補助金（再生医療等研究事業）

分担研究報告書

選択的増幅遺伝子の造血幹細胞治療への応用研究

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研究要旨

高率の遺伝子導入が必要である。選択的増幅遺伝子（SAG）を利用した造血系細胞の体内増幅法は、現在のベクター性能の限界を補う最良の方法だと考えられる。これまで実際の臨床に向け最適化した完全ヒト化SAGの構築を進めてきた。一方、X-SCID治療の副作用事故以来、ベクター遺伝毒性回避が強く求められている。遺伝毒性はプロウイルスが感染細胞のゲノムに挿入された位置に起因して起こる問題である従ってその位置情報を正確かつ迅速に解析する手段が必要である。本年度はSAGをサル免疫不全ウイルス（SIV）ベクターに搭載し、SIVの挿入位置情報を解析するための方法であるLAM-PCRを改良し、ヒト細胞株に感染させたあと50に及ぶ挿入位置の解析を行った。

A．研究目的

難治性血液疾患に対して、造血幹細胞を標的とした遺伝子治療が最も有効な治療手段である。しかし、現時点ではいくつかの事象が障壁となり、高い効果を得るまでに至っていない。その一つは、造血幹細胞に対し治療遺伝子の導入効率が、高くない点である。最終的な治療効果は、対象疾患によって異なる標的細胞の中で治療遺伝子を導入された率によって決まる。その率も疾患によって異なり、慢性肉芽腫症のように顆粒球の数パーセントが正常に戻れば治療効果が得られるものからHIV感染症のように50%以上のCD4陽性T細胞に遺伝子導入が必要なものもある。遺伝子導入を受けた細胞のポピュレーションを高めるには、最初の造血幹細胞への遺伝子導入効率を高める、遺伝子導入幹細胞を選択的に増幅させる、ニッチへの遺伝子導入幹細胞の生着率を向上させる、といった手段がある。遺伝子導入効率を高めるためには、ベクター選択、エンベロープの交換・改良、感染時にサイトカインや、感染向上用薬剤の使用等がある。現在、レトロウイルスベクター中心に遺伝子導入法の改良が進み、以前に比べて高い導入効率が得られるようになった。しかし、それでも限界があることと、せっかく遺伝子導入された造血幹細胞が体内に投与された後、ニッチに入り生着しなければターゲットの細胞に分化することなく、いつしか脱落してしまう。従って、遺伝子導入効率を高めることは、ベクターが細胞に対して遺伝子導入する効率を上げるよりも、むしろ移植後の幹細胞の生着を高めること、生着した遺伝子改変細胞を選択的に増幅することの方が重要であると考えた。われわれはそのために遺伝子の導入された細胞を増幅することで遺伝子導入効率を高める遺伝子-選択的増幅遺伝子（SAG）を

開発してきた。これまでにこのSAG導入遺伝子を導入したサル末梢CD34陽性細胞を骨髄抑制皆無動物に生着させるために移植直前に骨髄内を洗浄し、その骨髄内に移植するという骨髄置換法と併用することにより、末梢血の遺伝子導入効率を10%まで高めることに成功した。これは骨髄抑制の処置なしという条件で極めて高い遺伝子導入効率であり、このことから移植時の生着率を高めることができれば、より高い遺伝子導入効率が得られると考えた。そこで、SAGと骨髄置換法とMSCとの共移植の３つの技術を組み合わせることを案出した。これまでの経過から、SAGのデザインはほぼ固まっており、性能も安定しているため、生着の向上が現時点で遺伝子導入効率を上昇させる鍵となる。さらにカニクイザルを用いたSAGとiBMTの組み合わせで最初からEPOを投与して移植直後から増幅させた場合と、移植60日後にEPOを投与した場合で遺伝子導入効率が大きく違っていたことから、増幅効果が生着に影響することも考えられる。即ちこれらの組み合わせは単なる相加効果ではなく相乗効果を生むことが予想される。

一方、治療効果の問題とは別に安全性の課題も未だに解決されていない。即ち、レトロウイルスベクターを用いたときに発生するDNA組込に起因する遺伝毒性の問題である。昨年度この問題をを回避する目的で欠失型センダイウイルス（SeV）ベクターにEpoR-Mplを搭載し、応用の可能性について検討した。SeVは一本鎖RNAウイルスに属し、その感染-複製-出芽という生活環は、すべて細胞膜上から細胞質内で起こり、細遺伝情報が細胞核内への移行することはなく、レトロウイルスの様にホストの遺伝子配列に影響を与えるということは全く起こらない。しかし、造血幹細胞に感染しても増幅させないという課題が残った。本年度はレトロウイルスの中でも静止期にある細胞や、分裂速度の遅い細胞に対し遺伝子導入可能なレンチウイルスベクターの一つであるサル免疫不全ウイルス（SIV）ベクターにEPOR-Mplを搭載した。SIVベクターは由来となるSIVagmが、自然宿主に対し病毒性を持たないこと、HIVベクターと比較して野生株HIVと組み替えを起こしにくいという利点がある。また、このSIVベクターをヒト網膜色素細胞株であるARPE-19に感染させ、遺伝子挿入位置を解析法を改良し、50クローンの位置を決定した。

B．研究方法

（1）SIVベクターは遺伝子導入プラスミドと、ヘルパープラスミドとエンベローププラスミドを細胞に同時に導入しベクターを得るいわゆる第２世代ベクターで、遺伝子導入プラスミドは3’LTRの一部を欠失したSINベクターであり、WPRE配列、cppt配列を挿入したベクターである。内部プロモーターにはCMVプロモーターを用いこれにより挿入したEPOR-Mplを発現させるようにしてある。挿入したEPOR、Mplはいずれもヒト由来の遺伝子である。293T細胞にリポフェクトアミンプラス（Invitrogen社）を用い、トランスフェクトした。トランスフェクト８時間後、培地交換を行いトランスフェクト48時間後にベクターを回収した。ベクターはドットブロットにより、粒子力価を測定した。

（2）機能確認：SIV-EPOR-Mplにより導入されたEPOR-MplがEPO依存的な増幅信号を発生するかどうかをIL-3依存的プロB細胞株であるマウスBaF3に感染させ、IL-3とEPOそれぞれを添加した培地にて増殖・生存性を調べた。

(3) SIV ベクタープロウイルス挿入サイト解析：従来の LAM-PCR を変法し挿入位置付近の配列を単離し、大腸菌にクローニングした。これらDNA配列を解読し、インターネット上のブラストサイトに情報を入力し、挿入サイトを決定した。即ち、プライマーを従来LTR内に設計していたが、LTRは5'側と3'側の2つあること、配列の長さが短く制限があることなどにより、SIVagm のパッケージングシグナルの中の TAGGGGGTAGAGGACTTCTATGATTC の 3' 側にビオチン結合させた配列を合成し、アニールさせる。プライマーエクステンションを行い、一本鎖 DNA を合成し、それをストレプトアビジンビーズにより回収する。DNA依存DNA合成酵素により回収した1本鎖DNAを2本鎖にし、制限酵素処理後合成アダプターと結合させる。始めのPrimerより上流の配列を PCR リバースプライマーとしアダプター内の配列をフォワードプライマーEX Taq (TaKaRa-Bio)ポリメラーゼを用いとしてネストPCRを行う。

ポリメラーゼの特性である 3' 端に付加されるdTを利用して2nd PCR産物をプラスミドにサブクローニングし、さらにコロニーPCRあるいは制限酵素処理によりインサートの長さを決定し異なる複数のコロニーをピックアップし、シークエンス解析を行う。塩基配列データを Basic Local Alignment Search Tool (BLAST)にて解析を行った。具体的にはインターネットのNCBI、BLAST サイト（http://www.ncbi.nlm.nih.gov/blast/Blast.cgi）のヒト塩基配列の所に、データとなる塩基配列を入力し、マッチする配列のマッピングデータから染色体番号や位置を決定する。

（倫理面への配慮）

倫理上、特に問題となる点はなかった。

C．研究結果

(1) EPOR-MplをSIVへ搭載しても通常のSIV力価のベクターが生産された。10^8vg/ml程度の生産が確認できた。また、SIV-EPOR-Mpl感染BaF3は、IL-3非存在下ではEPO依存的な生存・増殖が確認された。しかし、対照となるSIV-EGFP感染細胞は、EPO存在下では生育できなかった。

(2) SIV-EGFPを感染させたヒト細胞株でLAM-PCRを行い、144の大腸菌コロニーを得た。これからコロニーPCR を行った結果72クローンでインサートを確認できた。インサートサイズをPCRと制限酵素処理により確認した69種類のクローンを得た。全ての塩基配列を決定し、組み込み箇所が判明できたのはそのうち52クローンであった（重複2箇所）。そのうちわけは22箇所（44%）がイントロン内で、28箇所（56%）が遺伝子外に挿入されていた。染色体としては1本あたり0ー7箇所挿入があり、挿入されなかった染色体は13、21、22番染色体で、7箇所と一番多く挿入されたのは5番染色体であった。

D．考察

(1) SAG と骨髄置換法と MSC との共移植の3つの技術を組み合わせによる造血幹細胞遺伝子導入効率の改善に関しては、サルによる評価を待たねばならず、現在そのデータ解析を行っている。これにより生着が高まればレトロウイルスベクター特に SIV ベクターによりより効率の良い遺伝子導入が可能になり、高い遺伝子導入細胞の生着が期待される。

(2) レトロウイルスベクターの問題点が XSCID 治療により明らかにされたが、LAM-PCR 方法の確

立によって、詳細なプロウイルスの挿入位置が決定は今後必要なデータとなることが予想できるMLV由来レトロウイルスベクタとの比較や、ターゲットの細胞の処理等でより安全な手法の開発においてその解析が重要になると思われる今回は細胞株を用いたが、造血幹細胞を用いたときどう変化するか、コロニー形成ユニットごとに比較するなどその用途は広いと思われる。この解析結果から安全性を立証できるわけではないが、より安全な手法の開発に不可欠な手法であると考えられる。

E．結論

レンチウイルスベクターは造血幹細胞遺伝子治療用ベクターとしても期待が高いベクターである。しかも国産のベクターであるSIVベクターに搭載した意義は高い。また依然として残るレトロウイルスベクターの持つ遺伝毒性軽減の検討などに不可欠な挿入位置の解析方法を確立したことは意義深い。

G．研究発表

1. 論文発表

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Ⅲ. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Ⅳ. 研究成果の刊行物・別刷

（主なもの）

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Review

Cell and gene therapy using mesenchymal stem cells (MSCs)

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Abstract

Mesenchymal stem cells (MSCs) are considered to be a promising platform for cell and gene therapy for a variety of diseases. First, in the field of hematopoietic stem cell transplantation, there are two applications of MSCs: 1) the improvement of stem cell engrafting and the acceleration of hematopoietic reconstitution based on the hematopoiesis-supporting ability; and 2) the treatment of severe graft-versus-host disease (GVHD) based on the immunomodulatory ability. Regarding the immunosuppressive ability, we found that nitric oxide (NO) is involved in the MSC-mediated suppression of T cell proliferation. Second, tumor-bearing nude mice were injected with luciferase-expressing MSCs. An in vivo imaging analysis showed the significant accumulation of the MSCs at the site of tumors. The findings suggest that MSCs can be utilized to target metastatic tumors and to deliver anti-cancer molecules locally. As the third application, MSCs may be utilized as a cellular vehicle for protein-supplement gene therapy. When long-term transgene expression is needed, a therapeutic gene should be introduced with a minimal risk of insertional mutagenesis. To this end, site-specific integration into the AAVS1 locus on the chromosome 19 (19q13.4) by using the integration machinery of adeno-associated virus (AAV) would be particularly valuable. There will be wide-ranging applications of MSCs to frontier medical treatments in the near future.

Keywords: Cancer gene therapy; GVHD; Mesenchymal stem cells; Site-specific integration; Tumor targeting

1. Introduction

In bone marrow, there are different types of tissue stem cells (adult stem cells); i.e. hematopoietic stem cells and mesenchymal stem cells (MSCs). MSCs account for a small population of cells in bone marrow as a non-hematopoietic component with the capacity to differentiate into a variety of cell lineages, including adipocytes, osteocytes, chondrocytes, muscles, and stromal cells [1]. Recent studies demonstrated that MSCs are capable of supporting hematopoiesis and of regulating immune response [2]. In addition, since MSCs can be readily isolated and expanded *in vitro*, they are expected to be a source of cell therapy. Interestingly, MSCs have the ability to accumulate at the site of: i) tissue/organ damage; ii) inflammation; and iii) cancer when administered *in vivo*. Therefore, MSCs can be utilized for: i) regenerative therapy; ii) treatment of graft-versus-host disease (GVHD) and Crohn disease; and iii) platform of cancer gene therapy (targeted delivery of anti-cancer agents). Another unique feature of MSCs is little or low immunogenicity due to the lack of expression of co-stimulatory molecules. This phenomenon makes it possible to administer MSCs without HLA matching for cell therapy. A single lot of expanded MSCs from one healthy donor can be utilized for treatment of many patients. Although clinical applications of MSCs have been conducted for the suppression of severe acute GVHD in allogeneic stem cell transplantation [3,4] and for regenerative therapy [5,6], molecular mechanisms underlying the biological effects of

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MSCs remains obscure. Finding key molecules for differentiation, immunosuppression, and hematopoietic support of MSCs would be valuable for further augmenting the efficacy of MSCs in a wide range of clinical applications. In this regard, development of the technology for genetic manipulation of MSCs is also important research project. Site-specific integration of a therapeutic gene into a safe locus in the genome should be investigated from the safety standpoint.

2. Microarray analysis of genes responsible for differentiation of mesenchymal stem cells

Genes regulating the differentiation of MSCs remain obscure and it is technically difficult to do high-throughput analysis using primary MSCs, because such cells contain heterogeneous populations. To overcome the problems related to the heterogeneity of primary MSCs, we utilized MSC-like cell lines. It has been shown that 10T1/2 cells, derived from C3H mouse embryo cells, differentiate into adipocytes, osteocytes, and chondrocytes with a treatment of 5-azacytidine. We previously established two sub-lines from 10T1/2, designated as A54 for a preadipocyte cell line and M1601 for a myoblast cell line [7]. Under appropriate culture conditions, A54 and M1601 cells terminally differentiate into adipocytes and myotubes, respectively, while parental 10T1/2 cells remain undifferentiated under the same culture conditions. Therefore, 10T1/2 cells can be utilized as a model of MSCs, and A54 and M1601 are used as committed mesenchymal progenitors. Gene expression profiles of these cell lines were compared by microarray analysis before and after differentiation.

Each of parent 10T1/2, A54, and M1601 cell lines showed a distinctive and unique gene expression profile despite morphological similarity (Fig. 1) [8]. Parental 10T1/2 cells had 105 elevated genes including ones encoding Activin, Dlk, Nov, Grb10, p15, and many functionally unknown molecules. Dlk and Nov are known to be involved in Notch signaling pathway and were reported to have the ability to inhibit differentiation into adipocytes and osteoblasts [9]. In preadipocyte A54 cells, 201 genes were up-regulated, including genes known to be involved in adipocyte differentiation such as genes encoding C/EBPα, C/EBPδ, PPAR-γ, PAI-I, and Frizzled-1 [10]. Myoblasts M1601 cells showed 137 up-regulated genes, including ones related to skeletal muscle differentiation such as genes encoding MyoD, MLC1F, α-skeletal actin, myosin heavy chain, and myosin light chain [11] as well as genes related to cardiac muscle differentiation such as genes encoding α-cardiac actin, cardiac troponin C, and troponin T2 [12].

Previous studies have shown that preadipocytes have a higher ability to support hematopoiesis than other kinds of stromal cell components *in vitro* [12,13]. Our results of gene expression profile revealed up-regulation of critical cytokines for hematopoiesis such as SCF and SDF-1 in preadipocyte A54 cells. In addition, many chemokines, such as CXCL-1 and CCL-7, were also up-regulated. Since Ang-1 was reported to be indispensable for the self-renewal of hematopoietic stem cells [14], we performed real-time PCR analysis of Ang-1 along with SCF, SDF-1, CEBP-δ, IGF-1, and CXCL-1. The expression of these genes was highest in A54 cells among the three cell lines. Moreover, protein expression of Ang-1 was only detected in A54 among three cell lines and the level of this protein decreased after adipocyte differentiation.

To examine the effects of these three lines on hematopoiesis, we co-cultured mouse hematopoietic stem cell fraction with these three stromal cell lines. The cells in Lin(−)Sca-1(+) fraction were plated on 10T1/2, A54, or M1601 cells.

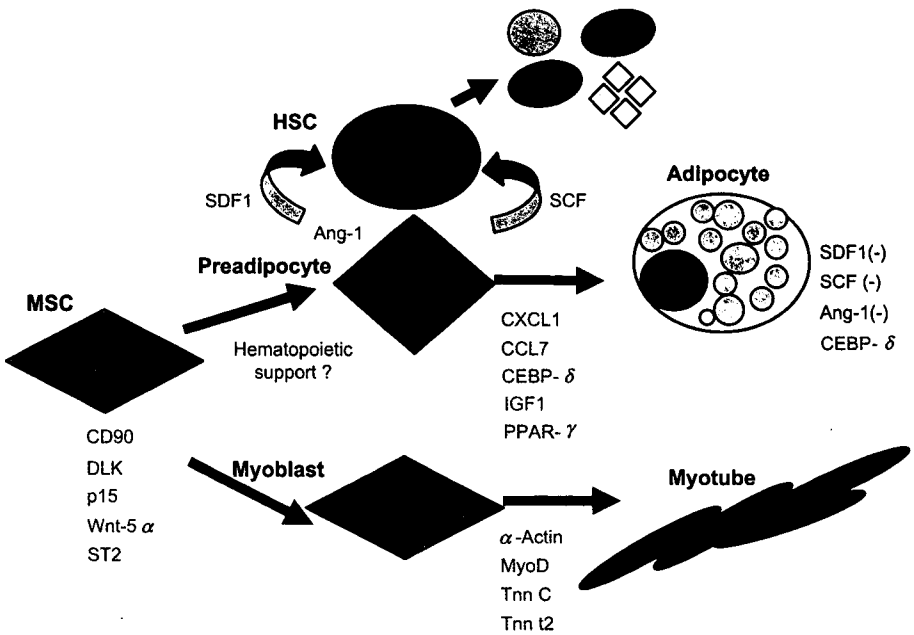

Fig. 1. Proposed model for the hierarchy of the bone marrow stromal system [8].

After 6 days of co-culture, hematopoietic progenitors were detected only on the A54 cells. These results suggest that only A54 cells have the ability to support hematopoietic cell growth among these three cell lines, consistent with the previous report. Hematopoietic cell proliferation was not observed on the layer of the terminally differentiated A54 adipocytes, suggesting that A54 cells lose the ability for hematopoietic cell support after adipocyte differentiation. To understand the molecular mechanisms of this observation, we examined the expression levels of SCF, SDF-1, and Ang-1 during adipocyte differentiation by RT real-time PCR. The expression levels of Ang-1 and SCF decreased immediately after the induction of adipocyte differentiation, and that of SDF-1 decreased gradually. In contrast to this, the level of adipocyte differentiation marker, CEBP-δ, was unchanged.

The analysis of functionally unknown molecules is currently underway. In addition, cell-to-cell contact is also believed to be crucial in the interaction between hematopoietic stem cells and MSCs. We are currently investigating the cellular and molecular events in the interactive communication between hematopoietic stem cells and MSCs.

3. Nitric oxide (NO) plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells

There is a case report of severe steroid-resistant GVHD after bone marrow transplantation, in which intravenous infusion of MSCs greatly improved clinical manifestations [3]. Moreover, multi-institutional clinical trial of MSC-treatment of severe grade III–IV acute GVHD in Europe revealed very high overall response rate (about 70%) (Le Blanc et al., ASH meeting 2006). The molecular mechanisms by which MSCs suppress T-cell proliferation are complicated, and whether a soluble factor plays a major role remains controversial. Transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), and prostaglandin E_2 (PGE_2) have been reported to mediate T-cell suppression by MSCs [15–17]. In addition, some reports have shown that a soluble factor is the major mediator of suppression, whereas some reports have demonstrated that T-cell-MSC contact is required for this suppression.

We also investigated the molecular mechanisms using primary murine MSCs, and focused on nitric oxide (NO), because it is known to inhibit T-cell proliferation. NO is produced by NO synthases (NOSs), of which there are 3 subtypes; i.e. inducible NOS (iNOS), endothelial NOS, and neuronal NOS. It has been known that macrophages suppress T-cell proliferation, and that this suppression is caused by NO-mediated inhibition of Stat5 phosphorylation [18]. We investigated whether MSCs can also produce NO and whether NO is involved in their ability to suppress T-cell proliferation [19].

T cells proliferated in response to PMA and ionomycin, which act downstream of the T-cell-receptor complex by activating protein kinase C and inducing Ca^{2+} influx, respectively. Such T-cell proliferation was suppressed by the presence of MSC, suggesting that MSCs influence signals downstream of protein kinase C and Ca^{2+} influx. The expression of the activation markers CD25 and CD69 on CD4 or CD8 T cells did not change even in the presence of MSCs. MSCs suppressed the production of IFN-γ but not IL-2.

Although T cells from $Stat5^{-/-}$ mice do not proliferate upon stimulation with anti-CD3, they up-regulate CD25. Because this phenotype is similar to the status of activated T cells in the presence of MSCs, we hypothesized that MSCs suppress Stat5 phosphorylation. Indeed, Stat5 phosphorylation in activated T cells was diminished in the presence of MSCs. We found that MSCs caused a significant and cell-dose-dependent production of NO only when co-cultured with activated T cells. The induction of iNOS was readily detected in MSCs but not in T cells. RT-PCR and Western blot analysis detected iNOS expression in MSCs cocultured with activated splenocytes but not in MSCs or splenocytes when cultured alone. The immunofluorescence studies showed that iNOS was exclusively expressed in $CD45^-$ adherent cells, which correspond to MSCs, but not in $CD45^+$T cells. Next, we investigated the effects of *N*-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of NOS. As expected, L-NAME dose-dependently inhibited the production of NO by MSCs in the presence of activated T cells. Importantly, L-NAME restored T-cell proliferation and Stat5 phosphorylation, indicating that NO is involved in the inhibition of T-cell proliferation and Stat5 phosphorylation. Moreover, MSCs from inducible $NOS^{-/-}$ mice had a reduced ability to suppress T-cell proliferation.

In the presence of direct interaction between T cells and MSCs, there was a high level of NO production accompanied by a strong suppression of T-cell proliferation. In contrast, both NO production and T-cell suppression were reduced in a transwell system, in which T cells were separated from MSCs by a 1-μm-pore membrane. There are two possible explanations for the difference in T-cell suppression between the presence and absence of the transwell system. First, the amount of NO produced in the transwell system was lower than that in the presence of direct interaction. This finding suggests that direct interaction is critical for efficient production of NO as well as for strong suppression of T-cell proliferation. A second possible explanation is that, because NO is highly unstable, it can lose its activity before it reaches T cells in the transwell system.

Because TGF-β, IDO, and PGE_2 were reported as mediators of T-cell suppression by MSCs, we compared the effects of L-NAME with inhibitors of each mediator. Indomethacin (inhibitor of PGE_2 production) but not 1-methyl-DL-tryptophan (1-MT: inhibitor of IDO) or an anti-TGF-β-neutralizing antibody restored T-cell proliferation as effectively as L-NAME; however, the effects of L-NAME and indomethacin were not additive, suggesting that the NO and PGE_2 share signaling pathways leading to T-cell suppression.

In summary, our hypothesis that NO is produced by MSCs and that it suppresses T-cell proliferation in part through inhibition of Stat5 phosphorylation was supported by the following facts: (1) NO was readily detected in the medium in the co-culture of MSCs and activated T cells; (2) L-NAME restored T-cell proliferation as well as Stat5 phosphorylation; and (3) MSCs from $iNOS^{-/-}$ mice had markedly

reduced ability to suppress T-cell proliferation. This hypothesis was further confirmed by the finding that iNOS expression was detected only in MSCs co-cultured with activated T cells.

In our scenario (Fig. 2), when MSCs are administered to the patients with severe acute GVHD, MSCs are considered to accumulate at the site of inflammation. Upon interaction with activated T cells, MSCs express iNOS and produce NO, which suppresses T-cell proliferation via inhibition of STAT5 phosphorylation. Systemic adverse effects of NO do not occur due to local production of NO with very short half-life. This is a very important point, because conventional treatment of acute GVHD causes severe systemic immunosuppression, which sometimes leads to life-threatening infections. Since MSC treatment causes just local immunosuppression, it should be much safer.

4. Interferon-γ and NF-κB mediate nitric oxide production by mesenchymal stem cells

Human MSCs were reported to suppress Th1 differentiation and augment Th2 differentiation. Therefore, we investigated whether mouse bone-marrow-derived MSCs and the 10T1/2 cell lines have the same effect on Th1 and Th2. We found a reverse correlation between NO production and T cell proliferation in Th1/Th2 conditions, where NO production was highly induced in the presence of MSCs in Th1 but it was only minimally induced in Th2. In particular, primary MSCs and the A54 preadipocyte cell line, which induce strong T cell suppression in Th1, produce high levels of NO in Th1 condition. These results suggest that NO also plays a major role in the preferential suppression of Th1 proliferation by MSCs.

To determine what inhibits the production of NO in Th2 condition, the two differentiation factors that support Th2 differentiation, anti-IFN-γ antibody and IL-4, were investigated. As a result, anti-IFN-γ antibody clearly inhibited the production of NO, whereas suppression by IL-4 was less evident. These results suggest that IFN-γ is a key regulator of NO production by MSCs.

Interestingly, cell supernatant collected from activated T cells had the ability to induce NO production by MSCs. IFN-γ is critical for NO production; however, in a T cell-free environment, IFN-γ alone does not induce NO production from primary MSCs. IFN-γ in combination with LPS, but not IL-2, stimulates NO secretion from primary MSCs, suggesting that both the IFN-γ and the signal from Toll-like receptor-4 (TLR4) are required for NO induction by MSCs. The addition of flagellin induced NO production in combination with IFN-γ. While, synthetic double strand RNA, poly(I:C), and CpG-oligonucleotide did not induce NO. Flagellin is a protein component of bacteria known to induce NO production from macrophages via TLR5 in the presence of either a TLR4 or IFN-γ signal. In addition to these factors, IL-1β and TNF-α induce NO when provided in combination with IFN-γ. As NF-κB is a downstream target of the signaling cascades activated by LPS, flagellin, IL-1β, and TNF-α, we hypothesized that activation of NF-κB is required for NO induction by MSCs. Bay-11-7085, a specific inhibitor of NF-κB, suppressed induction of iNOS in MSCs, thus suggesting that NF-κB is involved in NO production by MSCs as well as IFN-γ [20].

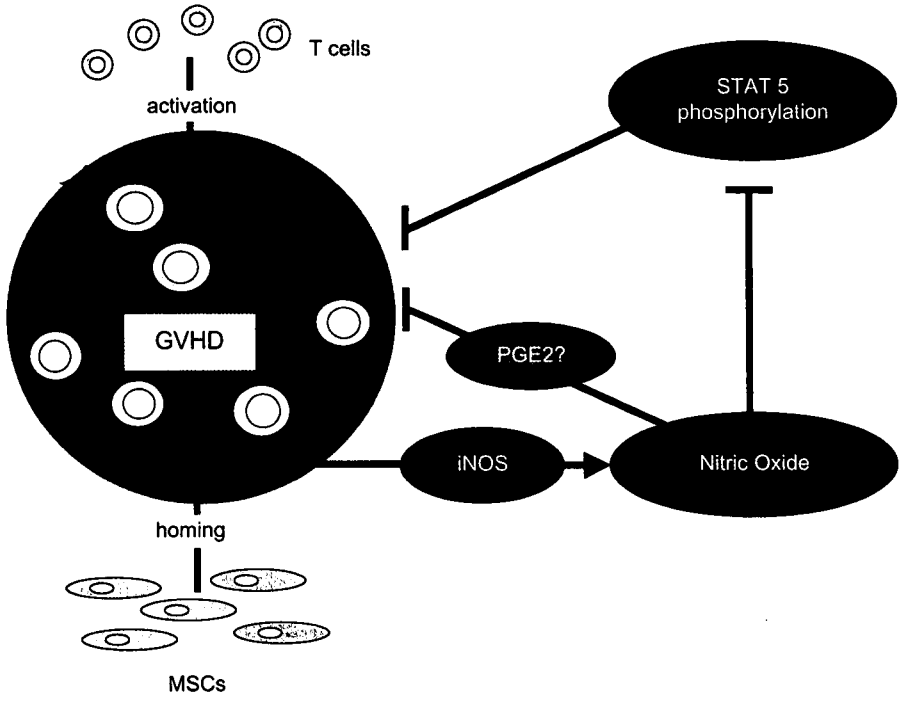

Fig. 2. MSC treatment of acute GVHD and the molecular mechanisms of T-cell suppression. MSCs are considered to accumulate at the site of inflammation and systemic adverse effects may not appear due to the local production of NO, which has very short half-life.

5. Retroviral vector-producing mesenchymal stem cells for tumor tracking and therapeutic gene amplification in suicide cancer gene therapy

MSCs are known to have a tendency to accumulate at the site of tumors, and therefore can be utilized as a platform for targeted delivery of anti-cancer agents [21–23]. The MSC-based targeted cancer gene therapy can enhance the therapeutic efficacy, because MSCs are considered to reach tumors including metastatic lesions and to deliver therapeutic molecules in a concentrated fashion. This targeted therapy can also reduce systemic adverse side effects, because the anti-cancer agents act locally at the site of tumors without elevating their systemic concentrations. We developed genetically-modified MSCs that produce retroviral vectors encoding HSV*tk*, aiming at augmenting therapeutic efficacy of systemic suicide cancer gene therapy (Fig. 3). The tumor tropism and anti-tumor effects of vector-producing MSCs (VP-MSCs) were examined by intravascular injection in tumor-bearing nude mice. MSCs isolated from the bone marrow of SD rats were transfected with plasmid DNA expressing luciferase alone (=non-VP-MSCs) or whole retroviral vector components (LTR-Luc or LTR-HSV*tk* with Gag-pol and VSV-G) (=VP-MSCs) by nucleofection. To assess tumor tropism of MSCs, nude mice were subcutaneously inoculated with 9 L rat glioma cells or Rat-1 fibroblasts, and were subsequently injected with luciferase-expressing MSCs through the left ventricular cavity. The transgene expression was periodically traced by using an *in vivo* imaging system. As a result, the transgene expression accumulated at the site of subcutaneous 9 L tumors, but undetectable at the site of Rat-1 fibroblasts. In addition, the injection of luciferase-expressing VP-MSCs caused much stronger signal of bioluminescence at the site of 9 L tumors compared with luciferease-expressing non-VP-MSCs. Immunostaining study showed that luciferase-positive cells (injected MSCs and transduced glioma cells) were detected at the periphery of tumors. To evaluate the therapeutic efficacy, tumor-bearing nude mice were treated with non-VP-MSCs or VP-MSCs combined with HSV*tk*/GCV system and then the size of subcutaneous tumors was periodically measured. In this model experiments, tumor growth was more efficiently suppressed by injecting VP-MSCs compared with non-VP-MSCs (Uchibori R, et al.: manuscript in preparation). This study suggests the effectiveness of VP-MSCs in suicide cancer gene therapy. The therapeutic benefit of this strategy should be further examined in orthotopic and metastatic tumor models.

6. Site-specific insertion of a therapeutic gene into the AAVS1 locus (19q13.4) in human mesenchymal stem cells by using adeno-associated virus integration machinery

Hematopoietic stem cells, ES cells, and MSCs are attractive targets for gene therapy and regenerative medicine, since they replicate themselves and differentiate into various cell lineages. To introduce genes in these stem cells, it is especially important to utilize a system that results in a minimal risk of insertional mutagenesis. To date, only one animal virus, the adeno-associated virus (AAV), is able to integrate into a defined site in human chromosome, AAVS1 (19q13.4), which is mediated by the activity of specific replicase/integrase protein, Rep. The Rep78 or Rep68 protein recognizes the GAGC motif on the viral inverted terminal repeat (ITR) sequence and a similar motif in AAVS1, leading to the site-specific integration of the AAV genome.

We and others have reported that a plasmid transfection system utilizing AAV derived components, the *rep* gene and ITR, could integrate the gene of interest preferentially into AAVS1 in epithelial or adherent cells (e.g., 293, HeLa, Huh-7 cells) [24–26]. Our system uses two plasmids, one harboring the transgene cassette flanked by the ITR sequences, and the other for *rep* expression, allowing only plasmid DNA harboring the ITR to integrate into the AAVS1 locus. In addition, this system can deliver DNA segments larger than the 4.5-kb packaging limit of AAV. As a first step toward establishing a method capable of integrating therapeutic DNA into the AAVS1 locus in MSCs, we tested this strategy in KM-102 cells, a cell line derived from human marrow stromal cells. KM-102 cells were co-transfected with a bicistronic plasmid containing a humanized GFP gene and a blasticidin S resistance gene (*bsr*) between the ITRs and a Rep68 plasmid. After transfection, single cell clones were grown in the presence of

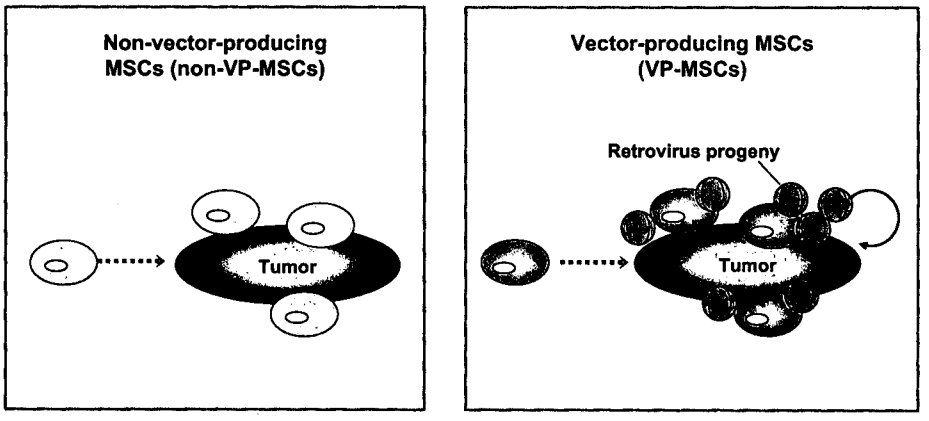

Fig. 3. Development of vector-producing tumor-tracking MSCs to augment suicide cancer gene therapy.

blasticidin S. Southern blot analysis of their genomic DNA revealed that three out of eight blasticidin S resistant clones showed site-specific integration of transgene into the AAVS1 site and that these clones had the GFP gene only at AAVS1. These results indicated that foreign DNA linked with ITR sequence could be targeted specifically into AAVS1 in KM-102 cells.

It is reported that the genome of myosin binding subunit 85 (MBS85) overlaps with the AAVS1 site [27]. To identify the junction between the transgene plasmid and the AAVS1 site, PCR was conducted using a transgene- and an AAVS1-specific primers. In two of the three clones the integration site was identified. In one clone the GFP gene was inserted at the first intron of MBS85 gene. The other clone had insertion of the GFP gene upstream of the first exon. Quantification of mRNA for MBS85 by real time PCR showed that the mRNA level decreased in these two KM-102 clones. The MBS85 is involved in the assembly of actin cytoskeleton. Although the outcome of allelic disruption of the MBS85 genome should be carefully evaluated, the system for AAVS1-specific integration of therapeutic DNA using AAV integration machinery is particularly valuable for *ex vivo* gene therapy applications for stem cells, such as ES cells and MSCs. For additional readings on the use of bone marrow cells for the treatment of autoimmunity, the reader is referred to companion papers published herein in this special issue of the Journal of Autoimmunity [28–38].

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