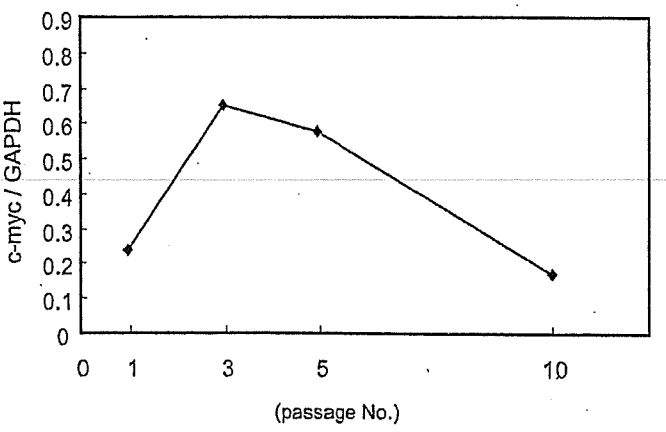

Fig. 3. Effect of the passage number on the mRNA expression c-myc oncogene in hMSC.

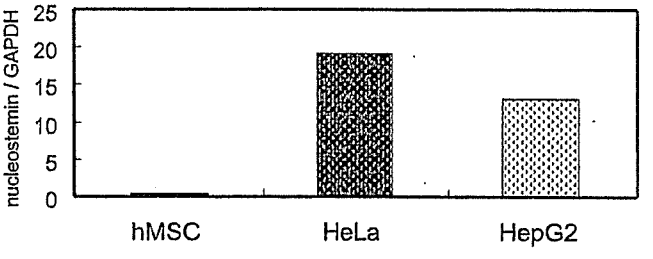

Fig. 4. The mRNA expression nucleostemin in hMSC, HeLa, and HepG2.

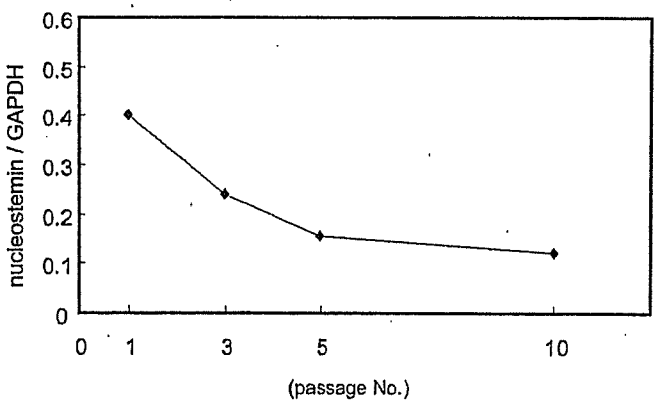

Fig. 5. Effect og the passage number on the mRNA expression of nucleostemin in hMSC.

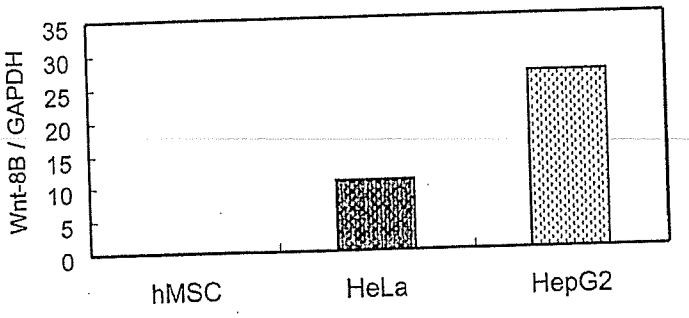

Fig. 6. The mRNA expression Wnt-8B in hMSC, HeLa, and HepG2.

4. DISCUSSION

In this study, effects of the passage number on the gene expression in hMSC were investigated. At first, c-myc oncogene and Wnt-8B concerned with cell proliferation and tumorigenesis were noticed by gene chip analysis (data not shown). Therefore, c-myc oncogene and Wnt-8B mRNA expressions in four kinds of passage numbers (#1, #3, #5, and #10) of hMSC were measured by quantitative real time RT-PCR. Furthermore, nucleostemin that concerned with proliferation of both stem cells and tumor cells (1) was also investigated. The proliferation speed of hMSC was lowered with the cell passage number (Fig. 1).

The mRNA expressions of c-myc oncogene, Wnt-8B, and nucleostemin in 1st, 3rd, 5th, and 10th passage of hMSC were investigated using quantitative real time RT-PCR. The mRNA levels of c-myc oncogene were decreased with the passage number from 3rd to 10th (Fig. 3). The mRNA expression of nucleostemin was decreased with the passage number (Fig. 5). In all three genes, their mRNA expressions of the stem cells (hMSC) were significantly lower than two kinds of tumor cells (HeLa and HepG2) (Fig. 2, 4, and 6). In hMSC, Wnt-8B was not expressed in any passage numbers. Although these results suggest that change in these expression levels are not directly related to the tumorigenesis of hMSC, it is discussed that mRNA expression levels of c-myc oncogene, nucleostemin, and Wnt-8B can be used as an index of hMSC tumorigenesis.

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EFFECT OF BIODEGRADABLE POLYMER POLY (L-LACTIC ACID) ON THE CELLULAR FUNCTION OF HUMAN ASTROCYTES

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Abstract: The objective of this study is to assay the efficiency and safety of poly (L-lactic acid) (PLLA) on human neural tissues. We used normal human astrocytes (NHA) to clarify effects of PLLA on their proliferation and differentiation. We cultured NHA with PLLA for one week, and determined NHA cell number and neural cell specific marker genes to assay their proliferation and development, respectively.

Cell proliferation was determined by tetrazolium salt (MTT) assay. The cell number of astrocytes cultured with 50 μg/ml PLLA was 70% of control. It has been suggested that a part of astrocytes had neural precursor cell activity that give rise to neuron, oligodendrocyte and astrocyte. We compared gene expression of neural cell specific markers. Expression of Nestin, a specific gene for neural precursor cell was decreased in a dose-dependent manner, while expression of specific genes for neuron markers and astrocyte markers were not different from that of control.

PLLA suppressed astocyte proliferation in dose dependent manner. A neural precursor cell marker decreased when astrocytes were cultured with PLLA. These findings suggest that PLLA reduces proliferation and developmental potential of astrocytes.

Key words: Astrocyte, PLLA, proliferation, development

S. Iijima and K.-I. Nishijma (eds.), Animal Cell Technology: Basic & Applied Aspects, 331–337.

1. INTRODUCTION

Brain and neural clinical hospitality have been rapidly advancing, including implantation techniques. Otherwise discreditable accidents sometimes happened. It is necessary to study efficiency and safety of techniques and materials for brain and neural cell proliferation and development. Precise mechanisms by which neurogenesis and gliogenesis are regulated in the central nervous system (CNS) remain to be elucidated. Telencephalic neuroepithelial cells contain neural precursors that give rise to the neuronal lineage and the glial lineage, which includes astrocytes and oliogodendrocytes (1, 2). The fate of neural precursors in the developing brain is believed to be determined by intrinsic cellular programs and by external cues, including implantation of biomaterials and cytokines (3). Doetsch et al. demonstrated that subventricular zone (SVZ) astrocytes act as neural stem cells in both the normal and regenerating brain (4). Neural stem cells, endogenously present in spinal cord *in vivo*, proliferate in response to injury, yet the vast majority of newly generate cells are glial fibrillary acidic protein (GFAP)-positive astrocytes (5). In addition, adult hippocampus-derived neural stem cells, when implanted into adult brain in such a region as cerebellum or striatum, have been reported to differentiate predominantly into glial cells (2, 6, 7).

Biodegradable polymers have been attractive candidates for scaffolding materials because they degrade and the new tissues are formed, although adverse events such as foreign-body reaction, inflammation and tumor formation were reported in clinical human and animal study. These scaffolds have shown great promise in the research of engineering a variety of tissues. Biodegradable polymer poly (L-lactic acid) (PLLA) is frequently implanted in cranial surgery etc. However, to engineer clinically useful tissues and organs is still a challenge. The understanding of the principles of scaffolding is far from satisfactory, still more its effect and safety on neural tissues are not known. We previously reported PLLA suppressed proliferation and differentiation of fetal rat midbrain neural precursor cells (8). In this report, we investigated the effect of PLLA on normal human astrocytes (NHA).

2. MATERIALS AND METHODS

Astrocyte cell culture

We used normal human astrocyte (Cambrex Bio Science, Walkersville, MD). NHA were seeded into 12-well plates for quantitative RT-PCR at a density of 2 x 10^4/well, or 24-well plates for MTT assay at a density of 1 x 10^4/well in ABM medium(Cambrex Bio

Science) supplemented with 5% FCS, rhEGF and IGF, and cultured in a humidified atmosphere of 5% CO_2 in 95% air at 37°C.

PLLA preparation

Stock solutions of PLLA were made in dimethyl sulfoxide (DMSO) and final concentration of DMSO was 0.1%; this concentration did not affect proliferation and development of NHA. Control cultures were incubated with 0.1% DMSO. Stock solutions of lactic acid and tin chloride were made directly in ABM medium.

MTT assay

After cell culturing for 1 week with PLLA, the viability of NHA cells was determined by MTT assay. The TetraColor ONE (Seikagaku Kogyo, Tokyo, Japan) was used to measure changes of cell numbers. This assay is a nonradioactive alternative to tritium-thymidine incorporation. The system measures the conversion of tetrazolium salt compound into a soluble formazan product by the mitochondria of living cells. NHA in 24-well plates were cultured as described above. One week after NHA cultured with vehicle or PLLA, the media were replaced with 300 µl of fresh medium containing 6 µl TetraColor ONE reagent. After 2h, samples were measured in a micro plate reader.

Expression of neural cell marker genes

Total RNA was prepared from NHA using a modified acid guanidium thiocyanate-phenol-chloroform method. The total RNA treated with RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) were subjected to reverse transcription using oligo d(T) primer (Toyobo, Tokyo, Japan) and superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) at 42°C for 30 min followed by RNase H treatment. Aliquots of the cDNA (1/20) were used as templates for PCR analysis using Lightcycler system (Roche, Mannheim, Germany). PCR amplification was performed in a total volume of 20 µl mixture including 1 µl of RT reaction, 2 µl Light Cycler-Fast Start Reaction Mix SYBR Green 1(Roche, Mannheim, Germany), 0.5 µM/liter of each primer, and 3 mmol/liter $MgCl_2$. The PCR program consisted of 40 cycles of 8 sec at 94°C, 5 sec at 65°C, 10 sec at 72°C. Primer sequences for amplification are 5'- CTAAGGAGGAGATTGGACAGG-3' and 5'-AGTGGTGGCAGTGATTT CAGT-3' for Nurr-1 amplification, 5'-TCCGCTGCTCGCCGCTCCTAC-3'and 5'-TCATCTCTGCCCGCTCACTGG -3' for GFAP amplification, 5'-TCGCCCTGCCCACTTGACTTC-3' and 5'-TTCCACACCTCCACGCTC TGA-3' for Id-3 amplification, 5'-GAGATCAGAGCCCAGGATGCT-3' and 5'-CTGAGGGGTGGTGCCAAGGAG -3' for Nestin amplification, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-

TCCACCACCCTGTTGCTGT A-3' for GAPDH. RNA preparation and RT-PCR in the present study were performed in triplicate.

Statistical analysis

The Fisher's PLSD was used to compare the PLLA concentration and relative expression levels of neural specific marker mRNA.

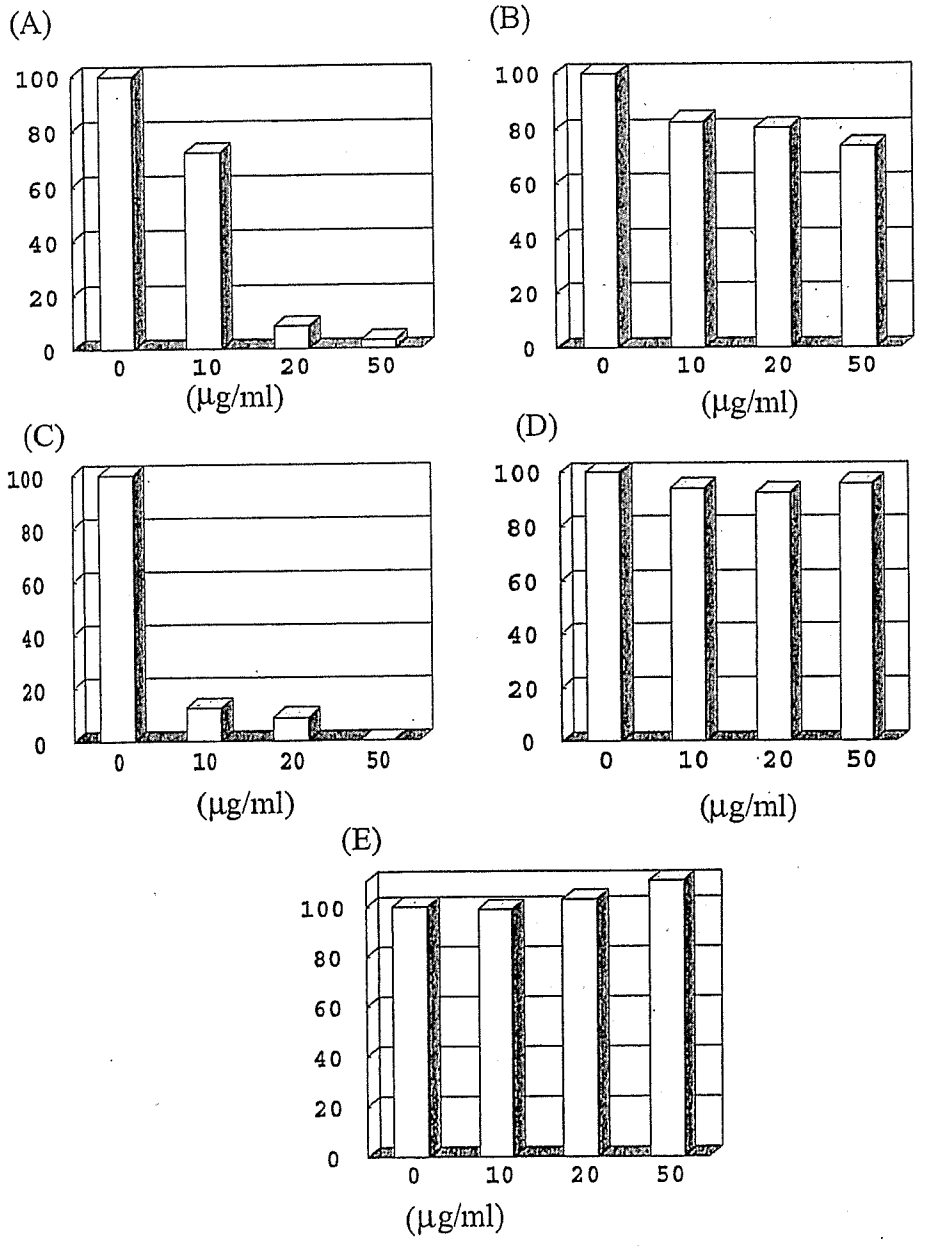

Fig. 1 Effect of PLLA on NHA proliferation (A) PLLA 3.000 (B) PLLA 5.000 (C) PLLA 11.000 (D) Lactic acid (E) Tin chloride

3. RESULTS AND DISCUSSION

NHA proliferation

We used three kinds of PLLA. PLLA 3000 (PLLA, Mw 3000) is made without catalyst. PLLA 6000 (PLLA, Mw 5000) is made with organic tin catalyst. PLLA 11000 (PLLA, Mw 11000) is made with catalyst tin chloride, contains 590 ppm tin. After a week culture with PLLA, we detected cell number of NHA using MTT assay. Cell numbers were decreased in a dose-dependent manner of PLLA (Fig. 1A-C). The cell number of NHA cultured with 50 μg/ml of PLLA 3000, PLLA 5000 and PLLA 11000 were 15%, 70% and 7.8% of that of control respectively.

Whether tin ion included in PLLA affected NHA proliferation or not, we added tin chloride to NHA culture medium (Fig. 1D). The concentration of tin chloride at 50 ng/ml did not affect NHA proliferation. PLLA is hydrolysed in medium, we assayed lactic acid (LA), a monomer of PLLA was also tested by the MTT assay using NHA cells. (Fig. 1E). There was no effect on the cell number of NHA culture with LA monomer. The cause of PLLA effect for NHA was neither included tin ion nor degraded LA monomer. It was probably the effect of PLLA itself and/or degraded LA oligomers.

Lam and his co-workers demonstrated that predegraded PLLA (P-PLLA; 25 kGy gamma-irradiation) caused signs of cell damage, cell death, and cell lysis due to phagocytosis of a large amount of P-PLLA particles (9). Phagocytosis of LA oligomers or degraded PLLA particles may affect the proliferation and development of NHA. It is necessary to know culture medium with PLLA contains how much PLLA particles, PLLA oligomer and organic tin.

Gene expression of neural cell specific markers

It has been suggested that a part of astrocytes contain neural precursor cell activity that give rise to neuron, oligodendrocyte and astrocyte itself. The recent discovery of stem cell populations in the CNS has generated intense interest, since the brain has long been regarded as incapable of regeneration (5, 10, 11). Neural stem cells (NSCs) have capability for expansion and differentiation into astrocytes, oligodendrocytes, and neurons in vitro (12, 13). NSCs have been suggested to have therapeutic potential for central nervous system regeneration (14-16).

They express their original specific genes, neural cell specific markers. Neural precursor cells express Nestin, a class IV intermediate filament protein. Differentiated neuron expresses Nurr-1, a transcription factor and Id-3, a transcription inhibitory factor. Astrocyte expresses

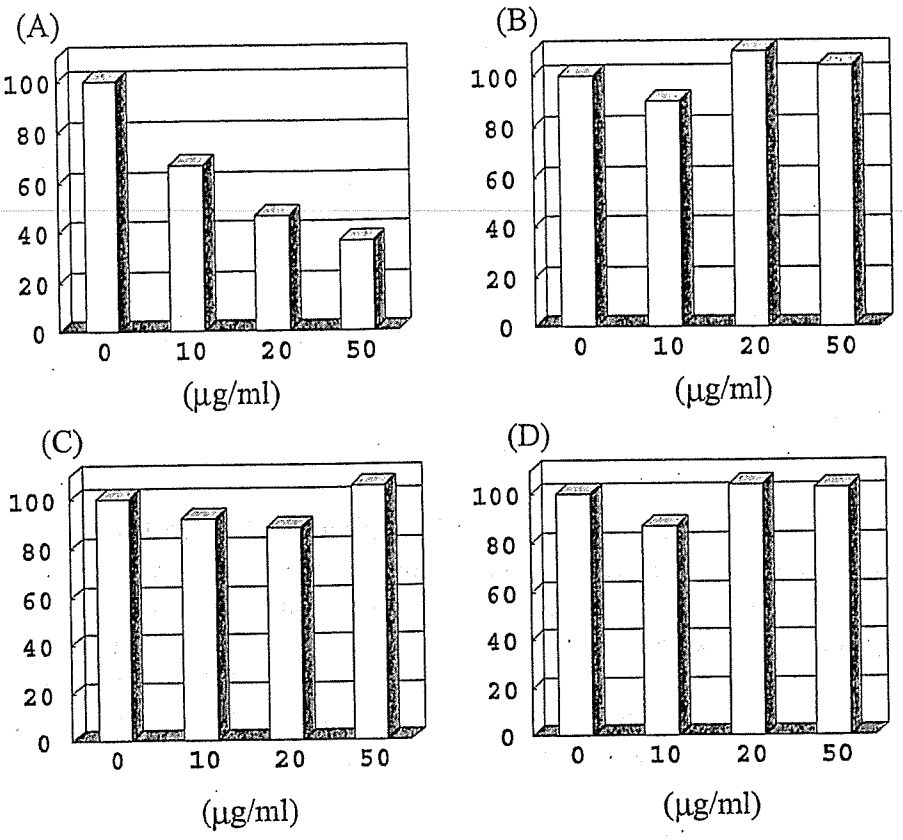

Fig. 2 Effect of PLLA on neural specific gene expression.
(A) Nestin (B) Nurr-1 (C) GFAP (D) Id-3

GFAP, a glial filamentous acidic protein. We compared gene expression of neural cell specific markers. Expression of Nestin, a neural precursor cell marker decreased with the dose of PLLA5000. The expression of Nestin in NHA cultured with 50 μg/ml PLLA was 30% of control (Fig. 2A). Expressions of the other genes that assayed in this study were similar to control (Fig. 2B-D).

Expression of Nestin was decreased when NHA were cultured with PLLA suggested that PLLA decreased population of neural precursor cells. There were two kinds of possibilities. (1) PLLA leads NHA to gliogenesis. Nakashima et al. reported that Gliogenesis significantly reduced the number of cells expressing Nestin and the number of cells expressing microtubule-associated protein 2 (MAP2), a neuronal marker. (2) When neural precursor cells specifically phagocytosed PLLA, they go to programmed cell death, apoptosis or loose their developmental potential as neural precursor cells. Lam et al. demonstrated that PLLA caused signs of cell damage, cell death, and cell lysis due to phagocytosis of a large amount of P-PLLA particles. Phagocytosis of PLLA may affect proliferation and development of NHA.

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特集：ナノテクノロジーと医療

ナノレベルイメージングによる分子構造と機能の解析

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ナノレベルイメージングによる分子構造と機能の解析

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Nano-level imaging for analyzing protein structure and function

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Abstract

The present manuscript outlines the nano-level imaging project, which is under promotion by the three national research institutes and supported by a research grant from the Ministry of Health, Labor and Welfare (nano-001). This research project targets collecting fundamental information regarding comprehensive understanding of cardiovascular, neurological and the other disorders, developing new diagnostic and therapeutic methods by visualizing protein structure and function in atomic (sub-nano level) or molecular (nano-level) resolution. The results of the current projects will be extended into drug design, clinical diagnostic technology and medical materials in near future.

Key words: nano-technology, structural biology, drug design, protein crystallography, tailor-made medicine

はじめに

21世紀の医療の社会的課題として提唱されているテーラーメード医療の達成には，標的となる蛋白の構造を患者ごとに確定し(分子診断)，最適な薬剤の構造を選択し(分子治療)，薬剤と生体蛋白の相互作用を分子レベルで観察する(分子評価)などの医療基盤技術の育成が求められる．ナノレベルイメージングプロジェクトでは，蛋白分子の構造と機能の解析を通じてテーラーメード医療実現のための基盤技術の形成を目指している．

本稿では蛋白構造イメージングを中心に概説する．

1. 創薬に貢献した分子構造イメージング

近年，放射光を用いたX線回折法の発達により原子レベルの解像度で蛋白結晶の構造を決定できるようになった．構造に基づく薬剤設計の具体的な成功例として，AIDS治療薬(HIVプロ

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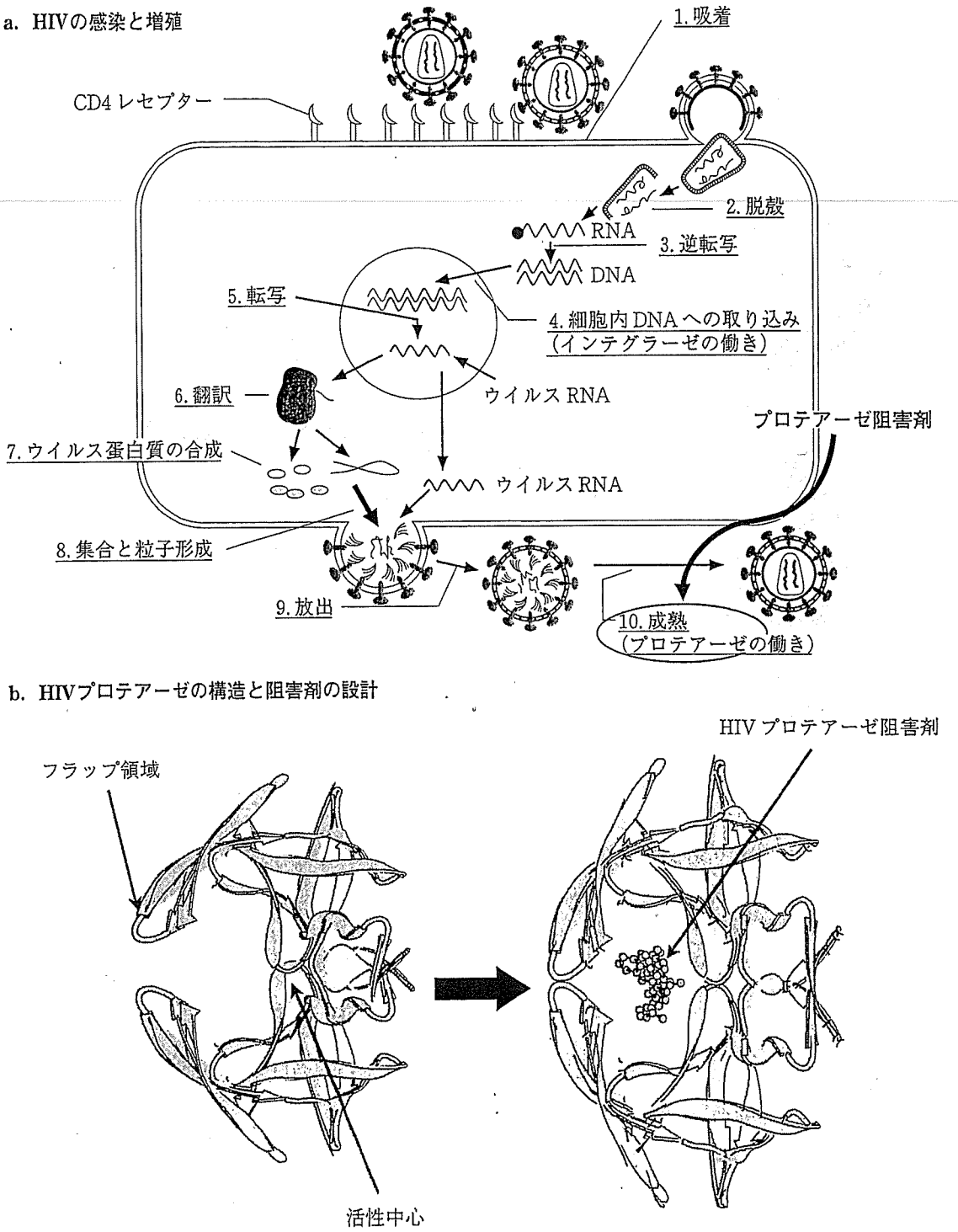


図1 AIDSウイルスの増殖過程と蛋白構造に基づくHIVプロテアーゼ阻害薬の作用機構

テアーゼ阻害薬), 白血病治療薬(グリベック)について以下に述べる.

AIDSウイルス, HIVは活性化外殻蛋白gp120によりCD4陽性Tリンパ球に感染し, 自己増殖をする. その際自己由来のプロテアーゼによって前駆体蛋白から活性化外殻蛋白を得る(図1-a). このHIVプロテアーゼの構造に基づいて設計され, その活性中心を選択的に阻害する目的で設計された薬剤がHIVプロテアーゼ阻害薬である(図1-b). 本剤はAIDSの発症を遅らせることに貢献した[1].

慢性骨髄性白血病ではフィラデルフィア染色

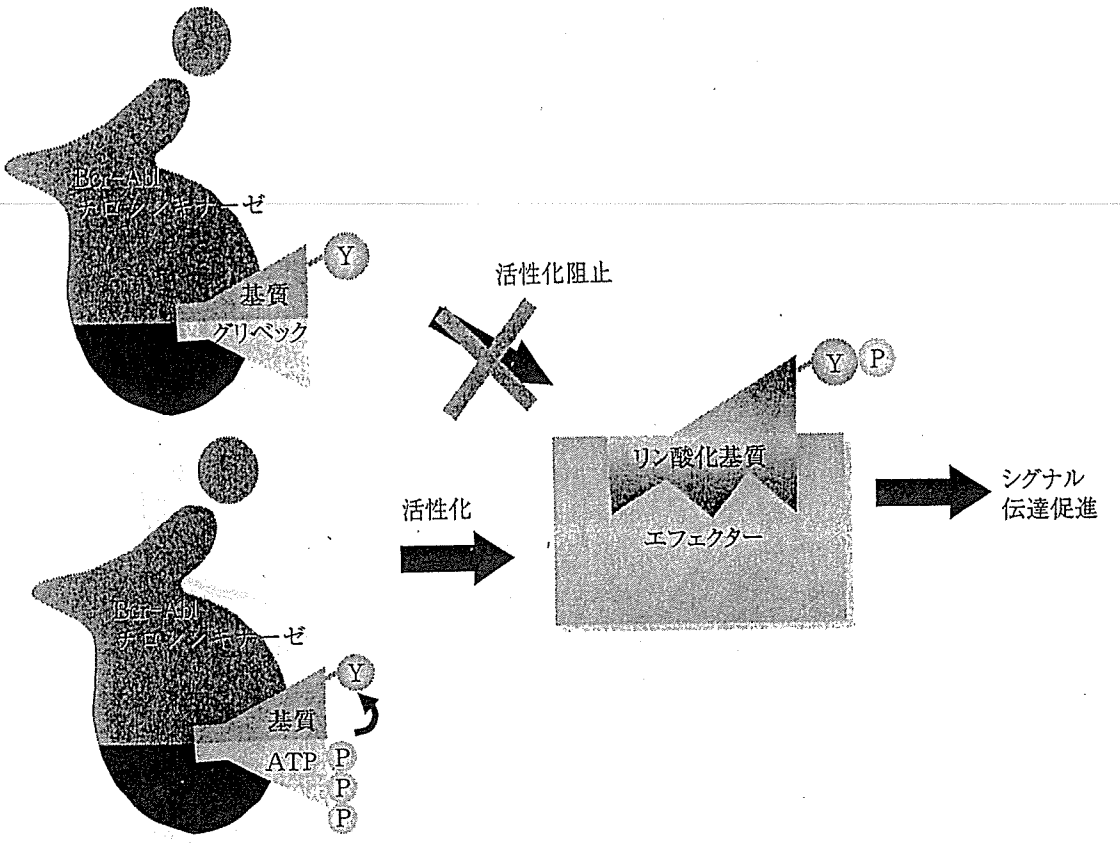


図2 慢性白血病治療薬(グリベック)の蛋白構造に基づく作用機構

体に由来するBcr-Ablチロシンキナーゼが恒常的な増殖シグナル伝達系の活性化を通じて慢性骨髄性白血病発症の原因になると考えられている．同酵素はATPと基質に結合し，ATPから切り離したリン酸基で基質のチロシン残基をリン酸化する．グリベックはBcr-Ablチロシンキナーゼの ATP結合部位の詳細な構造に基づいて設計され，基質のチロシンリン酸化を構造特異的に阻害して白血病化を防ぐ(図2)[2]．

このような構造に基づいて薬剤設計を行うことで標的蛋白との結合の特異性を高め，副作用を減少させることを期待できる．

2. ヒト心筋トロポニンの構造解析とそれに基づく創薬の可能性

心筋収縮を調節する心筋トロポニンの中核部分(コアドメイン)の構造は分担研究者である武田と理化学研究所の前田らによって解析され，Nature誌に報告された(Vol 424, 2003)[3]．前田らの総説[4]に基づき，トロポニンの筋収縮調節メカニズムについて述べる．

筋収縮はアクチンとミオシンの滑り運動による．アクチンフィラメントはアクチン，トロポニン，トロポミオシンを含む複合体であり，それらの3分子は7：1：1の存在比をもつ．トロポニンの存在下でアクチンとミオシンはカルシウムイオン濃度に応じた収縮と弛緩を行う．

図3に心筋トロポニンのコアドメインの構造を示す．トロポニンはTnC，TnI，TnTと呼ばれる3つのポリペプチド鎖からなる．これまでの研究により，TnIは収縮抑制因子，TnCは脱抑制因子，TnTはTnCの脱抑制を弱める因子(カルシウム濃度依存性の付加因子)であることが示されている[5]．

トロポニンのコアドメインは更に調節頭部とITアームの2つのサブドメインに分かれる．調節頭部はカルシウムイオンとの結合を通じてトロポニンの構造変化とそれに基づくアクチンとミオシンの滑り運動に対するスイッチの役割を果たす．ITアームは剛性を有するコイルドコイ

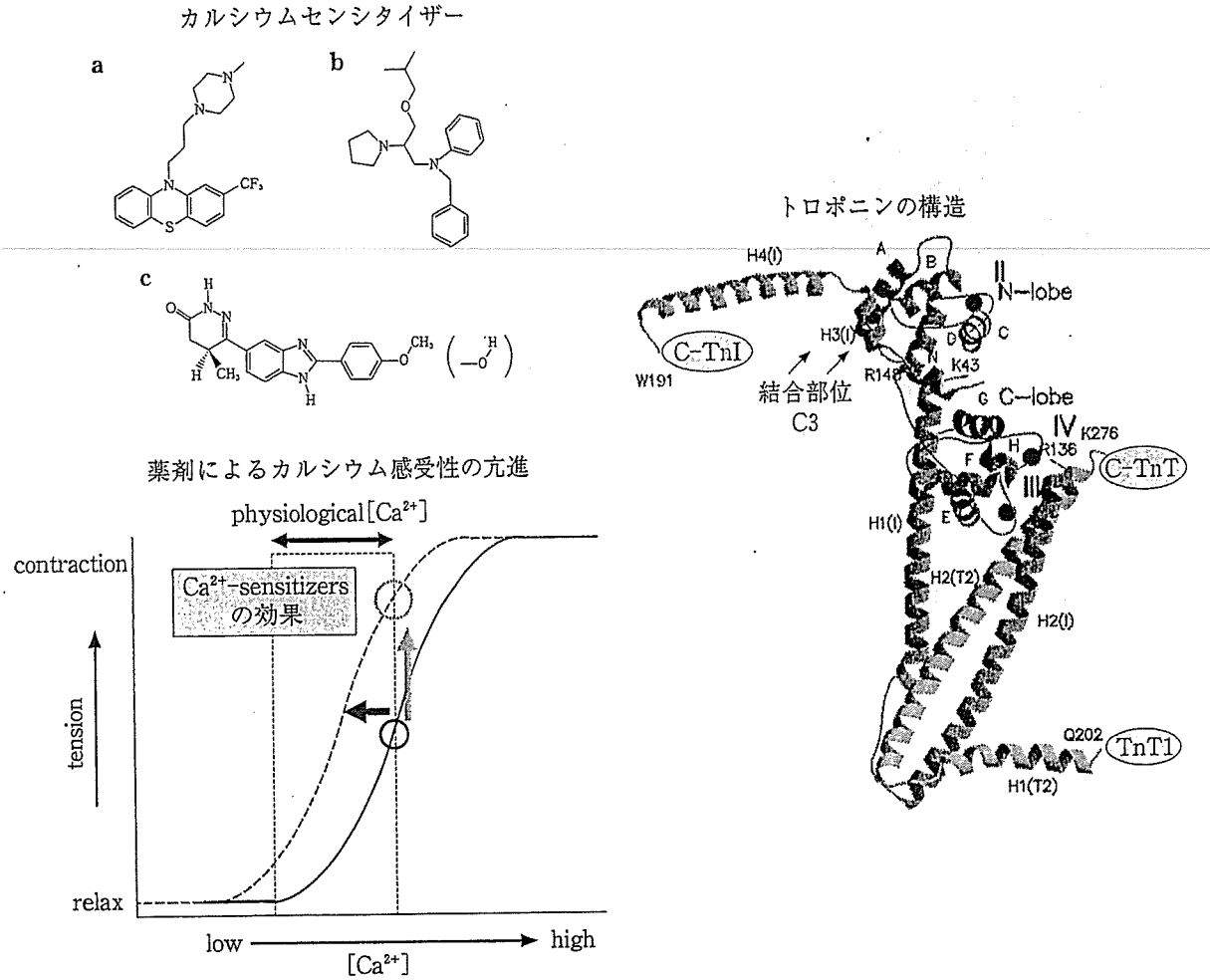
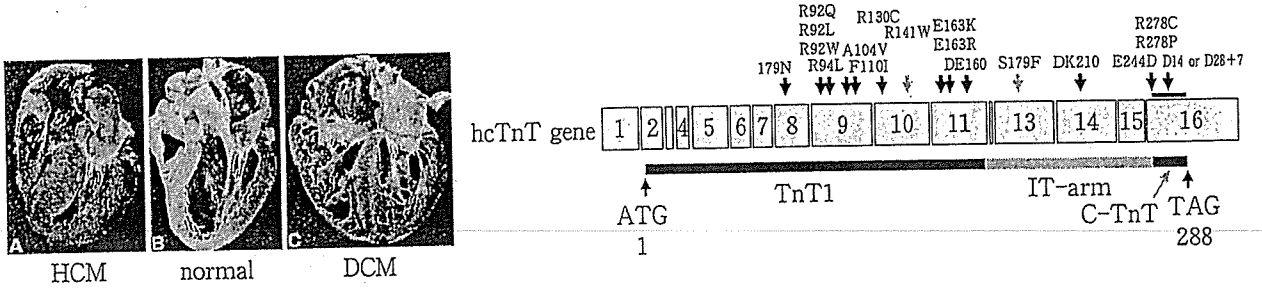


図3　トロポニンコアドメインの構造(文献[3]より改変引用)

ル構造からなる．TnCはN末端側とC末端側の2つの球状部がαヘリックスで連結された構造をもつ．カルシウム濃度にかかわらずC末側球状部はTnIに結合し，TnCをトロポニン分子内に常につなぎとめている．一方，TnCのN末端側球状部は細胞内カルシウム濃度が上昇した場合のみ構造が開き，TnIの第二結合部位(両親媒性αヘリックスH3)を結合する．これにより，TnIの調節領域全体がトロポミオシン/アクチンより解離し，アクチンとミオシンの滑り運動が始まる．

TnCのN末端側球状部にカルシウムセンシタイザーが結合すると，同球状部は開いた構造をとりTnIの第二結合部位を結合しやすくなる．すなわち，TnCによるTnIの脱抑制が起こりやすくなる．前述のようにTnTはTnCの脱抑制作用にカルシウム濃度依存性を付加することができるので，TnCとTnTの制御を組み合わせることで段階的な筋収縮の増強を実現できるかもしれない．近年循環器領域では血管作動性薬剤で優れた新薬が数多く開発されてきたが，ジギタリス以来，これを超える強心剤が生まれていない．従来の強心剤は細胞内カルシウムイオン濃度を高めて強心作用を誘導するために，細胞に対する負荷(カルシウムoverload)が不可避であった．1980年代後半に開発されたカルシウムセンシタイザーと呼ばれた薬剤群はカルシウムイオン濃度-張力関係を左方にシフトさせることにより，低い細胞内カルシウムイオン濃度で高い収縮力を得ることができる理想的な強心剤ではないかと期待された[6]．しかしながら，これらの薬剤の臨床使用経験から，短期的に心筋収縮力は高まるものの，心不全患者の長期予後の改善に役立つことはなかった．これらのカ



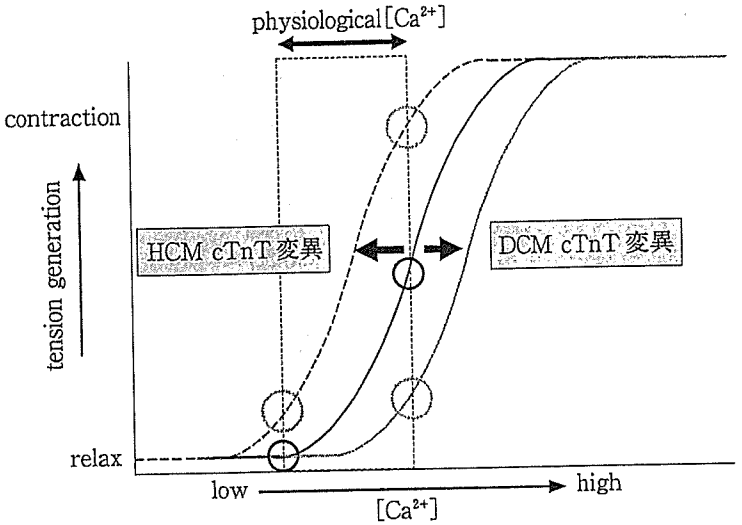


図4　心筋症におけるトロポニンの遺伝子変異と筋カルシウム感受性
心筋症の遺伝子変異はTnT1，C-TnTに多く，筋カルシウム感受性を修飾する．

ルシウムセンシタイザーはphoshodiesteraseの阻害作用も併せてもっており，細胞内cyclic-AMPの増加によって筋小胞体からのカルシウムイオン放出が増加し，ついにはカルシウムoverloadとなる可能性や[7]，構造が類似した他の蛋白と相互作用があるなど，薬剤としての標的特異性が低いことが原因として考えられる．拡張型心筋症例では，少なくとも一部の症例でカルシウム感受性の低下と収縮不全の関連が示唆されている．これらの事実はTnCやTnTを特異的に制御する化合物の設計により，新たな強心剤の開発の可能性を示している．

一方，肥大型心筋症(HCM)ではトロポニンの遺伝子変異によりカルシウム感受性が亢進することが発病に関連する可能性が示唆されている．同患者の遺伝子解析によると，約15％の患者にTnTの遺伝子変異が認められる．大槻らによれば[5]トロポニンがアクチン/トロポミオシンと直接接触する部分(TnT1，C-TnT，TnI調節領域)に変異が多く認められ，コアドメインには変異は少ないという(図4)．変異TnTの交換導入を行った心筋スキンドファイバーを用いた研究で，カルシウムイオン濃度-張力関係の左方シフト，すなわちカルシウム感受性の亢進が認められた．この結果からTnTの変異により，カルシウム感受性が亢進し，収縮増加と弛緩不全という肥大型心筋症に特有の症状が発症するという有力な仮説が生まれる．TnTの変異によるカルシウム感受性亢進のメカニズムを原子構造で解明すると，肥大型心筋症に特異的に作用する薬剤の設計を期待できる．原因となる遺伝子変異ごとに構造が異なる薬剤設計が求められる可能性もある．言い換えれば，心筋トロポニンの変異に基づく肥大型心筋症の治療法の開発はテーラーメード医療のモデルケースとなる可能性がある．

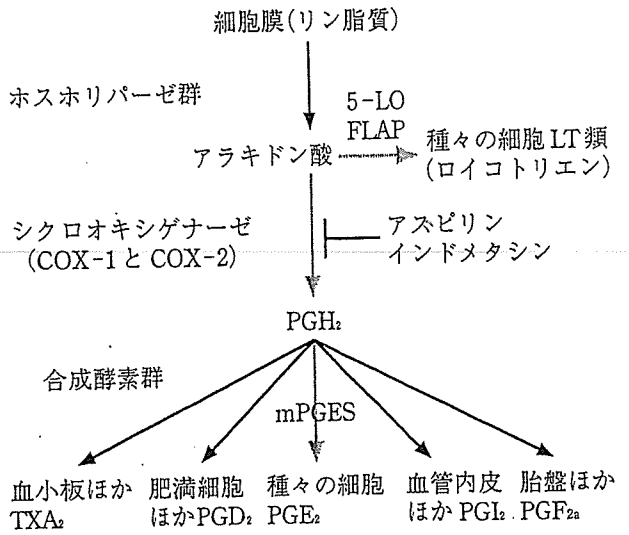


図5 プロスタグランジン産生系

3. 創薬の標的として注目されているプロスタグランジン合成酵素群の構造解析

シクロオキシゲナーゼ(COX)はプロスタグランジン(PG)を生合成する律速酵素として知られている(図5)2種類のアイソザイムが存在する．COX-1は constitutive enzyme と呼ばれ，ほとんどの細胞で常時発現しており，生体の安定性を維持する役割を果たす．一方，COX-2は inducible enzyme として，単球，線維芽細胞，滑膜細胞などの炎症にかかわる細胞で発現し，炎症性サイトカインなどによって誘導される．従来の非ステロイド系抗炎症剤は，COX-1とCOX-2の両方を阻害するために炎症巣のPGだけでなく，胃粘膜や腎でのPG(特にPGE_2)産生を抑制し胃や腎の副作用を合併する．そこで，炎症に深く関与していると考えられるCOX-2だけを選択的に阻害する薬剤の開発が進められてきた．このようにして開発されたCOX-2阻害薬は胃潰瘍を起こしにくい鎮痛剤として好んで投薬されていた．しかしながら，2004年末，米政府は，これらのCOX-2選択的阻害薬の3剤を心筋梗塞や脳梗塞の危険性を高める恐れがあるとして，心臓病患者への処方や多量の長期使用を避けるよう勧告した．COX-2の下流に位置するプロスタサイクリン合成酵素の作用も抑制するために，同酵素に由来する抗血栓性作用や血流増加作用が損なわれることが原因ではないかと考えられている[8]．図5に示したようにCOX-2の下流には多くの合成酵素があってそれぞれの作用を有する蛋白を合成している．個々の合成酵素を選択的に阻害する薬剤の開発が次世代の創薬の標的として注目される．PGE_2の産生にかかわるmPGESを阻害する薬物の開発は血管内血栓形成を伴わない理想的な抗炎症剤となる可能性がある．TXA_2産生を阻害する薬剤の開発は血管内血栓形成の予防，局所血流増加作用を通じて脳梗塞，心筋梗塞の予防薬や治療薬として期待できる．PGI_2は既に難病といわれた原発性肺高血圧症の治療に有効であることが知られている．PG関連薬剤の開発は構造に基づく創薬の最大の標的の一つになっており，ナノメディシンプロジェクトでも複数の関連酵素の構造解析に取り組んでいる．

4. ナノメディシンプロジェクトのそのほかの研究

本プロジェクトでは分子構造イメージングに関連して上記のほかに，細胞内イオン環境や，血管新生にかかわる蛋白など幾つかの蛋白構造についても研究を進めている(国立循環器病センター研究所)．国立精神神経センターではin-silicoスクリーニング法によるParkinson病の治療薬探索に蛋白構造情報を応用する研究を進めている．国立医薬品食品衛生研究所では原子間力顕微鏡を用いて蛋白表面の詳細な構造を解析することなどを通じて，医用材料作成に向けた応用研究に取り組んでいる．

一方，分子機能イメージングの領域では，国立循環器病センターの望月らが増殖因子(EGF)刺激に伴うRas分子の活性化をFRET法で可視化できることをNature誌に報告した[9]．ナノメディシンプロジェクト開始後も血管内皮の走化運動にかかわるRap1蛋白の可視化に関する研究などにFRET法による分子イメージングを展開している．国立精神神経センターの研究グループでは分子機能イメージング技術を応用してシナプス機能，プリオン蛋白質の機能の評価に

取り組みProc Natl Acad Sciなどの雑誌に研究成果を報告している[10].

おわりに

本ナノメディシンプロジェクトでは循環器治療の中核施設である国立循環器病センター内に構造生物学ラボを立ち上げ，分子特異的な治療薬の開発を目指している．ナノDDS技術や分子機能イメージング技術に関する研究を併せて推進することで，特異的分子治療薬の分子輸送技術開発と他の分子との相互作用の可視化技術を推進することが可能となる．これにより，分子診断・分子治療・分子評価を包含するテーラーメード医療の基盤形成に貢献したい．

謝辞　本原稿の執筆内容は本研究グループの成果を元にしております．国立循環器病センター研究所若林繁夫分子生理部長およびユーセフ・ベン・アマー同研究員，増田道隆循環器形態部室長，柴田洋之心臓生理部同室員，五十嵐智子同研究員，松原孝宜同研究員，大阪大学月原富武教授，理化学研究所宮野雅司主任研究員に感謝いたします．また，本原稿編集と英文作成に協力していただいた東本弘子女史，松尾千重女史に感謝します．

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ORIGINAL ARTICLE

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Toshie Tsuchiya, PhD

Changes in expression of genes related to cell proliferation in human mesenchymal stem cells during in vitro culture in comparison with cancer cells

Abstract We investigated the expression levels of several genes related to cell proliferation in human mesenchymal stem cells (hMSCs) during in vitro culture for use in clinical applications. In this study, we focused on the relationship between hMSC proliferation and transforming growth factor β (TGFβ) signaling during in vitro culture. The proliferation rate of hMSCs gradually decreased and marked changes in hMSC morphology were not observed in 3 months of in vitro culture. The mRNA expressions of TGFβ1, TGFβ2, and TGFβ receptor type I (TGFβRI) in hMSCs increased with the length of cell culture. There had been no change in the TGFβ3, TGFβRII, and TGFβRIII mRNA expressions by the 12th passage from the primary culture (at about 3 months). The mRNA expression of Smad3 increased, but those of c-myc and nucleostemin decreased with the length of hMSC in vitro culture. In addition, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of cancer cells. In conclusion, hMSCs derived from bone marrow seldom underwent spontaneous transformation during 1–2 months of in vitro culture for use in clinical applications. In hMSCs as well as in epithelial cells, growth might be controlled by the TGFβ family signaling.

Key words Stem cells · Cell proliferation · TGFβ signaling · TGFβ receptors

Introduction

Several recent studies demonstrated the potential of bioengineering using somatic stem cells in regenerative medicine.[1,2] Bone marrow includes both mesenchymal and hematopoietic stem cells. Adult human mesenchymal stem cells (hMSCs) derived from bone marrow have the pluripotency to differentiate into cells of mesodermal origin, e.g., bone, cartilage, adipose, and muscle cells.[1–5] Moreover hMSCs also have the capacity to differentiate into myocytes,[6,7] hepatocytes,[1,8] and neural cells.[3] In addition, because they are comparatively easy to expand ex vivo, hMSs have many potential clinical applications, not only in the field of orthopedic surgery but also for the treatment of cardiac infarction, cirrhosis, and diabetes. On the other hand, stem cells possess a self-renewal capability similar to that of cancer cells.[9] Recently Rubio et al.[10] reported spontaneous transformation of human adult stem cells derived from adipose tissue in long-term (4–5 months) in vitro culture. In practice, if hMSCs are to be used for clinical applications and tissue-engineered medical devices, they have to be expanded in vitro for about 1–2 months. The proliferation ability and the gene expression profile of hMSCs, however, might change during in vitro culture. In this study, we focused on the relationship between hMSC proliferation and transforming growth factor β (TGFβ) signaling during in vitro culture. TGFβ is a multifunctional protein that regulates cellular proliferation, differentiation, apoptosis, development, extracellular matrix formation, immunosuppression, and tumorigenesis. In humans, three TGFβ isomers have been identified: β1, β2, and β3. TGFβ signals through three high-affinity cell surface receptors: TGFβ type I (TGFβRI), type II (TGFβRII), and type III (TGFβRIII) receptors. TGFβRI and TGFβRII are serine-tyrosine kinases. TGFβRIII is known to be a betaglycan.[11] TGFβs are first bound to TGFβRII and TGFβRIII.[12] It has been considered that TGFβRIII regulates access to TGFβRII,[12–14] and then TGFβ signal transduction in the cellular pathway is started through stimulation of TGFβRI by TGFβRII. After that, activated TGFβRI phosphorylates Smad2 or Smad3, which are receptor-regulated Smads (R-Smad) activated by TGFβ and activin.[15,16] After Smad4, which is a common mediator Smad (C-Smad), is connected to phosphorylated R-Smads, the complex is transported to the cell nucleus and influences the transcription activity of TGFβ-dependent genes.[15,16] c-myc, which is one of the

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TGFβ-dependent genes, is regarded as an oncogene and regulates cellular proliferation. In the present study, therefore, we investigated whether the gene expression levels of three TGFβ isomers (TGFβ1, TGFβ2, and TGFβ3) and their receptors (TGFβRI, TGFβRII, and TGFβRIII), Smad3 and c-myc were changed in hMSCs during in vitro culture.

Wnt-8B is related to cell self-renewal and tumorigenesis,[9] and Wnt proteins can act as stem cell growth factors.[17] Wnt signaling activates the genes that promote proliferation (c-myc and others) by accumulating β-catenin in some kinds of stem cells and cancer cells.[9] Nucleostemin is involved in proliferation in both stem cells and cancer cells.[18] Therefore we also investigated the gene expression levels of Wnt-8B and nucleostemin in hMSCs.

In addition to investigating the expression of these genes r ing to cellular proliferation in hMSCs during in vitro culture, we compared them with those in two kinds of cancer cell lines, HeLa S3 (a human cervical cancer cell line) and HepG2 (a human hepatoma cell line).

Materials and methods

Cell culture. Human mesenchymal stem cells (hMSCs) derived from bone marrow were purchased from Cambrex Bio Science (Walkersville, MD, USA). Their donor was an African American woman aged 19 years. The cells that we obtained from Cambrex Bio Science were second-passage cells. The hMSCs were cultured in mesenchymal stem cell basal medium (MSCBM; Cambrex Bio Science) supplemented with mesenchymal cell growth supplement (MCGS; Cambrex Bio Science), L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C under a 5% CO_2 atmosphere. The cells were seeded at a density of 6000 cells/cm^2 ; were subcultured when they were just subconfluent (approximately 90% confluent) up to the 10th passage, corresponding to the 12th passage from when the hMSCs were collected from the donor. The human cervical carcinoma cell line HeLa S3 (JCRB Cell Bank, Osaka, Japan) was cultured using Ham's F-12 culture medium (Dainippon Pharmaceutical, Osaka, Japan) containing 10% fetal bovine serum (FBS) (Intergen, Purchase, NY, USA) and 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The human hepatoma cell line HepG2 (Riken Bioresource Center, Tsukuba, Japan) was cultured using minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1 mM nonessential amino acids (NEAA) (Invitrogen), 10% FBS (Intergen), and 100 U/ml penicillin-streptomycin (Invitrogen).

Preparation of total RNA. Because the purchased hMSCs had been expanded in the manufacturing process as described above, we express the 1st passage of the hMSCs in this study as the 3rd from the primary culture. For quantitative real time-polymerase chain reaction (RT-PCR), total RNA was extracted from hMSC cultures during the 3rd, 5th, 7th, and 12th passages from the donor with Isogen (Nippon Gene, Toyama, Japan). Total RNA was also extracted from HeLa S3 and HepG2 cells once only with Isogen (Nippon Gene).

Quantitative RT-PCR. RNA was then reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Basel, Switzerland). Primers and annealing temperatures for the c-myc oncogene, nucleostemin, Wnt-8B, transforming growth factor (TGF)β3, and TGFβRIII are summarized in Table 1. Amplifications were carried out for 10 s at 95°C, for 15 s at each annealing temperature, and for 12 s at 72°C for 40 cycles. Amplifications of TGFβ1, TGFβ2, TGFβRI, TGFβRII, and Smad3, plus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, were performed using Light Cycler Primer Sets (Roche Diagnostics). PCR was performed in Light Cycler Fast Start DNA Master SYBR Green I (Roche Diagnostics) in a Roche Light Cycler (software version 4.0).

Statistical analysis. All results are shown as means ± SD. The significance of the differences in mean values was evaluated by Student's *t* test.

Table 1. Primers and annealing temperatures used for real-time PCR

Gene name	GenBank accession number	Primer orientation	Nucleotide sequence	Starting sequence position	Size for the PCR amplicon(bp)	Annealing temp. (°C)
c-myc	V00568	Forward	5′- GCG AAC ACA CAA CGT C -3′	1626	315	50
		Reverse	5′- CAA GTT CAT AGG TGA TTG CT -3′	1940		
nucleostemin	X91940	Forward	5′- CCA TTC GGG TTG GAG TAA -3′	782	284	50
		Reverse	5′- CTG TCG AGC ATC AGC C -3′	1065		
Wnt-8B	NM_014366	Forward	5′- AGT GAC AAT GTG GGC T -3′	331	244	60
		Reverse	5′- CGT GGT ACT TCT CCT TCA G -3′	574		
TGFβ3	NM_003239	Forward	5′- AAA CAC CGA GTC GGA A -3′	535	284	60
		Reverse	5′- TGC CAC CGA TAT AGC G -3′	818		
TGFβRIII	NM_003243	Forward	5′- TCC CTA TCC CGC AAG C -3′	2369	189	60
		Reverse	5′- AGA TTA TCG AGG CGT CC -3′	2557		

PCR, polymerase chain reaction; TGFβ3, transfroming growth factor β3; TGFβRIII, TGFβ recepter type III

Results

The proliferation rate of hMSCs decreased with the length of in vitro culture (Fig. 1). The effects of the in vitro culture term on hMSC proliferation and the mRNA expressions of three TGFβ isomers (TGFβ1, β2, β3) and their receptors type I, II, and III (TGFβRI, RII, RIII) in hMSCs were investigated (Fig. 2). The mRNA expressions of TGFβ1, TGFβ2, and TGFβRI increased with the length of cell culture (Fig. 2A,B,D), but there had been no change in the TGFβ3, TGFβRII, and TGFβRIII mRNA expressions by the 12th passage (at about 3 months) (Fig. 2C,E,F). In addition, the mRNA expression of Smad3, which is one of the R-Smads activated by TGFβ and activin, in hMSCs was investigated. The mRNA expression of Smad3 decreased in the 5th and 7th passages of hMSCs but increased in the 12th passage (Fig. 3). The mRNA expressions of c-myc in hMSCs were higher in the 5th and 7th passages than in the 3rd and 12th passages (Fig. 4A). The mRNA expressions of nucleostemin in hMSCs decreased with the length of cell culture (Fig. 4B).

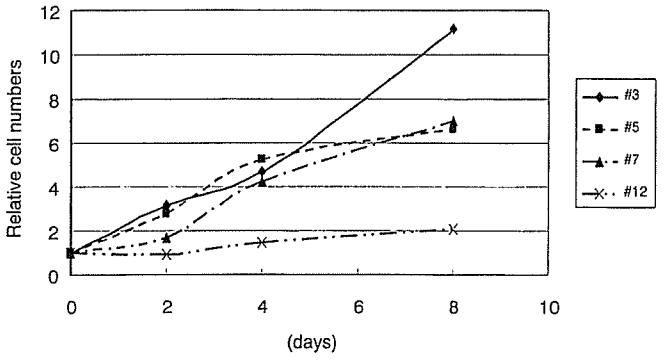

Fig. 1. Proliferation of human mesenchymal stem cells (hMSCs) in the 3rd, 5th, 7th, and 12th passages. hMSCs were seeded at 1.7×10^5 cells/F 60-mm dish (6000 cells/cm^2), and cells were counted after 2, 4, and 8 days. The initial cell number (0 days) is expressed as 1, and the other cell numbers (2, 4, and 8 days) are expressed relative to that of day 0. $n = 3$

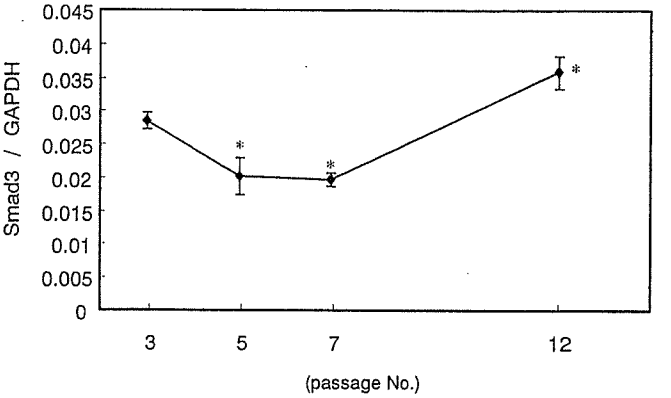

Fig. 3. Effect of in vitro culture length on mRNA expression of Smad3 in hMSCs. The expression of Smad3 relative to GAPDH in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages was investigated by quantitative RT-PCR. Mean values with SDs are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage ($^*P < 0.05$)

Fig. 2. Effect of in vitro culture length on mRNA expressions of transforming growth factor β1 (*TGFβ1*) (**A**), TGFβ2 (**B**), TGFβ3 (**C**), TGFβ receptor type I (*TGFβRI*) (**D**), TGFβRII (**E**), and TGFβRIII (**F**) in hMSCs. Expressions of the four genes, relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative real time-polymerase chain reaction (RT-PCR). Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage ($^*P < 0.05$)