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Introduction

“New Public Interest Incorporated Foundation”



Akimichi Kaneko, MD, PhD
Chairman of the Board of Trustees

This annual report includes research and meeting reports written by the 2010 grantees. Their tireless effort is the driving force to promote and to keep the high standard of the Japanese life science. I strongly believe that the aim of the Foundation is to help keeping their activity and we will do our best toward this goal. I sincerely appreciate the assistance and warm encouragement extended by the members of the Board of Trustees, the auditors, the Board of Councilors and the Selection Committee. The powerful support by the Novartis Pharma KK enabled us to sustain our activity without interruption.

The Foundation was reincarnated as a new public interest incorporated foundation on April 1, 2012. As you may know, the Foundation was originally established on September 4, 1987 with basic assets of JPY 1 billion donated by Ciba-Geigy AG, Switzerland for the purpose of contributing to academic development and thus improving public health and welfare by means of promoting creative research and international exchange in the field of life science and related chemistry. Since then, the Foundation has granted JPY 1.768 billion to 1,460 researches and international exchange activities.

In December 2008, the new law, “Act on Authorization of Public Interest Incorporated Associations and Public Interest Incorporated Foundations”, was put in force, demanding all associations and foundations in Japan to restructure along the new law. The Foundation started preparative discussion for the transition in February 2009. Although it took a longer period to settle arrangements, the Foundation finally applied to the Cabinet Office for the Public Interest Corporation Authorization on July 26, 2011. The Office reviewed and approved that the Foundation meets prescribed standards, then submitted the report on November 18, 2011. The certificate of authorization was issued on March 21, 2012 followed by the official registration on April 1, 2012.

Regardless of these organizational changes, the purpose of the Foundation remains unchanged. The Foundation will continue to support researchers and research communities. I hope that the Foundation’s activity will contribute to the development of life science in Japan.

はじめに

「新公益財団法人」

代表理事 金子章道

本年度もここに 2011 年度にノバルティス科学振興財団研究助成金を受けられた方々の研究報告を収録いたしました。受賞者の皆様の素晴らしい研究がまとめられたエッセイ集です。研究者お一人おひとりの努力の結晶は我が国の学術水準を発展させていく原動力です。こうした方々の研究に多少なりとお力添えをすることが本財団の事業の基本と考え、今後も努力を続けて行きたいと思っております。これらの優れた研究を選考していただいた選考委員の皆様をはじめ、財団の活動を支えて下さっている関係者の皆様に深く感謝いたします。とりわけノバルティスファーマ社の継続した強力な経済的支援に御礼申し上げたいと思います。

さて、2012 年の 4 月をもって、ノバルティス科学振興財団は新たな公益財団法人として生まれ変わりました。ご存じのように当財団は 1987 年 9 月 4 日、スイス、チバガイギー社からの 10 億円のご寄附をもとに、「生物・生命科学および関連する化学の領域において、創造的な研究ならびに国際交流への助成を行うことにより、学術の振興を図り国民の健康と福祉の向上に寄与する」ことを目的に設立されました。爾来 25 年間に 1,460 件、金額にして 17 億 6800 万円の助成を行ってまいりました。

2008 年 12 月に公益法人制度改革に関する新しい法律が施行され、現行のすべての法人が新組織へ移行することが必要となりました。当財団におきましては 2009 年 2 月の理事会・評議員会における審議をもって新組織への移行準備をスタートさせ、体制を整えるため少し時間がかかりましたが、2011 年 7 月末に内閣府へ移行申請を行いました。それに対し内閣府より 11 月 18 日付で公益法人認定の基準を満たしている旨の答申を得、本年 3 月 21 日に正式の認定証が交付され、4 月 1 日付の登記をもって新組織への移行が完了いたしました。

新しい組織になりましたも、当財団の事業の目的は変わることはありません。多くの研究者の方々のお役にたてる研究助成活動を続けて参ります。私どもの事業が少しでも日本の生命科学の発展に寄与できることを念願しております。

II.

Reports from the Recipients of Novartis Research Grants

Molecular analysis of human T cell leukemia virus type-1 (HTLV-1) HBZ gene in the pathogenesis of HTLV-1 associated myelopathy.

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Abstract

To investigate the pathological role of HBZ expression in HTLV-1 infection, we have generated monoclonal antibodies against HBZ, and analyzed the expression of HBZ protein and anti-HBZ antibodies in clinical samples from HTLV-1 infected individuals. Although we successfully detected HBZ protein in an enforced overexpressed cells and HTLV-1 infected cell lines by flow cytometry, ELISA, immunofluorescence and immunoblotting, we could not detect any HBZ protein or anti-HBZ antibodies in HAM patients and asymptomatic carriers by our system.

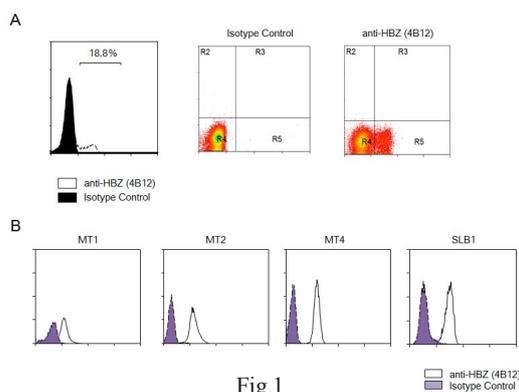
Key Words : HTLV-1, HTLV-1 associated myelopathy (HAM), HBZ, Chronic inflammation

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a human retrovirus etiologically associated with adult T-cell leukemia (ATL) and HTLV-associated myelopathy (HAM). HTLV-1 basic leucine zipper factor (HBZ), encoded on the negative strand, was suggested to play an important role in both HTLV-1-induced neoplastic and inflammatory diseases. We have previously reported that the HBZ gene is expressed in all HAM patients and its expression is strongly correlated with the HTLV-1 proviral load in infected individuals and with disease severity in HAM patients. However, there have been no reports about the relationship between HBZ protein expression and disease status.

Results

We generated rat monoclonal antibody (moAb) against a C-terminal peptide (HBZ193-206 : CVNYWQGRLEAMWLQ) of HBZ (clone 4B12). We also generated human moAbs against recombinant HBZ protein by a phage display system (clones 13405.1, 13407.1, 13413.1). Then we have developed the ELISA system for the detection of HBZ protein and anti-HBZ antibodies. Using these reagents, we analyzed the expression of HBZ protein in plasma and peripheral blood mononuclear cells (PBMCs) of HTLV-1 infected individuals including HAM patients as well as HTLV-1 infected cell lines. We successfully detected HBZ protein in an enforced overexpressed cells and HTLV-1 infected cell lines by flow cytometry (Fig.1), ELISA



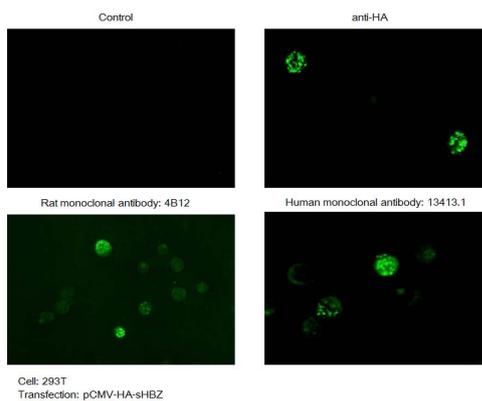


Fig.2

(data not shown), immunofluorescence (Fig.2) and immunoblotting (Fig.3).

However, we could not detect any HBZ protein or anti-HBZ antibodies in HAM patients and asymptomatic carriers (ACs) by our system. In contrast, HBZ mRNA was significantly elevated in HAM/TSP patients than ACs. Especially, rapidly progressive HAM/TSP patients showed extremely high expression of HBZ mRNA but not tax mRNA in their PBMCs (data not shown).

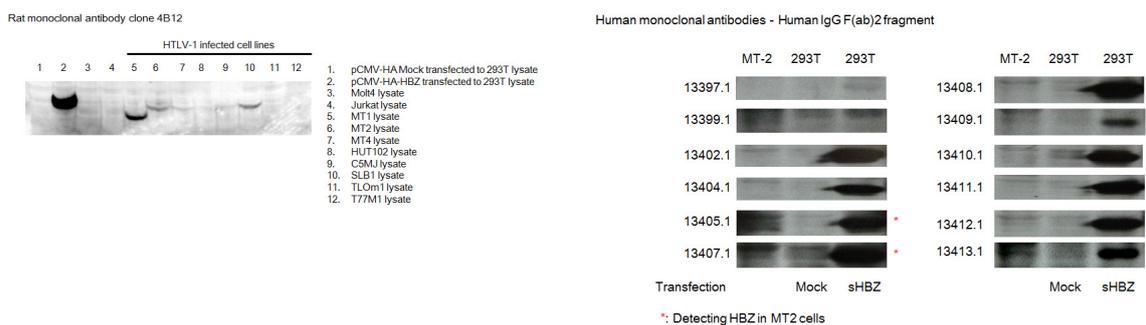


Fig.3

Discussion & Conclusion

For over two decades since the discovery of HTLV-1, the investigation of HTLV-1-mediated pathogenesis has been focused on Tax, an HTLV-1 encoded viral oncoprotein. Although Tax has been viewed as critical for leukemogenesis because of its pleiotropic effects on both viral and many cellular genes responsible for cell proliferation, dysregulation of the cell cycle and apoptosis, Tax expression is not detected in about 60% of freshly isolated samples from ATL cases. In contrast, the spliced form of HBZ gene is expressed in all ATL and HAM cases, and its expression is strongly correlated with the HTLV-1 proviral load in HTLV-1 infected individuals and with disease severity in HAM patients. Since there have been no reports about the relationship between HBZ protein expression and disease status, we generated the moAbs against HBZ and examined the HBZ protein expression in clinical samples. As a result, we could not detect any HBZ protein or anti-HBZ antibodies in serum or PBMCs of HAM patients and ACs, indicating that the expression of HBZ protein was suppressed in vivo in infected individuals. Low protein expression and low immunogenicity of HBZ may contribute to the low immune selective pressure and the persistence of HTLV-1.

References

Saito M, Bangham CR. Immunopathogenesis of Human T-cell leukemia virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP): Recent perspectives. **Leukemia Research and Treatment** Article ID 259045 (online journal), 2012.

一般の皆様へ

ヒト T 細胞白血病ウイルス 1 型 (HTLV-1) は、HTLV-1 関連脊髄症 (HAM) および成人 T 細胞白血病 (ATL) の原因ウイルスである。ATL は死亡者数が年間 1000 人を超え、HAM 患者では約 40% が経過中に歩行不能となるため、その制圧は重要である。我々は以前、HBZ 遺伝子 (mRNA) がすべての HTLV-1 感染者のリンパ球に発現し、発現量が HAM の病勢に関連していることを報告した。本研究では、抗 HBZ モノクローナル抗体を作製し、HTLV-1 感染者の血清・リンパ球における HBZ 蛋白、抗 HBZ 抗体の有無を調べたが、いずれも検出できなかった。すなわち、HTLV-1 は生体内での HBZ 蛋白発現を強く抑制して、宿主の免疫から逃避することで持続感染を維持している可能性が示唆された。HAM の治療・発症予防には、HBZ 蛋白でなく mRNA 発現を抑制することがより重要かもしれない。

The role of SUMOylation in visual phototransduction in the retina

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Abstract

SUMOylation, one of post-translational modifications (PTM), plays a crucial role in switching activities of the target proteins. Since we have shown that SUMOylation is responsible for photoreceptor subtype specification, we studied the functional role of SUMOylation in mature photoreceptors by using mice in which SUMO E2 ligase was conditionally disrupted. As a result, these conditional knockout mice were normal in immunohistochemical studies and electrophysiological recording, suggesting that SUMOylation is less important in phototransduction and maintenance of mature photoreceptors.

Key Words : Phototransduction, SUMOylation, photoreceptor, retina

Introduction

Vertebrate image-forming retinal photoreceptors are comprised of rod and cone subtypes. Rod photoreceptors are activated by dim light, whereas cone photoreceptors are activated by bright light. Correct expression of the subtype-specific genes is critical for normal vision, which is essential for many ecologically important behaviors. We have found that Pias3, a transcriptional coregulator and E3 SUMO ligase, plays a central role in photoreceptor subtype specification by SUMOylating a number of photoreceptor-enriched transcription factors. Since Pias3 is expressed in mature photoreceptors, we studied a role of SUMOylation in phototransduction.

Results

Pias3 (Protein Inhibitor of Activated Stat3) is an E3 SUMO ligase, catalyzing covalent linkage of SUMO proteins, which are distant homologs of ubiquitin, to specific lysine residues on target proteins. SUMOylation, which in many ways mechanistically resembles ubiquitination mediated by E1 (activation), E2 (conjugation) and E3 (ligation) enzymes. While there are a number of E1 and E3 enzymes reported, Ubc9 is the only SUMO E2 enzyme. Thus, we targeted Ubc9 to disrupt SUMOylation cascades. Since the targeted null mutation die prior to E7.5 due to major chromosome condensation and segregation defects (Dev Cell. 9:769-979), we created a line of mice in which loxP sites flank Ubc9 coding sequence (floxed) enable Cre recombinase-mediated disruption of SUMOylation cascade.

We first disrupt Ubc9 during retinogenesis, since strong inhibition of SUMOylation by injecting the SUMOylation-deficient Ubc9 resulted in small eyes in *Xenopus laevis* (Dev Biol. 347:180-194). We crossed the floxed mice with Chx10-Cre mice in which Cre recombinase is expressed in retinal

progenitor cells driven by Chx10 promoter. The conditional knockout mice resulted in small eyes, indicating that Ubc9 is also responsible for retinogenesis in mice.

To study SUMOylation function in phototransduction in photoreceptor cells, we then crossed the flow mice with Crx-CreERT2 (tamoxifen-dependent Cre recombinase fused with the modified ligand binding domain of the estrogen receptor) in which Cre-ERT2 is expressed in rod and cone photoreceptor cells. The conditional knockout mice were treated with tamoxifen at the age of 4 weeks and analyzed at 2 and 6 months of age. We first tried to detect immunohistochemical and morphological difference by section immunohistochemistry using antibodies localized at the outer segments (opsins and transducins) and the synaptic clefts (CtBP2, PSD95 and Pikachurin) of photoreceptors. We did not find significant morphological difference such as the length of outer segments, the size and localization of somas and the shape of synaptic clefts, while rod photoreceptor precursors whose SUMOylation activity is inhibited by knocking down of Pias3 at neonatal retinas turned cone photoreceptor cell-like morphology. There was not ectopic expression of rod or cone-specific genes (e.g. rhodopsin and cone opsins) in other photoreceptor subtypes. We also visualized synaptic connection between photoreceptor and bipolar cells by immunostaining mGluR6 and TRPM1 that are localized at dendritic tips of the ON bipolar cells and PKC α , a rod-bipolar cell-specific marker. But we did not found the significant difference. Next, we compared electrophysiological response between the conditional knockout and control mice by electroretinogram (ERG) that records field potentials of retinal neurons. We recorded ERG under scotopic condition (where the response of rod photoreceptors is dominant) and photopic condition (where rod response is saturated, thus cone response is recorded). We did not observe significant difference in amplitudes of responses of a- and b-waves, components of ERG that are derived from photoreceptors and ON-bipolar cells, respectively. In addition, since some of photoreceptor-enriched proteins (e.g. Phosducin, mGluR8 and Cask) are reported to be SUMOylated, we electroporated the mutant forms whose SUMOylation motifs are altered, but there were not significant immunohistochemical and morphological differences between the conditional knockout and the control mice.

Discussion & Conclusion

These results indicated that SUMOylation is less significant in phototransduction and maintenance in mature photoreceptor cells, while it plays a pivotal role in photoreceptor subtype specification. Since SUMOylation was disrupted after maturation of photoreceptor cells, we need to study SUMOylation focused on maturation period to fully understand SUMOylation in neural development and maturation.

一般の皆様へ

ゲノム解析により遺伝子数は予想より少ない事が判明し、遺伝子産物であるタンパク質に多様な機能を持たせる機構の一つとして翻訳後修飾が注目されています。その一つである SUMO 化の機能は

細胞増殖から分化・維持と多岐に渡り、近年はアルツハイマー病などの神経変性疾患に関与する事も報告されています。本助成研究では視覚刺激を受容する網膜の視細胞における SUMO 化の機能を解析しました。その結果、SUMO 化は視細胞の発生・分化に重要である一方、成熟後の視覚の情報伝達では SUMO 化の欠損の影響が低いことが示唆されました。今後、分化決定から機能発現に至るまでの成熟（細胞構造の構築や神経回路生成）の過程での SUMO 化の役割の解析を進めたいと思います。

Establishing a new methodology for genome mining and biosynthesis of natural products and their analogs through a yeast molecular genomics

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Abstract

Fungal genome sequencing revealed the presence of many genes coding for natural product biosynthetic enzymes, including polyketide synthases and nonribosomal peptide synthetases. However, it remains a challenge to characterize these enzymes and identify the compounds they synthesize, either in their original hosts or more tractable heterologous hosts, such as yeast. Here, we have developed a streamlined method for isolating biosynthetic genes from fungal sources and producing bioactive molecules using an engineered *Saccharomyces cerevisiae* strain as a host. We used overlap extension PCR and yeast homologous recombination technology to clone a desired fungal polyketide synthase or nonribosomal peptide synthetase gene (5–20 kb) into a yeast expression vector quickly and efficiently. This approach was used successfully to clone five polyketide synthases and one nonribosomal peptide synthetase from various fungal species. Subsequent detailed chemical characterizations of the resulting natural products identified 6 polyketide and 2 nonribosomal peptide products, some of which were new compounds. Our system should facilitate the efforts in mining the uncharacterized fungal biosynthetic potential for identifying novel natural products and rationally engineering in the biosynthetic pathways for production of analogs possessing desired bioactivity.

Key Words : drug discovery • fungal putative gene cluster • polyketide synthase (PKS) • yeast heterologous biosynthesis

Introduction

Polyketides (PKs) and nonribosomal peptides (NRPs) have been isolated from *Streptomyces* and many other source organisms. In recent years, numerous biosynthetic gene clusters encoding enzymes for biosynthesis of those compounds, such as PK synthases (PKSs) and NRP synthetases (NRPSs), have been discovered in fungal genomes. Despite the presence of 30 to 40 gene clusters in a single *Aspergillus* genome, only a handful of fungal polyketide, peptide and terpene products can be isolated from a culture grown under a typical growth condition. Therefore, a simple approach for activating these cryptic gene clusters is likely ineffective. Recent advancement of spectroscopic technology permits elucidation of the chemical structures of natural products even at a microgram level. Furthermore, recent advancements in high resolution LC–MS and NMR technology allow determination of the complex structures of these compounds from a very small amount of sample. However, it is still necessary to be able to activate the cluster to induce biosynthesis of the

corresponding compounds, even at a trace level, to be able to take advantage of these techniques.

An alternative approach in mining for novel secondary metabolites is to heterologously express genes from putative biosynthetic gene clusters that are encoded in the fungal chromosomal DNA. However, fungal expression systems often cannot accommodate the number of genes present in a PKS or a NRPS gene cluster. Additionally, heterologous expression of fungal biosynthetic genes from a 5- to 20-kilobase (kb) genomic DNA fragment using a typical host, such as *Aspergillus oryzae*, will require the host transcriptional system to splice together exons correctly for the production of functional biosynthetic proteins. Extraction of correct protein-coding segments by PCR is challenging due to inherent difficulty in predicting intron regions accurately. Likewise, there has been a significant progress made in *Escherichia coli*-based heterologous gene expression and metabolite biosynthesis. However, *E. coli* requires the fungal genes to be synthesized from mRNA isolated from the original host to allow correct splicing of the genomic copy of the fungal genes, and successful reverse transcription (RT) of full-length mRNAs of PKSs or NRPSs, which can be more than 20 kb in length, is challenging. Furthermore, difference in the codon usage between fungi and *E. coli* can also hinder efficient production of fungal enzymes, making reconstitution of fungal biosynthetic enzymes in *E. coli* very difficult.

Results

For our heterologous biosynthetic system, we developed an engineered *S. cerevisiae* strain SCKW5, whose genome is modified to include a G418 resistance marker *kanMX*, along with *matB* and *npgA* genes encoding for a malonyl-CoA synthetase and a phosphopantetheinyl transferase, respectively. We then prepared expression vectors carrying the target PKS and NRPS genes and transformed SCKW5 with these vectors for further studies. Initially, cDNA of each of the putative genes, namely CHGG_00542, CHGG_10027, CHGG_10128, CC1G_05377 and Afu6g12080, was synthesized by RT-PCR from the total RNA isolated from the corresponding fungal host. A full-length cDNA of these genes was assembled from multiple partial amplicons of the cDNA using the overlap extension PCR technique. Each of the resulting full-length gene was inserted into pKW1810 using in vivo recombination activity of yeast, yielding a series of five expression vectors, pKW14050 (CHGG_00542), pKW11002 (CHGG_10027), pKW11001 (CHGG_10128), pKW11000 (CC1G_05377) and pKW5022 (Afu6g12080). We introduced each expression vector into SCKW5 to confirm gene expression by western blotting analysis. We then looked for the biosynthesis of the corresponding polyketide or nonribosomal peptide compounds. Reverse-phase HPLC analysis of the crude organic extract of the culture revealed the presence of some late-eluting compounds that were present only in the culture extracts of the transformants. These results gave us a good indication that all of the transformants were capable of producing nonpolar metabolites, suggesting successful biosynthesis of the target compounds.

Discussion & Conclusion

In this study, we established an innovative approach for biosynthesizing bioactive compounds of fungal origin by focusing on previously uncharacterized biosynthetic gene clusters and using an engineered *S. cerevisiae* strain as a surrogate host. We exploited the overlap extension PCR method to quickly synthesize full-length PKS and NRPS genes, which are usually 5- to 20-kb or longer, using a pool of cDNA reverse-transcribed from a total RNA isolated from the source fungus. The amplified gene was subsequently cloned into a yeast expression vector using the recombination capability of yeast. This series of procedures allowed fast and efficient establishment of a yeast system for expression of biosynthetic genes of unknown function and production of corresponding natural products. Our results clearly demonstrated successful expression of four type I iterative PKS genes and one NRPS gene from three different fungal species in *S. cerevisiae*, all of which led to the production of a total of six compounds whose identities were characterized spectroscopically and verified the speculated functions of these biosynthetic mega-enzymes. Our plasmid-based system provides an advantage over fungal systems in terms of ease and speed of cloning the target genes, and it also tolerates handling of genes up to at least 12 kb in length. Also, the use of a plasmid-borne system simplifies the effort of engineering biosynthetic pathways for production of various analogs using traditional molecular biological techniques. By streamlining the process of translating uncharacterized fungal biosynthetic genes into structurally characterized compounds, our methodology should facilitate the efforts in isolating novel natural products and rationally engineering in the biosynthetic pathways for production of analogs possessing comparable or even more potent bioactivity.

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一般の皆様へ

研究の背景

人類が初めて手にした抗生物質であるペニシリンは人々を感染症から守り、それが発見されるまでは考えられないような膨大な数の生命を救ってきました。しかしながら、ペニシリンのように容易に得られる薬は既に取り尽くされ、今までのように見つけ出すことが難しくなってきました。そこで、解読されてきた様々な生物の遺伝子情報を活用して、新しい薬を見つけ出すことが期待されています。

研究の目標

この研究は、新しい抗ガン剤や新興感染症あるいは患者数の少ない難病治療薬などの薬を効率的に発見し、さらに安い価格で国民に提供することを目的としています。

Application of epigenetics to elucidate molecular mechanism of gene-environment interaction involving resistin in type 2 diabetes

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Abstract

The G/G genotype of the human resistin gene promoter single nucleotide polymorphism (SNP) at -420 increased type 2 diabetes susceptibility by enhancing promoter activity. We here analyzed methylation of five cytosines and other SNPs around SNP-420 in the human resistin gene in type 2 diabetes. Quantification of cytosine methylation could be useful as a marker for human resistin gene expression and could be of assistance to the evaluation of gene-environment interaction.

Key Words : resistin, SNP, methylation, epigenetics

Introduction

Resistin is a cytokine secreted from monocytes, which antagonizes insulin action. Previously, we reported that the G/G genotype of a promoter single nucleotide polymorphism (SNP) at -420 (rs1862513) was associated with type 2 diabetes susceptibility by enhancing promoter activity. Increasing evidence suggests that epigenetic mechanisms such as DNA methylation, which might change in the environmental state, could regulate the gene expression. In this study, we analyzed cytosine methylation of five cytosines around SNP-420 in human resistin gene.

Results

Genotype of SNP-420 in the promoter region of human resistin gene was strongly associated with plasma resistin level. We selected five cytosines around SNP-420 to analyze cytosine methylation. Cytosine methylation was assayed quantitatively by pyrosequencing after bisulfite treatment of genomic DNA. Genomic DNA was obtained from leukocyte of type 2diabete patients. We quantified methylation of five selected cytosines. The percentage of methylation was different among these cytosines (20~80% of methylation ratio). Furthermore, the percentage of methylation in each selected cytosine was different among individuals. So the methylation of these five cytosines could be a marker for evaluating resistin gene expression.

We are now analyzing methylation of these cytosines as well as plasma resistin level in the general Japanese population aiming for 2500 subjects, of which SNP in the promoter of resistin gene have been analyzed before. The resulting information about SNP, methylation ratio, and plasma resistin level will be helpful for the analysis of gene-environment interaction.

Discussion & Conclusion

We analyzed genotype of SNP-420 and cytosine methylation of five cytosines around SNP-420 in the promoter region of the human resistin gene. Genotype of SNP-420 was strongly associated with plasma resistin level and percentage of methylation in five cytosines was variable among these cytosines as well as among individuals. Quantification of cytosine methylation could be useful as a marker for human resistin gene expression and could be of assistance to the evaluation of gene-environment interaction.

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一般の皆様へ

2型糖尿病は、インスリン抵抗性やインスリン分泌不全に関わる遺伝子に、肥満、運動不足といった環境因子が作用して発症すると考えられている。従って、その発症予防やオーダーメイド医療の確立には、原因遺伝子に加え、遺伝子・環境因子相互作用の解明が鍵と考えられる。本研究ではレジスチンという2型糖尿病感受性遺伝子をモデルとして、エピジェネティクス機構の1つであるDNAのメチル化に焦点を当て、遺伝子-環境相互作用の解明を目的に研究を進めている。

Targeting at the interface between normal and transformed epithelial cells.

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Abstract

We have revealed that Scribble-knockdown cells undergo apoptosis and are apically extruded from the epithelium when surrounded by normal epithelial cells. This is the first demonstration that an oncogenic transformation within an epithelium induces cell competition in a mammalian cell culture system.

Key Words : Cell Competition, Scribble, p38MAPK, apoptosis

Introduction

In *Drosophila*, normal and transformed cells compete with each other for survival, a process called cell competition. However, it is not known whether comparable phenomena also occur in mammals. Scribble is a tumor suppressor protein in *Drosophila* and mammals. In this study we examine the interface between normal and Scribble-knockdown epithelial cells using Madin-Darby Canine Kidney (MDCK) cells expressing Scribble short hairpin RNA (shRNA) in a tetracycline-inducible manner.

Results

(Project 1: Demonstration of cell competition in mammalian cell culture systems)

To examine the interaction between normal and Scribble-knockdown epithelial cells, we established MDCK epithelial cells stably expressing Scribble shRNA in a tetracycline-inducible manner. We found that Scribble-knockdown cells undergo apoptosis when surrounded by normal cells (Figure). Importantly, when Scribble-knockdown cells alone were present, no cell death was observed, indicating that the presence of surrounding normal cells induces death of Scribble-knockdown cells. This is the first demonstration that knockdown of a tumor suppressor protein causes cell competition in mammalian epithelial cells. Bcl-2 homologous antagonist/killer (Bak) and Bcl-2 associated X protein (Bax) are proapoptotic members of the Bcl-2 family and promote apoptosis through their effect on mitochondria. We found that Bak and Bax were activated in

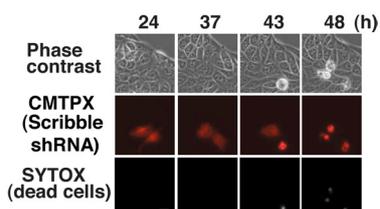


Figure Knockdown of Scribble expression induces cell competition in MDCK cells.

MDCK-pTR Scribble shRNA cells were fluorescently labeled with CMTPX dye (red) and mixed with normal MDCK cells at a ratio of 1:10, and cultured in the presence of tetracycline for the indicated times. Cells were incubated with SYTOX Blue to label dead cells.

apically extruded Scribble-knockdown cells, suggesting the involvement of the mitochondrial apoptosis pathway in their death.

Next we examined which signaling pathways are involved in this cell competition phenotype. p38MAPK belongs to the MAPK family that is activated by various types of cellular stresses. Immunofluorescence analyses with anti-phosphorylated p38MAPK antibody demonstrated that p38MAPK activity was significantly enhanced in Scribble-knockdown cells surrounded by normal cells, but not in normal cells or Scribble-knockdown cells cultured alone. Addition of the p38MAPK inhibitor SB202190 significantly reduced activation of caspase-3 and death of Scribble-knockdown cells surrounded by normal cells. Furthermore, expression of dominant negative form of p38MAPK strongly suppressed death of Scribble-knockdown cells surrounded by normal cells. Collectively, these data indicate that p38MAPK plays a crucial role in cell competition between normal and Scribble-knockdown MDCK cells. Finally, inhibition of p38MAPK significantly suppressed activation of Bak in Scribble-knockdown cells surrounded by normal cells, suggesting that the mitochondrial apoptosis pathway is one of the downstream targets of p38MAPK.

These data were published in *Journal of Cell Science* (Norman et al., 2012, *JCS*).

(Project 2: Identification of molecules that specifically function at the interface between normal and transformed epithelial cells)

To identify molecules that specifically function at the interface between normal and Src-transformed cells, we first cultured cells under three different conditions: i) normal MDCK cells alone, ii) 1:1 mix culture of normal and Src-transformed MDCK cells, and iii) Src-transformed MDCK cells alone. We found several molecules that predominantly immunoprecipitated with anti-phospho-tyrosine antibody under the mix culture condition. Among them, using mass spectrometry we identified the 280 kDa and 55 kDa proteins as filamin A and vimentin, respectively. Both filamin A and vimentin were accumulated in the surrounding normal cells at the interface with Src-transformed cells. In addition, when Src-transformed cells were surrounded by filamin A-knockdown or vimentin-knockdown cells, apical extrusion of the transformed cells from the epithelium was significantly suppressed, suggesting a positive role for filamin and vimentin in the elimination of the transformed cells. Furthermore, we showed that filamin acts upstream of vimentin in these processes. This is the first report demonstrating that neighboring normal epithelial cells are actively involved in the elimination of transformed cells and that filamin is a key mediator in the interaction between normal and transformed epithelial cells.

These data are now submitted to *Nature* (Kajita et al., 2012).

Discussion & Conclusion

In this study, we have shown that Scribble-knockdown MDCK cells and normal MDCK cells

compete with each other for cell survival and that the former undergo apoptosis when surrounded by the latter. This is the first demonstration that an oncogenic transformation within an epithelium induces cell competition in a mammalian cell culture system. The next important question is the molecular mechanisms of how cell death of Scribble-knockdown MDCK cells is induced in the process of cell competition. In my laboratory, we are now trying to identify molecules that play a crucial role in the intercellular recognition between normal and transformed epithelial cells or in the following signal transduction pathways.

Cell competition is a newly emerging field in cancer biology, which sheds light on the interaction between normal and transformed epithelial cells at the early stage of carcinogenesis. It is hoped that future studies will lead us to a novel type of cancer prevention and treatment.

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一般の皆様へ

がんは正常な細胞のがん遺伝子やがん抑制遺伝子に変異が蓄積することによって生じる。しかし、がんの初期段階において、正常細胞に最初の変異が起こった時に正常細胞と変異細胞の境界でどのような現象が起こるのかは明らかでなく、がん研究のブラックボックスとなっている。最近の我々の研究で正常細胞には変異細胞を認識し、それらを駆逐する能力があることが明らかになってきた。これらの研究をさらに発展させることにより、「周囲の正常細胞に変異細胞を攻撃させる」というこれまでになかった新規のがん予防・治療法の開発へつなげていきたいと考えている。

Experimental verification of the abnormal neurodevelopmental hypothesis of schizophrenia

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Abstract

The membrane-associated guanylate kinase with inverted organization (MAGI) proteins consist of three members, MAGI-1, -2 and -3. We here prepared a specific antibody against MAGI-1, and carried out biochemical and morphological analyses in rat neuronal tissues. By western blotting, MAGI-1 was highly detected in nervous tissues, especially in olfactory bulb. Biochemical fractionation clarified that MAGI-1 was relatively enriched in the synaptosomal vesicle and synaptic plasma membrane fractions. Immunofluorescent analyses revealed diffuse distribution of MAGI-1 in the cell body and processes of primary cultured rat hippocampal neurons. Immunohistochemistry demonstrated that MAGI-1 was mainly expressed in Purkinje cells and in hippocampal neurons.

Key Words : MAGI-1, synapses, neuronal development

Introduction

Schizophrenia is a mental disorder featured by various psychotic phenomena. Since schizophrenia could be regarded as a neurodevelopmental disorder resulting from abnormalities of synaptic connectivity, we attempted to clarify the function of MAGI-1 possibly involved in neuronal development. MAGI-1, a member of MAGI family, harbors six PDZ domains, a GUK domain and two WW motifs. Physiological functions of MAGI-1 have been investigated mainly in non-neuronal tissues; it is localized at cell-cell contact sites of various cells. MAGI-1 is likely to act as a scaffold at cell junctions. However, little investigation has been done as for the physiological significance and characterization of MAGI-1 in neuronal tissues. In the present study, we prepared a specific antibody for MAGI-1 and performed biochemical and morphological characterization of the protein in neuronal tissues.

Results

Production and characterization of an antibody against MAGI-1.

To characterize the feature of MAGI-1 in neuronal tissues, we attempted to prepare a rabbit polyclonal antibody for MAGI-1. We used a long fragment of human MAGI-1b (aa194-1256) as an antigen. The resultant antibody however cross-reacted with MAGI-2 and MAGI-3, maybe due to the amino acid sequence and structural similarities among MAGI proteins. We named this pan-MAGI antibody as anti-MAGI-1/2/3. We next affinity-purified a MAGI-1-specific antibody from the anti-MAGI-1/2/3 antiserum using GST-MAGI-1(aa194-295)-bound sepharose beads. The resultant

antibody, termed anti-MAGI-1, selectively recognized MAGI-1 as expected, since only 11 amino acids are shared among MAGI proteins in the 102 amino acids of MAGI-1(aa194-295) .

Biochemical characterization of MAGI-1 in the rat brain.

By western blotting with anti-MAGI-1, we analyzed the expression profile of MAGI-1 in a variety of rat tissues and compared the results with those obtained with anti-MAGI-1/2/3. MAGI-1 was expressed abundantly in brain tissues such as cerebrum, hippocampus and cerebellum. Anti-MAGI-1 demonstrated specific expression profiles of MAGI-1 in several rat tissues including liver, adrenal gland, uterus, extensor digitorum longus (EDL), ovary and testis. To characterize further the expression of MAGI-1 in brain, various brain regions were dissected from adult rats and subjected to western blotting using anti-MAGI-1. The results clarified that MAGI-1 is abundant in olfactory bulb. Biochemical subcellular fractionation of rat brain demonstrated that MAGI-1 was relatively enriched in the synaptosomal vesicle (LP2) and synaptic plasma membrane (SPM) fractions, suggestive of MAGI-1 localization at presynaptic vesicles. On the other hand, results with anti-MAGI-1/2/3 suggest MAGI-2 and/or MAGI-3 are enriched in the postsynaptic fraction.

Immunohistochemical characterization of MAGI-1 in the rat brain.

Sagittal sections stained with anti-MAGI-1 showed the protein localization in Purkinje cells in cerebellum. In hippocampus, MAGI-1 was enriched in the cytoplasm of pyramidal cells. We further performed immunohistochemical analyses to examine the MAGI-1 distribution. Consequently, we found that MAGI-1 is highly expressed in the glomerulus region of olfactory bulb, a spherical structure where synapses form between olfactory nerve terminals and dendrites of mitral, periglomerular and tufted cells. Additionally, MAGI-1 was recognized in a spinal cord in an embryonic rat: strong signal was detected in the dorsal root entry zone. These results imply that MAGI-1 is involved in the regulation of neurite outgrowth or guidance of peripheral neurons. We next examined subcellular distribution of MAGI-1 in primary cultured rat hippocampal neurons. Immunofluorescence analyses with 3 days in vitro (DIV) neurons revealed that MAGI-1 distributes diffusely in the cell body, axon and dendrites. When anti-MAGI-1/2/3 was used instead of anti-MAGI-1, very similar staining pattern was observed. We then looked into the distribution of MAGI-1 in 21DIV neurons, and it was distributed diffusely throughout the cell body, axon and dendrites. Notably, MAGI-1 was localized at synapses. On the other hand, MAGI-1 expression and localization were also observed at the neurite tip of dorsal root ganglia (peripheral) neurons. These results imply that MAGI-1 is involved in the regulation of neurite outgrowth or guidance of peripheral neurons.

Discussion & Conclusion

Our present observations suggest that MAGI proteins are expressed in region- and cell type-specific manners in brain tissues. Further investigation with molecular cellular approaches and in vivo analyses will be needed to clarify the physiological role of MAGI-1 in neuronal tissues.

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一般の皆様へ

統合失調症の発症の背景には神経組織の発達障害が関与するという仮説（発達障害仮説）があります。私共は、発達期の異常によって神経組織の形態形成障害が生じることで、神経細胞同士の情報伝達が阻害されることが統合失調症の病態と関連すると考えています。本研究では、神経細胞の相互作用と形態形成に重要な役割を果たすと考えられる蛋白質 MAGI-1 について解析をしました。

To detect susceptibility factor for mental disorders based on genome and mouse

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Abstract

Genome-wide association studies (GWASs) of schizophrenia have provided a small number of definitive risk genes, because this method requires very stringent level of significance. This indicates a large number of subjects is essential for GWASs, the cost being very expensive. Another way to solve this problem is to prioritize possible risk genes using different methods, such as transcriptomics. In this study we conducted transcriptomics of mouse model of schizophrenia and compared the results with those based upon GWASs. We found several risk genes for schizophrenia, which require further replication study.

Key Words : Schizophrenia, transcriptome, genome-wide association study

Introduction

Several lines of evidence suggest that genetic factor contributes to susceptibility for major psychiatric disorders, such as schizophrenia and bipolar disorder. Recently genome-wide association studies (GWASs) detected a small number of risk genes with strong evidence of association for these disorders. The effect size of such genes is relatively small, the odds ratio (OR) being less than 1.2. Therefore large number of subjects (case and controls, typically more than several thousands) should be examined to avoid false positive or false negative. Otherwise, another method to enhance the results by screening GWAS is required to obtain better candidate genes that should be followed-up in the independent replication set with larger number of subjects. Transcriptomics, that measures the comprehensive expression level of RNA in tissue, is one of the best methods to prioritize GWAS results. In this study, we conducted transcriptomic study of mouse brain exposed by several antipsychotics under the hypothesis where the susceptibility genes for psychiatric disorders involve in genes influenced by antipsychotics. Finally we check the GWAS results for the significant genes we obtained in the current study.

Results

1) Mouse transcriptomics

To generate mouse models of schizophrenia, mice were firstly exposed by methamphetamine (METH: 1mg/kg s.c for 1 week, N=5) a priori and then treated by a second generation antipsychotics, risperidone (N=5). As a comparison control, saline was treated instead of METH or antipsychotics (N=5). RNA from frontal cortex was extracted and Mouse Gene ST 1.0 chip

(Affymetrix) was used to evaluate the expression levels of all probes. Student's t-test was used to examine the difference of the expression level for each comparison (test1: METH-saline vs saline-saline, test2: METH-saline vs METH-risperidone, Figure1). In this analysis, we used the best P value of the probes, if multiple probes were examined within a certain gene.

2) Integration of transcriptomics data with GWAS result

We consulted our GWAS data for schizophrenia to check the association of the genes detected in the current transcriptomics study. We used gene-wide P values (not individual SNP-wise P value, because gene-wide analysis has more statistical power) by VEGAS software.

283 genes showed significant differences in the comparison both of test1 (METH-Saline vs Saline-Saline) and test2 (METH-saline vs METH-risperidone). 203 out of 283 genes were annotated to human genes, and SNPs in 15 genes showed significant association in the GWAS of schizophrenia.

Two genes out of 15 significant ones are histone-related genes, encoding responsible proteins for the nucleosome structure of the chromosomal fiber. Also it is of note that PTPRN which encodes protein tyrosine phosphatase, receptor type N is in the same pathway listed as candidate factor for schizophrenia based upon KO mouse (Takahashi et al, 2011, Biol Psychiatry).

Table1 shows that genes with significant P values in all analysis

Table1. Significant genes based upon transcriptomics and GWAS

Gene	1) METH-saline vs saline-saline	2) METH-saline vs METH-risperidone	SCZ GWAS
NAV1	0.0308	0.03517	0.0044
TRIM17	0.01742	0.00161	0.0118
HIST3H2A	0.01429	0.00146	0.0126
THBS1	0.02458	0.0369	0.0135
KSR2	0.00411	0.00718	0.0147
HERPUD1	0.00364	0.01061	0.0160
SORBS2	0.002	0.03905	0.0161
USP21	0.00856	0.01644	0.0265
GTF2IRD1	0.02934	0.04735	0.0297
CREB3L4	0.04269	0.02474	0.0311
HIST2H2AB	0.03449	0.02653	0.0372
PTPRN	0.0255	0.02286	0.0383
PSMD7	0.00154	0.00267	0.0395
FBXO3	0.01271	0.0357	0.0421

Discussion & Conclusion

In this study, we prioritized possible risk genes for schizophrenia based upon transcriptomic study of mouse expression analysis and GWAS. Specifically PTPRN is one of the promising candidate genes for susceptibility of schizophrenia, and further functional study will be essential.

The integration of independent data is one of the promising methods for selecting candidate genes, because the very stringent level of significance which is normally used in the GWAS (called genome-wide significant, $P < 5 \times 10^{-8}$) is not required. Therefore we can enhance the nominal significant SNPs (e.g. $P \sim 10^{-5}$ level) that should follow-up in the different set of samples in the genetic association study.

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一般の皆様へ

全ゲノムを半網羅的に検証することが可能な全ゲノム関連研究 (GWAS) は、疾患のリスク検出に大きな役割を果たした。しかし、精神疾患のリスク同定は、未だ完成したとは言えず、今後も様々な工夫が必要である。一つの理由として、GWAS は大量のサンプルが必要であるが、一つの研究施設などで収集できるサンプル数には限りがあり、どのようにリスク遺伝子の優先順位をつけていくかが課題となっている。本研究では、統合失調症の仮想のマウスモデルを用い、抗精神病薬投与して変化する遺伝子を同定し、これらと GWAS の結果を比較することを行った。このような複数の結果を統合することで、今後新たな統合失調症のリスク遺伝子同定につながると期待される。

Analysis of role of hypoxia inducible transcription factor in the regulation of metabolisms in liver and its pathological significance for the development of metabolic diseases

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Abstract

Lowered oxygen concentrations often occur in various kinds of metabolic disorders including fatty liver and diabetes, although its pathological significance remains elusive. To address this question, we generated mice lacking a gene encoding hypoxia inducible factor (HIF)- α , a master regulator of hypoxic response, in the liver. When exposed to ethanol, mice lacking HIF-1 α , but not HIF-2 α , showed severer fatty infiltration through derepressing lipid synthesis. HIF-1 α -deficient mice also displayed insulin resistance and glucose intolerance to greater extents than control mice in response to high fat/sucrose diet. These results suggest that HIF-1 α serves as a protective mechanism against fatty liver and diabetes.

Key Words : Hypoxia, liver, metabolism, HIF

Introduction

Liver is a central organ of metabolisms in the body, and its metabolic dysfunctions are associated with development of various metabolic diseases including diabetes and fatty liver. In these metabolic conditions, excess influx of nutrients disrupts balance of oxygen consumption and supply, leading to formation of hypoxic conditions in the liver. Metabolic adaptation to hypoxia is largely mediated by activation of HIFs, key transcription factors of hypoxic responses, and plays critical roles in the regulation of glucose and lipid metabolisms. Thus, hypoxic remodeling of metabolism seems to influence metabolic dysfunctions in disease conditions, however, pathological importance of hypoxia in the development of liver metabolic diseases remains elusive.

Results

To elucidate pathological roles of HIFs in liver-associated metabolic diseases, we inactivated HIF α gene in a hepatocyte-dependent manner based on Cre-loxP systems, and subjected these mice to either ethanol-containing diet (ETD) or high fat/sucrose diet (HFSD). When exposed to ETD for 4 weeks, control mice showed modest accumulation of lipids in pericentral hepatocytes, distribution of which were coincident with deposits of pimonidazole-protein adducts, marker of hypoxia. Consistent with these findings, HIF-1 α protein levels as well as its transcriptional activity were substantially elevated in the alcoholic fatty liver. On the other hand, loss of HIF-1 α gene was found to enhance lipid accumulation and deteriorate the fatty liver in response to ETD. The aberrant fatty infiltration

was associated with increased levels of sterol responsive element binding protein (SREBP)-1c and its downstream target, acetyl CoA carboxylase (ACC) 1. Serum adiponectin levels were compatible between the mutant and control mice, suggesting that the adiponectin-AMP kinase pathway is not involved in hyper-activation of SREBP-1c in the mutant mice. Differentiated embryo chondrocyte (DEC) 1, a well-known target gene of HIF-1, was greatly up-regulated by ETD exposure in control mice, whereas such an induction was completely abolished in the mutant mice. Forced expression of DEC1 in the mutant liver ameliorated alcoholic fatty liver concomitantly with decreased mRNA levels of SREBP-1c and ACC1. In addition, dimethyloxallyl glycine (DMOG), a HIF α prolyl hydroxylase inhibitor that inhibits oxygen-dependent HIF α degradation and subsequently activates HIFs transcriptional activity, reduced lipid accumulation by suppressing SREBP-1c expression in livers of control, but not HIF-1 α -deficient mice fed ETD. In a good agreement with these findings, a lack of HIF-2 α gene in the liver did not show any influences on the ethanol-induced lipid accumulation compared to control mice,

Results

although HIF-2 α protein was also induced in response to ETD. To further confirm effects of HIF-1 on hepatic lipid metabolism, we treated mice with choline-deficient diet (CDD), an animal model of non-alcoholic fatty liver disease. CDD induced large amounts of lipid accumulation predominantly in periportal hepatocytes in control mice, whereas such fatty infiltration was accelerated in HIF-1 α -deficient mice, although serum lipid levels did not differ between the two groups.

We next investigated effects of HIF-1 α deficiency on liver glucose metabolism. Under normal conditions, HIF-1 α -deficient mice showed no apparent abnormal phenotypes with compatible serum levels of glucose and insulin compared to control mice. In response to HFSD for 5 weeks, HIF-1 α expression was greatly induced predominantly in pericentral hepatocytes concomitantly with increased liver hypoxia. HIF-1 α -deficient mice still had compatible levels of glucose and insulin to control mice under both fed and fasted conditions. However, the mutant mice showed modest, but significant increase in blood glucose after oral glucose challenge without any difference in insulin resistance and insulin secretion when compared to control mice. Longer exposure to the diet for 20 weeks, substantial glucose intolerance and insulin resistance were observed in both mice, but such phenotypes were further deteriorated in HIF-1 α -deficient mice. Consistent with these alterations, phosphorylation of Akt was markedly reduced in response to insulin treatment in skeletal muscle and adipose tissues of the mutant mice compared to that in control mice. In addition, islet area in pancreas was observed to decrease in HIF-1 α -deficient mice, although the mice still had compatible potential to secrete insulin in response to glucose. We also found that hepatic glucokinase (GK) induction was totally abrogated with substantial decrease in hepatic glucose uptake in the mutant mice fed HFSD. However, other glycolytic and gluconeogenic enzyme expressions did not differ between the two groups.

Discussion & Conclusion

Our present study has clearly demonstrated protective roles of HIF-1 induction against alcoholic and non-alcoholic fatty liver. Although aberrant lipid accumulation is a common phenotype, distinct molecular mechanisms are involved in the fatty liver diseases. Enhanced fatty infiltration in HIF-1 α -deficient mice fed ETD is caused by DEC1-mediated desuppression of lipogenic transcription factor, SREBP-1c. In contrast, our data also suggest that HIF-1 activation is a prerequisite for the maintenance of gene expression involved in fatty acid β -oxidation in CDD-elicited fatty liver by unknown mechanism. Taken together, HIF-1 serves as a critical determinant for hepatic lipid metabolism by modulating both lipogenesis and lipolysis in the context-dependent manner. In addition, our present findings showing effects of a lack of HIF-2 induction on hepatic lipid metabolism strongly suggest that HIF has isoform-specific roles in the regulation of liver lipid metabolism. Our present study also sheds light on roles of HIF-1 as an anti-diabetic factor in the diet-induced metabolic disease. At present, we could not reveal molecular mechanisms of how loss of hepatic HIF-1 disrupts glucose metabolism, but HIF-1-mediated GK induction might serve as a protective mechanism against the diet-induced impairments of whole-body glucose metabolism.

In conclusion, HIF-1 functions as a critical regulator of glucose and lipid metabolism in the liver. Furthermore, present findings provide evidence that HIF-1 is a promising therapeutic target against fatty liver diseases and diabetes.

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一般の皆様へ

本研究では、肝臓における低酸素に応答した糖・脂質代謝リモデリングが、糖尿病・脂肪肝などの生活習慣病に対して病態抑制機構として機能していることを明らかにした。この知見は、低酸素を病態形成の根底に抱えるがん、動脈硬化、虚血性心疾患などのさまざまな疾患の病態形成メカニズムを、代謝リモデリングの観点から捉え直すことに繋がり、これらの疾患の形成や進展を理解する上でも新しい概念を生み出す可能性があると期待される重要な研究と言える。

Mechanism of self-recognition regulating the homeostasis of peripheral T cells

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Abstract

Constitutive recognition of self-peptide-MHC complexes is important for peripheral T cell homeostasis, but the nature of those self-peptides has been obscure. CD8 T cells are positively selected through recognition of self-peptides produced by thymoproteasomes. This study showed thymoproteasomes are not expressed in the peripheral tissues with which T cells can interact for their homeostasis. In addition, CD8 T cells in beta5t-deficient mice could not retain naïve phenotype after migration out of the thymus to the periphery. These findings suggested self-peptides used for CD8 T cell homeostasis may differ from those recognized during positive selection.

Key Words : Lymphocyte, T cell, positive selection, homeostasis

Introduction

Following the development in the thymus, mature T cells are maintained in the periphery depending on homeostatic cytokine signals and tonic T cell receptor (TCR) signals induced by the recognition of self-peptide-MHC complexes (1). While there is a general belief that self-peptides used for thymic positive selection and peripheral homeostasis of T cells are identical, it has not been directly elucidated. Thymoproteasomes are expressed in cortical thymic epithelial cells and play a central role in the production of self-peptides inducing positive selection of CD8 T cells (2,3,4). Here, we examined how the self-peptides for positive selection and homeostasis of CD8 T cells are related, using a mouse model deficient of thymoproteasom-specific proteolytic subunit, beta5t.

Results

1. Thymoproteasome is not expressed in the periphery

In the steady state, T cells circulate peripheral lymphoid organs including the spleen and lymph nodes through the blood and lymph. T cells interact with dendritic cells and stromal cells in these lymphoid organs to receive the signals for their homeostasis. We initially examined the expression of beta5t by highly sensitive RT-PCR in the spleens and lymph nodes and various cell subsets purified from these organs. Contrary to our expectations, the beta5t gene transcripts were not detected at all in the peripheral organs, whereas expression of beta5t was apparent in the thymus in accordance with earlier studies (2,3).

2. Abnormal phenotype of peripheral CD8 T cells in beta5t-deficient mice

In beta5t-deficient mice, CD8 T cells show a reduction (<25% of normal) in number and an alteration of TCR repertoire (2,3,4). In this study, phenotype of CD8 T cells in beta5t-deficient mice was investigated in detail by flow cytometry, and we found that CD8 T cells in the secondary lymphoid organs of beta5t-deficient mice were enriched with CD44hiCD122hi cells, which resembles memory T cells. Increased proportion of these memory-like cells was first observed in mice between 7 and 10 days after birth and gradually enhanced with aging of mice. This phenotypic abnormality of CD8 T cells was also seen in beta5t-deficient mice with OT-I TCR transgenic background, suggesting memory-like cells appear independently of exogenous antigen recognition. On the other hand, the proportion of memory-like cells were significantly lower in mice bearing T cells with monoclonal TCR than in mice with polyclonal T cells, presumably because of the competition for specific self-peptides among T cells with identical TCR (intraclonal competition) (1). Importantly, regression of phenotypic abnormality in monoclonal T cells in comparison of polyclonal T cells was observed in both beta5t-sufficient and -deficient groups, suggesting the requirement of tonic TCR signals for the emergence of memory-like CD8 T cells at high frequencies in beta5t-deficient mice.

3. Peripheral CD8 T cells in beta5t-deficient mice is a stable population

We examined whether increased frequency of memory-like cells in beta5t-deficient mice is attributed to 1) increased development of memory-like cells in the thymus, 2) decreased thymic egress of naïve cells, 3) differentiation of naïve cells into memory-like cells, 4) impaired survival of naïve cells, or 5) increased proliferation of memory-like cells. In beta5t-deficient mice, memory-like cells occupied a very small fraction of (less than 5%) of CD4-CD8+ mature thymocytes as in normal mice. There was no obvious changes in this frequency even after inhibiting thymocyte migration from the thymus to the periphery by treatment of mice with FTY720, suggesting that increased proportion of memory-like cells is neither due to increased development of memory-like cells in the thymus nor impaired thymic egress of naïve cells. Then, CD44loCD122lo naïve CD8 T cells obtained from the peripheral lymphoid organs of beta5t-deficient and -sufficient mice were adoptively transferred to normal mice and the phenotype and the survival of the donor cells were examined overtime. Until 4 weeks after transfer, donor cells retained naïve phenotype in both groups and the recovery of donor cells was similar. Normal survival of naïve cells were further supported by the results of in vitro culture performed in the presence or absence of IL-7, a primary survival factor of naïve T cells. In addition, donor cells including CD44hiCD122hi and CD44loCD122lo populations obtained from beta5t-deficient and -sufficient mice were labeled with carboxyfluorescein succinimidyl ester and were transferred into normal mice to examine the cell proliferation. In both beta5t-deficient and -sufficient groups, division of donor cells was not observed or very limited. These results indicated that CD8 T cells, regardless of naïve or memory-like, in the periphery of beta5t-deficient mice are quite stable in number and phenotype.

Discussion & Conclusion

This study revealed a phenotypic abnormality of peripheral CD8 T cells in beta5t-deficient mice. In beta5t-deficient mice, immunoproteasomes which are present in cortical thymic epithelial cells at low levels produce positive selection-inducing self-peptides on behalf of thymoproteasomes (2,4). Differently from thymoproteasomes whose expression is strictly restricted to the thymus, immunoproteasomes are systemically present in the immune cells. Therefore, in beta5t-deficient mice, CD8 T cells that have differentiated dependently of immunoproteasomes in the thymus are again exposed to the self-peptides produced by immunoproteasomes in the periphery. This recognition of identical self-peptides for positive selection and homeostasis may lead to the failure to maintain naïve phenotype of CD8 T cells. This hypothesis suggested from the findings in beta5t-deficient mice seems to be against the current T cell homeostasis model that self-peptides used for T cell homeostasis is identical to those for positive selection. However, the background studies which support the current homeostasis model were designed to examine CD4 T cells, not CD8 T cells (5,6,7). The further investigation of CD8 T cells in beta5t-deficient mice will reveal the characteristics of self-peptides for the development and the maintenance of T cells and lead to the better understanding of the mechanism of naïve T cell homeostasis.

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一般の皆様へ

免疫系の主役であるT細胞は、体内に侵入した病原体や異物に由来するペプチドを自分の体の成分とは違うもの(非自己)として認識し、排除することで体を守っています。T細胞がこれらのペプチドを非自己として認識するには、自分の体に由来するペプチド(自己ペプチド)を常に認識し続けることで、T細胞自身が健康な状態に保たれなくてはなりません。私達は、T細胞を健康に保つために必要な自己ペプチドの正体を解明したいと考えています。このような免疫系を健康に保つ仕組みを理解することは、免疫の異常に起因する様々な病気の予防や治療に役立つと期待されます。

In vitro generation mature oocytes through factitious activation of dormant primordial follicles

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Abstract

The goal of this project is to generate mature human oocytes capable for fertilization and subsequent development from in vitro cultured ovarian tissues. In this project, we identified several important factors that facilitate early follicle growth. Using these factors, we are trying to optimize the method for tissue cultures of human ovaries.

Key Words : Primordial follicle, oocyte, PTEN, PI3K

Introduction

Under in vitro culture, a trial to obtain mature oocytes from primordial follicles in cultured ovaries has only succeeded in mice in mammals. However, it has not established in human, because of lack of knowledge about the mechanisms of activation of dormant ovarian follicles and early follicular growth. Recently, we succeeded to activate dormant ovarian follicles by stimulation of the PI3K-Akt pathway (1). Using this method, we attempted to establish in vitro culture system of human ovarian follicles to generate mature oocytes.

Results

Although large follicles (antral stage) are known to grow in response to follicle stimulating hormone (FSH), it is not clear the mechanisms of the development of early stage of small follicles. Thus, we tried to identify important growth factors to stimulate early follicular growth based on ovarian microarray analyses (2, 3).

C-type natriuretic peptide (CNP) encoded by the NPPC (Natriuretic Peptide Precursor C) gene expressed in ovarian granulosa cells inhibits oocyte maturation by activating the natriuretic peptide receptor (NPR)B (NPRB) in cumulus cells. Real-time RT-PCR analyses indicated increased expression of NPPC and NPRB transcripts in somatic cells obtained from mice during prepubertal development (from day 7–19 of age) in an age-dependent manner with negligible levels in oocytes, associated with increases in ovarian CNP peptides in mice. In cultured somatic cells from infantile ovaries and granulosa cells from prepubertal animals, treatment with CNP, but not ANP, led to dose-dependent increases in media content of cGMP for both cell preparations. In contrast, no changes in cAMP production after CNP treatment were detected for both types of cells. Also, treatment with CNP led to dose- and time-dependent increases in follicle size, reaching levels comparable to those induced by a cGMP analog, 8-bromo-cGMP. In contrast, treatment with ANP

was ineffective. Furthermore, combined treatment with CNP and FSH led to further increases in follicle growth. Treatment of cultured ovarian explants from infantile mice with CNP, increased ovarian weight gain that was associated with the development of primary and early secondary follicles to the late secondary stage. In *in vivo* studies, CNP pretreatment led to a 56% increase in ovarian weight. Histological analyses indicated increases in the development of antral follicles after CNP pretreatment followed by eCG. Some of these eCG-treated animals were further treated with an ovulatory dose of human (h) CG to check ovulation efficiency. Higher numbers of ovulated oocyte were found in the oviducts of CNP-pretreated animals as compared with controls. These findings suggest the ability of CNP to promote the development of secondary/preantral follicles to the early antral stage, thus allowing efficient induction of ovulation by the subsequent eCG-hCG treatment. In prepubertal mice, CNP treatment alone also promoted early antral follicle growth to the preovulatory stage, leading to efficient ovulation induction by LH/human chorionic gonadotropin. Mature oocytes retrieved after CNP treatment could be fertilized *in vitro* and developed into blastocysts, allowing the successful pregnancy and delivery of viable offspring. To further elucidate molecular mechanisms underlying CNP stimulation of the development of preovulatory follicles capable of responding to the preovulatory hCG stimulation, we analyzed the expression of diverse ovarian genes important for follicle maturation, steroidogenesis, and ovulation. Treatment with CNP increased the expression of transcripts for LH receptor, progesterone receptor, inhibin-alpha (inhibin-a), IGF-I, tissue plasminogen activator, key transcriptional factors (GATA6, WT1, and Ccaat/enhancer-binding protein-alpha), and key steroidogenic enzymes (CYP11a1, CYP19, and steroidogenic acute regulatory protein) in a time-dependent manner.

Discussion & Conclusion

Other than CNP, we identified different factors capable to stimulate early follicle growth (under review). The stimulatory effects of CNP and other factors on early follicle growth were also confirmed in human samples obtained from patients (unpublished data). Using these factors in combination with the method for activation of dormant ovarian follicles, we are trying to establish *in vitro* cultures of human ovaries to generate mature oocytes. We are also trying to find a suitable method of large follicle culture in human without losing cell-to-cell (oocyte-to-cumulus cell) communications, which is important for optimal maturation of oocytes.

Some materials and a person who hired to help experiments were also used for other projects to avoid waste and two papers were published (Sato et al. *Mol Endocrinol* 2012, in press, Kawamura et al. *Endocrinology* 2012, in press).

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一般の皆様へ

本研究では、卵巣内で休眠状態にある原始卵胞を我々の開発した人為的活性化技術を用いて活性化させ、多数の活性化卵胞検体を得た上で、それらを検体として用いて体外培養によりヒト原始卵胞から成熟卵子を作出することを最終目的とした。本年度は初期卵胞発育に必要な新たな因子を見出すことに成功し、完全なる体外培養環境を構築すべく現在検討を進めている。

Critical molecular mechanisms to determine brain size and neuronal population

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Abstract

A hallmark of neurogenesis in the vertebrate brain is the apical-basal nuclear oscillation in polarized neural progenitor cells. Known as interkinetic nuclear migration (INM), these movements are synchronized with the cell cycle such that nuclei move basally during G1-phase and apically during G2-phase. Based on the results of time-lapse analyses and simulation model, I propose that the basal migration of G1-phase nuclei depends on a displacement effect by G2-phase nuclei migrating apically. Furthermore, I demonstrated that basal-to-apical active nuclear movement is inhibited by disruption of gene function of microcephaly causative gene.

Key Words : Brain development, Interkinetic nuclear migration, Neurogenesis

Introduction

A fundamental question of brain development is how the appropriate number of neurons, and ultimately, the size of brain are determined. This can be re-phrased as, how do neural stem cells know when to switch their mode of cell division from self-renewal proliferation to neurogenic differentiation in a coordinated manner within the neuroepithelium, a pseudostratified epithelial tissue in the developing brain. Dynamic movement of neural stem cells contributes to intermingling the neuroepithelium, where neural stem cells undergo both self-renewing expansion and commitment to neuronal differentiation. I propose that neural stem cells receive and exchange signals for their cell-fate choice through rather “dynamic interactions” during brain formation, which would ensure the regional homogeneity of the vertebrate brain. To understand how the dynamic environment is generated, I pursued to unravel the mechanism of the “interkinetic nuclear migration”, a nuclear migration associated with the cell cycle progression of neural progenitors in the developing brain.

Results

To analyze nuclear movement during INM, I established a system that enabled to quantitatively track the motion of individual nuclei in living tissue. Nuclei in the dorsal cortex of an E13.5 mouse brain were labeled by green fluorescent protein (GFP) containing a nuclear localization signal (NLS) using *in utero* electroporation. Labeled nuclei in cultures of brain slices were tracked using a video imaging system. First, nuclear “ratcheting,” a forward and backward motion of nuclei, occurs while the nuclei migrate toward the basal side during G1-phase. Second, during the basal-to-apical

migration before mitosis (G2-phase), the nuclei show linear movements and faster kinetics than nuclei that are moving in the opposite direction. Next, I demonstrated that INM in both directions is dependent on microtubules, which indicates that microtubule regulation coupled with the cell cycle might control the timing of nuclear migration. In this study, functions of Tpx2, a microtubule associated protein, for proper basal-to-apical nuclear migration and for the cell cycle dependent microtubule organization are determined. Tpx2 localizes on the apical process of G2-phase neural progenitor cell, but not on the G1-phase. Knocking-down of Tpx2 slows down the basal-to-apical nuclear movement, and the conformation of microtubule bundling in the apical process is altered.

Next, I found that cell cycle arrest at G1-phase by overexpressing p18^{Ink4c} induces the basal accumulation of G1-arrested nuclei. This result suggests that nuclei of G1-arrested neural progenitor cells migrate basally after mitosis at the apical surface under conditions in which surrounding cells undergo normal INM. This raised the possibility of a specific mechanism that conveys nuclei from the apical to the basal region. To test whether the translocation system in the VZ is cell-autonomous or dependent on the activity of surrounding cells, I observed the behavior of fluorescent microbeads in cultures of brain slices. As a result, beads showed basally-directed translocation. During the translocation, beads showed “ratcheting,” back-and-forth movement as observed in the migration of G1-phase nuclei. I thus conclude that there is a mechanism by which beads translocate from the apical surface to the basal region through intercellular space in the VZ.

Microbead translocation in the brain slice suggests that G1-nuclei are driven in the apical-to-basal direction by a non-autonomous mechanism. However, such a mechanism is not immediately apparent. One possibility is that active basal-to-apical migration of nuclei increases the nuclear density high on the apical side of the VZ. The resultant close packing of nuclei may crowd out free nuclei that have completed mitosis, so that they are pushed further away from the ventricular surface. If this is the case, apical-to-basal migration should be affected by the acute perturbation of basal-to-apical migration of nuclei in G2-phase. To test this hypothesis, I arrested the cell cycle at S-phase by drug treatment using hydroxyurea (HU) and performed time-lapse observations of migrating nuclei. Under this condition, the average velocity of nuclei moving in the basal direction was significantly decreased. I next tested whether microbead translocation is also perturbed by the same drug treatment. Indeed, most fluorescent beads incorporated from the apical surface translocated shorter distances after HU treatment.

Finally, to evaluate the possibility that apical-to-basal nuclear migration occurs as a result of the displacement of nuclei in the apical VZ, I constructed a computational model for INM, and qualitatively compared the outcomes predicted by the model with the experimental data (a collaboration with A. Kimura, National Institute of Genomics). This qualitative correlation supports a model in which apical-to-basal nuclear migration is mainly driven by the displacement of nuclear migration in the basal-to-apical direction during G2-phase.

From a mechanistic perspective, the role of centrosomes in INM is noteworthy. A close relationship between neurogenesis and centrosomal functions is suggested from the identification of causative genes of human autosomal-recessive primary microcephaly (MCPH genes). Indeed, knocking down

of one of such gene, *CDK5RAP2*, resulted in slowing down of basal-to-apical nuclear migration. Currently, studies how slowing down of basal-to-apical nuclear migration gives rise to impaired neurogenesis are taken place.

Discussion & Conclusion

The most remarkable finding of this study is that nuclear migration in the apical-to-basal direction during G1-phase involves a passive, non-autonomous process dependent on the migration of nuclei during G2-phase, which takes place toward the apical surface and results in a higher nuclear density in the apical region and a lower nuclear density in the basal region of the VZ. In a packed tissue such as the VZ of the embryonic brain, free, mobile nuclei redistribute so that the nuclear density becomes homogeneous. Based on our experimental results and computational modeling, I propose a displacement model of INM, in which, after mitosis, nuclei on the ventricular surface are driven away from this surface by a collective displacement effect generated by other nuclei migrating toward the ventricular surface. The model for INM explains how the dynamics of neural progenitors harmonize their extensive proliferation with the epithelial architecture in the developing brain.

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一般の皆様へ

神経前駆細胞の細胞核が細胞周期に従って組織内を往復運動するエレベーター運動は、発見以来 70 年以上その仕組みについて不明であったが、私は、神経管内腔に向かう核移行は微小管細胞骨格の細胞周期依存的な安定化による「能動的」な運動であることに対し、逆方向の移行は組織内の細胞密度勾配に従った「受動的」な運動であることを示した (Kosodo et al, 2011 ; Kosodo, 2012)。さらに、小脳症原因遺伝子の機能を胎生期マウス脳で阻害することで、エレベーター運動過程に異常が見られることが見出された。

Development of promising anticancer agents based on practical total synthesis of marine natural products

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Abstract

An improved total synthesis of neopeltolide, a potent anticancer macrolide natural product, was accomplished. The newly developed concise route was successfully applied to the synthesis of 8,9-dehydroneopeltolide, a designed analogue with more potent antiproliferative activity than the parent natural product against human cancer cell lines.

Key Words : macrolide, anticancer agents, total synthesis, natural products

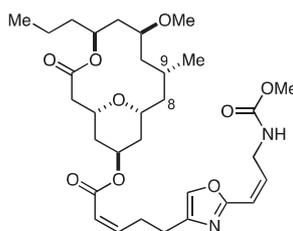
Introduction

Marine organisms are an enormously rich source of structurally diverse secondary metabolites of novel molecular architecture and potent biological activities. In particular, marine macrolide natural products represent a rich source of structurally novel and potent anticancer chemotherapeutic agents. However, many of these promising compounds are available only in extremely limited quantities and thus organic synthesis is the only way to address these problems. Neopeltolide, a marine macrolide isolated from a deep-water sponge, exhibits potent *in vitro* antiproliferative activity against several cancer cell lines with nanomolar IC₅₀ values and is expected to be a drug candidate for anticancer agent. We report herein a novel analogue of neopeltolide based on an improved synthetic route.

Results

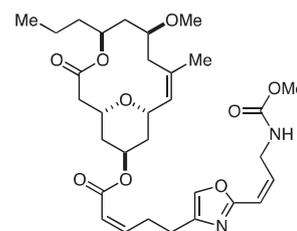
We have already reported a concise total synthesis of neopeltolide based on strategic application of olefin metathesis reactions [1]. In this research, we developed a more efficient route to the key intermediate in our earlier synthesis, tetrahydropyran-containing fragment. The synthesis features Nagao aldol reaction using a chiral 1,3-thiazolidine-2-thione derivative, Evans–Tishchenko reduction of a β -hydroxy ketone with SmI₂ to establish 1,3-*anti* diol, chemoselective cross-metathesis reaction, and construction of the tetrahydropyran ring through oxa-Michael reaction under thermodynamic control.

We designed a novel analogue of neopeltolide, 8,9-dehydroneopeltolide, on the basis of our earlier structure-activity relationship studies [2]. The



neopeltolide

A549: IC₅₀ 1.50 nM
MCF7: IC₅₀ 1.85 nM



8,9-dehydroneopeltolide

A549: IC₅₀ 0.50 nM
MCF7: IC₅₀ 33 nM

synthesis of 8,9-dehydroneoptolide was successfully accomplished by using the synthetic route described above. This dehydro analogue exhibited potent antiproliferative activity against A549 human lung adenocarcinoma and MCF7 breast cancer cell lines with IC_{50} value of 0.50 nM and 33 nM, respectively. The former activity was more potent than that of the parent compound (IC_{50} 1.50 nM).

Discussion & Conclusion

We have accomplished a concise synthetic route to neopeltolide, an antitumor marine macrolide. The synthesis features a convergent union of three fragments, of comparable complexity, in late stages of the total synthesis and was successfully applied to the synthesis of 8,9-dehydroneoptolide. The designed analogue was equipotent or more active than the parent natural product and thus has potential for further stereostructure-activity relationship studies. Further studies for the design and synthesis of more potent structural analogues and molecular probes for elucidating the biological functions of neopeltolide are underway.

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一般の皆様へ

海洋生物が産生する化合物（海洋天然物）の中には、顕著な抗がん作用を示すものが数多く発見されており、新たな抗がん剤の候補として注目されている。しかし、海洋天然物の多くは微量成分であり、薬剤開発には化学合成による実用的な化合物供給法の開発が必須である。深海に生息する海綿から単離された強力な抗がん活性マクロリド天然物ネオペルトリドの超効率的な化学合成法を開発し、天然物を凌駕する抗がん活性を示す人工類縁体を合成した。今後さらに、化合物の最適化や作用機構の解明により新しい抗がん剤開発に繋がる可能性がある。

Molecular mechanisms of biological signal transduction operated by gas molecules

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Abstract

We have studied the structural and enzymatic properties of a diguanylate cyclase from an obligatory anaerobic bacterium *Desulfotalea psychrophila*, which consists of the N-terminal sensor domain and the C-terminal diguanylate cyclase domain. The sensor domain shows an amino acid sequence homology and spectroscopic properties similar to those of the sensor domains of the globin-coupled sensor proteins containing a protoheme. This heme-containing diguanylate cyclase catalyzes the formation of cyclic di-GMP from GTP only when the heme in the sensor domain binds molecular oxygen. The hydrogen bonding network plays a crucial role for the selective O₂ sensing responsible for the regulation of the enzymatic activity.

Key Words : Oxygen sensor protein, heme-based sensor, diguanylate cyclase

Introduction

Hemoproteins are a typical metalloprotein, which show a variety of function including oxygen storage/transport, electron transfer, redox catalysis with various substrates. Besides these traditional functions of hemoproteins, a new function of hemoprotein has been found recently, which is a sensor of diatomic gas molecules or redox change. In these heme-based sensor proteins, the heme acts as the active site for sensing the external signal such as gas molecules and redox change. Our research interests are focused on the elucidation of the structure-function relationships of these heme-based sensor proteins.

Results

HemDGC was found to be a novel heme-based sensor protein that has the enzymatic activity of diguanylate cyclase. The enzymatic activity for the formation of c-di-GMP from GTP was measured for ferric, cyanomet, deoxy, oxy, CO-bound, and NO-bound HemDGC. Among them, only oxy HemDGC showed the activity of diguanylate cyclase for the formation of c-di-GMP from GTP, but ferric, cyanomet, deoxy, CO-bound, and NO-bound HemDGC did not at all. These results indicate that O₂ will be a physiological effector of HemDGC and will regulate the enzymatic activity of HemDGC via the formation of the oxy-complex of the heme. Thus, the globin domain of HemDGC acts as a sensor that can strictly discriminate O₂ among external ligands capable to bind to the heme.

The specific interaction between the heme-bound O₂ and surrounding amino acid residue(s) would be responsible for the discrimination of O₂ against other ligands. To elucidate the mechanisms by

which HemDGC discriminate O₂ against other ligands to switch on the enzymatic activity, we investigated the heme environmental structure of HemDGC by resonance Raman spectroscopy. The Fe-O ($\nu_{\text{Fe-O}}$) and O-O ($\nu_{\text{O-O}}$) stretching modes were observed at 566 and 1138 cm⁻¹, respectively. The $\nu_{\text{Fe-O}}$ band is a sensitive marker of the status of hydrogen bonding interaction on the heme-bound O₂. The $\nu_{\text{Fe-O}}$ frequency of HemDGC was similar to those of several non-vertebrate hemoglobins (Hbs) such as *Ascaris suum* Hb (566 cm⁻¹) [1], *Mycobacterium tuberculosis* HbN (562-566 cm⁻¹) [2-4], and *Paramecium caudatum* Hb (563 cm⁻¹) [5].

The Fe-CO stretching ($\nu_{\text{Fe-CO}}$), C-O stretching ($\nu_{\text{C-O}}$), and Fe-C-O bending ($\delta_{\text{Fe-C-O}}$) modes were observed at 506, 1944, and 579 cm⁻¹, respectively. The $\nu_{\text{Fe-His}}$ band was observed at 226 cm⁻¹ in deoxy HemDGC. These results indicate that a histidine is the proximal ligand of the heme in HemDGC.

We prepared Y55F, Y80F, and Q81A HemDGC and characterized their spectroscopic and enzymatic properties to elucidate the functional roles of these amino residues interacting with the heme-bound ligand in the distal heme pocket.

The spectroscopic and enzymatic properties of Y80F HemDGC were similar to those of wild-type HemDGC, indicating that the mutation of Tyr80 did not affect these properties. On the other hand, the mutation of Tyr55 or Gln81 resulted in changes in the stability of the oxy form and the enzymatic activity of these mutants. Y55F HemDGC did not show any activity at all for the production of c-di-GMP, partly because this mutant could not form a stable oxy form. Thus, Tyr55 plays an important role for the stabilization of the heme-bound O₂ to maintain the stable oxy form.

Though Q81A HemDGC formed a stable oxy form, it changed to the ferric form by autoxidation rapidly compared with wild-type HemDGC. Though oxy Q81A HemDGC was active for the formation of c-di-GMP, the activity was lower than that of wild-type HemDGC. The reaction stopped in 20 min due to the conversion of Q81A HemDGC from the oxy form to the ferric form by autoxidation.

The mutation of Gln81 resulted in a change of the resonance Raman spectrum of the CO-bound form, as shown in Fig. 5. While wild-type and Y55F HemDGCs showed the $\nu_{\text{Fe-CO}}$ band at 506 cm⁻¹, the $\nu_{\text{Fe-CO}}$ band of Q81 HemDGC down-shifted to 496 cm⁻¹. Taken together, Tyr55 and Gln81 are thought to form a hydrogen bonding network with the heme-bound O₂.

Discussion & Conclusion

HemDGC from *Desulfotalea psychrophila* has an N-terminal globin domain and a C-terminal GGDEF domain. In this work, we have showed that the N-terminal globin domain has a b-type heme with a ferrous heme that can binds external ligands such as O₂, CO, and NO, and that HemDGC shows the enzymatic activity of diguanylate cyclase that forms c-di-GMP from GTP only when the heme in the N-terminal globin domain binds O₂. These results indicate that HemDGC is a new member of globin-coupled O₂ sensor proteins in which O₂ regulates the diguanylate cyclase activity of the C-terminal GGDEF domain. HemDGC shows different hydrogen bonding patterns

between the O₂- and CO-bound forms. The formation of the hydrogen bond between Tyr55 and the heme-bound O₂, but not with CO, will play an important role for the discrimination of O₂ against CO.

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一般の皆様へ

近年、気体分子が生理的なシグナル分子として機能し、さまざまな生理機能の制御に関与していることが報告され、多くの研究者の注目を集めている。気体分子がシグナル分子として機能するためには、そのレセプター（センサー）タンパク質が必要不可欠である。本研究では、酸素分子のセンサー機能を有する新規なタンパク質 HemDGC を対象として研究を行い、HemDGC がどのようにして酸素分子をセンシングしているか、センシングされた酸素分子により HemDGC の機能がどのように制御されているかを明らかにした。

Regulatory mechanism of cell division by telomeres

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Abstract

In *Schizosaccharomyces pombe* closed mitosis, the telomeres, tethered to the NE during interphase, are transiently dissociated from the NE during mitosis. This transient dissociation from the NE is essential for accurate chromosome segregation, because forced telomere tethering to the NE caused frequent chromosome loss. We found that the phosphorylation of the telomere protein Rap1 during mitosis impedes the interaction between Rap1 and Bqt4, a nuclear membrane protein, thereby inducing telomere dissociation from the NE. We propose that the telomere dissociation from the NE promoted by Rap1 phosphorylation is critical for the fidelity of chromosome segregation in closed mitosis.

Key Words : chromosome, telomere, mitosis

Introduction

Efficient chromosomal movements are important for the fidelity of chromosome segregation; however, movements are constrained during interphase by the tethering of many domains to the nuclear envelope (NE). Higher eukaryotes undergo open mitosis accompanied by NE breakdown, whereas lower eukaryotes undergo closed mitosis in which NE breakdown does not occur. Although the chromosomal movements in closed mitosis are proposed to be restricted compared to open mitosis, the cells overcome this problem by an unknown mechanism to segregate the chromosomes accurately.

Results

To investigate the regulation of the physical interaction between telomeres and the NE during the cell cycle in *S. pombe*, we measured the distances between telomeres and the NE. Throughout the G₂ phase, most distances were <0.2 μm (the diameter of the *S. pombe* nucleus is 2.0 – 2.5 μm). Thus, the telomeres are continuously moving within the vicinity of the NE during G₂ phase. In contrast to the G₂-phase distance, the distance during M phase was markedly increased (> 0.4 μm). These data indicated that telomeres are transiently dissociated from the NE in M phase.

To clarify whether transient telomere dissociation from the NE during M phase is required for the fidelity of chromosome segregation in closed mitosis, we forced the tethering of telomeres to the NE by expressing the Taz1-Bqt4 fusion protein (Taz1 is a telomere DNA-binding protein, and Bqt4 is a nuclear membrane protein). We found that the Taz1-GFP-Bqt4ΔN-expressing strain lost mini-chromosome Ch16 after cell division at a markedly high frequency. These observations demonstrated that transient telomere dissociation from the NE during M phase is required for proper

chromosomal movements and thus the fidelity of chromosome segregation in closed mitosis.

To investigate the molecular mechanisms of telomere dissociation from the NE during M phase, the nature of Rap1, which binds to both Taz1 and Bqt4 for telomere tethering to the NE during interphase, was analyzed. We found that Rap1 is highly phosphorylated, particularly during M phase. Western blot analyses using phospho-specific antibodies have revealed that Rap1 is phosphorylated at Ser213, Thr378, Ser422, Ser456, and Ser513 during M phase *in vivo*. The alanine mutations (*rap1-5A*) increased the efficiency of the interaction between Rap1 and Bqt4, whereas the phospho-mimic mutations (*rap1-5E* and *rap1-5D*) decreased the efficiency, suggesting that Rap1 phosphorylation has an inhibitory effect on the Rap1-Bqt4 association.

Intriguingly, the distance in the *rap1-5A* mutant during G₂ phase was markedly less than that in the wild type (average, 0.13 μ m). In contrast, the distances were clearly greater in the *rap1-5E* and *rap1-5D* mutants (average, 0.37 μ m and 0.35 μ m, respectively) than in the wild type. These data suggest that Rap1 phosphorylation promotes telomere-NE dissociation by inhibiting the Rap1-Bqt4 interaction.

We investigated the physiological consequences of the abnormal kinetics of telomere-NE dissociation in the *rap1-5A* mutant. The *rap1-5A* mutant lost mini-chromosome Ch16 at a higher frequency (2.1-fold) than the wild type. Furthermore, the spindle assembly checkpoint, which blocks metaphase-to-anaphase progression, was hyper-activated in the *rap1-5A* mutant; a higher proportion (2.6-fold) of the mutant cells showed the kinetochore accumulation of the Bub1 protein, which recognizes tension-less kinetochores, than that of the wild-type cells. These results suggest that the shortened distances between telomeres and the NE by the *rap1-5A* mutation disturbs M phase progression.

We next determined which phosphorylated residue(s) of Rap1 is important for the telomere-NE dissociation. *rap1*^{513A}, but not any of the other single-alanine mutants, showed markedly shorter distances between telomeres and the NE in G₂-phase cells (average, 0.11 μ m). In contrast, the *rap1*^{513E} mutant showed moderate elongation of the distance (average, 0.30 μ m). These results suggest that phosphorylation at Ser513 is the key for Rap1-Bqt4 dissociation and thereby for telomere dissociation from the NE during M phase. Consistent with this mechanism, Ser513 phosphorylation was observed during the early to middle stages of mitosis, a time during which telomeres are dissociated from the NE. Ser513 is presumably phosphorylated by Cdc2 kinase, because Ser513 was efficiently phosphorylated by p13^{suc1}-associated kinases (the major kinase is Cdc2) *in vitro*.

Discussion & Conclusion

In our study, we discovered a novel regulatory mechanism involved in closed mitosis. Upon entry into mitosis, telomeres are dissociated from the NE. The release of telomeres from the NE is required for faithful chromosome segregation. Phosphorylation of the telomere-binding protein Rap1 presumably by Cdc2 kinase induces its detachment from the nuclear membrane protein Bqt4. This reaction, in collaboration with other unknown mechanisms, promotes telomere dissociation from

the NE. Hence, we propose a model in which the telomere plays an important role in the dynamism of chromosomes during M phase. In a sense, the release of chromosomes from the NE may be the common mechanism that is required for the proper movement of chromosomes in both closed mitosis and open mitosis. In closed mitosis, cells show breakdown of the tethering of the telomere (and probably other chromosomal regions) to the NE, whereas chromosomes become free in open mitosis following breakdown of the NE.

一般の皆様へ

生命の維持には、生命活動の司令塔である染色体（DNA がまとまって形作る構造体）が安定に維持されることが必要です。染色体が分配される細胞分裂期において、染色体の末端にあるテロメアという構造体が染色体の安定性において重要な役割を果たしていることを明らかにしました。（テロメアは、細胞の老化に関与することがよく知られています。）

Development of novel strategy for therapeutic angiogenesis

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Abstract

In this study, we performed both *in vivo* and *in vitro* investigations into the role of Sema3E/plexinD1 in postnatal angiogenesis, and we found that these two molecules inhibit angiogenesis by suppressing the vascular endothelial growth factor (VEGF) signaling pathway.

Key Words : angiogenesis, semaphorins, p53, diabetes

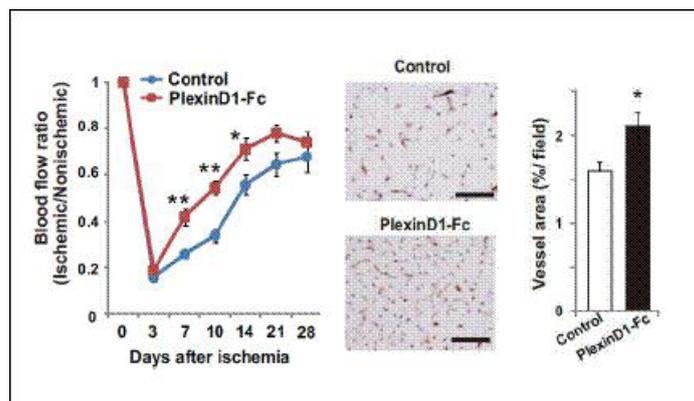
Introduction

The axon-guiding molecules known as semaphorins and their receptors (plexins) regulate the vascular pattern and play an important role in the development of vascular network during embryogenesis^{1, 2}. Semaphorin3E (Sema3E) is one of the class 3 semaphorins, and plexinD1 is known to be its receptor³. While these molecules have a role in embryonic vascular development, it remains unclear whether the Sema3E/plexinD1 axis is involved in postnatal angiogenesis. The objective of this study is to elucidate the role of Sema3E/plexinD1 in postnatal angiogenesis.

Results

Sema3E inhibited cell growth and tube formation by suppressing the vascular endothelial growth factor (VEGF) signaling pathway. Sema3E suppressed VEGF-induced phosphorylation of ERK and Akt. It is well accepted that both ERK and Akt are crucial for the intracellular signaling pathways stimulated by hepatocyte growth factor (HGF) or basic fibroblast growth factor basic (bFGF) to induce angiogenesis. Interestingly, Sema3E also inhibited bFGF or HGF-induced tube formation in a dose dependent manner. Moreover, Sema3E significantly inhibited bFGF-induced tube formation even in the presence of the anti-VEGF antibody, whereas it did not inhibit HGF-induced tube formation. These results suggest that besides the suppression of VEGF-induced angiogenesis, the anti-angiogenic effect of Sema3E was partially mediated by VEGF-independent mechanisms. Expression of Sema3E and plexinD1 was markedly up-regulated in ischemic limbs of mice (2.5 fold and 4.5 fold increase for Sema3E and plexinD1, respectively), and inhibition of this pathway by introduction of the plexinD1-Fc gene or disruption of Sema3E led to a significant increase of blood flow recovery (1.6 fold and 1.5 fold increase for the plexinD1-Fc gene treatment and Sema3E disruption, respectively). Hypoxia activated the tumor suppressor protein p53, thereby up-regulating Sema3E expression. Expression of p53 and Sema3E was enhanced in diabetic mice compared with normal mice (2 fold and 1.3 fold increase for p53 and Sema3E, respectively). Consequently,

neovascularization after VEGF treatment was poor in the ischemic tissues of diabetic mice, while treatment with VEGF plus plexinD1-Fc markedly improved neovascularization.



Discussion & Conclusion

The present study demonstrated that the *Sema3E/plexinD1* axis inhibits postnatal angiogenesis in a murine model of hindlimb ischemia. Our results suggested that *Sema3E* inhibits angiogenesis by blocking activation of the VEGFR-2 and its downstream signaling pathway. Our results also suggest that p53 has a crucial role in the induction of *Sema3E* expression in ischemic tissue, although the precise mechanism of how p53 regulates *Sema3E* expression remains unknown. Since the anti-angiogenic activity of p53 is important for tumor suppression, *Sema3E/plexinD1* could be a potential target for the treatment of malignancies with p53 mutations. It has been reported that hyperglycemia activates p53 by increasing the production of reactive oxygen species. Thus, p53-induced up-regulation of anti-angiogenic factors (including *Sema3E*) is likely to account for the impairment of angiogenesis in patients with diabetes. Therefore, *Sema3E/plexinD1* could also be a target for the treatment of ischemic cardiovascular disease in diabetic patients because conventional therapeutic angiogenesis is not very efficient in this patient population^{4,5}.

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一般の皆様へ

セマフォリンは元々神経ガイダンス分子として発見された分子であるが、最近胎児期における血管形成にも重要な分子であることがわかってきた。そこで本研究では、成人期のセマフォリンの血管新生に対する作用を明らかにすることによって、次世代の血管新生治療を開発することを目的とした。培養細胞やマウスモデルを用いた検討では、セマフォリンは血管内皮増殖因子の作用を抑制することによって、血管新生を負に制御していることがわかった。セマフォリンの発現は、老化分子 p53 によって正に制御されており、特に糖尿病マウスモデルなどでその発現の亢進がみられた。糖尿病モデルにおいては、血管増殖因子などによる血管新生治療効果は著しく障害されていたが、セマフォリンを併用することによって、その治療効果は著しく改善された。以上より、セマフォリンを用いた血管新生治療は、これまでの血管新生治療に抵抗性であった糖尿病患者などにおいて有効である可能性が示唆され、次世代の血管新生治療の標的となりうると考えられた。

Spatio-temporal regulation of sensory neural circuit formation

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Abstract

Elucidating the fundamental principles in the development of sensory neuronal circuits is one of the important questions in neuroscience. Here, we selectively visualized layer 2/3 neurons and examined their axonal organizations in the somatosensory cortex. We found that the axons of layer 2/3 neurons run preferentially in septal regions of layer 4. Immunohistochemical analyses revealed that at least two distinct steps are involved. Our results suggest that distinct developmental principles mediate the formation of axonal organizations of layer 2/3 neurons in the somatosensory cortex.

Key Words : somatosensory system, development

Introduction

Elucidating the fundamental principles in the development of sensory neuronal circuits is one of the important questions in neuroscience. Although the mechanisms underlying the formation of neuronal circuits between the sensory periphery and the cerebral cortex have been extensively examined, those of intracortical circuitries still remain elusive. To examine intracortical circuitries, here we selectively visualized layer 2/3 neurons and examined their axonal trajectories in the somatosensory cortex. We found a novel intracortical circuitry and developmental principles underlying the formation of the intracortical circuitry.

Results

To elucidate the intracortical axonal trajectories of layer 2/3 neurons, we selectively expressed GFP in layer 2/3 neurons of the mouse somatosensory cortex using in utero electroporation. Consistent with previous reports, when in utero electroporation was performed at E15.5, GFP-positive neurons were selectively located in layer 2/3.

Then we examined the axonal trajectories of layer 2/3 neurons in the somatosensory cortex. We found that the axons of layer 2/3 neurons preferentially located in the septal regions of layer 4. GFP signal intensities were significantly higher in the septal regions compared with barrel hollows. We then examined the distribution patterns of GFP-positive axons in tangential sections of flattened cortices. We found that GFP-positive axons were predominantly distributed all around barrel hollows in a whisker-related pattern in layer 4. Our confocal microscopic analyses demonstrated that there was a much higher density of GFP-positive axons in the septal regions. GFP-positive axons formed mesh-like structures, with the majority of axons running radially and the remainder running

obliquely.

It seemed possible that the axons of layer 2/3 neurons were just passing through the septal regions without making any synaptic contacts. Conversely, it also seemed possible that the axons of layer 2/3 neurons could make synaptic contacts in the septal region of layer 4. To address these possibilities, we examined the distribution pattern of a presynaptic marker protein. Using in utero electroporation, we transfected layer 2/3 neurons with GFP-tagged synaptophysin, which accumulates at presynaptic terminals when exogenously expressed. Intriguingly, we found a number of GFP-positive puncta, suggesting that the axons of layer 2/3 neurons in the septal regions contain presynaptic structures. This is indicative of a possibility that the spatial patterns of the axons of layer 2/3 neurons are involved in the functioning of the neuronal circuitry in the somatosensory cortex.

Next, we examined the developmental processes of the axonal trajectories of layer 2/3 neurons. We performed in utero electroporation at E15.5 and examined the distribution pattern of GFP-positive axons of layer 2/3 neurons at various time points during development. GFP-positive axons were distributed evenly without any specific preference until P6, and were found to be preferentially located in the septal regions at P10 and thereafter. This suggests that the formation of the axonal trajectories of layer 2/3 neurons consists of two distinct steps: initial axon projection without distinction of the septal and barrel regions, followed by the formation of axonal projections preferentially located in the septal regions. To further uncover the detailed processes underlying the formation of the axonal trajectories of layer 2/3 neurons, it would be interesting to investigate three-dimensional reconstruction images of single axon arbors derived from single layer 2/3 neurons.

To investigate the fine-scale structures of individual neurons in vivo, we developed and validated a rapid genetic technique that enables simultaneous investigation of multiple neuronal properties with single-cell resolution in the living rodent brain. Our technique PASME targets specific small subsets of sparse neurons in layer 2/3, layer 5 of the cerebral cortex and in the hippocampus with multiple fluorescent reporter proteins such as PSD-95-GFP and GFP-gephyrin. This technique, broadly applicable for probing and manipulating neurons with single-cell resolution in vivo, should provide robust means to uncover the basic mechanisms employed by the brain, especially when combined with in vivo two-photon imaging and/or optogenetic technologies.

Discussion & Conclusion

We identified the novel intracortical axonal trajectories of layer 2/3 neurons in the somatosensory cortex. The axons of layer 2/3 neurons were preferentially located in the septal region of layer 4. It would be intriguing to search for similar axonal trajectories in other cortical regions containing column-like structures such as ocular dominance columns. Because previous studies reported that rats do not have column-like structures in the visual cortex, higher mammals such as carnivores and primates might be appropriate for addressing this point.

It would be interesting to uncover the mechanisms underlying formation of the axonal trajectories we found during development. At P6 the axons of layer 2/3 neurons ran radially regardless of

barrels and septa. At P10, the axons started to locate preferentially in septa. This suggests that the formation of the axonal trajectories of layer 2/3 neurons may consist of two steps. It seems likely that each step requires distinct molecular mechanisms regulating axon pathfinding. Pinning down mechanisms underlying the formation of axonal trajectories of layer 2/3 neurons would lead to better understanding of the general principles of intracortical axonal development.

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一般の皆様へ

脳神経系を理解することは、ヒトを理解することと言っても過言ではありません。従って、高次脳機能の基盤となる複雑かつ精緻な脳神経回路の情報処理機構や形成メカニズムの解明は、神経科学の最重要課題の一つです。我々は、視覚や体性感覚（触覚）などの感覚系神経回路を用いて、この課題に挑んでいます。これまでに新たな神経回路の同定、神経回路形成メカニズムに2段階あること、さらには生体脳内で神経回路を可視化するための技術開発を行ってきました。今後は回路形成異常がもたらす病態についても解析していきたいと思っています。

PHD2-inactivation and new strategy for a treatment of Ischemia/ Reperfusion Injury

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Abstract

Prolyl-hydroxylase PHD1~3, also known as the “oxygen sensor”, negatively regulate hypoxic response via transcriptional factor hypoxia-inducible factor (HIF), a master regulator of hypoxic responses. PHDs are inactivated when oxygen is limited, which stabilizes HIF and turns on various hypoxic response. Among three PHDs, PHD2 is known to be a dominant hydroxylase for HIF *in vivo*. Therefore, inactivation of PHD2 is supposed to be protective against hypoxia and here we’ve hypothesized that PHD-inactivation might bring beneficial outcomes on ischemia/reperfusion (I/R)-injuries including myocardial infarction.

Key Words : Ischemia-reperfusion injury, PHD, HIF, Hypoxia

Introduction

Inactivating PHD2 shifts cellular metabolism toward “hypoxic mode” HIF-dependent manner, which enables cells to be survived in hypoxic environment. The purpose of this study is to test the potential beneficial effect of PHD2-inactivation in ischemia-reperfusion injuries, such as myocardial infarction, using PHD2 knockout mice that we’ve established recently.

Results

As a mouse I/R-injury model (acute myocardial infarction model), left anterior descending (LAD) artery was clamped for 15 minutes before reperfusion (LAD-ligation assay) in Wt or PHD2 conditional knockout mice (PHD2 KO). PHD2 was inactivated by orally given tamoxifen 72 hours prior to the surgery. Size of the infarction was significantly smaller in PHD2 KO mice than Wt mice. Left ventricular end-diastolic pressure (LVEDP) was elevated in both Wt and PHD2 KO mice during LAD-ligation, but rapidly decreased in PHD2 KO mice, suggesting cardiac function was maintained upon PHD2-inactivation. Taken together, inactivation PHD2 might be beneficial against I/R injuries including myocardial infarction. However, mechanism of this cardio-protective effect upon PHD2-inactivation was still unclear.

We have focused on cellular metabolism in PHD2-deficient heart and revealed these findings;

- (1) ATP levels of PHD2-KO hearts were significantly higher than Wt mice.
- (2) Oxygen consumption rate (OCR) of the isolated cardiomyocytes was significantly lower in PHD2 KO cardiomyocytes.
- (3) Mitochondrial mass is smaller in PHD2-KO hearts and copy number of mitochondrial DNA

was also significantly lower in PHD2 KO hearts.

- (4) Metabolome analysis revealed that glycolysis was activated and mitochondrial respiration was suppressed in PHD2 KO hearts.
- (5) Long term inactivation of PHD2 in cardiomyocytes failed to maintain cardiac function possibly due to remodeling of heart tissue, at least partially.

These data suggest that maintaining higher ATP levels with lower oxygen consumption and less mitochondria via anaerobic glycolysis in cardiomyocytes might contribute to better outcomes upon I/R-injuries upon PHD2-inactivation.

However, inactivation of PHD2 on I/R-injury must be limited to short term to avoid detrimental effect, such as cardiac failure.

Discussion & Conclusion

Here we showed the beneficial effect of hypoxic responses by PHD2-inactivation in mouse LAD-ligation model. This suggests that PHD2-inactivation might have global tissue protection effect against I/R-injuries.

I/R-injuries could be an issue not only in ischemic diseases including myocardial infarction or brain stroke, but in organ transplantation, as well. It is still critical how to maintain the organ function from the blockade of blood flow until the reperfusion. ATP-levels in the grafts have been a marker of tissue preservation. Our data showed that PHD2-inactivation can maintain the higher tissue ATP levels, at least in hearts. Also, as already reported, treating donor with PHD-inhibitor has better outcomes in rat kidney transplantation model. This could raise the possibility that PHD-inactivation is also tissue-protective in other organ transplantation including liver, islet or heart, where the long-term tissue preservation is difficult and donor is limited. Our mice model of PHD-inactivation would be a powerful tool to study the mechanism of tissue protection in organ transplantation, as well.

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一般の皆様へ

水酸化酵素 PHD は、我々の細胞内で“低酸素センサー”として機能している。この低酸素センサーが失活すると、細胞は自らが低酸素環境に陥ったものと勘違いして、低酸素環境でも生き延びることができるように、エネルギー代謝を低酸素モードに切り替える。この生理現象を逆手にとって、心筋梗塞などの虚血が関与する疾患の治療時に PHD を阻害する薬剤を使用すると、心筋梗塞サイズの縮小・心筋脳定価の抑制など、生体にとって有利な結果を導くことができるかもしれない。また、臓器移植など、血流の遮断と再灌流が関与する治療への応用、なかでも、ドナー不足や臓器保護時間が限られている心移植への応用が期待される。

Analysis of cell signaling-mediated phosphorylation and regulation of nucleocytoplasmic transport machinery

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Abstract

The nuclear pore complex (NPC) is composed of ~30 different nucleoporins (Nups), and about 10 Nups contain phenylalanine-glycine (FG) repeats that form a meshwork or brushwork barrier in the central channel of the NPC. We revealed that ERK and p38 MAP kinases efficiently phosphorylate various FG and non-FG Nups *in vitro* and *in vivo*. The phosphorylation of FG Nups by MAP kinases does not affect the overall architecture of the NPC but directly inhibits their interactions with NTRs and regulates the permeability properties of the NPC.

Key Words : signal transduction, phosphorylation, nuclear transport, nuclear pore complex

Introduction

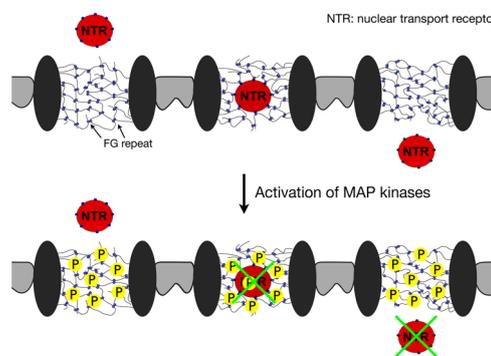
In eukaryotes, the nuclear pore complex (NPC) is a highly selective, bidirectional transporter for a wide range of material between the nucleus and cytoplasm. It is composed of about 30 unique proteins, collectively referred to as nucleoporins (Nups). Approximately a third of these Nups are classified as FG Nups due to their content of phenylalanine-glycine (FG) repeat region, which provides hydrophobic binding sites for nuclear transport receptors (NTRs) during the NPC passage.

Results

Recently, our multi-step phosphoproteomic approach combining IMAC (immobilized metal ion affinity chromatography) and 2D-DIGE (fluorescent two-dimensional difference gel electrophoresis) revealed a number of new ERK MAP kinase targets including Nup50 (also called Npap60). ERK phosphorylation of the FG repeat region of Nup50 specifically reduced its interaction with importin- β family proteins and regulated the permeability properties of the NPC in *in vitro* nuclear transport assays (Kosako *et al.*, *Nat. Struct. Mol. Biol.*, 2009).

In this research, we have shown that both ERK and p38 MAP kinases efficiently phosphorylate multiple FG and non-FG Nups *in vitro*. Phosphate-affinity SDS-PAGE using the Phos-tag ligand followed by Western blotting (Kosako, *Nat. Protoc.*, 2009) revealed that these Nups are stoichiometrically phosphorylated by ERK and p38 *in vivo*. By preparing phospho-specific antibodies to several Nups, we have detected the spatiotemporal changes in phosphorylation of these Nups in cells (Kosako and Imamoto, *Nucleus*, 2010). Quantitative phosphoproteomic analysis using phosphopeptide enrichment, chemical labeling with different stable isotopes, and LC-MS/MS (liquid chromatography-tandem mass spectrometry) was developed for simultaneous identification

and quantitation of individual phosphorylation sites (Kosako and Nagano, *Expert Rev. Proteomics*, 2011). As a result, we detected and quantified over 100 phosphorylation sites of all Nups in control, ERK-activated, and ERK-inhibited cell lysates. Then effects of phosphorylation of each FG repeat region on interaction with different NTRs (such as importins, Tap/NXF1, NTF2, and Hikeshi) were analyzed qualitatively and quantitatively. Affinity capture experiments from total cell lysates showed that interactions of FG Nups with various NTRs but not with other Nups are sensitive to phosphorylation of FG Nups. Finally, MAP kinase activation has been shown to inhibit nucleocytoplasmic translocation of fluorescent reporters in digitonin-permeabilized cells. These results suggest that MAP kinase signaling pathways phosphorylate multiple nucleoporins in the NPC to regulate nuclear transport.



Discussion & Conclusion

In higher eukaryotes, it has been well known that the phosphorylation of Nups causes the disassembly of the NPC during mitosis. However, its roles in interphase were ill defined. Our studies suggest that signal transduction-mediated phosphorylation and regulation of nuclear transport machinery may have a broad impact on cellular physiology. Until recently, many studies have focused on phosphorylation of individual cargo proteins, such as transcription factors. There is increasing evidence that Nups have multiple functions, some independent of their roles in transport processes such as gene expression, mitotic progression, differentiation, and aging. An understanding of the effects of signaling pathways on nuclear transport machinery is only beginning to emerge, and Nups and NTRs may function as molecular hubs for various signal transduction pathways.

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一般の皆様へ

ヒトなどの真核生物において多くの生命現象に関わる重要な細胞内での出来事の一つに、核膜孔と輸送運搬体との結合を介した、核と細胞質の間の選択的な物質輸送があります。私たちは細胞内での情報伝達に重要な MAP キナーゼという酵素が核膜孔タンパク質をリン酸化することにより、輸送運搬体との結合を抑制することを見出しました。細胞内情報伝達による核膜孔や輸送運搬体の調節の仕組みを明らかにすることは、核膜の突破が必要な遺伝子治療薬の開発などに役立つと考えられます。

Catalytic Asymmetric Synthesis of Tetrahydropyran Derivatives and Study of Its Quantitative Structure-Activity Relationship

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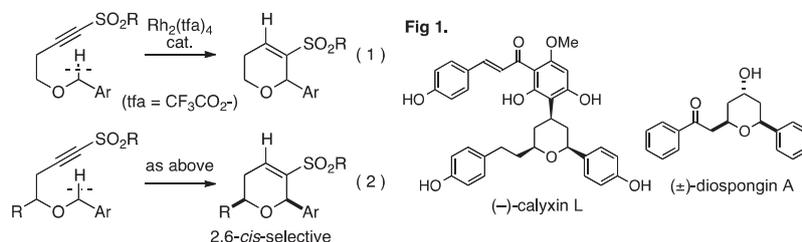
Abstract

We reported that Rh-catalyzed cyclization of alkynyl benzyl ethers via C–H bond activation afforded 2,6-*cis*-disubstituted dihydropyrans with high diastereoselectivity. This reaction was applied to syntheses of (–)-calyxin L (anti-cancer) and (±)-diospongins A (anti-osteoporosis).

Key Words : Asymmetric Synthesis, C–H Bond Activation, Rhodium Catalyst, Tetrahydropyran

Introduction

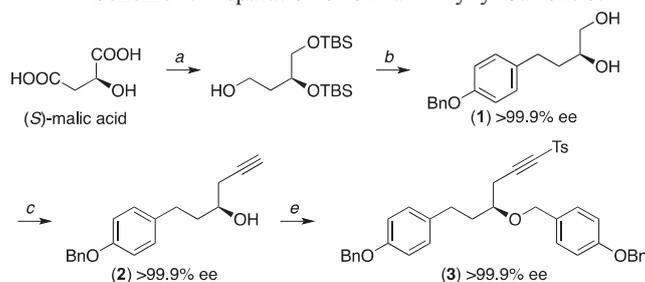
Transition-metal-catalyzed C–H bond activation is one of useful synthetic methods in organic chemistry. We previously reported that Rh-catalyzed alkynyl benzyl ethers via C–H bond activation affording dihydropyrans (eq 1).^[1] As shown in eq 2, diastereoselective ring closure is also viable and a substituent at the homopropargyl position controls the stereoselection to yield 2,6-*cis*-disubstituted dihydropyrans. Based on these findings, we examined the syntheses of (–)-calyxin L and (±)-diospongins A by the above Rh-catalyzed C–H bond activation.



Results

Chiral alkynyl sulfone **3** (>99.9% ee) was readily prepared from commercially available (S)-malic acid by several steps via diol **1** and propargyl alcohol **2** (Scheme 1).

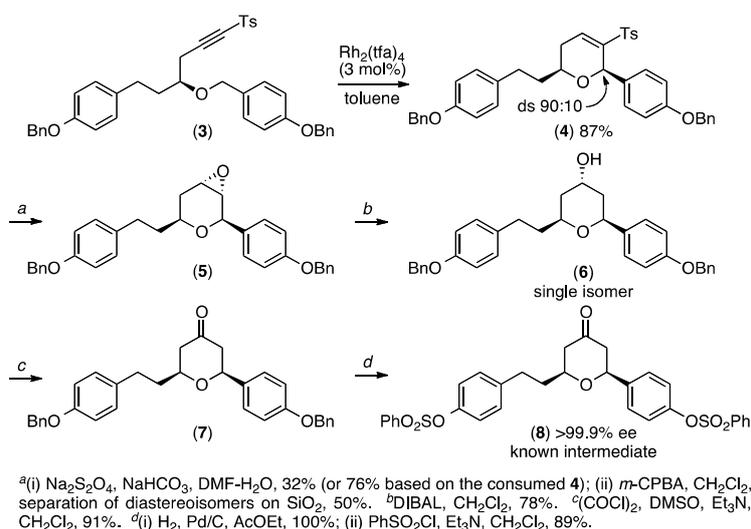
Scheme 1. Preparation of Chiral Alkynyl Sulfone **3**.



^a(i) H₂SO₄ cat., EtOH, 78%; (ii) BH₃·Me₂S, NaBH₄ cat., THF, 99%; (iii) TBSCl, imidazole, DMF, 93%; (iv) DIBAL, CH₂Cl₂, 94%. ^b(i) TsCl, pyridine, 98%; (ii) 4-benzyloxyphenylmagnesium bromide, CuI, THF; (iii) TBAF, THF, 55% (3 steps). ^c(i) MeC(OMe)₃, PPTS cat., CH₂Cl₂; (ii) Me₃SiCl, CH₂Cl₂; (iii) K₂CO₃, MeOH, 85%. ^dlithium acetylide-ethylenediamine, DMSO, 92%. ^e(i) NaH, [4-(benzyloxy)phenyl]methyl chloride, DMF, 93%; (ii) BuLi, (TolS)₂, THF, 90%; (iii) *m*-CPBA, CH₂Cl₂, 78%.

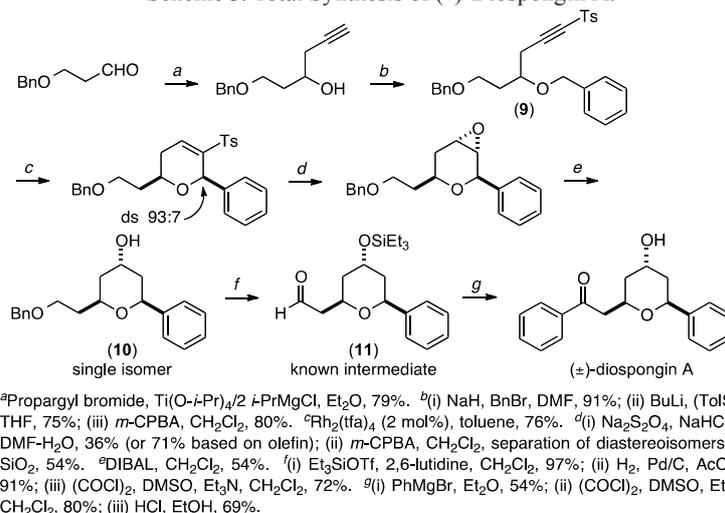
Treatment of **3** with a catalytic amount of the Rh catalyst gave the corresponding dihydropyran **4** with high diastereoselectivity. After the sulfone moiety in **4** was removed, epoxidation of the olefin and the treatment with DIBAL afforded the desired alcohol **6** as a single isomer via **5**. Chiral alcohol **6** was converted to ketone **7** by Swern oxidation, which was followed by hydrogenolysis and benzenesulfonylation to give an enantiomerically pure **8**, a known intermediate of chiral (–)-calyxin L^[2] (Scheme 2).

Scheme 2. Formal Total Synthesis of (–)-calyxin L.



As the above synthesis of (–)-calyxin L, alkynyl benzyl ether **9** by the Rh-catalyzed reaction, desulfonylation, epoxidation, and reduction afforded the desired alcohol **10** as a single isomer. After the hydroxyl group of **10** was protected with triethylsilyl group, hydrogenation and Swern oxidation provided the aldehyde **11**, a known intermediate of (±)-diospongins A.^[3] Actually, aldehyde **11** was transformed into (±)-diospongins A by a three-step sequence (Scheme 3).

Scheme 3. Total Synthesis of (±)-Diospongins A.



Discussion & Conclusion

In conclusion, we reported a formal total synthesis of (–)-calyxin L from chiral alkynyl sulfone **3** catalyzed by a rhodium salt without loss of ee value. Similarly, a total synthesis of (±)-diospongin A was completed by the above mentioned Rh-catalyzed C–H bond activation and cyclization. Further investigations on developing of chiral Rh-catalysts for asymmetric C–H bond activation and synthesis of their derivatives for researching quantitative structure-activity relationships are in progress.

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一般の皆様へ

炭素—水素結合 (C-H 結合) の活性化を用いる炭素—炭素結合形成反応は、反応基質を事前に官能基化する必要がなく、これまで官能基変換に要していた工程を削減できるため、天然有機化合物合成の効率化に有効である。我々は置換ベンジルアルキニルエーテルにロジウム触媒を作用させると、その C-H 結合活性化を経て環化し、ビニルスルホン部位を有する 2,6-ジ置換ジヒドロピランを立体選択的に与えることを既に報告している(*Journal of the American Chemical Society*, 2009年)。本研究では、この知見に基づき種々検討した結果、カリキシン L (抗ガン作用) の形式的不斉合成およびディオスポンジン A (抗骨粗鬆作用) のラセミ体全合成を達成した。

How does energy metabolism affect sex differentiation and stem cell regulation?

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Abstract

Early gametogenesis, especially the number of germ cells, was regulated cell-autonomously through stem-like germ cells by energy conditions. Interestingly it was found that these conditions affect proper sex differentiation. We also establish the transgenic medaka that could monitor an ATP amount in germ cells. These studies will collectively enhance the cellular and molecular studies of the effect of energy levels on the germline stem cell regulation.

Key Words : medaka, mutant, lipid, metabolism

Introduction

Using a model vertebrate, teleost fish, medaka, we have isolated mutants which shows the defect in gonadogenesis. One of the mutants, called *zenzai*, exhibits a phenotype of a gradual loss of stem-type germ cells and subsequently becomes sterile by a loss of germ cells. This suggests that energy metabolism is associated with stem cell regulation. Therefore we have analyzed how stem-like germ cells are regulated at molecular and cellular levels during the course of gametogenesis.

Results

In order to assess possible relations between germ cell regulation and energy levels, we have addressed the following four aspects.

1. First, we investigated if the developing gonad has germ cells that exhibit the character similar to germline stem cells identified in medaka ovary (ref.1). Incorporation of BrdU indicated the presence of quiescent type of germ cells, suggesting the presence of germ cells with the similar character of germline stem cells. We revealed that the proliferating type of stem-like germ cells, but not a quiescent type, was regulated by an ancient type of TGF β family, amh (anti-Mullerian hormone) (ref.2). The AMH system functions in an interfollicular manner but does not directly act on germ cells. Our result indicates that the activity of the AMH system modulates somatic cells and the unknown factor secreted from the somatic cells functions to attenuate the proliferation of mitotically active stem-type germ cells.
2. Next we examined how stem like germ cells were affected by an energy level. Using transgenic

medaka allowing us to observe germ cells and gonadal somatic cells by fluorescence (*sox9b-DsRed/olvas-EGFP*), the transgenic larva were left in the starved conditions without feeding after hatching. Gonadal development was then monitored for a month. Contrary to our anticipation, a preliminary result shows that the proliferation of stem-like germ cells was not severely impaired in the starved conditions but the germ cell number was reduced. This implies that not proliferation of stem-type germ cells but some subsequent process in gametogenesis is regulated with an energy level. Loss of germ cells lead to the sex reversal to male phenotype, which is consistent with our recent findings that germ cells are critical for proper manifestation of sex directed by sex determination gene (ref.3-5).

3. As described in Introduction, *zenzai* mutant gradually decreases the germ cell number. We have tried addressing the cellular mechanism affecting the mutant phenotype. In situ hybridization analysis demonstrated that the gene is expressed in both the germ cells and the surrounding somatic cells. We next generated chimera between wild-type and mutant medaka. Interestingly the mutant germ cells in the wild-type somatic environment were decreased gradually in number, recapitulating the mutant phenotype, while the wild-type germ cells surrounded by mutant somatic cells did not exhibit any phenotype found in the mutant. This clearly indicates that the gene functions germ cell-autonomously. Currently we are investigating if the mutant phenotype is rescued with some pharmaceutical activators and/or inhibitors, thus trying to identify the molecular pathway regulating stem-like germ cells. In addition, we are examining the change of expression levels of the genes at RNA and protein levels in the candidate pathway. The same type of experiment is also ongoing for the starved medaka (mentioned in above 2).
4. Lastly we are developing the transgenic medaka that senses and indicates an intracellular ATP level by fluorescence. For this purpose, we established medaka lines that express ATeam-fluorescent protein in germ cells. In the gonad of the transgenic medaka, CFP fluorescence is detected in the germ cells. We are now examining if the fluorescence shifts from short to long wavelength by FRET in vivo. Also we have successfully established the method where germ cells were isolated from the developing gonads with more than 98 % purity by FACS, which allows us to evaluate the correlation of fluorescence with an ATP levels in the culture system.

Discussion & Conclusion

We have demonstrated the presence of stem-like germ cells in the developing medaka gonads. The stem-like germ cells fall into two types, a quiescent type and mitotically active type. The mitotically active type is regulated by the activity of anti-Müllerian hormone and loss of this signal causes hyperproliferation of germ cells. The systemic energy levels do not seem to make a direct effect on proliferation but regulate some subsequent process. This results in a gradual loss of germ cells and the sex reversal to male phenotype, which is consistent with the analysis of *zenzai* mutant

where lipid metabolism is impaired in the germ cells. These analyses collectively indicate that gametogenesis is regulated by the systemic energy levels and the control of germ cells is necessary for proper manifestation of sex.

For the direct assessment to energy levels, we have established transgenic lines which express ATeam fluorescent protein (ATP sensing protein).

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一般の皆様へ

生き物にとって卵や精子産生のタイミングやその量など適切に制御することは、次世代を作り出すためには重要なことである。今回、メダカを用いた研究により、身体のエネルギーレベルが卵や精子を生み出す元の細胞（生殖細胞）を制御していることが明らかとなった。そしてその制御が適切でないと性転換を引き起こすことも示された。今後、エネルギーとして作用する物質を同定するとともに、その分子機構のより詳細な機構を明らかにし、生殖とエネルギーとの関係を解析していく予定である。

The role of CD11b⁺ myelomonocyte in tumor vasculature related to cancer recurrence

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Abstract

Radiotherapy and chemotherapy play a major role in the treatment of oral cancer as conservative therapy. However, tumors recur or grow after those treatments in many cases. In this study, we investigated if the irradiation and chemo drug affect the tumor vasculature using human oral cancer clinical samples and a xenograft mouse model. We found that irradiation induced recruitment of bone marrow-derived CD11b⁺ cells into tumors. This data suggests that recruited CD11b⁺ cells has a major role for restoring tumor vasculature in irradiated oral cancer cells.

Key Words : CD11b⁺ bone marrow-derived cells(BMDCs) vasculogenesis

Introduction

In past several decades, oral cancer patients have been continuously increased, and it will be estimated approximately 7800 in 2015, which accounts for 1~2% of all cancers or 40% of all head and neck cancers in Japan. Standard treatments for the patients with oral cancer are radiotherapy, chemotherapy, and surgery. To prevent the functional and sensuousness disorder such as chewing, swallowing, and articulator, social need of the conservative therapy has been growing. Despite the advances of radiotherapy and development of anti-cancer drug, tumors invaluablely recur within the radiation field, resulting in poor prognosis. Therefore, the local control of the primary tumor is very important.

Tumor vasculature is required for tumor relapse and growth. The tumor vasculature can arise from sprouting and proliferation of endothelial cells from local vessels (angiogenesis) or by colonization of circulating endothelial or other cells, primarily derived from bone-marrow (vasculogenesis).

In this study, we explored if the tumor vasculature after irradiation depends on vasculogenesis because tumor endothelial cells are not likely to survive the large doses of radiation.

Results

To investigate strong association between recurrence of oral cancers after chemo-radio treatment and bone-marrow derived CD11b⁺ cells, we demonstrated following experiments.

We first examined to see if the chemo-radio treatment induces the recruitment of CD11b⁺ cells in tumor recurrences using human clinical samples from the patients with oral cancer by immunohistochemistry (IHC). We determined the levels of CD11b⁺ cells in human oral cancers

both prior to therapy and when the tumors had recurred in the same patients. We observed higher levels of CD11b⁺ cells in recurrent tumors compared with those in the untreated tumors in 8 out of 11 paired samples. (Fig.1A,B)

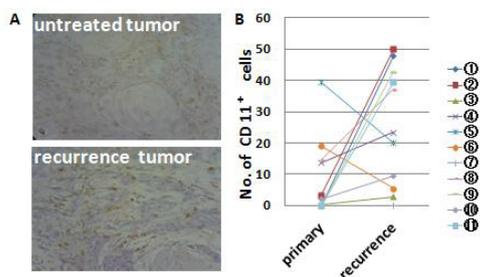


Fig.1
A : IHC of oral cancer clinical samples staining for CD11b.
B : Significantly increased levels of CD11b⁺ cells in the recurrent human oral cancers compared with the untreated tumors.

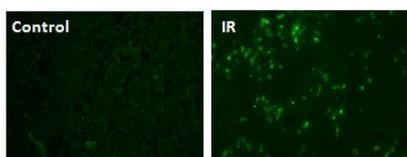


Fig.2
IHC staining for CD11b in OSC-19 s.c. tumors.

Next, we examined human oral cancer xenograft mice models with immunofluorescent staining. OSC-19 (human oral squamous cell carcinoma cell line) cells were inoculated s.c. at 1×10^7 cells/mouse on the back of the 6 weeks old, ♀, BALB/c nu/nu mouse approximately 1cm proximal to the base of the tail. Anesthetized mice were placed under the lead plate through which the established tumors (approximately 200 mm³ in volume) protruded for irradiation 15 Gy or 25 Gy. Tumor volume was calculated using the formula: $V = 0.5 \times \text{width} \times \text{length} \times \text{depth}$. Irradiation of 25 Gy eradicated the established tumors completely. The tumors irradiated 15 Gy were shrunk at once and then

showed the regrowth. When 15Gy-irradiated tumors reached approximately 200 mm³ again, tumors were collected by cardiac perfusion with 4% paraformaldehyde. As shown in Fig 2, we found that high levels of CD11b⁺ cells in irradiated tumors compared with untreated tumors.

In addition, we investigated if anti-cancer drugs also induce the recruitment of bone marrow-derived CD11b⁺ cells into tumor in a mouse xenograft model. When tumors had reached approximately 200mm³ in volumes, docetaxel (20mg/kg, 40mg/kg) or cisplatin (5mg/kg, 10mg/kg) were administered two times once a week. Currently these tumors have been underwent analyses.

Discussion & Conclusion

In this study, we showed that CD11b recruitment into tumor after irradiation was associated with tumor recurrence. CD11b expressing myelomonocytes are known to be associated with immunosuppressant and secret inflammatory cytokines. In recent years, it is reported that these cells have an important role on vasculogenesis in ischemic tissue, wound healing, and cancer, but the detailed mechanism of tumor vasculogenesis has not been understood. Our data suggest that irradiation induces recruitment of BMDCs into tumors, restoring the radiation-damaged vasculature by vasculogenesis and thereby allowing the growth of surviving tumor cells in oral cancer.

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Analyses of the pathophysiological elements of the deafness underlying disorder of the cochlear endolymph

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Abstract

Hearing loss caused by damage of cochlea in inner ear affects many people in the world but its pathophysiological sources remain largely unknown. To overcome such disease in the future, basis research that clarifies the mechanisms underlying sound-transduction in the cochlea is of importance. We have focused on the cochlear endolymph, an unusual extracellular solution essential for hearing, and analyzed its functional roles and properties. In this study, we have utilized theoretical approach and for the first time found that the circulation current, a continuous ion-flux that unidirectionally flows throughout the cochlea, plays key roles in formation of unique electrochemical environments of the endolymph. (30-100 語程度)

Key Words : (1-5 語程度) Hearing, inner ear, ion transport, deafness, computer simulation

Introduction

Sound-evoked mechanical stimuli permit endolymphatic K^+ to enter sensory hair cells. This transduction is sensitized by an endocochlear potential (EP) of +80 mV in endolymph. After depolarizing the cells, K^+ is secreted into perilymph and then circulated back to endolymph across the lateral cochlear wall. In theory, this entails a continuous and unidirectional current carried by apical K^+ channels and basolateral K^+ -uptake transporters in both the marginal cell and syncytial layers of the lateral cochlear wall. The transporters regulate intracellular and extracellular $[K^+]$, allowing the channels to form K^+ diffusion potentials across each of the two layers. These potentials govern the EP. What remains uncertain is how this current might affect the characteristics of the endolymph. To address this question, we developed an electrophysiological model that incorporates channels and transporters of the lateral wall and channels of hair cells that derive a circulation current.

Results

In our model, the circulation current and the concentrations of ions in lateral wall compartments were inferred from the function of ion channels and transporters as well as morphological features of the cochlea, which included three layers, i.e., the marginal cell and syncytial layers of the lateral wall and hair cell layer, and three extracellular spaces between these layers. We assumed that (1) ion transport apparatus interplay on each of the membranes of the lateral wall and hair cells and these components are serially connected in a closed-loop circuit to represent the circulation current,

and (2) the circulation current is coupled to all of the elements responsible for the EP including the membrane potentials of the lateral wall and its spatial ionic concentrations. By using this computational model, we calculated ionic concentrations and potential of intra/extracellular spaces in the lateral wall and the EP.

The simulation replicated normal experimental EP values. Therefore, the principle elements and concepts underlying the model would seem to be valid. We previously found by the experimental approach that, when basolateral K^+ -uptake transporters of marginal cells were blocked, intra/extracellular $[K^+]$ in the lateral wall were drastically changed. This process modulated K^+ -diffusion potentials of the lateral wall and reduced the EP. Our computational model reasonably reproduced these experimentally-measured changes in the EP and intra/extracellular $[K^+]$ in the lateral wall. Furthermore, the simulation revealed that, in such condition, the predominant carrier of the circulation current switched from K^+ to Na^+ and Cl^- at the basolateral membranes of marginal cell. These additional ionic fluxes directly contributed to the change of ionic environment of the lateral wall. On the other hand, when K^+ channels at the apical membranes of syncytial layers of the lateral wall are inhibited, the situation is completely different. In the experiments, this perturbation reduced the EP with minimum alternation of the ionic milieu of the lateral wall. Our model successfully mimicked such electrochemical properties in the cochlea. Of importance, when the K^+ channels' activity was reduced, the circulation current was immediately decreased but continued to be dominated by K^+ . These two lines of *in silico* simulation imply that the circulation current associates closely with the electrochemical properties of the lateral wall and the EP and affect them differently in different conditions. We further simulated the process underlying the EP loss in a mouse model of a chloride channelopathy called "Bartter syndrome" that is accompanied with deafness. The chloride channels are expressed at the basolateral membrane of marginal cell layer and known to dysfunction in the disease. Unexpectedly, in our model, strong blockage of the channels evoked additional flux of Na^+ through the concomitant non-selective cation channels at the basolateral membrane of marginal cells and thus enlarged the potential difference across this membrane compartment. This abnormality of electrical profile is likely to underlie the loss of the EP in the mouse model and could be responsible for the deafness of Bartter syndrome.

Discussion & Conclusion

In this simulation study, we demonstrated that the circulation current is closely correlated with formation of the EP in the cochlea. Also, our model showed how the EP would be reduced in chloride-channel null mice, which mimic Bartter syndrome associated with deafness. These observations were achieved only by analyzing the circulation current and membrane currents with the present computer-simulation approach, because these currents cannot be measured *in vivo* by the current experimental protocol. Therefore, the findings are striking. Simultaneously, the basic ion homeostasis and the main machineries establishing the endolymphatic properties including the EP have been successfully reproduced and theoretically understood. With our computational model, the

pathophysiological processes and mechanisms underlying other hearing disorders may be analyzed and clarified in the future. Such endeavors could open an approach to develop new drugs for deaf diseases.

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一般の皆様へ

音は外耳・中耳を経由して、内耳に到達します。我々は、内耳が原因で起こる難聴を克服するため、内耳の基礎的な研究を行っています。その中心として、内リンパ液という内耳を満たす体液を対象にしています。この液体は、他の体液には見られない電気現象があり、その成立の仕組みを、コンピューターシミュレーションを駆使して検討しました。すると、内耳全体を巡る「循環電流」という電流が内リンパ液の電気現象に重要であることが分かりました。また、循環電流を支えている普段は目立たない小さな電流が止まることで、遺伝性難聴が起こることも理論的に理解できました。この研究により、内耳が音を処理する仕組みがより深く解明されたのみならず、色々な難聴の病態理解や治療法開発に繋がると期待されます。

Synthesis of Welwitindolinone A Isonitrile and Its Analogues by Catalytic Cycloaddition of Allenynes

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Abstract

Synthetic studies of welwitindolinone A isonitrile, belonging to oxyindole alkaloid

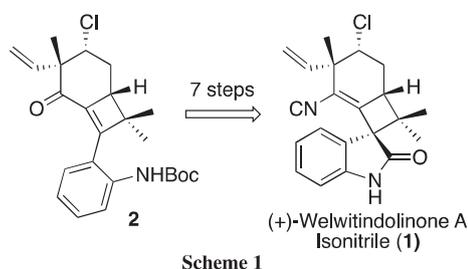
Key Words : welwitindolinone A isonitrile, ruthenium, allene, alkyne, [2 + 2] cycloaddition

Introduction

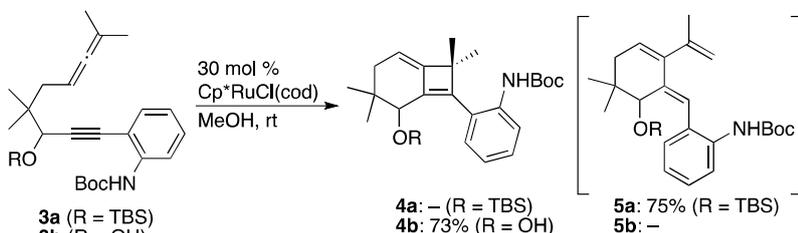
Welwitindolinone A isonitrile (**1**), isolated from blue-green algae, exerts fungicidal activity and is a densely functionalized oxindole including three all-carbon quaternary centers and a spiro-fused cyclobutane¹. Total synthesis of **1** was reported by Baran group² and Wood group³ to date. On the other hand, we recently reported ruthenium-catalyzed [2 + 2] cyclization of 1,7-allenyne leading to bicyclo[4.2.0]octa-1(8),5-diene skeleton⁴, found in **1** as core-structure. With this as a background, we planned total synthesis of **1** via our ruthenium-catalyzed [2 + 2] cyclization as a key step.

Results

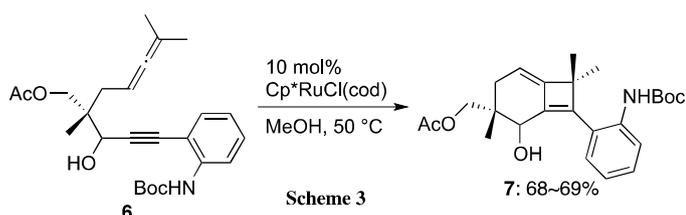
Wood demonstrated that bicyclic compound **2** could be converted into **1** in 7 steps (Scheme 1)³. Thus, we planned to synthesize **2** by our methodology. To examine the feasibility of our plan, we conducted model study first (Scheme 2). When allenyne **3a** having a silyloxy group at propargylic position was treated with ruthenium catalyst in methanol, desired product **4a** was



not obtained, but triene derivative **5a** was produced in 75% yield. On the other hand, the reaction of **3b** having hydroxy group gave desired bicyclic compound

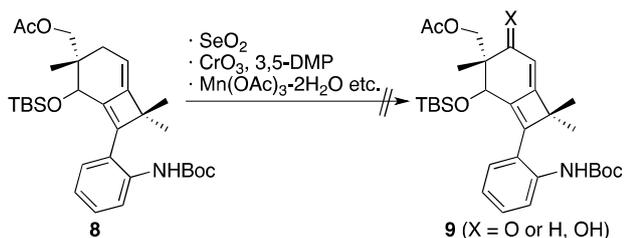


4b in 73% yield as a sole product. Encouraged by this result, the reaction of **6** having a quaternary carbon center was conducted in the presence of ruthenium catalyst (Scheme 3). As



a result, desired bicyclic compound **7** was obtained in good yield.

Next, we conducted further transformation of **7** into synthetic intermediate **2**. In order to install oxygen functionality into allylic position of six-membered ring, we examined allylic oxydation of compound **8** under various conditions. However, in all cases, the reaction gave complex mixture, and no desired product **9** was produced.

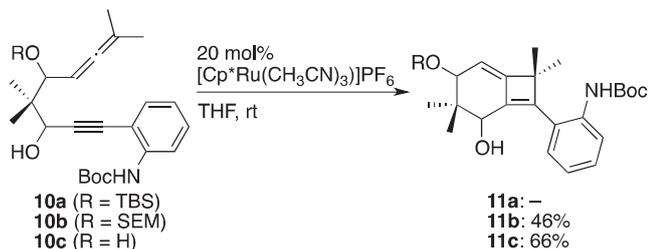


Scheme 4

Discussion & Conclusion

Based on above results, we decided to change the synthetic route. Thus, allenynes **10a-c** having an oxygen functionality on the allylic position were applied to [2 + 2] cyclization (Scheme 5). The reaction of **10a** bearing a silyloxy group did not give the desired product **11a** but complex mixture. On the other hand, allenyne **10b** having a smaller SEM group gave **11b** in 46% yield. Furthermore, diol **10c** was also applicable to the cyclization, desired **11c** was produced in 66% yield. These results indicated that size of the oxygen functionality at allylic position was crucial to progress of cyclization.

In conclusion, we conducted synthetic study of (+)-welwitindolone A isonitrile by ruthenium-catalyzed [2 + 2] cyclization of 1,7-allenyne. In the course of the study, we found that hydroxy groups in a chain remarkably affected on the progress of the cyclization. Further studies along this line are in progress.



Scheme 5

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一般の皆様へ

Welwitindolone A isonitrile (**1**) は天然からはごく少量しか得られないため、生物活性評価のために必要な十分量のサンプルを確保することが困難でした。従って**1**の効率的な全合成法の開発を通じ、**1**の大量供給が可能となると考えられ、未だ見出されていない薬理作用を探索する道を拓くことができると期待されます。一方、確立した**1**の合成法を応用することにより、**1**を基本骨格とした多様な誘導体を合成できると考えられます。これにより、全く新しい生理作用を示す誘導体を見出

すことができる可能性があります。従って、本研究を進めることにより新規医薬品リード化合物や新しいバイオツールの創製に繋がると期待されます。

Structural Basis for Oxidative Stress Sensing by Keap1-Nrf2 Cytoprotective System.

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Abstract

Keap1 is a substrate adaptor of a Cullin 3-based E3 ubiquitin ligase complex that recognizes Nrf2, and also acts as a cellular sensor for xenobiotics and oxidative stresses. We have succeeded crystallizing Keap1. X-ray data collection and structure determination are currently underway. We also analyzed interactions of Keap1 and Nrf2 by Surface Plasmon resonance method.

Key Words : X-ray structure

Introduction

The up-regulation of detoxifying enzyme reduces the risks of cancer and other life-style-related diseases caused by reactive oxygen species and electrophiles. The oxidative-stress sensor protein Kelch-like ECH-associated protein 1 (Keap1) and the transcription factor NF-E2-related factor 2 (Nrf2) play a major role for the induction of these protective enzymes through the antioxidant response element (ARE). In order to clarify the oxidative stress sensing mechanism, structural and biochemical analyses of Keap1 were performed.

Results

Upon exposure to oxidative and electrophilic stress, reactive cysteine residues in intervening region (IVR) and Broad complex, Tramtrack, and Bric-à-Brac (BTB) domains of Keap1 are modified by electrophiles. Previous reports suggest that cysteine residue 151 in BTB domain and cysteine residues 273 and 288 in IVR domain are important for the stress sensing by Keap1. Here we focused on the reactive cysteine 151 in BTB domain. Keap1 BTB domain forms homodimer and interacts with Cul3 protein. Biological function of Keap1 BTB is poorly understood due to the lack of structural information.

In order to obtain Keap1 fragment including BTB domain, we prepared various truncation mutant of mouse Keap1. 6x His-tagged protein was successfully expressed in *E. coli* and purified by using Ni-NTA column, and further purified by size exclusion chromatography. SDS-PAGE showed that the purity of resulting protein sample is >90%. We performed crystal screening by using TOPAZ 1.96 Chip (Fluidigm Corporation) and found that two deletion mutants were successfully crystallized (Fig. 1a). Initial crystallization conditions, obtained by using Free Interface Diffusion (FID) method, were further optimized for vapor diffusion methods. X-ray data was collected at beamline BL-1A at PhotonFactory Tsukuba (Fig. 1b). Using *HKL2000* the data was processed. Structure solution

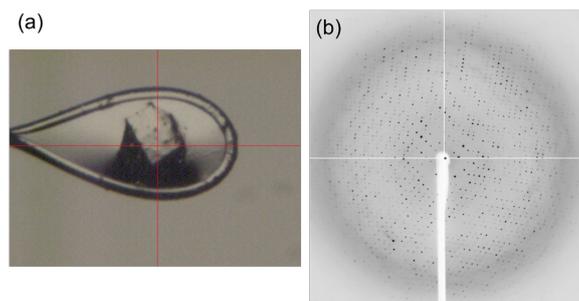


Figure 1. Structural analysis of Keap1. (a) Crystal of Keap1 used for X-ray data collection. (b) Diffraction image of Keap1 crystal.

was obtained by using molecular replacement methods by program Molrep. Further refinement and model building are currently underway by using program Coot.

In addition to the structure analysis of Keap1 BTB domain, we analyzed interactions of Keap1 DC domain and Nrf2. Nrf2 has two Keap1 binding site. Our single particle electron microscopy analysis showed that dimeric Keap1 binds monomeric Nrf2 at two sites. Somatic mutations were exclusively found in these two sites of Nrf2 in cancer patients. Roles of each Keap1 binding site in Nrf2 are, however, largely unknown.

In order to analyze binding mode of Nrf2 and Keap1, 6x His- and GST-tagged Nrf2 fragments including each Keap1 binding site were prepared. The His-GST-tagged Nrf2 samples were purified by using Ni-NTA column. The purified proteins were immobilized to the sensor chip. Purified Keap1 was used as analyte for Surface Plasmon resonance analysis by using Biacore X100 (GE healthcare). The kinetic analyses of Keap1 and Nrf2 binding showed distinct binding parameter of two sites.

Discussion & Conclusion

Cancer and other life-style-related diseases caused by reactive oxygen species and electrophiles can be prevented by the up-regulation of detoxifying enzymes. Nrf2 is a transcription factor controlling the inducible expression of these cytoprotective enzymes. Keap1, a regulator of Nrf2, is emerging drug target for various diseases including multiple sclerosis and chronic kidney disease (CKD) in type 2 diabetes. Small molecules, which inhibit Keap1-Cul3 E3 ligase, are potential drugs for these diseases. Lack of the structure data of Keap1, however, prevents us from understanding the inhibition mechanism of these small molecule against Keap1. Our structural analysis of Keap1 may offer an important insight of oxidative stress sensing mechanism as well as rational design of the inhibitors.

一般の皆様へ

酸化ストレスや親電子性分子と総称される毒性物質は癌や生活習慣病を引き起こすことが知られています。一方、生体にはこれを解毒・排出する生体防御システムが存在します。その中心が Keap1 と呼ばれるストレスを感知する生体分子で、近年、新たな創薬ターゲットとして注目されています。本研究では、Keap1 分子の詳細な三次元構造を明らかにします。本研究によって酸化ストレスを感知する分子機構が解明されれば、将来、Keap1 を標的とした生活習慣病の新たな予防薬開発が加速することが期待されます。

Pathological roles of a novel macrophage molecule M-mod in type-2 diabetes

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Abstract

We investigated anti-diabetes function of M-mod (Macrophage-modulator) using gene-engineered cells and mice. M-mod knockdown macrophage-like cells hampered insulin signaling of 3T3-L1 adipocytes accompanied by robust induction of type2 diabetes-deterioration molecules such as aP2 and PAI-1. On the other hand, macrophages from adipose tissues of obese M-mod transgenic mice produced less amount of aP2 and PAI-1 compared from control mice. In agreement, composition of immune cells in adipose tissues was dramatically affected in M-mod TG mice. Thus, M-mod seems to modulate progression of type2 diabetes.

Key Words : macrophage, type2 diabetes, gene-engineered mouse, obesity

Introduction

M-mod is a novel molecule whose expression is dramatically repressed during the macrophage-like differentiation of the leukemia cell lines. Our previous cell culture study indicated that M-mod is dispensable for macrophage-like differentiation. On the other hand, M-mod knockdown (KD) cells indicated selective increase of various type2 diabetes-deterioration molecules such as aP2 and PAI-1, indicating potential anti-diabetic effects of M-mod. In this study, we attempt to clarify roles of M-mod as an individual level, and also molecular mechanisms underlying function of M-mod.

Results

1. At first, we performed some experiments strengthening involvement of M-mod in pathogenesis of type2 diabetes.
 - A) Supernatant from HL-60 derivatives extensively expressing M-mod shRNA (M-mod KD cells) remarkably suppressed insulin sensitivity of 3T3-L1 adipocytes.
 - B) Macrophages isolated from obese ob/ob mice show lower expression of M-mod compared with control lean C57BL/6 mice.
2. To clarify roles of M-mod at an individual level, we assessed phenotypic changes of *M-mod* gene engineered mice.
 - C) We generated several lines of mice having M-mod transgene linked to a macrophage specific promoter. Peritoneal macrophages isolated from these mice markedly expressed M-mod compared with non-transgenic litter mates, indicating M-mod transgenic (TG) mice were

successfully generated.

- D) M-mod TG mice did not exhibit any obvious changes in fertilization, growth, and feeding under a normal feeding condition at least by 20 weeks.
 - E) When we gave high fat diet to M-mod TG mice, they increased body weight at the almost same degree as their litter mate control mice. Similarly, soft tissue masses of liver, visceral (omental and epididymal) and subcutaneous adipose tissue of M-mod TG mice was comparable to those of the control mice by 15 weeks.
 - F) M-mod TG mice did not display any decrease or increase in blood glucose, triglyceride, and cholesterol on both normal chow and high fat diet.
 - G) Fluorescence Activated Cell Sorting (FACS) analysis indicated that macrophage and other immune components in adipose tissues showed a unique pattern in M-mod TG mice.
 - H) Macrophages from adipose tissues of obese M-mod TG mice produced less amount of the type2 diabetes deleterious molecules such as *aP2* and PAI-1
 - I) Taking advantage of homologous recombination, we established more than 10 lines of recombinant mouse embryonic stem (ES) cells for generation of *M-mod* flox mice.
3. We also explore molecular mechanisms underlying function of M-mod.
- J) We monitored total and phosphorylated several protein kinases in M-mod KD cells by Western blotting analysis. We could not distinguished amount of phospho-MAPKs such as ERK, p38 and JNK between the control and KD cells both before and after differentiation stimuli. Moreover, M-mod KD cells did not modulated the PI3K cascade, verified by ratios of phospho-PI3K/total PI3K as well as phospho-Akt/total Akt. Similarly, M-mod KD cells failed to affect nuclear contents of PPARs (PPAR-alpha, gamma, and delta) and their binding partner RXR.
 - K) Intracellular FACS analysis represented that cellular contents of phospho-STATs and phospho-p65 NF-kappaB subunit in M-mod KD cells were almost identical to those of control cells.
 - L) Suspension arrays reproducibly indicated that knock down of M-mod did not affect binding activities of >80 transcription factors including PPAR and NF-κB.
 - M) Comprehensive chromatin-immunoprecipitation (ChIP)-PCR analysis revealed that lysine 4 residue of H3 histone was hypermethylated in M-mod KD cells at *aP2* locus. In contrast, lysine 9 residue was ignorably affected by M-mod KD cells. On the other hand, M-mod KD promoted acetylation of H4 histone at lysine 5 and 8.

Discussion & Conclusion

So far, we found M-mod KD promoted expression of some type2 diabetes exacerbation molecules, suggesting roles of M-mod in pathogenesis of type2 diabetes. In this study, we performed extensive experiments to validate this idea. As we expected, M-mod KD cells are capable to inhibit insulin

signaling in cultured adipocytes reinforcing the suppressor function of M-mod. In addition, M-mod TG mice slightly modulate accumulation of immune cells in adipose tissues accompanied by production of aP2 and PAI-1. In combination with the observation that macrophage from obese individuals expressed M-mod at a low level, expressional changes of M-mod is one of the major causes of pathogenesis of type2 diabetes.

In addition to verify pathophysiological roles of M-mod, we also attempt to uncover molecular mechanisms underlying function of M-mod in this study. We could found neither protein kinases nor transcription factors affected by M-mod KD, indicating M-mod did not employ these signaling molecules to regulate expression of type2 diabetes exacerbation molecules. On the other hand, M-mod KD cells show remarkable changes in histone H3 methylation as well as H4 acetylation. Thus, M-mod seems to be a novel epigenetic regulator affecting pathogenesis of type2 diabetes. Further studies are needed to clarify molecular events controlled by M-mod.

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一般の皆様へ

本研究では、マウスモデルを用いて、新たなマクロファージ分子 M-mod が2型糖尿病に特徴的な病変を軽減する知見を得た。細胞レベルの解析で、M-mod が2型糖尿病の進行に関わる分子の発現を抑えることを考え合わせると、M-mod が2型糖尿病治療の全く新たな抑制分子であることがより明らかになった。しかし M-mod が如何なる分子を介して標的分子の発現を制御しているのかなど依然課題は残る。ヒトでの M-mod の役割解明も合わせ研究を進め、抗2型糖尿病治療の分子標的としての M-mod の可能性をさらに探りたいと考えている。

Study of Inwardly Rectifying Potassium Ion Channels

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Abstract

The interaction of Kir3 channels with G proteins was analyzed with surface plasmon resonance.

Key Words : ion channel, membrane protein, structural biology, surface plasmon resonance

Introduction

Inwardly rectifying channels (Kir channels) are a family of potassium ion channels consisting of a tetrameric assembly of homologous subunits. There are seven subfamilies of Kir channels (Kir1-7). The activity of each subfamily is controlled by specific intracellular components. For instance, Kir3 opens in response to the release of G protein subunits $G\beta\gamma$ upon stimulation of G protein-coupled receptors.

The ion conduction pathway of these channels is formed at the center of the tetrameric assembly and is structurally divided into two parts, the cytoplasmic pore and the transmembrane pore. Especially, the cytoplasmic pore is important for regulation of Kir channels. In order to understand the mechanism of channel gating, it is necessary to obtain their structure in complex with regulatory factors. As an initial step toward this goal, it is worth characterizing their interaction with regulatory factors by employing other techniques besides electrophysiology.

Results

To begin with, I need to mention that some parts of the experimental result obtained from my study can not be described here in detail since they are yet to be published.

The Kir3 channels are activated by the binding of $G\beta\gamma$ subunits and phosphatidylinositol 4,5-bisphosphate (Logothetis *et al*, 1987; Huang *et al*, 1998). The binding site of G proteins has been proposed to reside primarily in their cytoplasmic portion and their dissociation constant for $G\beta\gamma$ has been reported in the order of nanomolar (Ivanova-Nikolova *et al*, 1998). However, it should be kept in mind that such information has been derived exclusively from experiments using electrophysiological techniques in which binding parameters were estimated indirectly by recording channel conductance. That means that, in such an experimental setting, it is unable to discern whether mutations introduced to the channel affect directly the interaction with G proteins or gating of the channel without impairing its association with G proteins.

Therefore, it is helpful to establish an experimental system in which physical association of Kir3 with G proteins or phosphatidylinositol 4,5-bisphosphate can be recorded directly. For this purpose, I have employed surface plasmon resonance. There are four subtypes of Kir3 that have been

identified so far in mammals (Kir3.1-4). I have analyzed the interaction of some of them with G proteins using their cytoplasmic portion. These were expressed in large quantity in *Escherichia coli* cells and purified to homogeneity with a combination of affinity and gel-filtration chromatography. Interestingly, some of these were purified as a tetramer that is a biological unit, whereas others were not stable in the tetrameric form and instead appeared to dissociate into monomers, dimers or trimers in the course of preparation. Accordingly, the inner surface of these channels, which is mostly hydrophobic and normally buried inside the intact tetramer, was exposed to solvent. As for G proteins, the dimeric G $\beta\gamma$ subunits were expressed in *Spodoptera frugiperda* cells and purified to homogeneity with a combination of affinity, ion-exchange and gel-filtration chromatography as well. The finally purified products were employed for the surface plasmon resonance experiment, in which the interaction between the channels and G $\beta\gamma$ subunits has been detected. Next, I will summarize how I interpret experimental sensorgrams of surface plasmon resonance.

Through the experiment, the proteins used as an analyte had been immobilized to a sensor chip by amine coupling. It is of notice that lysine residues are scattered broadly on the protein surface of both the channels and G $\beta\gamma$ subunits. Therefore, I do not exclude the possibility that the binding observed by surface plasmon resonance was caused in part by non-specific interactions between them. Even though, I suppose that the binding of a subtype that retains the tetrameric structure to G $\beta\gamma$ reflects physiological association inside cells upon stimulation of G protein-coupled receptors. In this case, the sensorgram indicates that the association kinetic of the channel with G $\beta\gamma$ is monophasic whereas the dissociation kinetic is biphasic. Obviously, the dissociation kinetic could be resolved into two contributions from fast and slow rates. At this stage, it is not clear whether this biphasic nature of dissociation was brought about by multiple conformations of the channel structure or the involvement of non-specific interactions.

On the contrary, the interaction of channel subtypes that had dissociated into monomers, dimers or trimers with G $\beta\gamma$ did not show this kind of property. In their sensorgrams, only a slow phase was observed during dissociation. Therefore, in these cases, I suppose that this slow process of dissociation is due mainly to non-specific interactions between the channels and G $\beta\gamma$.

Discussion & Conclusion

The dissociation constant of the channel for G $\beta\gamma$ estimated by this study is in the order of micromolar, which is weaker than that estimated by electrophysiological studies (Ivanova-Nikolova *et al*, 1998). It may be due to the absence of the transmembrane portion of the channel used for the surface plasmon resonance experiment. Considering this situation, it is important to employ in future experiments a full-length channel so as to evaluate the contribution of the transmembrane portion to binding to G proteins (Nishida & MacKinnon, 2007). It is also important to perform binding experiments in the presence of phosphatidylinositol 4,5-bisphosphate, which is an essential component for activation of Kir3 along with G $\beta\gamma$. In order to exclude the effect of non-specific interactions and enhance the contribution of physiological interactions in measurements, instead of

immobilizing the channel or G proteins to the sensor chip by amine coupling, they must be attached to the sensor chip via an antibody that recognizes epitope-tagged analytes. The understanding of biophysical characteristics of this protein-protein interaction should be improved significantly by taking these kinds of approach.

Although the interaction of Kir3 with G proteins has been analyzed mainly with electrophysiological techniques so far, some reports included biochemical experiments in order to complement the information derived from electrophysiological studies. In some of these reports, the interaction was analyzed by pull-down assay in which the cytoplasmic portion of the channel had been fused to glutathione S-transferase so that they can be immobilized to glutathione agarose beads without covalent reaction (Lei *et al*, 2000). However, for this assay, the channel seemed to have been prepared not as a tetramer that is physiologically relevant but as a monomer. My study suggests that the information obtained from such experiments should be interpreted very carefully since they might just detect hydrophobic interactions between the inner surface of the channel and G proteins.

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一般の皆様へ

本書類に述べた蛋白質間相互作用の解析手法を更に発展させ、これを参考としつつ、医療において重要な意義のあるチャンネル蛋白質の制御の機構を、X線結晶構造学的手法を用いて明らかにしたい。

また、本助成に採択される方々の研究は重要であり、そういった方々は関連した研究で他の助成団体や国からの研究費を複数得ている場合が多いと推察する。そういった場合、本助成の研究費はこれのみで大型の研究を推進するほど多額ではないので、おそらく他からの資金源より、本人を含む人件費や設備費等を支出しつつ、消耗品や旅費等に本助成金を使用するという場合が多いのではないだろうか。その場合、本助成による研究費は1年で使い切るというより、例えば、全額使用するのは5年間以内と延長するか、もしくは研究代表者を信頼し、思い切って無期限としてしまえば、大変使い勝手がよくなると思う。基礎研究は本来数年で完成する性質のものではない。従ってこれを長年かけて遂行する必要があるが、その際、諸事情によって、研究代表者が、必要な資金をど

ここからも得られなくなる年度も生じる可能性は充分ある。そこで研究を中断しなければならないとなると、これは非常に残念な事である。そういった際、もし本助成の期間が延長されており、これによる予算を随時使用できれば、例えば年度によって必要な消耗品が購入できなくなるといった事態が避けられる。あるいは、諸事情により本人を含む人件費すら捻出できない年度が生じた場合でも、予算をある程度額使わずにこれを保持しておけば、研究再開が可能となった年度より、ただちにこれを使用する事が可能となる。このような諸状況を想定すれば、私立の助成金で、たとえ小額であったとしても使い勝手の良い制度のものがあれば大変に便利であると思う。

Regulation of EGFR membrane trafficking by ROCO family kinase LRRK1

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Abstract

Key Words : EGFR, LRRK1, membrane trafficking, phosphorylation

Introduction

Endocytosis and subsequent delivery of EGFR to lysosomes are essential for the degradation of many membrane-associated proteins. This process is crucial for determining the amplitude of EGFR signaling, and is therefore tightly regulated. In spite of considerable effort researching EGFR intracellular transport, the detailed mechanisms underlying endosomal trafficking of EGFR is still not well understood. We have recently reported that Leucine-rich repeat kinase 1 (LRRK1) is involved in the trafficking of activated EGFR, specifically in the progression from early to late endosomes. In this study, we show that the EGFR-mediated tyrosine phosphorylation of LRRK1 plays an important role in the proper endosomal trafficking of EGFR.

Results

LRRK1 is phosphorylated at the Tyr-944 residue in response to EGF

We have recently shown that LRRK1 forms a complex with activated EGFR through a mutual interaction with Grb2. We further observed that EGFR is co-immunoprecipitated with LRRK1 and is tyrosine phosphorylated in cells stimulated by EGF. So, we attempted to identify the sites in LRRK1 that are tyrosine phosphorylated in an EGFR-dependent manner. The ROC-COR domain of LRRK1 contains one EGFR tyrosine kinase consensus site (Glu-Glu-Glu-Tyr-Phe) at residue 944. To examine whether this site is responsible for EGFR-mediated tyrosine phosphorylation, we generated a polyclonal antibody against a peptide encompassing phospho-Tyr-944. As a result, phosphorylation of LRRK1 Tyr-944 was detected by this antibody upon EGF stimulation.

Phosphorylation of LRRK1 at Tyr-944 negatively regulates its kinase activity

Since phosphorylation represents an important posttranslational modification that regulates kinase activities, we examined the effect of the non-phosphorylatable LRRK1(Y944F) mutation on these activities. We found that compared to wild-type LRRK1, LRRK1(Y944F) showed much higher autophosphorylation activity. We next examined the effect of EGF stimulation on LRRK1 kinase activity. Cos7 cells were transfected with wild-type GFP-LRRK1 and then treated with EGF for various times. LRRK1 proteins were immunoprecipitated and assayed for autophosphorylation activity. LRRK1 kinase activity was transiently activated by EGF stimulation, peaked at 10 min,

and then declined. In contrast, the kinase activity of the LRRK1(Y944F) mutant was more enhanced and was sustained for up to 120 min after EGF stimulation. These results suggest that LRRK1 is activated in response to EGF stimulation and subsequently inactivated by EGFR-mediated Tyr-944 phosphorylation in a negative feedback fashion.

LRRK1 kinase activity regulates the motility of EGF-containing endosomes

Since the kinase activity of LRRK1 is required for motility of EGF-containing endosomes, we examined the effect of LRRK1 hyper-activation on the dynamics of EGF movement *in vivo* using time-lapse confocal fluorescence microscopy. The movement of EGF-containing endosomes was followed in HeLa cells expressing GFP-LRRK1 at 15 min after a brief pulse of fluorescent EGF (Alexa 647-EGF). In addition to short-range movement, long-range rapid movement of GFP-LRRK1- and Alexa 647-EGF-double-positive endosomes was frequently observed. When HeLa cells were transfected with GFP-LRRK1(Y944F) and treated with Alexa 647-EGF, the frequency of EGF long-range movement was remarkably increased. These results suggest that hyper-activation of LRRK1 kinase activity enhances the long-range rapid movement of EGF-containing endosomes.

Hyper-activation of LRRK1 causes EGF/EGFR accumulation in perinuclear endosomes

Next, we investigated the effect of the LRRK1(Y944F) mutation on the intracellular distribution of EGF/EGFR after EGF stimulation. We expressed GFP-LRRK1 in HeLa S3 cells and assessed EGF localization by immunofluorescence using a fluorescently-labeled rhodamine-conjugated EGF (Rh-EGF). Cells expressing wild-type GFP-LRRK1 were briefly stimulated with Rh-EGF. By 10 min post-stimulation, Rh-EGF was distributed in a fine, punctate pattern that co-localized with GFP-LRRK1. After 30 min, weak punctate staining of Rh-EGF was co-localized with GFP-LRRK1 in the perinuclear region, suggesting that transport of EGF/EGFR from early to late endosomes. After 60 min, most of the Rh-EGF signal had disappeared and GFP-LRRK1 was diffusely distributed, suggesting that EGF/EGFR had been degraded in lysosomes and/or recycled. In GFP-LRRK1(Y944F)-expressing cells briefly stimulated with EGF, the distribution of Rh-EGF at 10 min was similar to that observed in cells expressing wild-type LRRK1. However, at 30 min, both Rh-EGF and GFP-LRRK1(Y944F) had accumulated in compartments in the perinuclear area and remained there up to 60 min after EGF stimulation. Thus, EGFR degradation appears to be impaired in these cells.

Discussion & Conclusion

Following clathrin-mediated endocytosis, activated EGFR is delivered to the lysosome for degradation. This transport and lysosomal degradation of activated EGFR occurs rapidly, presumably to limit excessive mitogenic EGFR signaling. The rapid kinetics of this process led us to postulate the existence of a more specific mechanism that regulates EGFR endosomal trafficking. Our findings here indicate that LRRK1 kinase activity plays a role in EGFR intracellular trafficking as a cargo-specific regulator of EGFR endosomal transport.

In this study, we find that LRRK1 is phosphorylated at Tyr-944 in response to EGF stimulation

and negatively regulated its kinase activity. Recent studies have shown that early endosomes move towards the cell center as they mature. The process of endosomal maturation is coordinated with the movement of endosomes along microtubules. This coordination appears to be disrupted by hyperactivation of LRRK1, as expression of LRRK1(Y944F) inappropriately promotes the motility of EGF-containing endosomes. Therefore, the regulation of LRRK1 kinase activity might be important to ensure the orderly and timely trafficking of endosomes containing EGFR.

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EGFR-dependent phosphorylation of leucine-rich repeat kinase LRRK1 is important for proper endosomal trafficking of EGFR. *Mol. Biol. Cell*, 23:1294-1306 (2012).

一般の皆様へ

活性化した上皮成長因子受容体 (EGFR) は、細胞増殖シグナルを発信します。過剰な EGFR シグナルは細胞の癌化を引き起こすことから、細胞は EGFR シグナルを素早く止める仕組みを備えています。リン酸化酵素 LRRK1 は、酵素活性依存的に EGFR の細胞内トラフィックを制御し、EGFR シグナルの抑制に機能していることを明らかにしました。

Analysis of the algal circadian clock for revealing the evolutionary history of plant-type circadian clocks

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Abstract

We investigated the molecular mechanisms of the circadian clock in the green alga *Chlamydomonas reinhardtii*. This study revealed that ROC75, a *Chlamydomonas* circadian clock protein, is a transcriptional repressor functioning as a generator of circadian oscillation and as a mediator for the timing information of the clock to many cellular biological processes.

Key Words : Circadian rhythm, Green alga, Clock gene, Plant

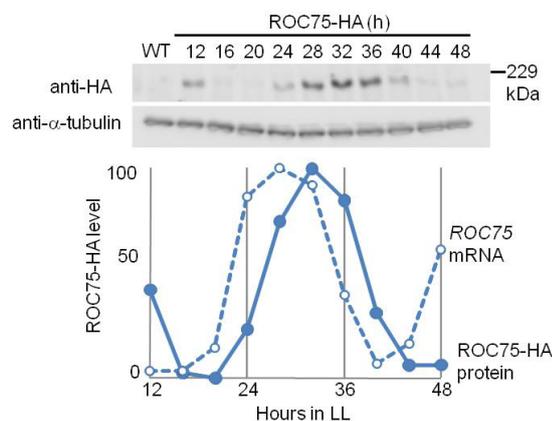
Introduction

Circadian clocks are self-sustained molecular machinery to generate 24-h rhythm (circadian rhythm) and control timing of various biological processes to adapt the organisms to daily environmental cycles. We identified the clock genes, which are core components of the molecular machinery of clock, in the green alga *Chlamydomonas reinhardtii*. In this study, we investigated the molecular mechanisms of the *Chlamydomonas* circadian clock and compared them with those of higher plants circadian clocks.

Results

At first, we analyzed the mRNA expression patterns of six clock genes of *Chlamydomonas* in the clock mutant genetic backgrounds, and found that the clock gene mutations affect expressions of other clock gene expressions. Especially, a mutation of a clock gene termed *ROC75* was strongly affect the expression of another clock gene *ROC40*. The *ROC40* mRNA level was constantly elevated in *roc75* mutant throughout the circadian cycles. Since *ROC75* encodes a protein having the GARP DNA-binding motif, it was expected to be a transcription factor controlling *ROC40* expression. Thus, in this study, we focused on *ROC75*.

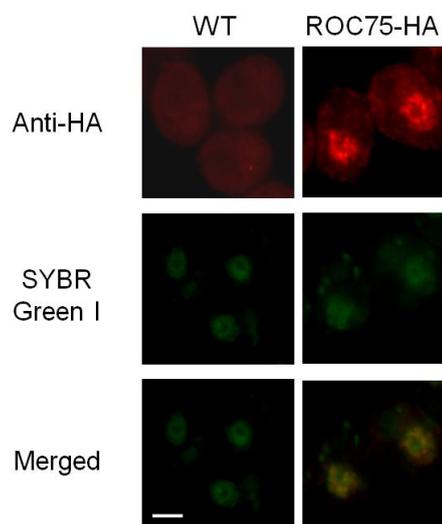
To analyze the *ROC75* protein expression, we introduced a hemagglutinin (HA) tag just prior to the stop codon of the *ROC75* gene on a genomic DNA fragment, and introduced it into *roc75* mutant. Since the tagged-*ROC75* fully complemented the *roc75* mutant phenotypes, the *ROC75*-HA fusion protein would be



functional in the *Chlamydomonas* circadian system. We analyzed circadian expression profile of the ROC75-HA protein by western blot. The ROC75 mRNA has been known to be expressed circadianly peaking at the subjective early-mid day. ROC75-HA protein level also showed strong circadian rhythm peaking at mid to late subjective day, which is slightly delayed from its mRNA oscillation. The ROC75-HA rhythm was clearly antiphase to the mRNA oscillation of *ROC40* gene, suggesting ROC75 is a repressor for *ROC40*.

Immunocytochemical staining using a HA antibody indicated that this protein is a nuclear protein. Double-staining with SYBR Green I or an antibody for the nuclear pore complex revealed that ROC75-HA is localized to the nuclear periphery. No obvious changes of localization were detected throughout the circadian cycles.

To investigate direct interaction of ROC75 with *ROC40* gene, we purified the DNA-binding motif of ROC75 expressed in *E. coli* and analyzed binding to the DNA fragment of the *ROC40* promoter region by the electrophoresis mobility shift assay. The DNA-binding motif clearly bound to the *ROC40* promoter. By analyzing binding to a series of DNA fragments of *ROC40* promoter region, we identified binding site of ROC75. In addition, we analyzed *in vivo* binding sites of ROC75 by the chromatin immunoprecipitation combined to deep sequencing (ChIP-Seq). ChIP-Seq analysis revealed ROC75 binding sites more than one thousand, including not only *ROC40* but also other clock genes (*ROC15*, *ROC66*) and genes related to various biological processes. This result suggests that ROC75 regulates not only clock oscillation itself but also many biological processes to output the timing information of the circadian clock.



Discussion & Conclusion

Our results suggest that ROC75 is a transcriptional repressor functioning as a generator of circadian oscillation and as mediator for the timing information of clock to many cellular biological processes. The expression profile and the target DNA motif of ROC75 suggest a part of molecular mechanisms of circadian clock is conserved between green algae and land plants. The large dataset of ChIP-Seq analysis will be useful for further investigation of the circadian-clock-regulation for various cellular physiological pathways, including growth, photosynthesis, lipid metabolism, which will be involved in creation of basic technology for new bioenergy production.

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一般の皆様へ

本研究では、これまでよく解っていなかった緑藻の生物時計の分子機構に関する新たな知見を多数得ることに成功しました。この成果は植物の生物時計の進化の過程を解明する手がかりとなります。また、近年盛んに研究が進められている緑藻を使ったバイオエネルギー生産の効率を高める手がかりにもなると期待されます。

The role of Cathepsin C in generating active IL-1 beta in vivo.

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Abstract

The aim of this study is to reveal how the active IL-1 β is produced in vivo. IL-1 β is transcribed as an inactive pro protein and cleaved to become active form which can transmit signal via IL-1 receptor. Here we identified Cathepsin C is responsible for generating active IL-1 β in vivo in accordance with Caspase-1.

Key Words : cell death, inflammasome, IL-1, NLRP

Introduction

The sterile inflammatory response to cell death and irritant crystals is medically important because it causes disease. Although these stimuli are structurally distinct, they cause inflammation through a common pathway that requires the cytokine IL-1. In vitro the inflammasome, and in particular its generation of active caspase 1, is absolutely required to produce bioactive IL-1 β . However, here we report that caspase 1 is not required in vivo for much of the IL-1 β -dependent sterile inflammatory response.

Results

We find that cathepsin C, which controls the activity of a number of leukocyte serine proteases capable of processing IL-1 β , plays a major role in this caspase 1-independent pathway. Mice that are deficient in cathepsin C have reduced inflammatory responses to dying cells and silica crystals. In the absence of cathepsin C, caspase 1 becomes rate limiting so that mice that are doubly deficient in both of these proteases make little IL-1 β in vivo and have markedly attenuated inflammatory responses to the sterile stimuli. In contrast, these mutant mice generate normal inflammation in response to exogenous IL-1 β , indicating that cathepsin C and caspase 1 function upstream of IL-1 β , and in their absence, all components of the pathway downstream of mature IL-1 β are intact.

Discussion & Conclusion

We believe that understanding how IL-1 β is produced during sterile inflammation is important because this process underlies a number of diseases. The inflammation that occurs in response to cell death can cause tissue damage and disease. Sterile inflammation to crystals underlies a number of diseases including silicosis, gout, pseudogout, asbestosis and possibly also even atherosclerosis. IL-1 β has also been implicated in metabolic syndrome and type II diabetes among other diseases. Our findings have implications for potential therapeutic targets to block IL-1 production and may help to explain why caspase 1 inhibitors were not effective in clinical trials.

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一般の皆様へ

痛風や関節リウマチなどの熱や痛みを伴ういわゆる炎症性の病気に、サイトカイン IL-1 β が重要な役割を果たしていることは知られていました。これまで代謝性疾患と考えられてきた動脈硬化や糖尿病も実は炎症性の病態が重要な役割を果たしており、IL-1 β が重要な役割を果たしていることが最近判明しました。IL-1 β の活性化機構についてはよく知られていませんでしたが、本研究においてこれまで知られていた caspase-1 に加えて cathepsin C という分子が IL-1 β の活性化に必要と判明しました。

Functional characterization of NADPH oxidases and their regulators of endophytic fungus

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Abstract

We investigated morphological characteristics of knockout mutants of NoxA, NoxB and their regulators, NoxR, RacA and BemA. On water agar, *noxA*, *noxB* and *noxR* mutants showed increased hyphal branching and irregular curving, indicated that Noxs and regulators are involved in polarized growth under starvation condition. In *noxA* mutant, formation of asexual conidia was increased. Interestingly, mutation of regulators has no effect on conidiation. Hyphae of *noxA*, *noxB*, *noxR* and *racA* mutants lost the ability to form hyphal fusion. These results indicated that NoxA, NoxB and their regulators have distinct or overlapping functions for the regulation of different hyphal morphogenesis.

Key Words : Conidiation, Endophyte, Hyphal fusion, NADPH oxidase, Reactive oxygen species.

Introduction

The endophytic fungus *Epichloë festucae* systemically colonizes the intercellular spaces of host grass to establish a mutualistic symbiotic association. We have previously shown that reactive oxygen species produced by a specific NADPH oxidase isoform, NoxA, and associated regulators, NoxR and RacA, have a critical role in regulating hyphal growth in the host plant to maintain symbiotic infection. We also have identified BemA and Cdc24, homologues of polarity establishment proteins of yeast, as interactors of NoxR. In this study, we further investigated the morphological characteristics of knockout mutants of *NoxA*, *NoxB* and their regulators, *NoxR*, *RacA* and *BemA*.

Results

E. festucae wild type (WT), *noxA*, *noxB*, *noxA/noxB*, *noxR*, *racA* and *bemA* deletion mutants were grown on PDA or water agar, and hyphal growth of these strains were observed. On nutrient rich medium, most of tested mutants showed normal growth like WT except *racA* mutant, which showed hyphal swelling and increased branching. On water agar, *noxA*, *noxB* and *noxR* mutants showed increased hyphal branching and irregular curving, indicated that both Noxs and regulators are involved in polarized growth under starvation condition.

Epichloë endophyte WT grown on PDA hardly produced conidia, but a few conidia were observed when it was grown on thin-layer PDA.

In *noxA* mutant, formation of asexual conidia was significantly increased, and *noxA/noxB* double

mutant showed further increase of conidiation. Interestingly, mutation of regulators, *NoxR* and *BemA*, has no effect on conidiation (Fig. 1).

Hyphae of wild type *E. festucae* showed frequent hyphal fusion. *noxA*, *noxB*, *noxR* and *racA* mutants lost the ability to form hyphal fusion. *bemA* mutant showed significant reduction on the frequency of cell fusion. These results indicated that NoxA, NoxB and their regulators have distinct or overlapping functions for the regulation of different hyphal morpho- genesis (Fig. 2).

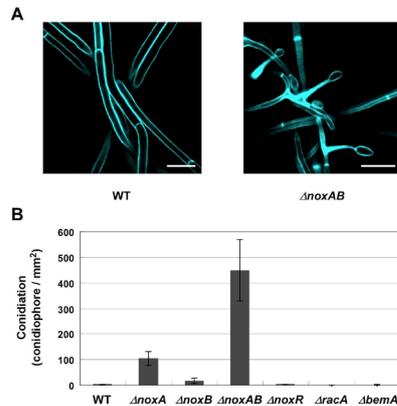


Fig. 1. Conidiation of nox mutants

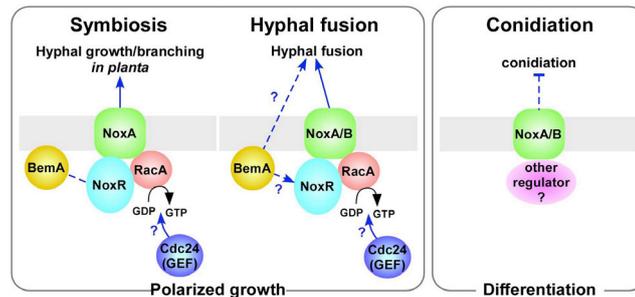


Fig. 2. Models of involvement of Noxs and their regulators in hyphal differentiations.

Discussion & Conclusion

It was found that NoxA, NoxB, NoxR and RacA are essential for polarity related events, such as establishment of polarized growth and apical dominance under starvation condition and formation of hyphal cell fusion. BemA would have supportive role in ROS production for polarity establishment. Phenotype of these mutants in symbiotic infection might be related to the phenotype observed on water agar, as intercellular space of plant cells, where endophyte is growing, is generally poor in nutrient, and hyphal growth and branching of endophyte is restrictedly controlled to establish symbiosis.

Interestingly, *noxA/noxB* mutant showed significant increase of conidiation, whereas knockout of *E. festucae noxR* have no effect on productivity of conidia, despite the previous reports indicating that NoxR is required for the function of both NoxA and NoxB. Therefore, alternative mechanisms or unknown regulator might be involved in NoxR-independent regulation of *E. festucae* NoxA and NoxB for control of conidiation. Altogether, Nox activity for polarity establishment require regulators like NoxR, whereas role of Nox for priming or suppression of differentiation might be regulated by another mechanism (Fig. 2).

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their regulators in polarized growth, conidiation and hyphal fusion of symbiotic fungus *Epichloë festucae*. (Submitted).

一般の皆様へ

共生糸状菌 *Epichloë festucae* は牧草、芝草の地上部組織の細胞間隙で伸長し、共生関係を保っている。この共生菌は植物内で種々の生理活性物質を生成し、植物に動物や昆虫による補食の抑制、耐乾性、耐病性などの効果をもたらすことが知られている。本研究では、共生菌が植物と共生するために必須な活性酸素生成酵素 (Nox) とその制御因子の機能解明を進めた。NoxA、NoxB および制御因子 (NoxR、RacA、BemA) の遺伝子を欠損した変異株を詳細に観察したところ、これら因子が、植物との共生確立、胞子の形成、菌糸の極性伸張および菌糸融合において異なる組み合わせで機能していることが解った。

Pharmacological and clinical significance of the enhanced protein farnesylation induced by the inactivation of retinoblastoma tumor suppressor gene

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Abstract

DNA microarray and subsequent gene ontology analysis indicated pRB targets many genes involved in the mevalonate pathway. Further analysis indicated pRB plays crucial roles in tumor progression by affecting farnesylation, geranylgeranylation or synthesis of cholesterol and its derivatives. Our studies suggested that pRB affects tumor cell differentiation and DNA damage response via regulating farnesylation, tumor cell invasion via geranylgeranylation and cancer stem cells via cholesterol synthesis.

Key Words : cancer, farnesylation, geranylgeranylation, cholesterol, cancer stem cell

Introduction

RB mutation is frequently found in the initiation of retinoblastoma, osteosarcoma or small cell lung carcinoma. However, in majority of cancers, pRB inactivation is found during cancer progression. This implicates that beyond its previously appreciated functions in cell cycle control or terminal differentiation, pRB plays many more functions during tumor progression. We in this study focus on the novel transcriptional targets of pRB, which contains many of genes involved in mevalonate pathway. Findings obtained by our current study indicate that pRB affects malignant behaviors of cancer cells through regulating lipid metabolism.

Results

We previously reported that Rb status affects Ras activation status through regulation of protein farnesylation. We then determined pRB transcriptional targets in cells in which the presence of pRB does not affect cell proliferation, and found many of genes involved in mevalonate pathway as novel pRB transcriptional targets. pRB via E2F family transcription factors targets SREBP transcription factors or SREBP targeted genes. Protein farnesylation, geranylgeranylation and cholesterol synthesis and subsequent steroidogenesis and other pathways are innervated by mevalonate pathway. We first observed increased level of total protein farnesylation in Rb-negative cells. In addition, we recently observed that pRB depletion in mouse cells lead to increased level of fatty acid synthesis. All these observation supported that pRB targets mevalonate pathway. In the current study, we focused on the effect of pRB inactivation on the cancer stem-cell-like behaviors seen in cancer cells. We found that in cells in particular genetic background, pRb depletion efficiently induces

sphere formation which is thought to be relevant to self-renewal activity exhibited by cancer stem cells. First, we tested whether total shut-down of mevalonate pathway by statins antagonizes pRb depletion-induced sphere formation. The result was positive. Then we determined which downstream event of mevalonate pathway is critical for pRb depletion-induced sphere formation. Our current results suggest that cholesterol synthesis pathway is the most critical in pRb depletion-induced sphere formation. Accordingly, we are currently trying to determine which cholesterol derivative is critical in this pathway. Oxy-LDL which exogenously supplies cells with oxy-sterols have been implicated in cell transformation. Elevated serum cholesterol level is known to higher risk and worsen prognosis in various cancers. We also determined the effect of geranylgeranylation and farnesylation in other facets of malignant behaviors of cancer cells. Geranylgeranylation mediates pRB to affect Rho family members thus this interaction is implicated in tumor cell invasion and metastasis. As we reported previously, farnesylation mediates pRB to affect Ras protein maturation, thus this interaction appeared to be implicated in cell differentiation, DNA damage, DNA damage response, cellular senescence, etc.

In addition, these studies disclosed a novel mode of cooperation between p53 and pRB. Currently p53 is implicated in the control of aerobic glycolysis. We found pRB controls the expression of many genes involved in lipidogenesis. Aerobic glycolysis and enhanced lipidogenesis consist two major metabolic perturbations featured in cancer cells. Therefore, inactivation of both p53 and pRB, which is frequently observed in human cancers, efficiently couples these two metabolic disorders by promoting production and consumption of NADPH. Indeed, we observed a dramatic “metabolic reprogramming” occurs in cells lacking both p53 and pRb. These cells exhibited increased levels of lipid anabolism and catabolism; the latter was indirectly proved by the increased expression of UCP1 in these cells. UCP1 is also known to frequently predict poor prognosis in prostate cancers. These observations suggest that inactivation of p53 and pRB contributes to malignant behavior of cancer cells through regulating cell metabolism. We are currently confirming these hypothesis by using Rb-p53 DKO mice simultaneously lacking one of genes involved in cholesterol synthesis or other pathways for example SREBP1.

Discussion & Conclusion

25 years have passed since the first molecular cloning of human RB tumor suppressor gene. The functions of pRB have been implicated in cell cycle control and terminal differentiation. However, researchers predicted more multi-faceted functions of pRB based on pathological and biological studies. Our study revealed that pRB inactivation contributes to carcinogenesis via perturbing lipid metabolism, and further that this role is more critical in the absence of p53 function which controls aerobic glycolysis. Our studies provide strong genetic evidence that metabolic perturbation in cancers is bona fide malignant phenotypes linked from tumor suppressor inactivation. Our studies also have shown that malignant phenotypes induced by inactivation of such tumor suppressors can be pharmacologically targeted. Based on findings accumulated in the current research, we are

establishing in vitro cancer stem cell models that are optimized for unbiased and high-throughput siRNA, drug or chemical screening. Our future study will identify more number of possible targets in cancer therapy.

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一般の皆様へ

RBという古くから知られているがん抑制遺伝子の新規標的がファルネシル化経路を含むメバロン酸経路であることを突き止めました。これは、脂肪合成やコレステロール合成も司る代謝経路です。がん抑制遺伝子が、脂肪代謝の経路を制御するという働きによって、発がんやがんの悪性を抑制する可能性があるわけです。近年、脂質異常と様々な疾病の関連に注目が集まっておりますが、この研究を契機に、がんとの関連も今後重要視されていくと考えます。

Analysis of leukemogenic mechanisms induced by Trib1

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Abstract

RAS/MAPK signaling and C/EBP α are important in myeloid leukemogenesis. Trib1 interacts with MEK1, enhances MEK1-dependent ERK phosphorylation and promotes degradation of C/EBP α . The Trib1 function in C/EBP α regulation has been also evident in bone marrow of *Trib1* knockout mouse where significant increase of mature granulocytes and accumulation of the C/EBP α protein was observed. We have identified TRIB1 point mutation in a human case of Down syndrome-related acute megakaryocytic leukemia, and found that the mutation R107L enhanced Trib1 function on both ERK phosphorylation and C/EBP α degradation. These studies suggest that Trib1 is a good candidate of a novel molecular targeted therapy for cancer.

Key Words : Trib1, leukemia, oncogene, signal transduction, C/EBP α

Introduction

Intracellular signal transduction is important for regulating proliferation, survival and differentiation, and the dysregulated signaling is an important hallmark of cancer. Mutations within the Ras/mitogen activated kinase (MAPK) pathway have been identified in human leukemias. Dysregulated transcriptional control is another important mechanism in leukemogenesis. C/EBP α acts as an anti-proliferative molecule and a tumor suppressor in myeloid cells. In human AML patients, loss-of-function mutations of C/EBP α are frequently observed. The tribbles family of proteins, Trib1 and Trib2, are adaptors that link the MAPK pathway and C/EBP transcriptional regulation, and affect hematopoietic cell growth and leukemogenesis.

Results

1. Analysis of tribbles mutant mice

In *tribbles* family genes *Trib1* and *Trib2* are expressed in bone marrow while *Trib3* is not. Bone marrow fractionation revealed that both *Trib1* and *Trib2* are expressed in LSK cells as well as myeloid progenitors. In search of the tribbles function in hematopoiesis, we have generated a novel *Trib1* conditional knockout mouse by homologous recombination. The *Trib1* null homozygous mutants were viable but sterile, and no major defects were observed in systemic organs. Similar phenotypes were observed in the *Trib2* null homozygous knockout mouse that was created by Dr. Ryuichi Nishinakamura (Kumamoto University). However, significant increase of segmented granulocytes was observed in the *Trib1* homozygous mutant, and polyI:polyC treatment of the *Trib1*^{flox/flox}/Mx1-Cre mouse induced identical changes in bone

marrow (BM). The increase of mature granulocytes was associated with accumulation of the C/EBP α protein and upregulation of C/EBP α target genes such as *Il6r*, *Csf3r* and *Mpo*. Interestingly, neither increased granulocytes nor C/EBP α accumulation was observed in the *Trib2* knockout mouse.

To assess the role of Trib1 in leukemogenesis and Ras/MAPK signaling, *Trib1* KO BM cells were introduced with upstream gain-of-function mutants such as *BCR-ABL*, *FLT3-ITD* and *KIT* DA. Wild type BM cells were immortalized by introduction of the mutants, however, the oncogenic effect of the mutants was abrogated when *Trib1* KO BM cells were used. The result suggests that Trib1 may be an important downstream effector to support leukemic cell growth bearing mutations in RAS/MAPK signaling.

2. *TRIB1* mutation in human leukemia

The Down syndrome (DS) patients are predisposed to developing myeloid leukemia, and those patients frequently exhibit *GATA1* mutations. However, it is proposed that the *GATA1* mutation is important for transient leukemia in DS but not sufficient for full blown leukemia, suggesting that additional genetic alterations are needed. Therefore, it is important to search the subsequent genetic changes for DS-related leukemia (ML-DS) to predict malignant transformation and prognosis of the patients.

We identified a *TRIB1* somatic mutation in a human case of Down syndrome-related acute megakaryocytic leukemia (DS-AMKL) (Figure 1). The mutation was observed at well conserved arginine 107 residue in the pseudokinase domain.

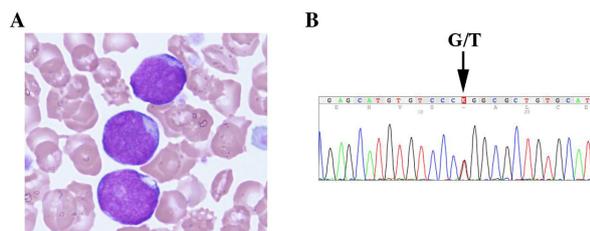


Figure 1. *TRIB1* mutation in DS-AMKL.

This R107L mutation remained in leukocytes of the remission stage in which *GATA1* mutation disappeared, suggesting the *TRIB1* mutation is an earlier genetic event in leukemogenesis. The bone marrow transfer experiment showed that AML development was accelerated by transducing murine bone marrow cells with the R107L mutant in which enhancement of ERK phosphorylation and C/EBP α degradation by *Trib1* expression was even greater than in those expressing wild type. These results suggest that *TRIB1* may be a novel important oncogene for DS-AMKL.

3. Genetic interaction of *Trib1* in leukemogenesis

Trib1 was identified as a cooperative gene for *Hoxa9* and *Meis1* in myeloid leukemogenesis. We have recently found that *Bcl11a* encoding a C₂H₂ zinc finger transcription factor is another cooperative gene in *Trib1*-induced leukemogenesis like *Hoxa9/Meis1*. Gene expression profiles were compared between *Trib1/Hoxa9/Meis1*- and *Trib1/Bcl11a*-induced AML. Genes involved in the nuclear co-repressor complex as well as cell cycle promoters/regulators are differentially expressed, and the function of *Bcl11a* as an epigenetic modulator in leukemogenesis was suggested.

Discussion & Conclusion

It was proposed that degradation of target proteins by tribbles is mediated by the E3 ubiquitin ligase COP1. Trib3 promotes ubiquitination of acetyl-coenzyme A carboxylase (ACC) in adipose tissue. Trib3 interacts with both ACC and COP1 using distinct domains, promoting COP1-dependent ubiquitination of ACC. The COP1 binding consensus sequence [D/E]QXVP[D/E] has been well conserved among the three tribbles during evolution, suggesting that COP1 binding by the tribbles family of proteins is essential for their targets in protein degradation. Post-translational regulation of C/EBP α by Trib1 is thus one of important mechanisms to control differentiation and growth suppression of myeloid cells.

Inhibition of cellular transformation by oncogenic signaling molecules including BCR-ABL and FLT3-ITD in Trib1 deficient hematopoietic cells underscores the importance of Trib1 in the Ras/MAPK pathway. Molecular targeted therapy using tyrosine kinase inhibitors (TKIs) has shown great success in leukemia, however, specific inhibitors are required for each mutants and secondary mutations make leukemic cells resistant to TKIs. Therefore Trib1 is an excellent candidate for the novel molecular target not only in leukemia but also in cancer with Ras/MAPK abnormalities.

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一般の皆様へ

私たちは、マウスの白血病モデル系において Trib1 遺伝子を同定した。Trib1 は RAS/MAPK シグナル系の活性化促進と C/EBP α 転写因子の抑制の二つの機能をもつことから、強力な白血病誘導効果を持っていると考えられた。Trib1 遺伝子をノックアウトすると、C/EBP α 蛋白量が骨髄で増加し、分化した好中球の数が増えた。したがって、Trib1 は正常骨髄でも C/EBP α を制御することで血球の分化に重要な働きをもつことが考えられた。これらの結果を合わせると、Trib1 はがん治療において新たな分子標的として有望である可能性が考えられた。

The roles of C-type lectin family in the maintenance of immunological homeostasis.

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Abstract

Dectin-1 and Dectin-2 are type II transmembrane proteins of the C-type lectin family with single carbohydrate-recognition domains (CRDs) in their extracellular region. They are expressed mainly in dendritic cells (DCs) and macrophages. In this study, the functional roles of Dectin-1 and Dectin-2 in host defense against fungal infection were examined, and it was elucidated that Dectin-1 is important in protection against *P. carinii* by inducing reactive oxygen species, whereas Dectin-2 plays important roles in host defense against *C. albicans* by preferentially inducing Th17 cell differentiation.

Key Words : C-type lectin, Interleukin-17, Dendritic cell, fungal infection

Introduction

The C-type lectins are one of the pattern recognition receptors (PRRs) with lectin-like CRDs in their extracellular carboxy-terminal domains. Some C-type lectin family members recognize the carbohydrate structures of microbes as pathogen-associated molecular patterns (PAMPs), whereas some members on natural killer cells recognize endogenous ligands and discriminate self from non-self. Similar to other PRRs such as Toll-like receptors (TLRs), C-type lectins that recognize PAMPs are also involved in host defense mechanisms against infection. However, different from TLRs, which recognize various PAMPs such as lipopolysaccharides, proteoglycans, DNAs, and RNAs, C-type lectins mostly recognize carbohydrate structures in pathogens (1).

Dectin-1 and Dectin-2 are members of the C-type lectin family, whose genes are located in the telomeric region of the mouse chromosome 6 and human chromosome 12q, where many C-type lectin genes are located and form several clusters. Both Dectin-1 and Dectin-2 are glycosylated type II transmembrane proteins with single CRDs, which are highly conserved in mice and humans (amino acid identities: Dectin-1 63%; and Dectin-2 75%). Whereas Dectin-1 has an ITAM-like motif in its intracellular region, Dectin-2 has no known signaling motif in its shorter cytoplasmic region. These structures are shared by mice and humans (2).

By using Dectin-1 and Dectin-2 deficient mice, we studied their roles in innate and acquired immunity.

Results

The ligands for Dectin-1 and Dectin-2

β -Glucans are an important cell-wall component of fungi; the backbone structure consists of polymerized β -1, 3-linked β -D-glucopyranosyl units with β -1, 6-linked side chains. β -Glucans are found in a wide range of edible mushrooms, seaweeds, yeasts, and pathogenic fungi including *C. albicans* and *P. carinii*, and various chain lengths and branchings are characteristics of each fungal species. It is widely accepted that β -glucans are located in the inner layer between the mannan and chitin layers as skeletal components of the candida cell wall, and they are exposed on the cell surface at budding sites.

Cytokine (e.g. TNF and IL-12) production occurred in bone marrow-derived DCs (BMDCs) stimulated with β -glucans purified from edible mushroom (*Sparassis crispa* glucan) or with NaClO-oxidized zymosan, was completely abolished when BMDCs or macrophages were prepared from Dectin-1-deficient mice, indicating that Dectin-1 is the functional receptor for β -glucans.

On the other hand, it was clearly shown that Dectin-2 is the functional receptor for α -mannans, because cytokine production was completely abolished in BMDCs from Dectin-2-deficient mice upon treatment with *C. albicans* cell-wall α -mannans.

The roles of Dectin-1 and Dectin-2 in host defense against fungal infection

Dectin-1-deficient mice were more susceptible to *P. carinii*. ROS production was abolished in Dectin-1-deficient alveolar macrophages. On the other hand, production of proinflammatory cytokines such as TNF or IL-12 was not affected by the Dectin-1 mutation upon treatment with *P. carinii*, suggesting that the Dectin-1-induced oxidative burst is important for fungal eradication. However, the mechanism of ROS production via activation of Dectin-1 remains to be elucidated. ROS induces maturation of IL-1 β through inflammasome-mediated activation of Caspase-1, that is important for the development of inflammation and Th17 cell differentiation.

Dectin-2-deficient mice showed decreased survival of the infection of three different strains *C. albicans* due to the increased fungal growth in the kidney. Thus, Dectin-2 seems to play more important roles for the defense against *C. albicans*, probably because mannans, but not β -glucans, are exposed on the outer most layer of the fungal cell wall. Because human Dectin-2 also has EPN motif which is responsible for mannose recognition, it is likely that Dectin-2 also plays an important role for the anti-fungal immunity in humans.

Interestingly, it was shown that Dectin-2 is involved in the differentiation of Th17 cells upon infection with the yeast form of *C. albicans*; Th17 cells were preferentially induced by the *C. albicans*-treated BMDC culture supernatant, whereas Th17 cell differentiation disappeared in Dectin-2-deficient BMDCs.

The importance of IL-17A and/or IL-17F in host defense against *C. albicans* has been suggested using IL-17RA-deficient mice and IL-23p19-deficient mice. We demonstrated that IL-17A-

deficient mice, but not IL-17F-deficient mice, show increased susceptibility to systemic *C. albicans* infection, indicating the importance of IL-17A for protection against this pathogen. The importance of Th17 cells for the defense against fungal infection is also suggested in humans; hyper-IgE syndrome patients with autosomal dominant mutations in the signal transducer and activator of transcription 3 (STAT3) gene, by which Th17 cell differentiation is suppressed, are associated with chronic mucocutaneous candidiasis (3).

Discussion & Conclusion

In this study, we have demonstrated that Dectin-1 is a functional β -glucan receptor and Dectin-2 is a functional α -mannan receptor to produce a large amount of cytokines. Although, it was suggested that the receptors may contribute to host defense against *C. albicans*, because both carbohydrates are important cell wall components of the fungal pathogen, Dectin-1 deficient mice showed normal host defense against this fungus, whereas Dectin-2 deficient mice were more susceptible. This may be because of the structure of the cell wall, it is believed that β -glucan is located inner layer. However, another group reported that Dectin-1-deficient mice also showed increased susceptibility to intravenous *C. albicans* infection, with enhanced fungal dissemination in the gastrointestinal tract and kidney using another fungal strain. Thus, the importance of Dectin-1 in the host defense against *C. albicans* may differ depending on the strains.

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一般の皆様へ

デクチン1とデクチン2は、C型レクチンと呼ばれるファミリーに属する膜タンパク質で、糖鎖と結合することが知られていました。今回の研究で、デクチン1は β グルカンと呼ばれる真菌細胞壁を構成する糖鎖を、デクチン2は α マンナンと呼ばれる別の真菌細胞壁構成糖鎖と結合し、炎症性の液性分子を分泌させることで、感染防御に関与していることが明らかとなりました。

p53 targeted medicine against heart failure

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Abstract

Mechanism of p53 elevation in the failing heart.

Key Words : Heart failure, p53, posttranslational modification

Introduction

p53 protein is a multifunctional protein that works as one of the most well-known tumor suppressors. p53 protein is often called as ‘a guardian of the genome’ for its function to conserve genomic stability and to prevent genomic mutation. The cellular level of p53 protein is usually kept low but is increased when the cells are damaged or stressed. Protein level of p53 is also kept low in the heart but is elevated when the cardiac cells are exposed to stresses including genotoxic stress after adriamycin treatment and hypoxic stress after pressure overload and coronary artery ligation.

Results

The amount of p53 protein in cells is determined mainly by the rate of its degradation, rather than the rate of transcription/translation. p53 protein is degraded through ubiquitin-proteasomal pathway, which is mediated by E3 ubiquitin ligases such as MDM2. Importantly, the expression of MDM2 is positively regulated by p53, forming a negative-feedback loop and preventing constitutive p53 elevation.

Adriamycin is one of the chemotherapeutics against many cancers but also notorious for its cardiotoxicity. Adriamycin is an anthracycline antibiotics and works by intercalating DNA. Adriamycin also produces reactive oxygen species (ROS) and indirectly damages mitochondria or genomic DNA. We identified that ROS-mediated indirect genomic DNA damage is critically involved in the mechanism for p53 elevation in the heart after adriamycin treatment. After intraperitoneal injection of adriamycin, p53 protein elevation was preceded by ROS production, DNA damage and activation of DNA damage pathway, as evidenced by increased DHE fluorescence, positive γ -H2AX staining, and increased phosphorylation of ATM (ataxia telangiectasia mutated) kinase. ATM kinase is one of the kinases that phosphorylate p53 at its amino terminus and stabilizes p53 protein by blocking MDM2 binding. ROS scavenger and inhibitor of ATM kinase both prevented p53 accumulation after adriamycin treatment in vitro, underscoring the importance of ROS-DNA damage-ATM kinase pathway on p53 accumulation in adriamycin-induced cardiomyopathy.

Hypoxia is another stress that causes p53 accumulation in the heart [48] and cardiomyocytes.

Hypoxia also induces p53 accumulation in other cell types but the mechanism of p53 accumulation was thought to be distinct from other types of stresses, such as DNA damaging reagent. Hypoxia-inducible factor-1 (Hif-1) is a protein that becomes stabilized under hypoxic condition and induces the transcription of various hypoxia-associated genes such as *erythropoietin* and *vascular endothelial growth factor (VEGF)* gene. Hif-1 is also known to be required for hypoxia-induced p53 accumulation and potentiates p53-dependent transcription through direct binding. In the heart, we identified that CHIP (carboxyl terminus of Hsp70-interacting protein) as one of the E3 ubiquitin ligases that ubiquitinate p53 and induce its proteasomal degradation. Unlike other p53 ubiquitinating enzymes, such as MDM2, CHIP expression is not induced by p53 to form a negative-feedback loop, but rather inversely correlated with p53 expression in cardiomyocytes under hypoxic condition. We further demonstrated that Hif-1-mediated transcriptional repression of *CHIP* gene is responsible for p53 accumulation under hypoxic condition in cardiomyocytes and after myocardial infarction in the heart, presenting a novel mechanism for hypoxia-induced p53 accumulation.

Stability and transcriptional activity of p53 is determined by various post-translational modifications, such as phosphorylation, acetylation, and methylation. Phosphorylation of p53 affects p53 stability through preventing and promoting ubiquitination by MDM2. Acetylation affects transcriptional activity of p53 and methylation affects both stability and transcriptional activity of p53. We found that phosphorylation and methylation, but not acetylation of p53 was increased in the heart of pressure-overloaded mice. We are currently investigating the mechanism why p53 become phosphorylated and methylated during heart failure and identified a unique DNA damage response pathway and protein lysine methylation enzyme which may end in phosphorylation and methylation of p53.

Discussion & Conclusion

Elevation of p53 is observed in the failing heart of various etiology such as adriamycin, coronary artery ligation, and pressure-overload, suggesting that p53 elevation may become a common therapeutic target against heart failure, irrespective of its etiology.

In the present study, we investigated the mechanism of p53 elevation during in the failing heart of various etiologies. We found that p53 protein level in the heart become elevated by many mechanisms and the mechanism varied depending on the etiology of heart failure.

Therefore, intervention against the mechanism of p53 elevation may not be suitable for the general treatment of heart failure. Rather, intervention against p53 per se, through modifying its post-translational modification may be more suitable as a therapeutic strategy against heart failure.

一般の皆様へ

食生活の欧米化および高齢者の増加と共に日本でも心不全患者数は増加している。心不全に対して様々な薬物療法が開発されているものの、重症心不全患者の予後はきわめて不良であり、新しい治療法の開発が望まれている。我々はガン抑制遺伝子の一つである p53 が心不全で増えていることから、心不全に対する新たな治療法として p53 の増加を防ぐ、あるいはその作用を減らす、とい

う戦略を考えている。p53 はガン抑制遺伝子でもあるため、p53 の作用を抑制してしまうことでガンを誘導してしまう可能性が我々の治療戦略の欠点であると考えており、この欠点を防ぐため今後は心臓で特異的に p53 の増加を防ぐ、p53 の作用を減らす手法を開発していく必要がある。

Investigation on chronic kidney disease through signal transduction between vascular endothelial cells and podocytes.

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Abstract

Diabetes has a constantly growing prevalence and leads to a number of complications including diabetic nephropathy. In the present study, we aimed to investigate the role of cell-to-cell communication via the endothelin system on the development of diabetic nephropathy using genetically modified mice. Although our study has not been completed yet, we could demonstrate several results, suggesting that blocking specifically ET-1 actions in the podocytes from the renal glomerulus should prevent diabetes-induced proteinuria.

Key Words : endothelin, podocyte, endothelial cells, chronic kidney disease

Introduction

Diabetes has a constantly growing prevalence and leads to a number of complications including diabetic nephropathy. A novel therapeutic approach is needed to limit the renal and also the cardiovascular complications linked to diabetic nephropathy.

Endothelin-1 (ET-1) plays a major role in the development of DN. ET-1 antagonists reduce proteinuria in DN patients but induce fluid retention.

We hypothesized that these adverse secondary effects are probably due to the diuretic function of ET-1 in the renal tubular system. We believe that blocking specifically ET-1 actions in the podocytes from the renal glomerulus should prevent diabetes-induced proteinuria without adverse effects.

In the present study, we aimed to investigate the role of cell-to-cell communication via the endothelin system on the development of diabetic nephropathy using genetically modified mice.

Results

1) Generation of podocyte-specific endothelin receptor A knockout mice

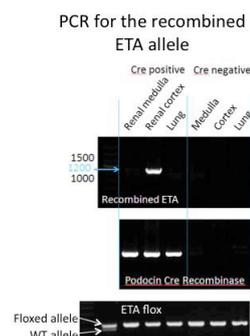
We have therefore generated mice knock-out for the ET-1 receptor A (ETA) specifically in the podocytes, the PodoETA KO mice. I have achieved this by crossing mice in which the ETA allele was floxed and mice expressing the Cre recombinase under the promoter of the podocin gene. The specific recombination of ETA has been proven by PCR.

Podocyte specific endothelin receptor A knockout mice (PodETA KO)

ETA floxed
X
Podocin Cre Recombinase

PodETA KO: ETA^{flax/flax}; Pod Cre (+)

Wild Type: ETA^{flax/flax}; Pod Cre (-)



2) Generation of diabetic model mice

We induced type 1 diabetes in seven weeks old endothelial cell-specific endothelin-1 knockout (VEETKO) male mice, podocyte specific endothelin receptor A knockout (PodETAKO) male mice and wild type (WT) littermates using streptozotocin (i.p., 50mg/kg, five consecutive days). Eight mice per group were analyzed.

Diabetes was defined as starving blood glucose level more than 300mg/dL. Body weight was monitored weekly.

3) Analysis of the phenotype of diabetic mice

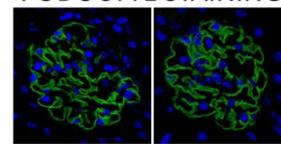
We measured blood pressure in awoken mice using the tail cuff method at 8 (early stage diabetes) and 28 weeks (late phase). Following this, we placed the animals in metabolic cages in order to collect 24h urine sample, measured urine volume, and monitored food and water consumption. At the end of the metabolic cages experiment, blood was withdrawn from the retro-orbital sinus for creatinine and hematocrit measurement.

At week 22, mice were sacrificed. Kidneys and hearts were harvested. From the left kidney, cortex and medulla was separated and both were frozen for protein and mRNA extraction. Half right kidneys were mounted in freezing medium or fixed in paraffin for immunofluorescence and histology.

We found that WEETKO mice had lower blood pressure than WT mice with and without diabetes. At base line, PodETAKO mice are similar to WT mice in terms of general appearance, kidney function and morphology. At the age of 28 weeks, the PodETAKO mice present a lower diastolic blood pressure and a higher serum creatinine concentration for which the reason is still unknown.

We have observed that after 8 weeks diabetes, proteinuria was lower in PodETAKO mice compared to WT mice, showing that ETA on podocytes is responsible for hyperglycaemia-induced proteinuria in vivo and participates in the development of DN. We are currently analysing the data after 28 weeks diabetes.

PODOCYTE STAINING



Wild type PodETAKO

Discussion & Conclusion

Analysis of the phenotypes of VEETKO mice and PodETAKO mice revealed that these mice are resistant to diabetes-induced renal damage. Although the data presented has not been completed yet, currently available observations suggest that the communications between endothelial cells and podocytes via ET-1–ETA receptor are involved in the development of diabetic nephropathy. Targeting the endothelin system by specific antagonist in kidney may provide the novel therapeutic

intervention towards diabetes-induced complications including diabetic nephropathy and cardiovascular disorders.

In cooperation with some pharmaceutical companies, we will test the efficacy of a new endothelin antagonist in our model. This experiment will investigate the benefit of blocking specifically the ETA on podocytes compared to a systemic unspecific blockade. Moreover, it will help determine the way of action of endothelin receptor antagonist in experimental diabetic nephropathy.

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一般の皆様へ

糖尿病の合併症のひとつである糖尿病性腎症は、わが国においても増加の一途をたどっており、有効な治療法の開発が社会から望まれています。今回、私たちはエンドセリンという分子が糖尿病性腎症の発症や進展に重要な役割を果たしていることを、遺伝子改変マウスを用いて明らかにし、エンドセリンの作用を抑制することが新しい治療法となることを示唆する成果を得ることができました。エンドセリン作用を抑制する薬剤は肺高血圧症という疾患に対しすでに臨床応用されており、今後糖尿病性腎症に対しても有効性を検討されることが期待されます。

Development of immunotherapy against disseminated *Candida* infection

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Abstract

Candida albicans is a major cause of invasive fungal infection. We studied if NKT cells play a role in the host defense against systemic *C. albicans* infection and if glycolipid mediated NKT cell activation affect on the course of *C. albicans* infection in diabetic mice. Our data show that NKT cells play a limited role in controlling systemic *C. albicans* infection and glycolipid mediated NKT cell activation exacerbates *C. albicans* infection in the diabetic mice. These results suggest that the regulation of NKT cell function may be beneficial for controlling inflammation during systemic *C. albicans* infection.

Key Words : NKT cell, *Candida albicans*, glycolipid

Introduction

Candida albicans is a major cause of invasive fungal infection. The mortality attributable to candidemia is very high, despite of the use of antifungal agents. Therefore, it is important to investigate the mechanisms of the immune response to *C. albicans* and develop a new therapy. NKT cells, innate lymphocytes that recognize glycolipid antigens, participate in the response to various microbes. We have shown that NKT cells play protective roles in the host defense against certain microbial infection. However, it is not known if NKT cells play a role in controlling *C. albicans* infection and if glycolipid mediated NKT cell activation affect on the course of *C. albicans* infection. In this study, we addressed these points.

Results

1. The effect of glycolipid treatment on the course of *Candida albicans* infection in mice with diabetes mellitus

In order to induce diabetes mellitus (DM), female C57BL/6 mice were injected intraperitoneally with streptozotocin (STZ) at 210 mg/kg. The mice with 200 - 500 mg/dL of glucose level in blood (on six days after STZ injection) were selected as DM mice. The DM mice were treated with antibiotics in drinking water (Gentamicin 0.1 mg/ml and Clindamycin 0.24 mg/ml) for more than 1 week in order to facilitate the colonization of *Candida albicans* in intestine.

The DM mice were inoculated intragastrically with *C. albicans* (2×10^8 cells) and 2 μ g of α -galactosylceramide (α -GalCer), a glycolipid that stimulates NKT cells potently, was intraperitoneally injected into these mice on either day 0, 1 or 2 after infection. The body weight

and fungal burden in feces were measured once a week, and the survival was monitored daily for 25 days.

All the mice treated with α -GalCer (regardless of the time point of injection; day 0, 1 or 2 after infection) lost their body weight up to 15%, whereas the body weight of control mice that had received Vehicle (Tween 20 containing buffer, solvent without α -GalCer) was not significantly changed. The fungal burden in feces was not significantly different between the control mice and the α -GalCer treated mice. Although all of the control mice were survived, some mice that had received α -GalCer on day 0 or 1, died within 25 days. Surprisingly, all of the mice that had received α -GalCer on day 0 died.

2. The role for NKT cells in controlling systemic *C. albicans* infection

To determine if NKT cells play a role in the host defense against *C. albicans*, we employed NKT deficient *J α 18KO* mice (provided by Dr. M. Taniguchi, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) and control wild type (WT) mice. We first examined the survival of *J α 18KO* mice and WT mice after intravenous infection with *C. albicans*. We infected these mice with the different doses of *C. albicans* and monitored the survival in three independent experiments. However, the mean survival time of WT mice and *J α 18KO* mice was equivalent in all the experiments and there was no significant difference between the two groups.

We next examined the fungal burden in the mouse kidneys at two different time points after *C. albicans* infection. The fungal burden in the kidneys was not significantly different between *J α 18KO* mice and WT mice at both time points. We also examined fungal burden in the livers of these mice, but there was no difference between the two groups.

Discussion & Conclusion

These results suggest that glycolipid mediated NKT cell activation exacerbates *C. albicans* infection in the DM mice. We speculate that NKT cell activation augments inflammatory response during systemic *C. albicans* infection in the DM mice.

Our data show that NKT cells do not play a major role in the clearance of the fungal pathogen in systemic *C. albicans* infection. It was reported that NKT cells did not contribute to microbial clearance during *Salmonella* infection, although these cells produced IFN γ in response to this pathogen. Similarly, NKT cells may not stimulate major effector cells to enhance clearance of the pathogen during *C. albicans* infection.

In conclusion, our data show that NKT cells play a limited role in controlling systemic *C. albicans* infection and glycolipid mediated NKT cell activation exacerbates *C. albicans* infection in the DM mice. These results suggest that the regulation of NKT cell function may be beneficial for controlling inflammation during systemic *C. albicans* infection.

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一般の皆様へ

カンジダは、免疫力の低下した方において菌血症(病原微生物が血中に侵入)をおこす重要な真菌で、致死率が高いことから、その病態解析及び治療法の開発は重要です。私達は、特定の細菌や真菌の感染において、感染防御に関与するNKT細胞というリンパ球に着目して、解析を行いました。その結果、カンジダ感染防御におけるNKT細胞の役割は限定的であること、糖尿病マウスにNKT細胞を活性化する糖脂質を投与すると病態が増悪することが分かりました。今後、NKT細胞の機能を抑制することで、真菌感染の増悪を制御できるか検討する必要があると考えられました。

Construction of chemical library of calyculin A and development of subtype selective phosphatase inhibitor

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Abstract

Calyculin A is a specific and potent inhibitor against protein phosphatases 1, 2A and 2B. We therefore tried to develop selective inhibitors against these subtype taking advantage of the scaffold of this natural product. Two different methods were applied to the synthesis of platform and fluorescent probe was also synthesized.

Key Words : calyculin A, marine sponge, protein phosphatase,

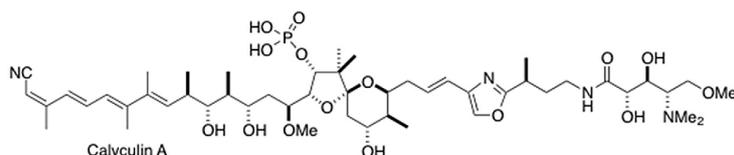
Introduction

Calyculin A is a major cytotoxic metabolite of a Japanese marine sponge *Discodermia calyx*. It has been known that this marine natural product is specific and potent inhibitor against protein phosphatase 1 and 2A as well as calcineurin (PP2B). These enzyme are potential targets of drug development for the purpose of antitumor and immunosuppressant. PP2A is the target enzyme of antitumor fostriecin, and moreover PP2B is well known as the target molecule of immunosuppressants such as tacrolimus and cyclosporine. Therefore, we launched the development of subtype-selective inhibitor against PPs on the basis of calyculin A.

Results

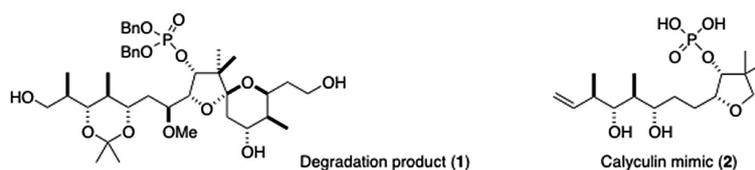
1) Degradation of Calyculin A

We first attempted to transform natural calyculin A to a small fragment containing spiroketal moiety as a platform of library construction. Taking advantage of olefins located at both sides of spiroketal core fragment, ozonolysis after protection of 1,3-diol and phosphate group successfully yielded the core fragment versatile for further introduction of side chains. However, the selective protection or modification of each hydroxyl group in the core fragment (**1**) obtained as triol was difficult. The selective modification of each terminal of core fragment is currently under investigation.



2) Chemical synthesis of calyculin mimics

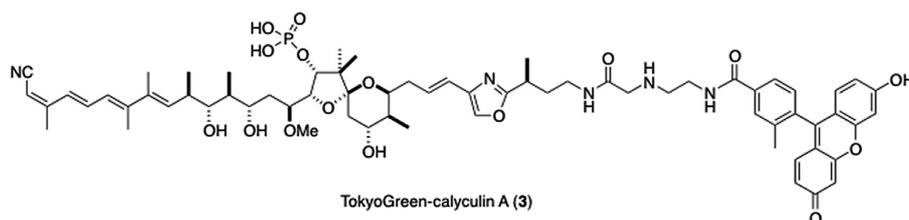
Another approach is chemical synthesis of the platform of library construction. In order to synthesize the platform easier, we designed the target molecule mimicking the partial structure of calyculin A corresponding to spiroketal moiety. Since the stereoselective synthesis of spiroketal moiety would require the multi-step transformations, the tetrahydrofuran ring bearing phosphate group was set as our target molecule (2). Starting from (*R*)-(-)-5-Oxotetrahydrofuran-2-carboxylic acid, diester generated from methanolysis was subjected to DIBAL reduction after protection of secondary hydroxyl group. The resulting aldehyde was subjected to Indium-mediated alkylation with prenyl bromide to introduce geminal dimethyl group characteristic for calyculin A. This reaction proceeded stereoselective manner and afforded desired alcohol, which will be phosphorylated in later step. Further chemical synthesis will be performed in due course.



3) Fluorescent probe of calyculin A

In the course of chemical transformation of calyculin A, we synthesized new analog having terminal aldehyde, which was versatile functionality for selective modification. In addition, the fluorescent probe of protein phosphatases had not been reported so far. Therefore, fluorescent probe derive from calyculin A was

designed and synthesized (3). The aldehyde was ligated with ethylenediamine and succeeding reductive amination successfully afford calyculin derivative bearing terminal amino group. Except for newly introduced amino groups, there is no other amino groups in calyculin A. Therefore, selective modification became possible despite of polyfunctional groups. Indeed, by the treatment with NHS ester of TokyoGreen, TokyoGreen was consequently introduced into terminal amino group.



Discussion & Conclusion

We tried to synthesize the platform of chemical library of calyculin A by two different methods. One was degradation of natural calyculin A and another was synthesis of calyculin mimic. Both methods are currently investigated and either of them will be applied to the construction of chemical library. In addition, fluorescent probe of calyculin A was successfully synthesized from natural calyculin A. In fact, this probe is the first fluorescent probe of protein phosphatases 1 and 2A. The

efficiency to visualize the localization and function of protein phosphatases will be examined in detail.

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一般の皆様へ

海綿 *Discodermia calyx* に含まれる Calyculin A は極めて選択的かつ強力なタンパク質脱リン酸化酵素の阻害物質として知られている。タンパク質脱リン酸化酵素のサブタイプは抗がん剤や免疫抑制剤の標的分子として知られている。そこで、我々は Calyculin A をもとに新たな医薬品のリード化合物を見出すことを目的として、サブタイプ選択的な化合物の創製を目指した。実際に Calyculin A の分解反応によって土台を合成するとともに、より簡略化した模倣分子の合成にも取り組んだ。また、タンパク質脱リン酸化酵素を可視化する蛍光プローブの合成にも成功した。

Roles of axon guidance protein, draxin in the corpus callosum formation

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Abstract

The correct selection of axonal path toward to their target is known accomplished by extracellular guidance molecules. We previously found a novel axon guidance protein, draxin, which is essential for the corpus callosum and thalamocortical projections. In this study, We addressed the importance of draxin expression in neocortical neurons for corpus callosum formation and thalamocortical projections by rescue experiments using transgenic mice. We found that the thalamocortical phenotype in *draxin*^{-/-} mice is sufficiently rescued by transgenic expression of *draxin* in neocortical neurons, suggesting that draxin from neocortical pioneer neurons is responsible for thalamocortical projections from the internal capsule to the neocortex.

Key Words : Axon guidance, draxin, corpus callosum, thalamocortical axon

Introduction

The correct wiring of the nervous system relies on the ability of axons and dendrites to locate and recognize their appropriate synaptic partners. The correct selection of axonal path toward to their target is known accomplished by extracellular guidance molecules such as the netrins, slits, semaphorins and ephrins. We have identified a novel secreted protein, draxin (dorsal repulsive axon guidance protein), which acts as a roof plate-derived chemorepellent for commissural axons in chick spinal cord. In this study, we focus on draxin functions in corpus callosum formation and thalamocortical projections in mouse brains.

Results

We previously found that *draxin* deficient mice show severe defects in axonal projection of the corpus callosum and thalamocortical axons. First, we checked the expression patterns of *draxin* by β -galactosidase staining of *draxin*^{+/-} mice. We found that draxin is expressed in the neocortical neurons and midline glial cells during corpus callosum formation. We also observed that draxin is strongly expressed in the neocortex and weakly expressed in the ventral telencephalon and dorsal thalamus during thalamocortical projections. Next, to examine the importance of draxin expression in neocortical neurons for corpus callosum formation and thalamocortical projections, we performed rescue experiments making use of transgenic mice (*Z/draxin*) in which Cre-mediated recombination results in the stable expression of *draxin* and *EGFP*. Induction of draxin expression in the neocortex of *draxin*^{-/-} mice was achieved by crossing *Z/draxin*^{+/+}; *draxin*^{-/-} mice with *Emx1*^{Cre/+}; *draxin*^{+/-} mice,

which show specific Cre expression in the dorsal telencephalon. Compared with *draxin* KO (*Emx1*^{+/+}; *Z/draxin*^{+/-}; *draxin*^{-/-}) mice, the neocortex of *Emx1*^{Cre/+}; *Z/draxin*^{+/-}; *draxin*^{-/-} (*Ctx-draxin*) mice was greatly reduced in size, suggesting that proliferation and/or cell death of neocortical progenitors might be affected. We examined the corpus callosum and thalamocortical projections in *Ctx-draxin* mice. We could not observe the rescue of corpus callosum in the mice. Importantly, we found that some of the thalamocortical axons turned into the neocortex in the *Ctx-draxin* mice, even though the number of corticofugal axons was greatly reduced. In agreement with this observation, Dil injection into the dorsal thalamus revealed that pathfinding errors of thalamocortical axons at the PSPB were sufficiently rescued in *Ctx-draxin* mice. These results suggest that *draxin* from neocortical neurons is critical for the guidance of thalamocortical axons into the neocortex.

Discussion & Conclusion

Our data demonstrate that *draxin* from neocortical neurons is critical for the guidance of thalamocortical axons into the neocortex, suggesting that *draxin* is a responsible guidance molecule from the neocortical neurons for thalamocortical axons. These data offer important clues to understanding the molecular mechanisms underlying the interaction between neocortical pioneer axons and thalamocortical axons, which is essential for thalamocortical projections. I believe that this finding will not only deepen our understanding of thalamocortical projections but also provide insights into axon guidance mechanisms by heterotypic axon interactions, which are necessary for the formation of complex neural networks in the brain.

一般の皆様へ

脳では膨大な数の神経細胞がそれぞれ適切な位置に配置され、精密な神経回路網を形成しています。これらの神経回路形成には、発生過程に神経軸索ガイダンス分子が担う神経細胞の選択的な軸索形成が必須です。本研究では、我々が独自に発見したガイダンス分子 *draxin* に着目した脳神経回路形成メカニズムの解明を目指しています。特に、既知の軸索ガイダンス分子ではなく、*draxin* が特有の機能を持つと考えられる脳梁と視床皮質軸索投射の分子機構を調べています。こうした研究から複雑な脳回路がどのようにして形成されるかを理解したいと考えています。

III.

Reports from the Recipients of Grants for International Meetings

The 13th TNF International Conference

1. Representative

Shigekazu Nagata, Ph.D.

Professor, Kyoto University, the Graduate School of Medicine

2. Opening period and Place

May 15 (Sun) - 18 (Wed), 2011

3. Number of participants / Number of participating countries and areas

Participants: 208

Countries: 19 (Australia, Belgium, Canada, Czech Republic, France, Germany, Israel, Italy, Japan, Romania, Russia, Singapore, Slovenia, Spain, Switzerland, Taiwan, The Netherlands, United Kingdom, USA)

4. Total cost

JPY28,201,247

5. Main use of subsidy

Travel expenses for invited speakers from abroad

6. Result and Impression

208 scientists from 19 different countries attended the 13th International TNF Conference in spite of the challenging circumstances after the tragedy on May 11, 2011. As previous TNF congresses, the discussion was very active. We were very glad to see many young scientists including Japanese graduate students actively engaged in the discussion. We are sure all the participants have enjoyed Science, and this meeting would trigger a new collaboration or new friendship among the participants.

7. Additional description



10th International Family Nursing Conference

1. Representative

Kazuko Ishigaki

President of the Japanese Association for Research in Family Nursing

2. Opening period and Place

June 25-27, 2011

Kyoto International Conference Center

3. Number of participants / Number of participating countries and areas

1,007 individuals from 34 countries

4. Total cost

¥ 48,424,123

5. Main use of subsidy

International invitation & program printing

6. Result and Impression

Although we have agonized until the last minute in deciding whether we should go ahead and have this conference after the Great East Japan Earthquake, the conference was a great success with the total number of participants exceeding 1,000 from as many as 34 countries. We have added an educational session on “Family in Disaster Nursing.” We have received many positive comments from the participants from Japan and overseas.

7. Additional description

Total cancellation from overseas due to earthquake and nuclear power plant accident was about 10% of the original applications.

We appreciate your generous grant for this conference.

XV International Congress of Molecular Plant-Microbe Interactions

1. Representative

Ko Shimamoto

Professor at Nara Institute of Science and Technology

2. Opening period and Place

July 29 to August 2, 2012

Kyoto International Conference Center

3. Number of participants / Number of participating countries and areas

993 participants from 42 countries

4. Total cost

JPY 69,232,990

5. Main use of subsidy

Printing and extra prize for poster awards

6. Result and Impression

XV IS-MPMI 2012 Kyoto was held as an interdisciplinary conference with attendance of a number of scientists of related fields including biochemistry, molecular biology, cell biology and genetics.

Research presentations have been made to find solutions to such problems as food shortage and energy supply on a global scale. For stabilization of food production, discussions were made on countermeasures against plant diseases that cause damage to various crops the world over. As approaches to energy problems, presentations were made to discuss possibilities of improvement of production of such plants for bio-based fuels as corn and sugar cane.

In spite of importance and rapid growth of study of this field, population of researchers in the field is very small in Japan compared to Europe and the United States. In addition, other Asian nations have even smaller population of researchers in this field, because advanced method is required for research. Therefore, Japanese scientists are expected to play a leading role among Asian countries in the coming future. As one of most advanced fields of study, our study would have great influence on other scientific fields of plants, so it is meaningful to have an international meeting like this in this country.

For this conference, close to 1000 people from 42 countries gathered to Kyoto. Most of participants were from abroad indicating that this field of study has importance on a global basis. While this conference has been held so far in Europe or the United States, this time we held it for the first time outside Europe and the United States. Characteristically, the number of participants from China, Korea and Taiwan came close to 150, having increased significantly compared to the

last meeting. This would create an opportunity for this field of study to make drastic progress in Asian region. Although this meeting was to be held in August 2011 originally, we postponed it by one year due to the Great East Japan Earthquake. Despite concern for a decrease in participants, we had as many participants as ever before.

7. Additional description

To encourage active participation of young researchers, we discounted students' registration fee as well as offered travel awards for students. On the other hand, in an effort to foster researchers in future generations we had workshops chaired by young researchers on the first day of conference.

The 6th International Conference of Neurons and Brain Diseases (ICNBD)
(by Association for Neuron and Diseases)

1. Representative

Dr. Kaoru Inokuchi

(Professor; University of Toyama, Graduate School of Medicine & Pharmaceutical Sciences)

2. Opening period and Place

3th August - 5th August 2011

ANA Crowne Plaza Toyama

3. Number of participants / Number of participating countries and areas

121 / 8

4. Total cost

9,650,636 JPY

5. Main use of subsidy

Conference room charge

6. Result and Impression

ICNBD Toyama, the joint conference of the 6th International Conference of Neurons and Brain Diseases and Japan Science and Technology Agency, JST, was held in the city of Toyama, Japan. AND has been established as a nonprofit organization to promote scientific exchange among us. ICNBD Toyama was the first annual meeting of AND since the establishment of AND. In this memorable conference, we have an excellent program of 47 talks and 53 poster exhibitions. All the attendees developed new understanding and insight that would lead to the creation of new concept in the neuroscience field. Attendees were highly interactive both in the formal scientific sessions and in the informal social events. That we hold an international conference such as ICNBD Toyama in recent adverse circumstances in Japan caused by the huge earthquake in North East region of Japan on March 11, 2011 would, I believe, has encouraged Japanese scientists, students, and postdoctoral fellows.

Finally, I would like to express our sincere thanks to The NOVARTIS Foundation (Japan) for the Promotion of Science for financial support.

7. Additional description

This conference, ICNBD, will be annually held, and next year venue will be Montreal, Canada.

*XXV Symposium of the International Association for Comparative Research on
Leukemia and Related Diseases (IACRLRD)*

1. Representative

Watanabe Toshiki

The University of Tokyo Professor

2. Opening period and Place

September 15 (Thu)-17 (Sat), 2011

3. Number of participants / Number of participating countries and areas

184 participants / 14 countries and areas

4. Total cost

31,530,332 Yen

5. Main use of subsidy

The cost of conference website production

6. Result and Impression

This was the jubilee symposium for the 50 years anniversary of the IACRLRD. Although the number of participants was not so large, all of them agreed that this symposium was a great success. The speakers were the leaders of the hematology/oncology in the world. Lectures were all those that report the “state of arts” of the diagnosis and treatments of leukemias and lymphomas, thus this symposium was an exceptional opportunity for the participants to update their knowledge in the field. In addition, on September 17th, the symposium co-organized an international symposium of the HTLV-1 and related diseases. This was also an exceptional chance to learn the recent achievements in the HTLV-1 and related diseases covering both basic and clinical studies.

7. Additional description

For the 50th anniversary, 5 special lectures were programmed, in addition to John Moloney Memorial Lecture and Yohei Ito Memorial Lecture. These lectures covered the areas of basic sciences in the oncology such as oncogenic retroviruses and the most recent achievements in the treatment of acute and chronic myelogenous leukemias. Thus, this symposium provided a rare opportunity to study both the history and most updated information of basic and clinical sciences of hematology and oncology.

8th AFMC International Medicinal Chemistry Symposium (AIMECS11)

1. Representative

Masakatsu Shibasaki

Symposium Chairperson. Institute of Microbial Chemistry, Tokyo, Japan

2. Opening period and Place

29 November- 2 December, KEIO PLAZA HOTEL TOKYO

3. Number of participants / Number of participating countries and areas

1100 participants, 23 countries

4. Total cost

¥66,000,000

5. Main use of subsidy

Printing – Program & Abstract book

6. Result and Impression

The eighth AFMC international medical chemistry symposium (AIMECS11) was held from Tuesday, November 29 to Friday, December 3 at Keio Plaza Hotel (Shinjuku). Dr. Masakatsu Shibasaki (Emeritus Professor of University of Tokyo and Director of Microbial Chemistry Research Center) was a symposium chairperson. This symposium is organized jointly by the Asian federation for Medicinal Chemistry (AFMC) and the Pharmaceutical Society of Japan (PSJ) in collaboration with the Medicinal Chemistry Division of the America Chemical Society (ACS-MEDI) and the European Federation for Medicinal Chemistry (EFMC).

Emphasis of this symposium is focused on the “Frontier of Medicinal Science”, and there are 6 plenary lecturers by globally distinguished scientist, 27 invited lectures in 21 sessions, and 578 poster presentations along with 1,125 participants. The numbers of foreign people were 174 from 22 countries. The number of participants and presentations of this symposium was highest among the past of this symposium.

7. Additional description

The most important result is that this symposium could be shown the high scientific level and possibility of drug discovery on Japanese pharmaceutical industry even after the earthquake on March, 2011.

25th Grant Report (FY2011)

The foundation has been conducting public interest activities such as research grant, meeting grant and international exchange program since its establishment on Sep. 4, 1987 in Japan under authorization of the Ministry of Education, Science, Sports and Culture. The grants conducted in FY 2011 are as follows.

25th Novartis Research Grant: 41 Researchers (JPY 1 mil.), Subtotal JPY 41 mil.
 Research Meeting Grant: 6 Meetings (JPY 0.4 mil.), Subtotal JPY 2.4 mil.
Total JPY 43.4 mil.

25th Novartis Research Grant (FY2011)

The grant is to aim supporting creative research in Japan in the field of bio, life science and relevant chemistry.

(Order received, Honorifics omitted, * Great East Japan Earthquake supporting)

#	Name	Institution	Title	Research Project
1	Yoshikazu Ugawa*	School of Medicine, Fukushima Medical University	Professor	Ageing effects on the neuroplasticity of human brain and Alzheimer's disease
2	Takahiro Suzuki	Faculty of Pharmaceutical Sciences, Tokyo University of Science	Assistant Professor	Synthetic study of non-alkaloidal diterpene isolated from the genus Aconitum
3	Hayato Ishikawa	Graduate School of Science and Technology, Kumamoto University	Associate Professor	Synthetic Study of Polymeric Pyrrolidinoindoline Alkaloids via Biomimetic Route
4	Takayuki Shindo*	Shinshu University Graduate School of Medicine	Professor	Regulation of vascular integrity by AM-RAMP system and its application
5	Hideaki Kano	School of Science, The University of Tokyo	Associate Professor	Development of novel molecular imaging method for visualization of clinical condition of intravital lipids
6	Masatake Osawa	Gifu University, Graduate School of Medicine	Professor	Role of autophagy in the induction of cornification of epidermal cells
7	Yasuhiko Horiguchi	Research Institute for Microbial Diseases, Osaka University	Professor	Analysis for the molecular mechanisms causing paroxysmal cough in pertussis
8	Akihiro Harada	Graduate School of Medicine, Osaka University	Professor	Analysis of mice deficient in molecules involved in intracellular polarized transport
9	Kentaro Nakano*	Graduate School of Life and Environmental Sciences, University of Tsukuba	Assistant Professor	Investigation of molecular signal output from a dividing nucleus to cell cortex
10	Takashi Maruyama*	Graduate School of Life Sciences, Tohoku University	Assistant Professor	The Role of transcription factor I κ B- ζ in immunity homeostasis
11	Tetsushi Sadakata*	Advanced Scientific Research Leaders Development Unit, Gunma University	Assistant Professor	Study of dense-core vesicle synthesis on the membrane of Golgi complex
12	Toshiharu Sakurai	Kinki University, Faculty of Medicine	Assistant Professor	Stress response, homeostasis and cancer
13	Hidenori Suzuki	Mie University Hospital, Department of Neurosurgery	Associate Professor	Regulation of cerebral vasospasm by growth factors and extracellular proteins
14	Daiju Kitagawa	National Institute of Genetics	Associate Professor	Towards elucidating the mechanisms of the step-wise formation of centrioles by using AID system
15	Akinori Akaike	Graduate School of Pharmaceutical Sciences, Kyoto University	Professor	The role of specific cell adhesion molecules in dopaminergic innervation of striatal neurons

#	Name	Institution	Title	Research Project
16	Satoshi Nishimura*	Department of Cardiovascular Medicine, The University of Tokyo	Assistant Professor	In vivo imaging reveals molecular mechanisms of adult common diseases based on chronic inflammation
17	Hitoshi Kurumizaka	Waseda University, Graduate School of Advanced Science & Engineering	Professor	Structural study of the human centromeric chromatin
18	Takuya Suzaki	National Institute for Basic Biology	Assistant Professor	An intrinsic molecular mechanism governing the development of root nodule primordia
19	Yasuhiro Izumiya	Faculty of Life Sciences, Kumamoto University	Assistant Professor	Identification and characterization of novel skeletal muscle-derived angiogenesis growth factor
20	Kazuhiko Ikeda*	Department of Cardiology and Hematology, Fukushima Medical University	Assistant Professor	Molecular mechanism of self-renewal and proliferation of a hematopoietic cell due to HMGA2 expression
21	Keizo Kanasaki	Kanazawa Medical University, Diabetes & Endocrinology	Senior Assistant Professor	The biological and pathological significance of Stox1 transcriptional factor
22	Tomoe Nishitani	National Cerebral and Cardiovascular Center	Laboratory Chief	Novel role of neuronal Ca ²⁺ sensor-1 as a cardioprotective factor in stressed heart
23	Masayo Kagami	The National Research Institute for Child Health and Development	Senior Investigator	Elucidation of the physiological function of imprinted genes on human chromosome 14
24	Hiroyuki Niida	Hamamatsu University School of Medicine	Associate Professor	Mechanism of dNTPs supply at beginning of DNA replication
25	Kentaro Nakagawa	Tokyo Medical and Dental University	Assistant Professor	Characterization of mammalian homologue of Unc119 as a tumor suppressor
26	Shu Shimada	Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University	Assistant Professor	Clarification of the mechanism and development of the treatment of diffuse-type gastric cancer using our mouse model
27	Kenji Osafune	Center for iPS Cell Research and Application, Kyoto University	Associate Professor	Characterization and generation of organ-specific vascular cells from human iPS cells towards development of novel in vitro models for intractable vascular diseases
28	Kuninori Suzuki	Frontier Research Center, Tokyo Institute of Technology	Associate Professor	Role of lipid droplets during autophagy in <i>Saccharomyces cerevisiae</i>
29	Takanori Iwasaki	Graduate School of Engineering, Osaka University	Assistant Professor	Synthesis of fatty acids having various carbon frameworks
30	Hisao Tsukamoto	National Institute for Molecular Sciences	Research Assistant Professor	Analyses of structural dynamics of a G protein-coupled receptor using site-directed fluorescent labeling
31	Naoto Ishii*	Tohoku University Graduate School of Medicine	Professor	Regulation of the generation and maintenance of memory T cells
32	Takaya Satoh	Graduate School of Science, Osaka Prefecture University	Professor	Studies on the regulatory mechanisms underlying cell cycle-dependent centrosome replication
33	Yuichiro Mishima	Graduate School of Science, Kobe University	Research Scientist	microRNA-mediated regulation of vasculogenesis
34	Akiko Hashimoto-Tane	RIKEN Research Center for Allergy and Immunology	Researcher	The role of molecular motor for regulating T cell activation
35	Wataru Ise	Immunology Frontier Research Center, Osaka University	Associate Professor	Regulation of humoral immune response by the transcription factor BATF
36	Yuzuru Imai*	Juntendo University Graduate School of Medicine	Associate Professor	Study on the molecular mechanisms of mitochondrial regulation by the genes responsible for early-onset familial forms of Parkinson's disease
37	Tomoaki Tanaka	Chiba University Graduate School of Medicine	Assistant Professor	Fundamental study for long intergenic RNA (linc RNA) and epigenetic regulation by tumor suppressor p53 using ES/iPS cells to control cancer stem cells
38	Takaaki Abe*	Tohoku University Graduate School of Biomedical Engineering	Professor	Drugs discovery for CKD patients using transporter manipulation

#	Name	Institution	Title	Research Project
39	Terukazu Nogi	Association of International Arts and Science, Yokohama City University	Associate Professor	Molecular mechanism of semaphorin receptor activation implicated in axon-pathfinding
40	Masakiyo Sakaguchi	Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences	Associate Professor	Promoter-based super improvement of gene expression in an antibody production
41	Yoshihiro Kawasaki	Institute of Molecular and Cellular Biosciences, The University of Tokyo	Lecturer	Analysis of a novel mechanism of tumorigenesis involving c-Myc proto-oncogene product

FY 2011 Research Meeting Grant

The grant is to aim supporting international research meetings in Japan in the field of bio, life science and relevant chemistry.

#	Meeting	Date (Place)	Representative	
			Institution / Title	Name
1	The 13th TNF International Conference	2011.5.15 ~ 5.18 (Awaji)	Graduate School of Medicine, Kyoto Univ. Prof.	Shigekazu Nagata
2	10th International Family Nursing Conference Making Family Nursing Visible	2011.6.25 ~ 6.27 (Kyoto)	Chiba Prefectural Univ. of Health Sciences Prof.	Kazuko Ishigami
3	The 15th Congress on Molecular Plant-Microbe Interactions	2011.8.2 ~ 8.6 (Kyoto)	Nara Institute of Science and Technology	Ko Shimamoto
4	The 6th International Conference of Neurons and Brain Diseases	2011.8.3 ~ 8.5 (Toyama)	Faculty of Medicine, Univ. of Toyama Prof.	Kaoru Inokuchi
5	XXV Symposium of the International Association for Comparative Research on Leukemia and Related Diseases	2011.9.15 ~ 9.17 (Tokyo)	Graduate School of Frontier Sciences, Univ. of Tokyo Prof.	Toshiki Watanabe
6	8th AFMC International Medicinal Chemistry Symposium	2011.11.29 ~ 12.2 (Tokyo)	Toho Univ. Prof.	Yusaku Yokoyama

第 25 期 (2011 年度) 助成事業報告

当財団は、文部大臣の認可を得て 1987 年 9 月 4 日に設立されて以降、研究助成を中心とした公益事業を行っております。2011 年度は、下記の総額 4,340 万円の助成事業を実施しました。

第 25 回ノバルティス研究奨励金 41 件 (1 件 100 万円) 4,100 万円
 研究集会助成 6 件 (1 件 40 万円) 240 万円
 総額 4,340 万円

第 25 回ノバルティス研究奨励金 (2011 年度)

この事業は、生物・生命科学および関連する化学の領域において、我が国で行われる創造的な研究の助成を目的としています。

(受付順・敬称略、所属・職位は申請時、贈呈額は 1 件 100 万円、* は震災支援 10 件)

No	氏名	所属	職位	研究課題
1	宇川 義一*	福島県立医科大学	教授	ヒト中枢神経可塑性に対する加齢の影響とアルツハイマー病
2	鈴木 孝洋	東京理科大学薬学部	助教	新規骨格を有するトリカブト属由来の非アルカロイド性ジテルペンの全合成研究
3	石川 勇人	熊本大学大学院自然科学研究科	准教授	多量体型ピロリジノインドリンアルカロイド類の生合成模倣的全合成研究
4	新藤 隆行*	信州大学大学院医学系研究科	教授	AM-RAMP システムによる血管制御機構の解明と応用展開
5	加納 英明	東京大学大学院理学系研究科	准教授	生体内脂肪の病態を可視化する新しい分子イメージング法の開発
6	大沢 匡毅	岐阜大学大学院医学系研究科	教授	皮膚上皮角質層形成の分子的機構の解明
7	堀口 安彦	大阪大学微生物病研究所	教授	百日咳における発作性咳嗽の発症メカニズムの解析
8	原田 彰宏	大阪大学医学系研究科	教授	細胞内の極性輸送を司る蛋白のノックアウトマウスの分子細胞生物学的解析
9	中野 賢太郎*	筑波大学大学院生命環境科学研究科	講師	細胞表層と分裂核を結びつける分子シグナルの解明
10	丸山 貴司*	東北大学大学院生命科学研究科	助教	免疫恒常性における転写因子 I κ B α の役割
11	定方 哲史*	群馬大学先端科学研究指導者育成ユニット	助教	有芯小胞はゴルジ体にてどのように生まれるか?
12	櫻井 俊治	近畿大学医学部	講師	ストレス応答蛋白による恒常性維持及び発癌制御機構の解明
13	鈴木 秀謙	三重大学医学部附属病院	講師	増殖因子とマトリックス細胞間蛋白に着目した脳血管攣縮の病態解明
14	北川 大樹	国立遺伝学研究所新分野創造センター	特任准教授	AID 法を用いた中心小体の段階的構築原理の解明
15	赤池 昭紀	京都大学大学院薬学研究科	教授	ドパミンニューロンによる線条体神経支配における特異的細胞接着因子の役割
16	西村 智*	東京大学医学系研究科	特任准教授	生体分子イメージングを用いた慢性炎症を背景とする生活習慣病の病態解明
17	胡桃坂 仁志	早稲田大学理工学術院先進理工学部	教授	染色体の均等分配を保証しているセントロメア領域の高次クロマチンの立体構造解析

No	氏名	所属	職位	研究課題
18	寿崎 拓哉	自然科学研究機構 基礎生物学研究所	助教	根粒原基形成を司る生体内分子機構
19	泉家 康宏	熊本大学大学院 生命科学研究部	助教	骨格筋由来の新規血管新生因子の同定 とその機能解析
20	池田 和彦*	福島県立医科大学	助教	HMG2A2 発現による造血細胞の自己複製・増殖における分子機構の解明
21	金崎 啓造	金沢医科大学	講師	転写因子 Stox1 の生物学的・病理学的意義の検討
22	西谷 友重	国立循環器病 研究センター	室長	新たな心筋保護因子としての Ca ²⁺ センサー NCS-1 の役割とその調節
23	鏡 雅代	国立成育医療 研究センター研究所	上級 研究員	14 番染色体インプリンティング遺伝子の生理学的機能の解明
24	丹伊田 浩行	浜松医科大学	准教授	DNA 複製開始時における dNTPs 供給機構
25	中川 健太郎	東京医科歯科大学	助教	網膜色素変性症原因遺伝子産物、線虫 Unc119 哺乳類ホモログの腫瘍抑制機能の解析
26	島田 周	東京医科歯科大学大学院医歯 学総合研究科	特任 助教	胃がんマウスモデルを利用した未分化型胃がんの発症機構の解明と新規治療方法の開発
27	長船 健二	京都大学 iPS 細胞研究所	准教授	難治性血管疾患に対する新規疾患モデル開発を目指した臓器特異的血管細胞の同定とヒト iPS 細胞からの分化誘導
28	鈴木 邦律	東京工業大学 フロンティア研究機構	特任 助教	出芽酵母のオートファジーにおける脂肪滴の役割
29	岩崎 孝紀	大阪大学大学院 工学研究科	助教	多様な炭素骨格を有する脂肪酸類の合成
30	塚本 寿夫	自然科学研究機構 分子科学研究所	特任 助教	部位特異的蛍光標識を用いた G タンパク質共役受容体の動的構造変化の解析
31	石井 直人*	東北大学大学院 医学系研究科	教授	記憶 T 細胞の産生と維持の制御機構の解明
32	佐藤 孝哉	大阪府立大学大学院 理学系研究科	教授	細胞周期に同調した中心体複製機構の解明
33	三嶋 雄一郎	神戸大学大学院 理学研究科	学術 研究員	microRNA による血管形成制御機構の研究
34	多根 彰子	理化学研究所免疫・アレルギー 科学総合研究センター	研究員	細胞内分子モーターによる T 細胞抗原応答制御メカニズムの解明
35	伊勢 渉	大阪大学免疫学フロンティア 研究センター	特任 准教授	転写因子 BATF による抗体産生応答の制御
36	今居 譲*	順天堂大学医学研究科	専任 准教授	若年性パーキンソン病原因遺伝子によるミトコンドリア機能制御の分子メカニズムの解明
37	田中 知明	千葉大学大学院 医学研究院	講師	がん幹細胞制御を目的としたヒト ES/iPS における linc RNA 探索と p53 によるエピゲノム制御機構の解析
38	阿部 高明*	東北大学大学院 医工学研究科	教授	トランスポーターを介した腎不全治療薬開発
39	禾 晃和	横浜市立大学学術院 国際総合科学群	准教授	神経軸索の伸長をガイドするセマフォリン受容体活性化の分子機構
40	阪口 政清	岡山大学大学院 医歯薬学総合研究科	准教授	抗体大量産生用超高効率遺伝子発現ベクターの開発
41	川崎 善博	東京大学 分子細胞生物学研究所	講師	癌原遺伝子産物 c-Myc による新たな発癌機構の解明

2011 年度 研究集会助成

この事業は、生物・生命化学および関連する化学の領域において、我が国で開催される国際色豊かな研究集会の助成を目的としています。

(受付順・敬称略、所属・職位は申請時、贈呈額は1件40万円)

No	研究集会名	開催日 (開催地)	代表者	
			所属・職位	氏名
1	第13回 TNF 国際会議	2011.5.15～5.18 (淡路)	京都大学大学院 医学系研究科 教授	長田 重一
2	第10回国際家族看護学会	2011.6.25～6.27 (京都)	千葉県立 保健医療大学 教授	石垣 和子
3	第15回国際分子植物微生物 相互作用学会	2011.8.2～8.6 (京都)	奈良先端科学技術 大学院大学 教授	島本 功
4	第6回ニューロンと脳疾患 国際カンファレンス	2011.8.3～8.5 (富山)	富山大学大学院 医学薬学研究部 教授	井ノ口 馨
5	第25回国際比較白血病学会・ 国際シンポジウム	2011.9.15～9.17 (東京)	東京大学大学院 新領域創成科学研究科 教授	渡邊 俊樹
6	第8回 AFMC 国際医薬化学 シンポジウム	2011.11.29～12.2 (東京)	東邦大学薬学部 教授	横山 祐作

25th Financial Report

Balance Sheet

As of March 31, 2012

(Unit: JP Yen)

Account	Amount
I Assets	
1. Current Assets	
Total Current Assets	66,425,041
2. Fixed Assets (Long-term)	
(1) Fundamental Property	
Investments in Securities (Designated)	1,000,000,000
Investments in Securities (General)	100,000,000
Total Fundamental Property	1,100,000,000
(2) Other Long-term Assets	
Total Other Long-term Assets	39,893,756
Total Long-term Assets	1,139,893,756
Total Assets	1,206,318,797
II Liabilities	
1. Current Liabilities	
Total Current Liabilities	41,093,720
Total Liabilities	41,093,720
III Equity (Net Assets)	
1. Designated Net Assets	
Total Designated Net Assets	1,000,000,000
(Amount appropriating to Fundamental Property)	(1,000,000,000)
2. General Net Assets	165,225,077
(Amount appropriating to Fundamental Property)	(100,000,000)
Total Equity (Net Assets)	1,165,225,077
Total Liabilities & Equity	1,206,318,797

Income & Expenditure Statement

April 1, 2011 - March 31, 2012

(Unit: JP Yen)

Account	Amount
I Business Activity	
1. Business Activity Income	
Donation	40,048,000
Business Activity Income Total	75,376,374
2. Business Activity Expenditure	
Project Expense	54,377,469
Grant Expense	43,400,000
Administrative Expense	4,691,785
Business Activity Expenditure Total	59,069,254
Business Activity Balance	16,307,120
II Investment Activity	
1. Investment Activity Income	
Investment Activity Income Total	10,070,000
2. Investment Activity Expenditure	
Investment Activity Expenditure Total	9,830,294
Investment Activity Balance	239,706
III Financial Activity	
1. Financial Activity Income	
Financial Activity Income Total	0
2. Financial Activity Expenditure	
Financial Activity Expenditure Total	0
Financial Activity Balance	0
Balance of Current Period	16,546,826
Balance brought from Previous Period	8,784,495
Balance to be carried to Next Period	25,331,321

第25期(2011年度)財務報告

貸借対照表

2012年3月31日現在

(単位:円)

科 目	金 額
I 資産の部	
1. 流動資産	
流動資産合計	66,425,041
2. 固定資産	
(1) 基本財産	
投資有価証券(指定)	1,000,000,000
投資有価証券(一般)	100,000,000
基本財産合計	1,100,000,000
(2) その他固定資産	
その他固定資産合計	39,893,756
固定資産合計	1,139,893,756
資産合計	1,206,318,797
II 負債の部	
1. 流動負債	
流動負債合計	41,093,720
負債合計	41,093,720
III 正味財産の部	
1. 指定正味財産	
指定正味財産合計	1,000,000,000
(うち基本財産への充当額)	(1,000,000,000)
2. 一般正味財産	165,225,077
(うち基本財産への充当額)	(100,000,000)
正味財産合計	1,165,225,077
負債及び正味財産合計	1,206,318,797

収支計算書

2011年4月1日～2012年3月31日

(単位:円)

科 目	決 算 額
I 事業活動収支の部	
1. 事業活動収入	
寄付金収入	40,048,000
事業活動収入計	75,376,374
2. 事業活動支出	
事業費支出	54,377,469
助成金支出	43,400,000
管理費支出	4,691,785
事業活動支出計	59,069,254
事業活動収支差額	16,307,120
II 投資活動収支の部	
1. 投資活動収入	
投資活動収入計	10,070,000
2. 投資活動支出	
投資活動支出計	9,830,294
投資活動収支差額	239,706
III 財務活動収支の部	
1. 財務活動収入	
財務活動収入計	0
2. 財務活動支出	
財務活動支出計	0
財務活動収支差額	0
当期収支差額	16,546,826
前期繰越収支差額	8,784,495
次期繰越収支差額	25,331,321

List of Board Members

[Board of Trustees]

As of Oct. 1, 2012

Post	Name	Title
Chairman	Akimichi KANEKO	Dean, Prof., MD, Grad. School of Health Science, Kio Univ., Emer. Prof., Keio Univ.
Trustee	Shigetaka ASANO	Prof., MD, School of Science & Engineering, Waseda Univ., Emer. Prof., Univ. of Tokyo
	Masao ENDOH	Emer. Prof., MD, Yamagata Univ.
	Hiroko ISHIKAWA	Managing Director, Novartis Pharma K.K.
	Toshio SUDA	Prof., MD, Keio Univ., School of Medicine
Auditor	Tokuzo NAKAJIMA	Certified Public Accountant
	Masanori FUSE	Head, Accounting Dept., Novartis Pharma K.K.

[Board of Councilors]

Post	Name	Title
Chairman	Tsuneyoshi KUROIWA	Director, Research Information Center of Extremophile, Prof., Grad. School of Science, Rikkyo Univ., Emer. Prof., Univ. of Tokyo
Councilor	Norio AKAIKE	Director, Kumamoto Health Science Univ., Emer. Prof., Kyushu Univ.
	Max M. BURGER	Vice Chairman, Novartis Science Board Prof., MD, Univ. of Basel
	Hiroyuki KAWASHIMA	Former Prof., Grad. School of Medical & Dental Sciences, Niigata Univ.
	Shigeo KOYASU	Prof., Keio Univ., School of Medicine
	Tadanori MAYUMI	Univ. Emer. Prof., Osaka Univ.
	Hiroyuki MITANI	President & CEO, Novartis Pharma K.K.
	Miwako MORI	Prof., Health Sciences Univ. of Hokkaido, Emer. Prof., Hokkaido Univ.
	Akihiko NAKANO	Prof., Univ. of Tokyo, Science Dept., Team Leader, RIKEN (Inst. of Physical & Chemical Research)
	Masakatsu SHIBASAKI	Director, Microbial Chemistry Research Center, Microbial Chemistry Research Foundation
	Takao SHIMIZU	Prof., Univ. of Tokyo, School of Medicine
	Toshifumi WATANABE	Corporate Officer, Head, Scientific Affairs Division, Novartis Pharma K.K.

[Grantee Selection Committee]

Post	Name	Title
Chairman	Masataka SATA	Prof., MD, Inst. of Health Biosciences, Univ. of Tokushima
Member	Hidenori ICHIJO	Prof., Dentist, Grad. School of Pharmaceutical Sciences, Univ. of Tokyo
	Nobuya INAGAKI	Prof., MD, Grad. School of Medicine, Kyoto Univ.
	Akihiro UMEZAWA	Director, MD, National Inst. for Child Health & Development
	Shinichi OKA	Director, MD, Aids Clinical Center, National Center for Global Health & Medicine
	Kozo KAIBUCHI	Prof., MD, Grad. School of Medicine, Nagoya Univ.
	Masanobu KANO	Prof., MD, Grad. School of Medicine, Univ. of Tokyo
	Takeo KISHIMOTO	Prof., Grad. School of Bioscience & Biotechnology, Tokyo Inst. of Technology
Member	Hiroaki SASAI	Prof., Inst. of Scientific & Industrial Research, Osaka Univ.
	Makoto SASAKI	Prof., Grad. School of Life Sciences, Tohoku Univ.
	Toshiharu SHIKANAI	Prof., Grad. School of Science, Kyoto Univ.
	Hiroshi TAKAYANAGI	Prof., MD, Grad. School of Medical & Dental Sciences, Tokyo Medical & Dental Univ.
	Tsutomu TAKEUCHI	Prof., MD, School of Medicine, Keio Univ.
	Hiroyuki TSUTSUI	Prof., MD, School of Medicine, Hokkaido Univ.
	Atsushi NISHIDA	Prof., Grad. School of Pharmaceutical Sciences, Chiba Univ.
	Mitsuyasu HASEBE	Prof., National Inst. for Basic Biology
	Masabumi MINAMI	Prof., Grad. School of Pharmaceutical Sciences, Hokkaido Univ.
	Takashi MINEGISHI	Prof., MD, Grad. School of Medicine, Gunma Univ.
	Kohei MIYAZONO	Prof., MD, Grad. School of Medicine, Univ. of Tokyo
	Toyoaki MUROHARA	Prof., MD, Grad. School of Medicine, Nagoya Univ.

公益財団法人ノバルティス科学振興財団

役員名簿

2012年10月1日現在（順不同、敬称略）

職名	氏名	現職	就任年月日	常勤・非常勤
代表理事	金子 章道	畿央大学大学院健康科学研究科教授 慶應義塾大学名誉教授	1997年6月2日	非常勤
理事	浅野 茂隆	早稲田大学先端科学・健康医療融合研究機構長 東京大学名誉教授	1997年6月2日	非常勤
	石川 裕子	ノバルティス ファーマ株式会社 常務取締役 人事・コミュニケーション本部長	2004年6月7日	非常勤
	遠藤 政夫	山形大学名誉教授	1997年6月2日	非常勤
	須田 年生	慶應義塾大学医学部教授	2012年4月1日	非常勤
監事	中嶋 徳三	中嶋徳三公認会計士事務所 公認会計士	2006年6月5日	非常勤
	布施 正則	ノバルティス ファーマ株式会社 経理部長	2012年4月1日	非常勤

評議員名簿

2012年10月1日現在（順不同、敬称略）

職名	氏名	現職	就任年月日	常勤・非常勤
評議員長	黒岩 常祥	立教大学極限生命情報センター長 東京大学名誉教授	2002年2月7日	非常勤
評議員	赤池 紀扶	熊本保健科学大学リハビリテーション学科教授 銀杏学園理事・副学長 九州大学名誉教授	1999年6月4日	非常勤
	川島 博行	元新潟大学大学院医歯学総合研究科教授	2001年6月1日	非常勤
	小安 重夫	慶應義塾大学医学部教授	2012年4月1日	非常勤
	柴崎 正勝	公益財団法人微生物化学研究会 微生物化学研究所長	2005年6月13日	非常勤
	清水 孝雄	東京大学大学院医学系研究科特任教授	2012年4月1日	非常勤
	中野 明彦	東京大学大学院理学系研究科教授	2012年4月1日	非常勤
	マックス・ブルガー	ノバルティス サイエンスボード副議長 バーゼル大学教授	1987年9月16日	非常勤
	眞弓 忠範	大阪大学名誉教授	2004年6月7日	非常勤
	三谷 宏幸	ノバルティス ファーマ株式会社 代表取締役社長	2007年9月5日	非常勤

職名	氏名	現職	就任年月日	常勤・非常勤
評議員	森 美和子	北海道医療大学客員教授 北海道大学名誉教授	2005年6月13日	非常勤
	渡邊 敏文	ノバルティス ファーマ株式会社 執行役員 サイエントフィック アフェアーズ本部長	2012年4月1日	非常勤

選考委員名簿

2012年10月1日現在（順不同、敬称略）

職名	氏名	現職	就任年月日	常勤・非常勤
選考委員長	佐田 政隆	徳島大学大学院 ヘルスバイオサイエンス研究部教授	2009年6月19日	非常勤
選考委員	一條 秀憲	東京大学大学院薬学系研究科教授	2011年6月17日	非常勤
	稲垣 暢也	京都大学大学院医学研究科教授	2012年6月15日	非常勤
	梅澤 明弘	国立成育医療研究センター 再生医療センター長	2012年6月15日	非常勤
	岡 慎一	国立国際医療センター エイズ治療・研究開発センター長	2011年6月17日	非常勤
	貝淵 弘三	名古屋大学大学院医学系研究科教授	2009年6月19日	非常勤
	狩野 方伸	東京大学大学院医学系研究科教授	2009年6月19日	非常勤
	岸本 健雄	東京工業大学大学院生命理工学研究科教授	2011年6月17日	非常勤
	笹井 宏明	大阪大学産業科学研究所教授	2010年6月18日	非常勤
	佐々木 誠	東北大学大学院生命科学研究科教授	2012年6月15日	非常勤
	鹿内 利治	京都大学大学院理学研究科教授	2011年6月17日	非常勤
	高柳 広	東京大学大学院医学系研究科教授	2011年6月17日	非常勤
	竹内 勤	慶應義塾大学医学部教授	2009年6月19日	非常勤
	筒井 裕之	北海道大学大学院医学研究科教授	2012年6月15日	非常勤
	西田 篤司	千葉大学大学院薬学研究科教授	2009年6月19日	非常勤
	長谷部 光泰	自然科学研究機構 基礎生物学研究所教授	2009年6月19日	非常勤
	南 雅文	北海道大学大学院薬学研究院教授	2011年6月17日	非常勤
	峯岸 敬	群馬大学大学院医学系研究科教授	2010年6月18日	非常勤
	宮園 浩平	東京大学大学院医学系研究科教授	2009年6月19日	非常勤
	室原 豊明	名古屋大学大学院医学系研究科教授	2011年6月17日	非常勤

事務局便り

ご寄附のお願い

当財団は、自然科学における創造的な研究の奨励等を行うことにより、学術の振興を図り、国民の健康と福祉の向上に寄与することを目的にしております。

当財団の事業は、基本財産の運用益並びに寄付金によって賄われており、財団では趣旨にご賛同いただける皆様からのご寄附を募っております。

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個人：年間寄附金の合計額もしくは年間所得の40%相当額のいずれか低い方から2千円を引いた金額が、所得税の寄附金控除額となります。

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詳しくは、財団事務局までお問合せ下さい。

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事務局より

本年度もお陰様で、財団年報を発行できる運びとなりました。これも偏に、助成を受けられた皆様および財団関係者の皆様のご尽力の賜物と感謝申し上げます。助成事業は財団設立以来、総数で1,460件、総額17億6千8百万円余りに達しました。

公益法人の制度改革につきましては、当財団は内閣府より、事業が社会一般の公益に資するとの認定を受け、2012年4月1日付で公益財団法人へ移行いたしました。

新しい公益を担う財団として、今後とも事務局は学術の進展に寄与するべく、公益事業の遂行に邁進いたします。

引き続きご指導、ご支援の程よろしくお願い申し上げます。

事務局長 松田光陽

公益財団法人 ノバルティス科学振興財団

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