Annual Report (2013)

No.25
The NOVARTIS Foundation (Japan)
for the Promotion of Science

平成25年度
財団年報 第25号

公益財団法人 ノバルティス科学振興財団

Contents

Introduction

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

This booklet includes research and meeting reports written by the 2012 grantees. 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 nearly JPY 1.8 billion to approximately 1,500 researches and international exchange activities. The Foundation supports basic researches in the field of life science and related chemistry. They may not develop directly into applications, but accumulation of new findings and theories will open a road to application research and business someday.

The tireless effort of researchers including the present grantees 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. We are encouraged by warm acknowledgements to the Foundation written in many publications of grantees.

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.

はじめに

代表理事　金 子 章 道

ここに 2012 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。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 26 年間に約 1,500 件、金額にしておよそ 18 億円の助成を行ってまいりました。当財団がご提供する研究費は、研究に要する総費用のうち微々たるものかもしれませんが、研究をスムーズに遂行するための役に立てていただくのがその目的です。事務局に寄せられた発表論文の謝辞の中にも受賞者のそうしたお気持ちが垣間見られ、大変嬉しく思っております。

当財団が助成の対象とする研究は生物・生命科学および関連する化学の領域における基礎的な研究です。その成果が即応用につながらなくても、新発見や新しい理論が応用研究のアイディアとして役立つことになるもしれません。基礎的な発見や理論を積み上げることが、いつの日か新分野を開き、新産業を生み出すもとになるものと信じております。

この年報は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。

II.

Reports from the Recipients of Novartis Research Grants

Ageing effects on the neurophysiological parameters of human brain

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Abstract

To study ageing influence on the corticospinal tract in humans, we measured the corticoconus motor conduction time (CCCT) in 100 healthy volunteers. Age was significantly correlated with L1-level latency reflecting total peripheral nerve conduction, but it was not significantly correlated with CCCT. Central motor conduction was found to be relatively less affected by aging compared with peripheral motor conduction. This is probably because the peripheral nerves are more often affected by several accidental influences, such as injury, compression and metabolic disorders effects (diabetes mellitus and so on) as compared with the central nervous system protected by bones (skull or spine).

Key words : ageing, corticospinal tract, peripheral nerves, transcranial magnetic stimulation

Introduction

Anti-ageing medication is one big problem, especially in Japan, because of high average life-time. The central nervous system is usually affected by ageing and its involvement often produces clinical symptoms, such as memory loss, dementia, difficulty in locomotion and so on. Motor system involvement may be a serious issue to be assessed. The corticospinal tract, main central motor pathway, must be affected by ageing. The objective assessment of its function is requisite for assessing the ageing effect. In the present investigation, therefore, we investigated aging effects on the corticospinal function by measuring the corticoconus motor conduction time (CCCT) in various aged normal subjects.

Results

Subjects.

Subjects consisted of 100 healthy volunteers (41 men and 59 women) with age and body height (mean 6 SD) of 52.9 6 21.6 (range 20–90) years and 160.4 6 10.4 (132–189) cm, respectively. All subjects were able to engage in normal tasks of daily life without assistance, even the most elderly. Informed consent to participate in our study was obtained from all subjects. The protocol used was approved by the ethics committee of the Faculty of Medicine, University of Tokyo, and the experiments were conducted in accordance with the ethical standards of the Declaration of Helsinki.

Methods.

Motor evoked potentials (MEPs) were recorded from the right tibialis anterior muscle (TA) with subjects sitting comfortably on a bed.

Magnetic stimulation was conducted using a monophasic stimulator (Magstim 200; Magstim Co., Ltd., Whitland, UK). For cortical stimulation, the edge of a round coil (20-cm diameter, 0.98T; Magstim) was placed over Cz (international 10–20 system), with induced currents flowing mediolaterally over the contralateral leg motor area.16,17 MEP onset latency was measured in the active condition (cortical latency).

For the conus medullaris stimulation, the magnetic augmented translumbosacral stimulation (MATS) coil was always placed from the midline to the contralateral side of the body (the opposite side from the recorded muscle) to prevent non-target parts of the coil from activating distal peripheral nerves for the target TA.

The CCCT, conventional CMCT, and cauda equina conduction time (CECT) values were obtained as follows. The CCCT was obtained by subtracting the L1-level latency from the cortical latency, the conventional CMCT by subtracting the L5-level latency from the cortical latency, and the CECT by subtracting the L5-level latency from the L1-level latency.

Results

The correlations between each conduction parameter and age are depicted in Figures 1and 2. All measured conduction parameters except CCCT had a significant positive, linear correlation with age. On the other hand, no significant correlation was found between CCCT and age (P: 0.946).

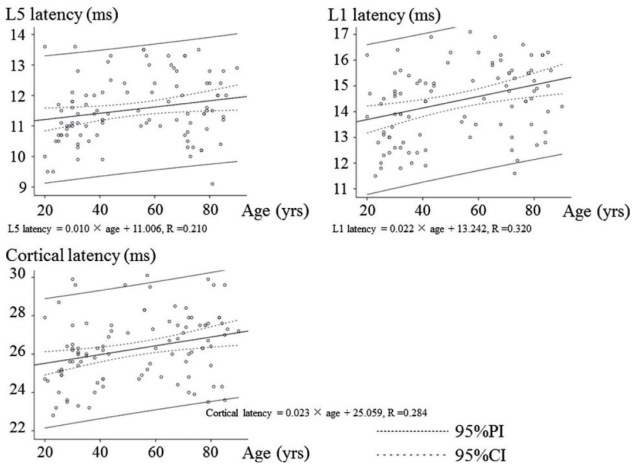

Figure 1

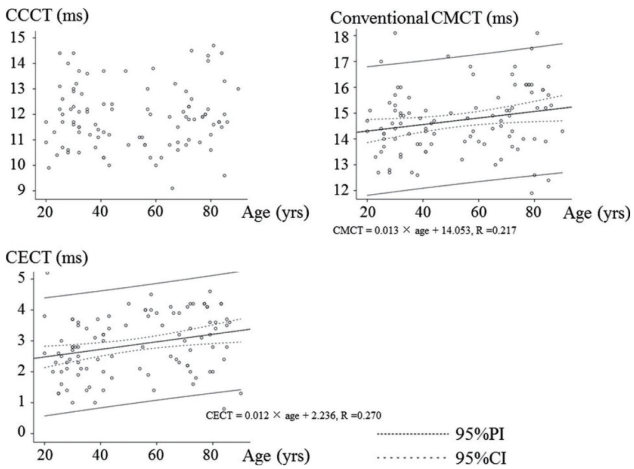

Figure 2

Discussion & Conclusion

In this study we obtained the following findings: L5- and L1-level latencies, cortical latency, conventional CMCT, and CECT depended on age, whereas CCCT was relatively independent of these variables. Thus, CCCT, a measure most directly reflecting corticospinal tract conduction, did not depend on age. In contrast, L1-level latency, reflecting the entirety of peripheral motor nerve conduction, depended significantly on age. Based on these findings, we conclude that, although corticospinal tract conduction is affected slightly by aging, peripheral motor conduction is strongly affected by aging.

One plausible explanation for the lack of ageing effects on the corticospinal tracts is that the brain and spinal cord are protected by cranial bones and the vertebral column, respectively. Moreover, the corticospinal tract is surrounded by central nervous system tissue, except for the pyramidal decussation portion, and bathed in cerebrospinal fluid. The blood–brain barrier may also be involved in the protection of the corticospinal tract. In contrast, peripheral nerves can be directly affected by minor trauma or injuries, due to their relative lack of protection. Based on these anatomical features, we believe that the corticospinal tract is less likely to be damaged by the various events occurring on a daily basis over the course of a long lifespan.

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一般の皆様へ

加齢が人の神経系にどのような影響を与えるかは、一般国民の関心事の一つである。認知症など高次機能に関する影響が重要であるが、歩行・姿勢などに影響を及ぼす運動神経系に対する加齢の影響も非常に重要である . しかし、ヒトの中枢運動系を客観的に評価できる検査法は従来存在しなかった。ところが、近年磁気刺激を用いた検査により、ヒトの中枢運動系を客観的に評価できるようになった。

本研究では、その方法を用いて様々な年齢の被験者で中枢運動系の評価を行い、ヒトの運動系に対する加齢の影響を検討した。その結果、末梢運動神経系は加齢とともに機能が低下する事が証明されたが、中枢運動系は加齢による大きな影響はなかった。

この理由としては、物理的に骨・髄液・脳血管関門に守られている脊髄・脳に比べて、末梢神経は特に守られてないため、外傷・代謝性影響など影響を受けやすい事が大きなファクターになっているのではないかと考えた。

Synthetic study of non-alkaloidal diterpene isolated from the genus *Aconitum*

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Abstract

We have achieved the construction of an unprecedented pentacyclic framework of non-alkaloidal diterpene, atropurpuran, by an intramolecular reverse electron demand Diels-Alder reaction of masked *o*-benzoquinone. We also have achieved the first synthesis of a tetracyclo[5.3.3.$0^{4,9}$.$0^{4,12}$] tridecane skeleton and development of a concise approach to the atropurpuran skeleton.

Key Words : Total synthesis, Diels-Alder reaction, Diterpene

Introduction

Aconitum is a genus of flowering plants that produce a variety of poisons and compounds of medicinal importance. The fascinating bioactivities of these compounds arise from C19 and C20 diterpene alkaloids, such as aconitine, hetisine, and atisine. Isolation of non-alkaloidal diterpenes from the genus Aconitum, however, has rarely been reported. In 2009, Wang and co-workers reported the isolation of the structurally unique non-alkaloidal diterpene atropurpuran from Aconitum hemsleyanum var. atropurpureum.[1] The structure of atropurpuran features an unprecedented cage-like skeleton, tetracyclo[5.3.3.$0^{4,9}$.$0^{4,12}$]tridecane, which includes two bicyclo[2.2.2]octane units.

Results

Despite the intriguing biosynthetic and structural properties of the tetracyclo[5.3.3.$0^{4,9}$.$0^{4,12}$] tridecane skeleton, there are no previous reports that describe efforts to synthesize this compound. Consequently, at the outset of our synthetic investigation on atropurpuran, we attempted to establish a methodology for the construction of the tetracyclic skeleton. Our strategy towards the synthesis of the tetracyclic skeleton was to utilize a reverse electron demand Diels–Alder (REDDA) reaction of masked o-benzoquinone (MOB). The REDDA reaction with MOB has recently emerged as a powerful tool for the construction of highly functionalized complex molecules. We envisaged that the intramolecular REDDA reaction of MOB **3** would directly provide an *anti*-Bredt compound **4**. (Fig. 1)

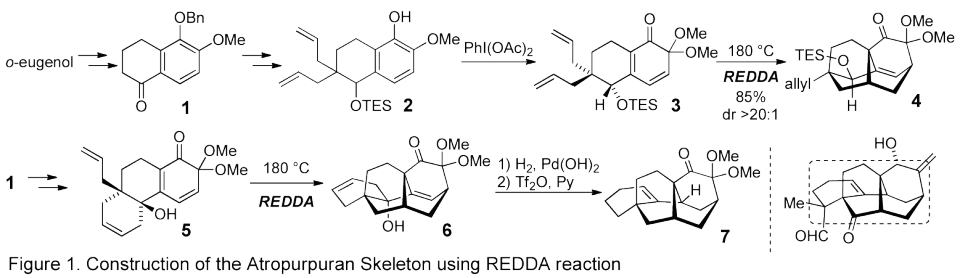

Figure 1. Construction of the Atropurpuran Skeleton using REDDA reaction

The preparation of REDDA precursor was started from *o*-eugenol (Fig. 1). Easily scalable tetralone **1** was successfully converted to phenol **2** by removal of Benzyl group, reduction with DIBAL, and silyl etherification. Oxidative dearomatization of phenol **2** with hypervalent iodine reagent, $PhI(OAc)_2$ afforded MOB **3**. The REDDA reaction of MOB **3** (mesitylene, 180 °C, 1h) afforded Diels-Alder adduct **4** in high yield almost as a single diastereomer. This result established that we had achieved the first artificial synthesis of the tetracyclo [5.3.3.$0^{4,9}$.$0^{4,12}$]tridecane skeleton. Next, we proceeded to the construction of pentacyclic carbon framework of atropurpuran. Deprotection of common intermediate **1** with BCl_3 in CH_2Cl_2 and addition of allylmagnesium bromide gave tri-allylated compound, of which ring-closing metathesis with Grubbs 2nd generation catalyst and oxidative dearomatization with $PhI(OAc)_2$ gave MOB **5**. REDDA reaction of the tricyclic MOB **5** was achieved to construct the pentacyclic Diels-Alder adduct **6** in high yield (75%). Finally, hydrogenation of 6 with Pearlman's catalyst and subsequent dehydration (Tf_2O, pyridine) afforded **7** to complete the construction of the pentacyclic framework of atropurpuran, despite the lack of some functionality.[2]

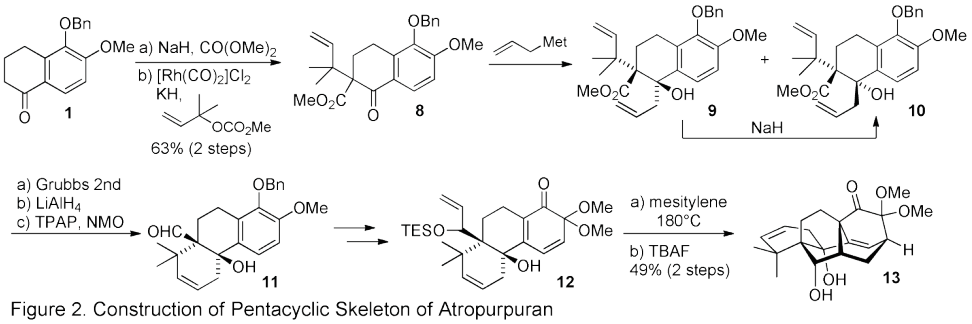

Figure 2. Construction of Pentacyclic Skeleton of Atropurpuran

Next, we were embarked on the synthetic study toward the total synthesis of atropurpuran (Fig. 2). Carboxylation of common intermediate **1** afforded the corresponding β-keto ester, which was subjected to Rh-catalyzed reverse prenylation of active methyne position to give ketone **8**. Addition of allylmagnesium bromide to ketone **8** gave anti-adduct **9** in excellent yield (95%). However, all attempts for further transformation from anti-adduct **9** was failed due to the steric hindrance of dimethyl group. Despite of our efforts to obtain syn-adduct **10**, any condition of allyl group addition was failed resulting in low yield of **10** (up to 27%). Fortunately, we found that anti-adduct **9** underwent base-promoted isomerization to give syn-adduct **10** by treatment of NaH. We postulated that the isomerization of syn-adduct **10** was caused by a retro-aldol/aldol reaction. Ring-closing metathesis of syn-adduct **10**, reduction of ester with $LiAlH_4$, and TPAP oxidation of the resulting alcohol afforded aldehyde **11**. Introduction of vinyl group as a dienophile to aldehyde **11** was successively proceeded by Grignard addition in diastereoselective manner. TES protection of the secondary alcohol, removal of Bn group with lithium naphthalenide, and oxidative dearomatization of the resulting phenol provided MOB **12**. The REDDA reaction of MOB **12** afforded the Diels-Alder adduct, which was treated with TBAF to give diol **13** in 49% overall yield. The pentacyclic structure was determined by NOESY experiment of diol **13**. At the same time, the stereochemistry of secondary alcohol generated by addition of vinylmagnesium bromide was also determined.

Discussion & Conclusion

In summary, we have achieved the construction of the pentacyclic framework of atropurpuran by an intramolecular REDDA reaction of MOB. Characteristic features of the present study are: 1) the first synthesis of a tetracyclo[5.3.3.$0^{4,9}$.$0^{4,12}$]tridecane skeleton and 2) a concise approach to the atropurpuran skeleton. These results demonstrate the power of the REDDA reaction by using MOB for the construction of *anti*-Bredt and cage-like complex molecules. Use of this methodology in synthetic efforts toward atropurpuran is currently underway. Further synthetic plan is that oxidation of methyl group would be achieved by Barton reaction and Wittig reaction of acetal carbonyl group would give atropurpuran.

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一般の皆様へ

トリカブト (Aconitum) 属の植物は、強力な神経毒であるアコニチンをはじめとして、根に多くのアルカロイドを含み、古来より狩猟の際の毒や、神経痛などの鎮痛薬としても広く用いられていた。2009 年にトリカブト属の植物から単離が報告されたアトロプルプランは、珍しいことにトリカブト由来であるにもかかわらず、窒素を含まないテルペノイドである。また構造的にも、前例のない複雑な 5 環性骨格を有している。その構造と生合成経路についての興味から、アトロプルプランの合成研究を開始した。現在までに炭素骨格の合成を達成しており、今後は官能基変換により全合成を達成したい。

Bio-Inspired Total Synthesis of Tryptophan-Based Dimeric Diketopiperazine Alkaloids

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Abstract

Direct bio-inspired dimerization reactions along with an originally proposed biosynthetic pathway from commercially available amine-free tryptophan derivatives in aqueous acidic media were developed to provide symmetric and asymmetric dimeric compounds. In addition, concise two-pot or three-step syntheses of naturally occurring dimeric diketopiperazine alkaloids (+)-WIN 64821 (**1**), (–)-ditryptophenaline (**2**) and (+)-naseseazine B (**3**) were accomplished in total yield of 20%, 13% and 20%, respectively.

Key Words : alkaloids, biomimetic synthesis, ditryptophenaline, naseseazine B, WIN 64821

Introduction

A large number of tryptophan-based dimeric diketopiperazine alkaloids, which show fascinating biological activities, have been isolated from fungi. For instance, (+)-WIN 64821 (**1**) and (–)-ditryptophenaline (**2**) were isolated from *Aspergillus sp.* and reported to be a competitive substance P antagonist with respect to human neurokinin-1 and the cholecystokinin B receptor [1]. In 2009, (+)-naseseazine B (**3**) possessing a $C3_{sp3}$–$C7_{sp2}$ bridge was isolated from Fijian actinomycete *Streptomyces sp* [2]. Herein, we describe direct bio-inspired dimerization reactions along with our proposed biosynthetic pathway from commercially available amine-free tryptophan derivatives in aqueous acidic media and concise three-step total syntheses of **1**, **2** and **3**.

Results

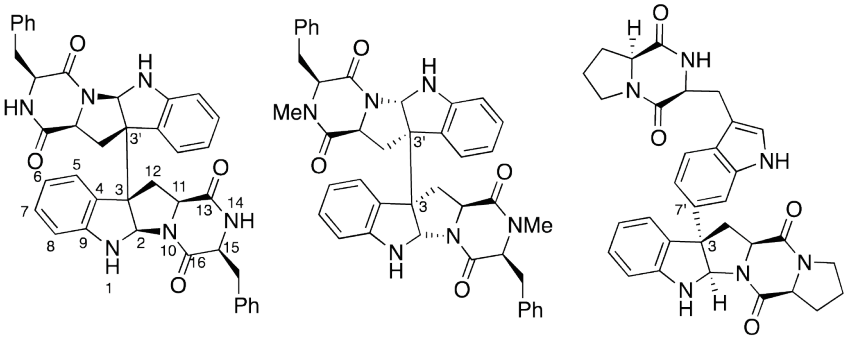


Figure 1. natural tryptophan-based dimeric diketopiperazine alkaloids

To achieve bio-inspired dimerization reaction in aqueous acidic media for synthesis of **1**, **2** and **3**, suitable oxidants and acids were screened with commercially available *L*-tryptophan ethyl ester (**4**)

as substrate (Figure 1, Table 1). When the reaction was carried out in aqueous CH_3SO_3H solution in the presence of 1.2 equivalent of $Mn(OAc)_3$ as oxidant, high conversions were observed (entry 1). Thus, dimeric compounds **5**, **6**, and **7** were obtained in 19%, 11%, 28% yield, respectively. CH_3SO_3H was also a suitable acid for VOF_3 as oxidant, providing almost the same result as $Mn(OAc)_3$ (entry 2). Interestingly, V_2O_5 in aqueous CH_3SO_3H solution was the most effective combination for forming C3 symmetric compounds (entry 3). Thus, when **4** was treated with 0.65 equiv of V_2O_5 at –15 °C for 129 h, C3 asymmetric compounds **5** and **6** were obtained, each in 28% yield. The dimerization reactions described above supplied the desired dimeric compounds in multigram amounts by a one-step procedure.

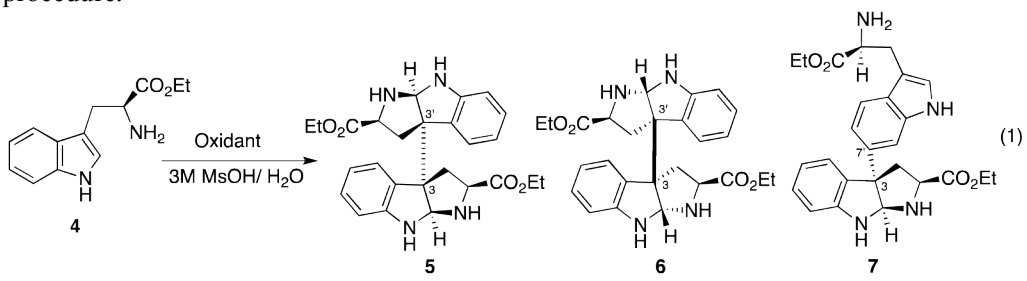
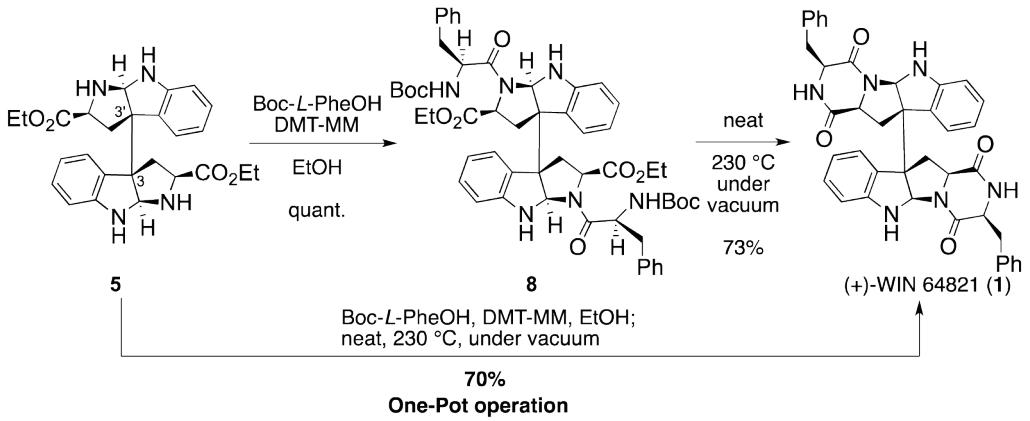


Table 1. The screening of oxidants for bio-inspired oxidative dimerization reactions

					yields		
entry	oxidant	equiv.	temp.	time	**5**	**6**	**7**
1	$Mn(OAc)_3$	1.2	0 °C	40 min.	19%	11%	28%
2	VOF_3	1.1	–10 °C	30 min.	19%	11%	28%
3	V_2O_5	0.65	–15 °C	129 h	28%	28%	14%

With large amounts of these key dimeric intermediates of proposed biosyntheses in hand, our focus moved to concise syntheses of (+)-**1**, (–)-**2** and (+)-**3**. For the dehydration condensation reaction toward the synthesis of (+)-**1**, 4-(4, 6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), developed by Kunishima and co-workers [3], was employed because the reagent can be used in environmentally friendly alcohol as solvent without any additives such as bases. Thus, treatment of resolved **5** in ethanol with 2.0 equiv of *N*-Boc-phenylalanine in the presence of 2.0 equiv of DMT-MM (0 °C, 4 h) in an open flask cleanly provided **8** in superb conversion (Scheme 1). Initial attempts to convert **8** directly to **1**, including removing the Boc group and diketopiperazine formation in a single step provided (+)-**1** in 59% yield under an argon atmosphere at 230 °C in neat conditions. Further improvement of conversion was accomplished under vacuum, because the

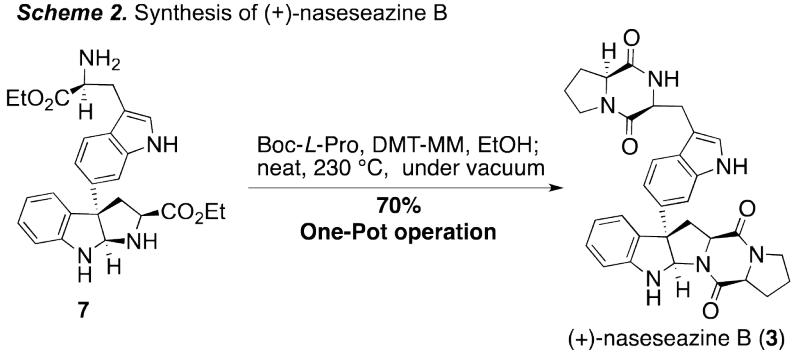
Scheme 1. Synthesis of (+)-WIN 64821



reaction side products such as isobutene, carbon dioxide and ethanol could be removed immediately from the reaction vessel (0.1 mbar, 30 min, 73%). Furthermore, the dehydration condensation reaction and Boc deprotection followed by intramolecular amide formation could be conducted in a one-pot operation (70%).

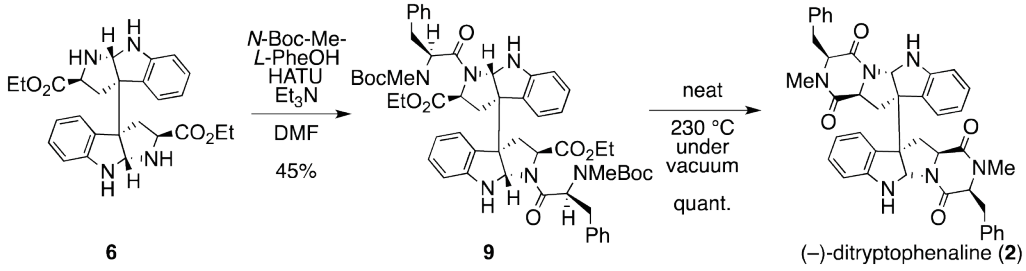
After completing the synthesis of (+)-**1**, the same procedure was applied to the synthesis of (+)-**3** (Scheme 2). Thus, the dehydration condensation reaction with **7** (1 equiv) and *N*-Boc-*L*-proline (2 equiv) in the presence of DMT-MM (2 equiv, EtOH, 0 °C, 4 h) was followed by diketopiperazine formation (neat, 230 °C, 30 min, 0.1 mbar) to provide (+)-**3** in superb yield (70%).

Scheme 2. Synthesis of (+)-naseseazine B



On the other hand, DMT-MM was not an effective coupling reagent for **6** to synthesize (–)-**2**. Surprisingly, when the same reaction conditions were applied to **6** with *N*-Boc-methyl-*L*-phenylalanine, the starting material **6** was completely recovered. In addition, *N*-Boc-methyl-*L*-phenylalanine ethyl ester was obtained as the only isolatable product. After several attempts to convert **6** to **9**, we found that *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and Et_3N in combination gave the desired coupling reaction in moderate yield (Scheme 3). Thus, treatment of **6** with *N*-Boc-methyl-*L*-phenylalanine (2 equiv) in the presence of HATU (2 equiv, 5 equiv of Et_3N, DMF, 0 °C, 72 h) provided **9** in 45% yield. Deprotection and diketopiperazine formation of **9** (neat, 230 °C, 30 min, 0.1 mbar) then furnished (–)-**2** (quantitative yield).

Scheme 3. Synthesis of (–)-ditryptophenaline



Discussion & Conclusion

We have developed direct bio-inspired dimerization reactions along with our proposed biosynthetic pathway from commercially available amine-free tryptophan derivatives in aqueous acidic media to provide symmetric and asymmetric dimeric compounds. Then, concise two-pot or three-

step syntheses of naturally occurring dimeric diketopiperazine alkaloids (+)-WIN 64821 (**1**), (–)-ditryptophenaline (**2**) and (+)-naseseazine B (**3**) were accomplished in total yields of 20%, 13% and 20%, respectively. The present synthesis has several noteworthy features. 1) Tryptophan-based symmetric and asymmetric dimeric key intermediates can be prepared on a multigram scale in one step. 2) The developed oxidation reaction was carried out in acidic aqueous solution without deactivation of metal oxidants. 3) Protection of the primary amine can be avoided by salt formation in acidic water in the synthesis scheme. 4) In two-pot operations, the reaction media are only environmentally friendly water and ethanol for the preparation of (+)-**1** and (+)-**3**. 5) Satisfactory total yields are obtained compared with previous reported synthesis. 6) No special care is needed to exclude water or air. 7) Effective one-pot diketopiperazine formation was discovered using the Kunishima coupling protocol followed by Boc deprotection and intramolecular amide formation under vacuum.

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一般の皆様へ

天然から得られる含窒素天然有機化合物（アルカロイド）には、多種多様な化学構造および顕著な生物活性を有するものも数多く、新規医薬品開発の観点からも注目されている。本研究ではこれらアルカロイド類の効率的かつ統一的な全合成法を確立し、量的供給並びに誘導体合成研究から新たな医薬品シーズとして発展させる事を目的とした。WIN 64821、ditryptophenaline、naseseazine B は細菌類から見出されたアルカロイドであり、その特異な構造と生物活性が注目されている。今回、これらアルカロイドの生合成が酸性水溶液中で進行しているという着想を得、フラスコ内で再現する事に成功した。本工程を鍵段階として、市販のトリプトファン誘導体から全行程 3 段階合成を達成した。

Regulation of vascular integrity by AM-RAMP system and its application.

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Abstract

Adrenomedullin (AM) is a peptide involved in both the pathogenesis of cardiovascular diseases and circulatory homeostasis. We have shown knockout mice of RAMP2 (RAMP2-/-), one of the accessory proteins of AM-receptor, are lethal at mid-gestation with the similar vascular abnormalities of AM-/-.

We generated vascular endothelial cell-specific RAMP2 knockout mice (E-RAMP2-/-). A small number of E-RAMP2-/- can survive until adulthood. In adult E-RAMP2-/-, spontaneous occurrence of vasculitis lesions was detected throughout the body. Furthermore, E-RAMP2-/- showed severe organ fibrosis with enhanced oxidative stress.

We next generated drug-inducible E-RAMP2-/- (DI-E-RAMP2-/-). After the induction of the gene-deletion in adult, DI-E-RAMP2-/- showed remarkable vascular leakage and malformation of endothelial cells.

In conclusion, RAMP2 is the key determinant of the vascular functions of AM; RAMP2 is essential for the vaso- and organ-protective effects of AM.

Key Words : Endothelial cell, Adrenomedullin, RAMP2

Introduction

Revealing the mechanisms underlying the functional integrity of the vascular system could make available novel therapeutic approaches. Adrenomedullin (AM) is a peptide involved in both the pathogenesis of cardiovascular diseases and circulatory homeostasis. We have shown RAMP2, one of the accessory proteins of AM-receptor, plays critical roles in AM's vascular functions.

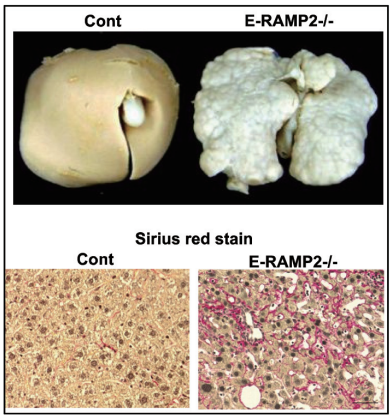
Results

We previously showed that knocking out the AM or RAMP2 causes vascular abnormalities and is embryonically lethal. In this study, to analyze the pathophysiological roles of vascular AM-RAMP2 system directly, we generated vascular endothelial cell (EC)-specific RAMP2 knockout mice (E-RAMP2-/-).

Most of E-RAMP2-/- were lethal at later-gestation with systemic edema. The surviving E-RAMP2-/- adults exhibited thinning of the aortic wall and enlarged aortic diameters. Electron microscopic observation revealed that the aortic smooth muscle layer was in disarray in E-RAMP2-/- mice, and that the endothelial cells were detached from the basement membrane and severely deformed.

Vasculitic lesions developed spontaneously in the surviving E-RAMP2-/- adults, and severe infiltration and accumulation of inflammatory cells was observed in blood vessels within the major organs, including the live, kidneys, and lungs.

In 6-month-old E-RAMP2-/- mice, we detected elevated levels of oxidative stress in the major organ. Levels of 4-HNE immunostaining, which reveals the presence of peroxides of unsaturated fatty acids, were increased in E-RAMP2-/-. More interestingly, organ damage developed spontaneously in E-RAMP2-/- adults. In particular, the livers of E-RAMP2-/- mice appeared cirrhotic. Pathological analysis revealed that the cirrhosis-like changes were not caused by damage to parenchymal hepatocytes, but by severe fibrosis along the vasculature within the liver, most likely due to dysfunction of the sinusoidal ECs (Fig).



We next used EAhy926 ECs to generate EC lines stably overexpressing RAMP2. H_2O_2-treatment has been shown to induce EC senescence. We found that after incubation for 24 h in the presence of H_2O_2 plus AM, control cells were strongly stained by SA-β-gal, but cells overexpressing RAMP2 were resistant to staining.

As only a small number of adult E-RAMP2-/- was obtained, next we generated drug-inducible E-RAMP2-/- (DI-E-RAMP2-/-). With this model, we are able to selectively delete RAMP2 gene from the ECs of adult mice through administration of tamoxifen. Interestingly, the tamoxifen-treated mice showed remarkable body weight gains with systemic edema. The cause of the edema was enhanced vascular permeability and plasma leakage, as evidenced by the extravascular leakage of Evans Blue dye.

We also confirmed that at the chronic stage after the gene-deletion, DI-E-RAMP2-/- mice exhibited vascular damage with enhanced perivascular inflammation that was the same as that of adult E-RAMP2-/-. With DI-E-RAMP2-/-, therefore, we were able to clarify both the acute and chronic effects of RAMP2 deletion in the adult, and demonstrate that RAMP2 is essential for EC viability, vascular integrity, and homeostasis.

Discussion & Conclusion

In this study, we were able to clarify both the acute and chronic effects of RAMP2 deletion in the adult, and to demonstrate that the AM-RAMP2 system is essential for vascular integrity and organ homeostasis. Early after RAMP2 gene deletion, ECs show morphological changes. The resultant EC deformation causes detachment, barrier dysfunction, enhanced vascular permeability and edema, which in turn promote the attachment and infiltration of inflammatory cells. Chronic vascular inflammation induces vascular damage and accelerated senescence, and enhances oxidative stress and organ fibrosis. Finally, the accumulated disorders cause chronic organ dysfunction with aging. The results obtained using these models demonstrate that vascular EC integrity is essential for organ homeostasis.

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一般の皆様へ

アドレノメデュリン (AM) は、血管をはじめ、全身の組織で広範に産生され、多彩な生理活性を有する因子あり、臨床応用も期待されている。我々は、AM による血管保護作用、臓器保護作用が、調節タンパク RAMP2 によって制御されていることを見出した。RAMP2 は、新たな治療標的分子として期待される。

Development of novel molecular imaging method for visualization of clinical condition of intravital lipids

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Abstract

The regulation of lipolysis in adipocytes involves coordinated actions of lipid droplet (LD) and LD-associated proteins such as perilipin. Here, we studied the cellular origin and physiological significance of micro LDs (mLDs) that emerge in the cytoplasm during active lipolysis in differentiated 3T3-L1 adipocytes. Multiplex coherent anti-Stokes Raman scattering (CARS) microscopy demonstrated that mLDs receive the fatty acid (FA) moiety of triglyceride from pre-existing LDs during lipolysis through vibrational imaging of CD bonds.

Key Words : Lipolysis, lipid droplet, CARS, Raman

Introduction

Coherent anti-Stokes Raman scattering (CARS) microscopy has been applied widely to cell and tissue imaging *ex* and *in vivo* [1]. CARS microscopy, however, provides only a monochromatic image using a single vibrational frequency such as CH_2 stretch vibrational mode. In order to obtain full spectral information on vibrational modes, we used a novel light source, nanosecond supercontinuum or white-light laser, which facilitated ultra-broadband (>3000cm^{-1}) multiplex CARS microspectroscopy. Owing to the narrow spectral bandwidth of the pump/probe laser in the CARS process, the vibrationally resonant CARS imaging in the fingerprint region is performed with high molecular specificity [2].

Results

The light source is a Q-switched microchip Nd:YAG laser. Fundamental output of the 1064- nm radiation is firstly divided into two. One is used as a pump laser for the CARS process, and the other is introduced into a photonic crystal fiber to generate white-light supercontinuum, which is also used as a Stokes laser for the CARS process. Although the spectral profile of the white-light supercontinuum ranges from visible to near infrared (NIR), only NIR spectral components are used for the sample excitation. After removing redundant components from excitation pulses, The two radiations are introduced into a modified inverted microscope. The sample is placed upon a PZT stage for raster scanning. Forward propagating CARS signal is collected and introduced into a spectrometer.

Figure shows the results of multi-modal and multiplex CARS and third-order sum-frequency (TSF) imaging of a living adipose cell, which is cultured with deutrated (D-) palmitate. In the basal

state, D-palmitate was detected in the preexisting large LDs. During lipolytic stimulation, mLDs containing both CH and CD bonds appeared in the cytoplasm, and increased with time. This result suggests that mLDs receive FAs from preexisting LDs, directly by fragmentation of large LDs and/or indirectly by re-esterification of FAs. Similar behavior is also found in the TSF images (bottom). The dynamical behavior of the lipolytic process is clearly visualized with multi-modal and multiplex CARS and TSF imaging.

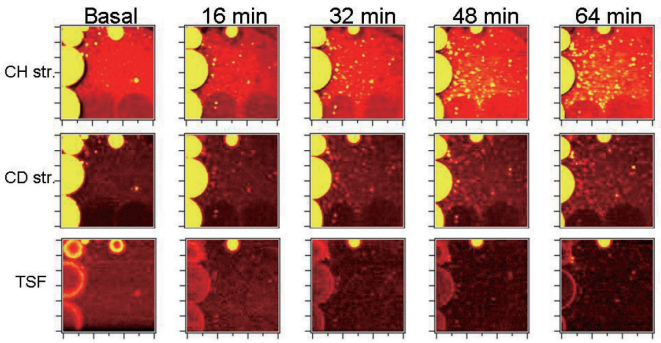

Figure. Multiplex CARS and TSF imaging of a living adipose cell before (basal) and after lipolytical stimulation. The images at the top and middle rows correspond to the CARS images at CH and CD stretch vibrational modes, respectively. The image at the bottom row shows the TSF image. Image acquisition time is about 8 min.

We next examined whether mLDs are formed as a consequence of the fragmentation of large LDs or formed by FA re-esterification. To address this issue, we inhibited the FA re-esterification and TAG synthesis by an acyl-CoA synthetase (ACS) inhibitor, triacsin C, and a DAG acyltransferase (DGAT) inhibitor, 2-bromooctanoate, and visualized the appearance of mLDs. mLDs did not appear even after 32 min or 64 min of lipolytic stimulation, when re-esterification of FAs was inhibited. This result indicates that mLDs are formed by re-esterification of FAs derived from central LDs or somewhatand possibly in part from the media[3].

Discussion & Conclusion

The present study provides important findings in understanding molecular mechanisms of lipolysis in adipocytes: during lipolysis, significant amount of FAs generated by TAG hydrolysis are re-esterified, leading to formation of mLDs. Multiplex CARS microspectroscopy elucidated the origin of the TAG contents in mLDs due to its high sensitivity to lipids abundant in long-chain hydrocarbons by tuning the laser wavelengths to CH stretch vibrational mode, and has proven to be an excellent tool for LD tracking. The deuterium in a CD bond is heavier than hydrogen, placing CD vibrational stretching frequencies into near 2100 cm^{-1}, which is an otherwise silent region of the biological Raman spectra. By incubating differentiated adipocytes with deuterium-labeled palmitate and replacing some CH bonds with CD bonds, it is possible to selectively image a specific chemical bond (i.e. CD bond) using CARS contrast. The multiplex CARS microspectroscopy enabled us to obtain simultaneous imaging of CD and CH stretch vibrational modes, and revealed that newly appearing mLDs are formed as a consequence of the fragmentation of large LDs and/or the re-esterification of intracellular FAs.

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一般の皆様へ

近年、肥満による糖尿病、高血圧、脂質異常症など生活習慣病が増加しています。肥満は脂肪細胞に脂肪が過剰に蓄積した状態であり、それによる細胞機能異常が、脂肪組織そのものに炎症性変化を引き起こすこと、そして炎症の慢性化がメタボリックシンドロームと関連があることが、最近の研究でわかってきました。しかしながら、細胞の機能異常と組織の病態との関連については、未だ不明な点が多いのが現状です。そこで本研究では、生体内脂肪そのものの機能を捉え、脂肪の病態を可視化できる新しい方法を開発することで、脂肪組織の病態を見る、新しい研究アプローチを試みました。代謝産物である脂肪は、これまで生きたまま可視化することは困難でしたが、今回開発したコヒーレント・ラマン (CARS) 分光イメージングを用いることで、非標識にて脂肪の分子構造及びその機能を可視化することができました。本手法により、細胞内の脂肪代謝過程において、細胞質に可溶化した脂肪酸が、再度リサイクルされる経路がある、という知見を得ることが出来ました。

Molecular mechanisms of cornification of the epidermis.

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Abstract

Epidermal barrier formation requires a coordinated process where keratinocytes undergo drastic morphological and biochemical changes to form highly keratinized denucleated cell layers. Despite critical importance of epidermal barrier in maintaining healthy skin, the underlying molecular mechanisms are poorly understood. Here we found that autophagy is activated in differentiating epidermal keratinocytes *in vivo* and this activation is partly mediated by the induced expression of Nix (Bnip3l), a member of the BH3-only family, which selectively induces mitochondrial autophagy (mitophagy). Induction of terminal differentiation in cultured keratinocytes results in significant upregulation of Nix expression and subsequent activation of mitophagy. Conversely, knockdown of Nix attenuates keratinocyte differentiation and autophagy induction. Taken together, our data strongly suggest an important role of Nix in promoting keratinocyte terminal differentiation and subsequent mitochondria clearance by mitophagy. Our data would provide a new insight into understating molecular basis of skin barrier formation.

Key Words : Epidermis, Autophagy, Skin barrier formation, Atopic dermatitis.

Introduction

The skin epidermis serves as a critical barrier that protects the body from various environmental insults and dehydration. Defective skin barrier formation represents a common cause of variety of skin inflammatory diseases including psoriasis and atopic dermatitis, which are affecting about 10-30 percent of the human population. Therefore, understanding molecular details of skin barrier formation has huge implications for developing effective therapeutic treatments for people suffering such skin disorders.

Terminal differentiation of keratinocytes within the epidermis involves extensive cellular remodeling, whereby keratin filaments and lipids are cross-linked to form rigid bundles and cell organelles including nucleus and mitochondria are eliminated to induce apoptosis-like cell death, which all together result in formation of an impenetrable barrier in the outermost layer of the epidermis. Despite extensive studies of this process, the mechanism by which epidermal keratinocytes are allowed to achieve the dramatic cellular remodeling is largely unclear. One candidate mechanism that enables such quick remodeling might be autophagy, a process that promotes cellular self-cannibalization. Autophagy is an intracellular bulk degradation system that delivers cytoplasmic organelles and proteins to the lysosome, where they are degraded to be quickly eliminated damaged and unnecessary cell components[1]. Accumulating evidence indicates

that autophagy has been implicated in wide range of the cellular remodeling that occurs during organogenesis[2], thereby enabling developing cells to rapidly differentiate or change their fate in order to acquire new functions. Based on these facts, we hypothesize that the induction of autophagy may play an important role in promoting epidermal terminal differentiation. Therefore, the focus of this study is to obtain an experimental proof for the involvement of autophagy in the regulation of epidermal terminal differentiation and barrier formation. Our study will uncover a novel role of autophagy in regulating epidermal barrier formation and provide new insights into development of novel therapeutic strategies for skin disorders.

Results

To test whether autophagy is induced during epidermal development, we performed immunohitochemical staining of the mouse embryonic skin using a specific antibody against an autophagosomal protein, LC3. Whereas LC3 positive cells were hardly detectable in E15.5 epidermis, strong LC3-positive cells were readily detected in the granular layer of E17.5 epidermis and these positive cells showed the accumulation of LC3 in cytoplasmic puncta, which is a typical sign of autophagy. To further confirm the induction of the autophagy, westernblot analysis was carried out using the LC3 antibody that distinguishes the cytosolic (LC3-I) and autophagosome membrane bound forms of LC3 (LC3-II). Consistent with the immunohistochemical analysis, the accumulation of LC3-II was seen in freshly isolated E17.5 epidermis. Hence these data clearly indicate that autophagy is induced in the granular layer cells of developing epidermis.

To further gain insights into understanding molecular mechanisms of the autophagy induction, we explored the upstream molecule responsible for in the induction of the autophagy in the epidermis. Previously, we performed trasncriptome analysis of developing epidermis to identify genes whose expression is changed in associated with epidermal differentiation[3]. Among dozens of such molecules, Nix (also called as Bnip3l) became focus of our study due to its important roles in the induction of autophagy and autophagy-mediated mitochondria clearance. In fact, immunohistochemical staining of embryonic skin revealed that Nix expression was detected in the granular layers, the region where coincided with autophagy induction. Thus these data suggest potential correlation between Nix expression and autophagy induction in the epidermis.

Previously, Nix has been implicated in mitochondria clearance in reticulocytes. As elimination of mitochondria also occurs during epidermal cornification, we hypothesized that Nix-mediated autophagy might contribute to mitochondria clearance in the epidermis. In support of this possibility, immunofluorescence confocal microscopic examination revealed colocalization of Nix protein and mitochondria within LC3-positive autophagosome. While the exact function of Nix is not clarified, our observation strongly suggests that epidermal autophagy may participate in the mitochondria clearance in the differentiating epidermal cells.

To address the function of Nix in epidermal keratinocytes, loss-of-function analysis was performed using cultured keratinocytes. To recapitulate terminal differentiation process of keratinocytes *in*

vitro, we carried out keratinocytes suspension culture. Consistent with the *in vivo* observation, the induction of keratinocyte differentiation caused dramatic upregulation of Nix expression and subsequent activation of autophagosome formation. In contrast, knockdown *Nix* expression by specific shRNAs resulted in attenuation of keratinocyte differentiation and impaired autophagosome formation. Hence, it has been clearly suggested from these data that Nix plays an important role in epidermal differentiation and autophagy.

Discussion & Conclusion

Here, we show that autophagy is activated in differentiating epidermal keratinocytes *in vivo*. Although physiological function of autophagy is still unclear, our in vitro analyses suggest a potential link between epidermal development and autophagy formation. In support of this notion, the importance of autophagy in organogenesis has already been demonstrated in various tissues development including cardiogenesis, hematopoiesis and osteogenesis, where rapid cell remodeling is required for executing proper development. As epidermal development involves extensive cell remodeling, it is conceivable that autophagy may also play a role in this process. However, without *in vivo* functional analysis, it could be hard to exclude the possibility that, despite the extensive autophagy activation, autophagy exhibits limited function in epidermal development. In fact, from an evolutionary perspective, autophagy is thought to be evolved as a means for surviving against various stresses, and it has been also demonstrated that autophagy play a crucial role in maintaining quality control of cells by eliminating damaged organelles and misfolded proteins. These functions of autophagy are particularly important for post-mitotic or long-lived cells. As in the epidermis, the cells undergoing autophagy are mostly confined to the granular layer, a layer consisting of post-mitotic cells, an alternative scenario that may explain the role of autophagy would be that autophagy plays a role in conferring quality control and stress resistance to granular layer cells. This possibility is also supposed to be likely because cells within the outermost layer of the epidermis are continuously exposed to environmental stimuli and stresses such as UV damage, oxidative stress, toxic chemical exposure and microbe infection. If this is the case, autophagy may be needed for the epidermal cells to cope effectively with such stresses, and defective autophagy might result in cell damages and impaired homeostasis, which ultimately causing skin abnormalities such as cancerous transformation or chromic inflammation.

In either case, further studies are needed to clarify the physiological role of autophagy and molecular function of Nix in the epidermis. To address these issues, generation of combinational knockout mice of Nix and its related gene, Bnip3, has been currently underway in our laboratory.

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一般の皆様へ

皮膚の健康は皮膚の角質層のバリア機能によって保たれており、その異常はさまざまな皮膚病の原因になります。角質層は上皮細胞がダイナミック変化を起こすことによって形成されますが、その詳細なメカニズムは明らかにされていません。私たちは、オートファイジーという現象に注目し、角質層形成機構を明らかにすることを目標にして研究を進めています。この研究から得られる成果は、皮膚病の新たな治療法開発に役立つと思われます。

Analysis for the molecular mechanisms causing paroxysmal cough in pertussis

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Abstract

Key Words : Whooping cough, *Bordetella*, Animal model

Introduction

Pertussis (whooping cough) is a highly contagious, acute respiratory disease caused by the pathogenic bacterium, *Bordetella pertussis* (1). Clinical manifestations of the disease include paroxysmal coughing, pneumonia, apnea, and hypoxia. The mechanism by which the bacteria establish the disease is poorly understood. It is not known either how the organisms cause the paroxysmal coughing. A major obstacle to analyzing the course of whooping cough is a lack of suitable animal models. Notably, hardly any models that reproduce the paroxysmal cough in whooping cough have been described (2). In this study, we tried to establish an animal model that can be readily utilized and reproduce the coughing in human whooping cough, and by using the model, we analyzed the course of the disease including the coughing.

Results

In this study, we carried out a series of experiments for animal infections with the combinations between various experimental animals and species and strains of *Bordetella*, and eventually, could establish an appropriate model for whooping cough by using *B. bronchiseptica* and a commonly-used small animal (hereinafter referred to as "Ax"). *B. bronchiseptica*, which is closely related to *B. pertussis*, producing nearly identical virulence factors except pertussis toxin, has Ax as its natural host. Axs infected with 10^3 cfu of *B. bronchiseptica* had a persistent cough which was observed as early as day 5 postinfection and its frequency reached a peak at day 9. The total number of coughs for 5 days from day 6 to 11 of infection ranged from 56 to 242/30 min (5 min x 6 days). The frequency of coughing of the animal infected with wild-type *B. bronchiseptica* was 140 coughs/30 min on average. We considered this coughing to be paroxysmal. Histological screenings revealed no apparent pathological changes in the trachea of infected animals. In contrast, Axs given SS medium or the nonpathogenic phase III variants of *B. bronchiseptica* hardly exhibited any coughing. Mutant strains deficient in genes for known major virulence factors caused the paroxysmal coughing indistinguishable from that caused by wild type strain, implying that unknown factors might be involved in the coughing. Similar coughing was also observed in Axs infected with *B. pertussis* inoculated at 10^8 cfu/Ax. During the course of these experiments, we unexpectedly isolated a

mutant strain of *B. bronchiseptica*, tentatively named *Δ*C, which colonized the trachea but did not cause coughing paroxysms. To identify the mutated gene in *Δ*C, which might be responsible for the cough in infected Axs, we sequenced the whole genome of the mutant and compared it with that of the parental strain. The first screening of mutation sites revealed single-base substitutions at 41 sites and deletion/insertion mutations at 6 sites. These regions of the *Δ*C and parental strain genomes were directly sequenced and eventually, we found a single-base insertion and a single-base substitution in *geneA* and *geneB* (tentative names) of *Δ*C, respectively. It was predicted that the former mutation should result in a translational frameshift and the latter should cause an amino-acid substitution. When *geneA* but not *geneB* in the wild type strain was disrupted by insertional mutagenesis, the resultant variant failed to cause coughing paroxysms. On day 15 postinoculation, the *geneA*-deficient strain was recovered from the trachea to a similar extent to the wild-type strain, indicating that the strain colonized the trachea but did not cause coughing. Moreover, when geneA was complemented, *Δ*C regained the ability to cause the paroxysmal coughing. From these results, we concluded that geneA, directly or indirectly, to be involved in the coughing in infected Axs.

Discussion & Conclusion

We utilized Ax model focusing on coughing paroxysms in this study, by using *B. bronchiseptica* instead of *B. pertussis* as the pathogen. *B. bronchiseptica* shares with *B. pertussis* various virulence factors that are highly homologous in amino-acid sequence. Several animal species including pigs and dogs exhibit a persistent cough when infected with *B. bronchiseptica*. This prompted us to use *B. bronchiseptica* for the rat model to explore the pathogenesis of *B. pertussis*.

In addition, Axs were found to respond to *B. bronchiseptica* infections with a persistent cough. A large amount of *B. pertussis*, although the infection did not persist, caused a similar cough, which assured us that Axs infected with *B. bronchiseptica* could be a model of human whooping cough. We also isolated *Δ*C, a spontaneous mutant strain, which did not cause coughing in Axs. A comparison of the genomes of *Δ*C and the parental strain revealed DNA mutations in two distinct genes, tentatively designated *geneA* and *geneB*. Notably, the present results imply that *geneA* is the more probable candidate involved in the coughing of infected Axs. The coughing is a common manifestation of the diseases caused by *Bordetella*e including *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. In a future study, we hope to clarify the mechanism responsible for the coughing paroxysms associated with *B. bronchiseptica* infections and eventually those seen in human whooping cough by analyzing the functions of *geneA*-like genes in *B. pertussis*.

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一般の皆様へ

百日咳は百日咳菌（*Bordetella pertussis*）の上部気道感染によって起こる伝染性の疾病です。百日咳の特徴的な症状は激しい咳発作ですが、この咳発作の原因も発症機序もこれまで全く不明でした。本研究課題では、咳発作を再現する動物モデルを作製し、百日咳菌類縁の気管支敗血症菌の感染による咳発作発症機構の解析を行いました。その結果、気管支敗血症菌の特定のある遺伝子が、咳発作発症に関与していることが明らかになりました。この遺伝子は百日咳菌にも存在するため、現在、この遺伝子の機能を解析して、咳発作の発症機構を理解すべくさらに研究を進めています。

Analysis of mice deficient in molecules involved in intracellular polarized transport

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Abstract

Polarized intracellular transport is required for the establishment and maintenance of cell polarity. We generated knockout mice of various genes involved in polarized transport to know how they are involved in this process. Several knockout mice showed unexpected results which are being summarized for publication.

Key Words : Cell polarity, knockout mice, polarized transport

Introduction

Cell polarity is crucial for the development and function of epithelial cells and neurons. To maintain cell polarity, polarized intracellular transport is essential, but the research of polarized transport has been carried out using cell lines, thus, how it relates to the development, function, and pathogenesis of tissues is largely unknown. To elucidate this, we generated and analyzed knockout mice of various genes involved in polarized transport.

Results

We have already generated knockout mice of Rab8a, Rab8b, syntaxin3, SNAP23, annexin13, MAL2, FAPP1, FAPP2, PKD1, and PKD2. We are now mainly analyzing Rab8a, Rab8b, syntaxin3, SNAP23, FAPP2, PKD1, and PKD2 knockout mice regarding their function in polarized transport.

Rab8a,b:

We have already analyzed and published about Rab8a single knockout mice in Nature. They showed abnormalities in apical transport. However, Rab8a has a closely related molecule, Rab8b. Thus, we generated Rab8b knockout. As Rab8b knockout mice were almost normal, we generated Rab8a and Rab8b double knockout mice to know their redundancies. As expected, we obtained data which implied their redundancies and we are currently submitting the manuscript for publication.

Syntaxin3:

Syntaxin3 has been known to be involved in apical and axonal transport in the epithelial cells and neurons, respectively. When we generated knockout mice which lacked syntaxin3 in the whole body, we were not able to find adult mice, suggesting syntaxin3 null mice were embryonic lethal. We are currently analyzing when and how they die during development. On the other hand, we generated neuron- and small intestine- specific knockout mice and currently analyzing their phenotype. Some results of knockout mice supported the previous observations, but other results apparently

contradicted the previous ones. We are currently analyzing why these contradictions occurred.

SNAP23:

SNAP23 has been known to be necessary for membrane fusion and secretion of enzymes and hormones. When we generated knockout mice of SNAP23, they were embryonic lethal. Thus, we generated neuron-, endocrine pancreas-, and exocrine pancreas- specific knockout mice. Like in syntaxin3 knockout mice, some results of knockout mice supported, but others did not support the previous findings. We are currently submitting the manuscript for publication.

FAPP2:

FAPP2 has been known to be necessary for apical transport. It has also known to be necessary for transport of glycolipids to the trans-Golgi network. Though FAPP2 knockout mice showed no gross phenotype, it showed some defects in lipid transport. Analysis of this knockout mice was done with Dr. DeMatteis (TIGEM, Italy) and the result will be published shortly.

We also generated FAPP1, a closely related molecule to FAPP2. To know the possible functional redundancies, we are now crossing FAPP1 and FAPP2 knockout mice.

PKD1, 2:

PKD1, 2 have been known to be necessary for basolateral transport and other biological activities. When we generated knockout for each molecule, we were not able to see gross phenotype. We are currently generating double knockout mice, but the double knockout mice seemed to be embryonic lethal. To know their function in vivo, we are now making double floxed mice (PKD1 floxed/floxed; PKD2 floxed/floxed). After establishment of double floxed mice, we will delete PKD1 and PKD2 genes altogether by infection of adenovirus which encodes cre recombinase or by crossing double floxed mice with transgenic mice which express cre in a tissue-specific manner. Since they are expressed abundantly in the lung, we will introduce lung explant culture for infection of Cre by adenovirus to see the developmental defects in the lung.

In addition to analyzing knockout mice, we established a gene trap system which could identify novel genes involved in axonal elongation or distribution of synaptic vesicles. In this system, we used PC12 cell, which is a cell line derived from rat pheochromocytoma. We infected retrovirus to PC12 and screened infected cells by puromycin which was expressed only when the inserted retrovirus was within the transcriptionally active regions.

After infection, drug-resistant colonies were picked up and they were further processed for morphological abnormalities. By this strategy, we identified several genes whose loss affected the morphology of PC12 cells or the distribution of synaptic vesicle-like small vesicles. These results were published in FASEB Journal.

Discussion & Conclusion

We identified essential genes for polarized transport or cell polarity by gene targeting in the mouse and the gene trap in the cell lines. Using gene targeting, we were able to confirm the previous results in some cases, but in most cases of gene targeting, our results contradicted the previous

results. Above all, we were not able to see in many knockout mice. This clearly showed that there is a functional redundancy between genes, but in some cases such as Rab8a, the previous results were not correct probably by technical limitations. We will pursue which genes are truly involved in cell polarity by continuing our efforts. We will, at the same time, search for the novel essential genes in cell polarity by screening C. elegans. We already identified a number of genes which caused defects in intestinal cell polarity in their absence. We are currently generating knockout mice for these genes to confirm their general importance in cell polarity and we hope the results will be published in a few years.

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一般の皆様へ

細胞の向き（極性）はその細胞や組織、個体の機能や発生に重要であるが、細胞の極性がどのようにして生じて維持されるかは良く分かっていない。本研究の目的は細胞の極性に関わる遺伝子を欠損するマウスを作製解析し、細胞の極性の出来るメカニズムを解明するものである。解析により、ある遺伝子については以前通りの役割が確認されたが、大多数については以前報告された機能が見られなかったため、その原因を解明中である。更に他にも重要な遺伝子が予想されるため、線虫を用いて新たな遺伝子をスクリーニングし、それらの遺伝子の欠損マウスも作製解析中である。

Investigation of molecular signal output from a dividing nucleus to cell cortex

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Abstract

The existence of all life is based on cell proliferation, which usually accompanies the process of cell division. After replicating its genome and enlarging its body, the mother cell divides into daughter cells without fail. Here we have studied the molecular systems supporting this fundamental process for life using fission yeasts and ciliate *Tetrahymena*. We found that two molecular systems were involved to ensure cytokinesis in fission yeast *Schizosaccharomyces pombe*. On the other hand, it was demonstrated that *Tetahymena* divides into two cells by unexpected and unidentified molecular mechanism. In future comparative study on cytokinesis using evolutionary divergent organisms will be indispensable to understand the fundamental cellular phenomenon for life.

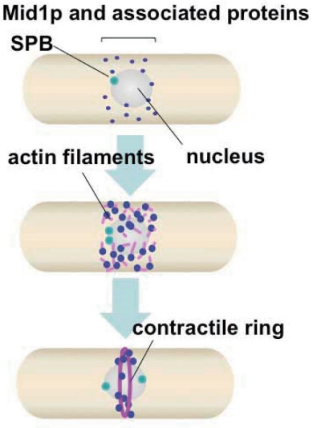
Key Words : actin, myosin, cytokinesis, fission yeast, *Tetrahymena*

Introduction

In the final stage of the cell cycle, cleaving a mother cell into two daughters ensures completion of both genome segregation and organelle distribution for life. This step called as cytokinesis in that requires the cell to solve a spatial problem (to divide in the correct place, orthogonally to the plane of chromosome segregation) and a temporal problem (to coordinate cytokinesis with mitosis). Defects in the spatiotemporal control of cytokinesis may cause cell death, or increase the risk of tumor formation. The principal focus of this study will be the molecular mechanisms of cytokinesis in the mitotic cycle using unicellular model organisms.

Results

To investigate the molecular mechanism of cytokinesis, we mainly used the fission yeast *Schizosaccharomyces pombe*. Because, this simple model has contributed significantly to our understanding of how the cell cycle is regulated, and serves as an excellent model for studying aspects of cytokinesis. The position of the division site is set by the location of the interphase nucleus and depends on a PH-domain protein Mid1p and proteins that confer cell polarity. A contractile ring (CR) is assembled at the center of the cell during mitosis at the end of anaphase, the constriction of this ring is thought to guide synthesis of the septum that bisects the cell (see the right cartoon). In this great opportunity given



by the Novartis found (Japan), I have firstly tried to reveal the molecular mechanism how actin and myosin II assemble into a ring under the control of Mid1p. Previous reports from our lab and others have mentioned that Rng2p is possible to link myosin II accumulation to the cortical Mid1p in the prospective division site. A member of my lab and I have investigated a physiological interaction among those proteins using a pull-down assay of cell extracts and Yeast Two-hybrid system. However, at this moment, their interaction has not been fully uncovered yet for reporting here. On the other hand, even in cells lacking Mid1p-activity, myosin II can associate with a cable-like actin filaments (F-actin) elongating from the cell cortex during anaphase, resulting in CR formation at the significant rate. In the course of study, we have succeeded to characterize the N-terminal actin-binding domain of Rng2p that can induce myosin II binding to F-actin in vivo and in vitro (manuscript in preparation). Thus, in *S. pombe* cell division is accomplished with Mid1p-dependent and –independent systems. This duplicate system for ensuring cell division may be conserved in another organism. To investigate this possibility, we have isolated genes encoding a counterpart of those proteins form another species of fission yeast *S. japonicas* and are now trying to characterize their cellular function.

In addition, we studied the molecular mechanism of cytokinesis in the ciliate *Tetrahymena*. Eukaryote can be broadly classified into opistkont and bikont. Animal cells and fungi including yeasts are grouped in the former, while plant cells, algae and ciliate belong to the latter. Interestingly, no gene corresponding to myosin II is conserved in bikont. However, it had well been known that many ciliate divide like animal cells. To challenge to solve this mystery-shrouded issue, we have studied function of actin and its binding proteins, and myosins unrelated to myosin II in *Tetrahymena*. Actin-depolymerizing factor (ADF) is one of the most important proteins controlling actin dymamics in eukaryotic cells. Suppressing cellular function of ADF causes severe defect in cytokinesis in opistkont. We have evaluated ADF-activity in cytokinesis of *Tetrahymena* cell by deleting the corresponding gene. As a result, the cells continue to divide by binary fission even in lacking ADF-activity (Shiozaki et al., in press). Moreover, administration of actin-inhibitory drug did not affect on cytokinesis of this organism (manuscript under revision). After determination of cellular localization using green fluorescence protein, all of 13 gene products of myosin were not found in a cleavage furrow in a dividing cell of *Tetrahymena* (manuscript in preparation). Taken together, we concluded that this organism probably accomplishes binary fission independently of the actomyosin-based system.

Discussion & Conclusion

In this study we arrive at the conclusion that *S. pombe* adopts two independent mechanisms, but closely related each other, in CR formation for cytokinesis. Rng2p is involved in both molecular pathways via its different structural domains. On the other hand, *Tetrahymena* cell seems to divide without the actomyosin-based system. At this moment we do not have a clue as to this unexpected phenomenon. Very recently, a foreign group has reported that the kinase complex called Sid2p-

Mob1p, a member of the NDR kinase family which is implicated in growth control and cytokinesis in multicellular eukaryotes as part of the Salvador-Warts-Hippo tumor suppressor network, may be involved in positional control of division site in *Tetrahymena*. This kinase family plays important role for CR maturation following the Mid1p-dependent actin and myosin II recruitment in *S. pombe*. Therefore, identification of downstream substrate of Sid2p-Mob1p may shed light on some of the mysteries about the conserved molecular mechanism of spatio-temporal control for cytokinesis in eukaryote.

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一般の皆様へ

核ゲノムを複製して細胞が増殖する際に、細胞は適切な位置で分裂する。分裂中の細胞のくびれにみられる「収縮環」は、骨格筋と同様にアクチン繊維とミオシンを主成分とした分子装置であり、細胞体を分裂させる力を発生させると考えられている。分裂細胞では、分配された核ゲノムを確実に娘細胞に伝承するために、適切な位置に一過的に収縮環が形成される。細胞表層から離れた空間に存在する分裂核が、どのようにして細胞表層での収縮環形成の位置を規定しうるのか、それらを結びつける分子シグナルの実体は不明な点が多い。そこで本研究では分裂酵母などの単細胞真核生物の実験系の利点を活用し、核と細胞表層をつなぐ分子シグナルの実体の解明を目指した。この研究成果は、生命活動の根本を支える細胞増殖のしくみや、生物史を通して母細胞から娘細胞へとゲノムが伝承されてきた過程を深く理解するのに貢献できる。

The role of transcription factor IκB-ζ in immune homeostasis

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Abstract

IκB-ζ plays an important role in immune homeostasis. This study showed that IκB-ζ is important in the immune suppressive function of Tregs. Furthermore, IκB-ζ is not required for the stability of forkhead box p3 (*Foxp3*) gene expression.

Key Words : IκB-ζ, *Foxp3*, Treg

Introduction

Nuclear transcriptional regulator IκB-ζ–deficient mice develop Sjögren's syndrome (SS) with age. Several studies have shown that SS is a T-cell dependent autoimmune disease. An Id3-deficient SS mouse model exhibits defects in the generations and suppression of the $Foxp3^+$ Tregs. This study focused on the role of IκB-ζ in immune homeostasis through the generation, function, and stability of Tregs.

Results

A previous study (Okuma *et al*., *Immunity*. **3**: 450-460, 2013) showed that IκB-ζ–deficient mice developed SS and exhibited splenomegaly and lymphadenopathy. In addition, these mice showed absolute numbers of T cells, whereas the number of $CD44^{high}$ memory/effector-type T cells in the peripheral lymph nodes significantly increased. These phenotypes can be found in each generation of the IκB-ζ–deficient mice. These observations indicate that IκB-ζ-deficient mice were unable to maintain immune homeostasis.

The $CD4^+$ Tregs express *Foxp3* and play an important role in maintaining immune homeostasis. Scurfy mice deficient in functional *Foxp3* undergo massive inflammation of the whole body and die within 4 weeks.

Unexpectedly, this study showed that the numbers of $Foxp3^+$ Tregs and $CD4^+$ T cells in the peripheral lymph nodes were similar to those in the wild type (WT) and IκB-ζ–deficient mice. To check the transcriptional activity of IκB-ζ in relation to *Foxp3* gene expression, a Foxp3 reporter assay was performed. The results showed that the overexpression of IκB-ζ did not alter the Foxp3 reporter activity in human embryonic kidney (HEK)293 cells.

To analyze the suppression ability of Tregs, naïve $CD4^+$ T cells were co-cultured with Tregs for 3 days, which showed that IκB-ζ–deficient Tregs suppressed the expansion of naïve $CD4^+$ T cells, although slightly lower than that in WT Tregs. Then, the immune-suppression associated with co-stimulatory molecules (cytotoxic T-lymphocyte antigen 4 [CTLA-4] and glucocorticoid-

Induced tumor necrosis factor receptor [GITR]) on Tregs were examined to address whether the low suppressive ability of IκB-ζ–deficient Tregs could be attributed to the expression levels of co-stimulatory molecules. This experiment showed that the expression levels were similar in the WT and IκB-ζ–deficient Tregs. In addition, production of the immune regulatory cytokine, Interleukin (IL)-10, was similar between WT and IκB-ζ-deficient Tregs.

Next, the stability of the Foxp3 gene expression in Tregs was examined. This is considered as one of the mechanisms behind the alteration in immune homeostasis in IκB-ζ–deficient mice. Carboxyfluorescein succinimidyl ester (CFSE)-labeled $CD4^+CD25^+$ Tregs cultured for 3 days under TCR and a low dose of IL-2 stimulation showed that the Foxp3 expression level was similar in both WT and IκB-ζ–deficient Tregs at each cell division. To examine the Foxp3 gene expression stability *in vivo*, adoptive WT or IκB-ζ-deficient Tregs were transplanted into $Rag2^{-/-}$ mice. Four weeks later, the Foxp3 expression levels in $CD4^+$ T cells was similar in both the WT and IκB-ζ-deficient Tregs. This study also found that natural Tregs express IκB-ζ, but the expression level was significantly lower than that in the Th17 cells. The SOCS1 expression level, which is required for the Foxp3 gene expression stability, was similar in both the WT and IκB-ζ-deficient Tregs. These results indicated that IκB-ζ is not required for the Foxp3 gene expression stability.

Discussion & Conclusions

Tregs strongly expressed CD25 (IL-2 receptor α); therefore, these cells can predominantly proliferate in inflammatory environments (IL-2–rich regions) such as the lymph nodes. The percentage of Tregs was similar in the WT and IκB-ζ–deficient mice; however, the role of IκB-ζ in the generation of Tregs from naïve $CD4^+$ T cells remains unresolved.

IκB-ζ-deficient Tregs have less suppressive ability; thus, it is necessary to check the suppressive ability *in vivo* by using an animal model, such as that showing inflammatory bowel disease. However, the molecular mechanisms responsible for this low suppressive ability in IκB-ζ–deficient Tregs remain unclear.

The stability of *Foxp3* gene expression in IκB-ζ–deficient Tregs is similar between WT and IκB-ζ–deficient mice.

Another interesting results is regarding IκB-ζ and Th17. It was previously shown that IκB-ζ interacts with RORγt to promote Th17 differentiation. Accordingly, in IκB-ζ-deficient mice, naïve $CD4^+$ T cells failed to generate Th17 cells. Thus, IκB-ζ accelerates the differentiation of both Tregs and Th17 cells.

一般の皆様へ

本研究では、シェーグレン症候群様の自己免疫疾患を引き起こす遺伝子欠損マウス (IκB-ζ 欠損マウス) において、その原因の 1 つとして免疫抑制能力の低下が示唆された。今後、その詳しい分子メカニズムについて明らかとする必要がある。

Study of dense-core vesicle synthesis on the membrane of Golgi complex

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Abstract

We found that somal Ca^{2+}-dependent activator protein for secretion 2 (CAPS2) is associated with the Golgi membrane, and mediates binding and recruitment of the GDP-bound form of ARF4 and ARF5 to the Golgi membrane. CAPS2 knockdown and over-expression of CAPS2-binding-deficient ARF4/5 both induced accumulation of the dense-core vesicle (DCV) resident protein chromogranin A around the Golgi apparatus. CAPS2 knockout mice have dilated trans-Golgi structures when viewed by electron microscopy. These results for CAPS2 strongly support our idea that the CAPS family proteins exert dual roles in DCV trafficking, mediating trafficking at both the secretion site for exocytosis and at the Golgi complex for biogenesis.

Key Words : CAPS, chromogranin, Golgi complex

Introduction

The CAPS family consists of two members (CAPS1 and CAPS2) and regulates exocytosis of catecholamine- or neuropeptide-containing DCVs at secretion sites such as nerve terminals. A large fraction of CAPS1 protein, however, is localized in the cell soma, and we have recently shown the possible involvement of somal CAPS1 in DCV trafficking in the trans-Golgi network (TGN). CAPS1 and CAPS2 are differentially expressed in various regions of the mouse brain but exhibit similar expression patterns in other tissues such as the spleen. Thus, in the present study we analyzed whether CAPS2 displays similar subcellular localization and functional roles in the cell soma as CAPS1.

Results

An exogenously expressed CAPS2 PH domain fused with the fluorescent protein YPet and syntaxin 6 (Stx6), a TGN membrane trafficking protein were colocalized in αT3-1 pituitary gonadotrope cells. We found that the CAPS2 constructs containing both C2 and PH domain (full-length and ΔC-Terminal) exhibited a diffuse distribution pattern throughout the cytoplasm, but ΔN-Terminal and ΔC2, which contain a PH domain but lacked the C2 domain, accumulated around the Golgi in a pattern similar to that of the PH domain alone.

To verify whether, like CAPS1, endogenous CAPS2 associates with the Golgi membrane, we analyzed CAPS2-immunoaffinity-purified microsomal fractions from P21 mouse cerebellum by western blotting with antibodies for various Golgi membrane-associated proteins. The western blot results indicated that CAPS2-immunoaffinity purification enriched the Golgi marker proteins

p115, GM130, GS28, vti1a, vti1b, Stx6, and VAMP4 as well as vesicle-trafficking-related proteins SNAP25 and ARF.

We next investigated the ability of CAPS2 to bind to the small GTPase ARF family (ARF1–6) using a co-immunoprecipitation assay. We found that only the class II ARFs (ARF4 and ARF5) were co-immunoprecipitated with anti-CAPS2 antibody. Interestingly, the binding of CAPS2 to ARF4 and ARF5 was preferentially observed with the GDP-locked form (T31N) but not with the GTP-locked form (Q71L).

Finally, we analyzed the in vivo effect of CAPS2 knockout on the Golgi appearance by utilizing CAPS2 knockout mice. Electron microscopic analyses showed that in the CA1 pyramidal cell layer of the hippocampus, approximately half of the identifiable cisternae of the Golgi stack, which were predominantly on the trans side, were dilated in CAPS2 knockout, compared with wild-type mice.

Discussion & Conclusion

Our findings indicate that CAPS2 binds to the Golgi membrane and interacts with the class II ARF small GTPases ARF4/5 in a GDP/GTP-state dependent manner, demonstrating the involvement of CAPS2 in the recruitment of ARF4/5 onto the Golgi membrane. Our data demonstrate that the recruitment of ARF4/5 via CAPS2 is indispensable to DCV trafficking. Firstly, CAPS2 knockdown resulted in the accumulation of ChgA around the Golgi apparatus. Secondly, overexpression of CAPS2-binding-deficient ARF5 mutants induced accumulation of ChgA in the Golgi. Thirdly, CAPS2 knockdown caused abnormal ARF5 distribution around cell soma, and finally CAPS2 knockout mice had dilated structures in the Golgi cisternae. Collectively, these findings suggest that CAPS2 is required for DCV trafficking in the Golgi complex by recruiting the class II ARF small GTPases, in a GDP-bound dependent manner, to the Golgi membrane.

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一般の皆様へ

有芯小胞はインスリンやドーパミンなどを内包し、生命活動や疾患に関わる重要な構造物である。しかし、有芯小胞による細胞からの分泌メカニズムについては詳しく解明されていない。今回我々は CAPS2 タンパク質が有芯小胞の分泌にどのように関与しているかを細胞生物学的に明らかにした。今後様々な応用発展の可能性を秘めた結果であると考えている。

Stress response, homeostasis and cancer

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Abstract

Most hepatocellular carcinomas (HCCs) develop in the context of severe liver fibrosis caused by chronic liver inflammation, which also results in accumulation of reactive oxygen species (ROS). We examined whether the stress activated protein kinase p38α and cold-inducible RNA-binding protein (CIRP) control ROS metabolism and development of fibrosis and cancer in mice given thioacetamide (TAA). Liver-specific p38α ablation enhanced ROS accumulation, which was exerted through the reduced expression of anti-oxidant protein heat shock protein (HSP) 27. CIRP-deficiency decreased ROS accumulation and attenuated TAA-induced hepatocarcinogenesis. Intriguingly, the risk of human HCC recurrence is positively correlated with ROS accumulation in liver.

Key Words : CIRP, p38, liver fibrosis, ROS, SOX2, Gankyrin

Introduction

HCC is the most common form of liver cancer and the third leading cause of cancer deaths worldwide and is usually associated with a very poor prognosis (1). The risk of hepatocarcinogenesis depends on background liver factors, of which chronic inflammation is a major one. Stress activated protein kinases (MAPK/SAPK) play a pivotal role in modulating numerous cellular responses, including cell proliferation, and metabolism (2,3). One of the SAPKs, p38α, the major p38 isoform, is activated in response to inflammation and oxidative stress (4-7). CIRP is induced by cellular stresses such as UV irradiation and hypoxia (8-10). In response to the stress, CIRP migrates from the nucleus to cytoplasm, and affects expression of its target mRNAs posttrascriptionally (11-13).

Results

TAA model may be suitable for studying the relationship between inflammation and cancer.

TAA can induce liver cirrhosis and cancer of the bile ducts when given to rats over a period of several months (14). However, as described here we found that mice given TAA for 10 months develop HCC rather than cholangiocellular carcinoma subsequent to appearance of severe liver fibrosis, thus providing a model that closely mimics the natural history of human HCV-related liver disease. In addition, the histology of the TAA-exposed rat liver was reported to resemble human liver cirrhosis (15). Thus, the mouse TAA model may be suitable for studying the relationship between ROS accumulation, liver fibrogenesis and hepatocarcinogenesis.

Enhanced ROS accumulation and fibrogenesis in p38α$^{\Delta hep}$ mice.

We used a conditional *p38α* "floxed" strain to generate *p38α*$^{\Delta hep}$ mice, lacking p38α in liver

parenchymal cells. After 8 weeks of TAA treatment, we observed inflammation, HSC activation and formation of fibrotic septa as assessed histologically or by immunohistochemistry with a specific antibody against α-SMA. *p38α*$^{\Delta hep}$ mice exhibited more TAA-induced liver damage assessed by ALT release, and hepatocyte apoptosis measured by a TUNEL assay, relative to controls. In addition, there were higher numbers of α-SMA-positive cells, higher levels of hydroxyproline and larger fibrotic areas in *p38α*$^{\Delta hep}$ mice compared with control mice. Loss of p38α significantly enhanced expression of the mRNAs for col1α1, TIMP1, TGF-β1 and PDGFb. *p38α*$^{\Delta hep}$ mice were found to have higher levels of oxidized protein in comparison to controls. To evaluate the contribution of oxidative stress to TAA-induced liver damage and fibrosis, we placed a group of mice on chow diet supplemented with the antioxidant butylated hydroxyanisole (BHA). *p38α*$^{\Delta hep}$ mice kept on this diet showed a significant reduction in TAA-induced liver injury and fibrosis. Thus, loss of p38α enhances TAA-induced cell death and fibrogenesis through mechanisms that may depend on ROS accumulation. Adenoviral transduction of HSP27 in *p38α*$^{\Delta hep}$ liver prevented TAA-induced ROS accumulation, liver damage and fibrogenesis. Loss of p38α significantly enhanced expression of stemness factor SOX2 mRNA and protein. *p38α*$^{\Delta hep}$ mice exhibited a significant increase in Gankyrin expression.

Enhanced hepatocarcinogenesis in p38α$^{\Delta hep}$ mice and attenuated hepatocarcinogenesis in CIRP-deficient mice.

Tumor size and area were considerably larger in *p38α*$^{\Delta hep}$ mice relative to similarly treated controls. In contrast, TAA-treated CIRP-deficient mice harbored less tumor load than control mice.

Association between risk of HCC recurrence and ROS accumulation in human liver.

Intrahepatic HCC development after hepatectomy is caused by de novo HCC development and/or metastasis from the resected HCC. The risk of the former depends on background liver factors such as liver fibrosis, while the risk of the latter mainly depends on characteristics of resected HCC (16). In mouse models, HSP25-mediated inhibition of ROS accumulation is involved in liver fibrogenesis and can subsequently affect de novo HCC development. We examined whether this hypothesis is applicable to humans, focusing on non-cancerous liver tissues rather than cancers to assess the potential for de novo HCC development or rapid progression of lesions that were undetectable or pre-neoplastic at the time of resection. In patients exhibiting HCC recurrence after hepatectomy, ROS levels in the non-tumor tissues, but not in tumors, were significantly higher than in those without HCC recurrence. In addition, patients with low ROS accumulation in non-cancerous liver had a prolonged recurrence-free survival. (17-19)

Discussion & Conclusion

p38α plays a critical role in liver fibrogenesis and hepatocarcinogenesis through the control of HSP27 expression and ROS accumulation in the mouse TAA model which may be suitable for studying the pathogenesis of inflammation-related HCC development. CIRP also controls ROS accumulation and hepatocarcinogenesis in mice given TAA. Correspondingly, elevated ROS accumulation in the liver are associated with increased risk of human HCC development or

recurrence. Pluripotency-associated transcription factors such as SOX2 and Nanog are known as regulators of cellular identity in embryonic stem cells (20). Gankyrin, a liver oncoprotein, was reported to mediate dedifferentiation and facilitate the tumorigenecity of rat hepatocytes (21). Deletion of p38α upregulates expression of SOX2 and Gankyrin, which may be involved in cancer stem cell maintenance.

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一般の皆様へ

ストレス応答蛋白は外敵から細胞を守るのに重要な役割を果たしています。p38 という蛋白はストレス応答蛋白 HSP27 の量を増加させ活性酸素種を抑制することで、肝臓における慢性炎症と癌の発生を抑制します。また別のストレス応答蛋白 CIRP は活性酸素種の蓄積を増幅させることで癌の発生を促進します。ヒトにおいて肝臓がんの再発率と活性酸素種が相関します。将来、HSP27 や CIRP の蛋白量を増やしたり、減らしたりする薬剤を開発することで肝癌を予防することが可能になるかもしれません。

Regulation of cerebral vasospasm by growth factors and matricellular proteins

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Abstract

The purpose of this study was to assess whether imatinib mesylate (imatinib), an inhibitor of the tyrosine kinases of platelet-derived growth factor receptors (PDGFRs), prevents cerebral vasospasm after subarachnoid hemorrhage (SAH) in rats, and to elucidate if tenascin-C (TNC), a matricellular protein, is involved in the mechanism. Imatinib significantly prevented post-SAH neurological impairments and vasospasm. SAH caused PDGFR-β upregulation, PDGFR activation, mitogen-activated protein kinases activation, and TNC upregulation in the spastic cerebral arteries, all of which were significantly suppressed by imatinib treatment. Recombinant TNC reversed the anti-vasospastic effects and protein expression changes by imatinib.

Key Words : vasospasm, subarachnoid hemorrhage, platelet-derived growth factor, tenascin-C

Introduction

Cerebral vasospasm remains a major cause of poor outcome after subarachnoid hemorrhage (SAH). We reported that tenascin-C (TNC) was induced after SAH in a clinical setting, associated with cerebral vasospasm. TNC is a matricellular protein, and exerts diverse functions through direct binding to cell surface receptors, other matrix proteins, and soluble extracellular factors such as growth factors and cytokines. As platelet-derived growth factor (PDGF), which is a potent inducer of TNC, has been involved in the pathogenesis of vasospasm, we hypothesized that the inhibition of tyrosine kinases of PDGF receptors (PDGFRs) prevents experimental vasospasm after SAH via inhibiting TNC induction.

Results

First, imatinib mesylate (imatinib; 10 or 50mg/kg body weight), an inhibitor of the tyrosine kinases of platelet-derived growth factor receptors (PDGFRs), was administered intraperitoneally to rats undergoing subarachnoid hemorrhage (SAH) by endovascular perforation, and the effects were evaluated by neurobehavioral tests and India-ink angiography at 24-72 hours post-SAH. Western blotting and immunohistochemistry were performed to explore the underlying mechanisms on cerebral arteries at 24 hours post-SAH. Recombinant tenascin-C (TNC) was administered intracisternally to imatinib-treated SAH rats, and the effects were evaluated by neurobehavioral tests, India-ink angiography and immunohistochemistry at 24 hours post-SAH. Both dosages of imatinib significantly prevented post-SAH neurological impairments and vasospasm at 24-72

hours. SAH caused PDGFR-β upregulation, PDGFR activation, mitogen-activated protein kinases activation, and TNC upregulation in the spastic cerebral arteries, all of which were significantly suppressed by imatinib treatment. Osteopontin (OPN), another matricellular protein, was also upregulated mildly after SAH, but its levels were not significantly affected by imatinib. In SAH rats, TNC immunoreactivity was markedly induced in the smooth muscle cell layers of spastic cerebral arteries at 24 hours post-SAH, but not in control animals. The TNC immunoreactivity decreased at 72 hours, as vasospasm improved: OPN immunoreactivity, on the other hand, was more induced in the arterial wall at 72 hours after SAH. Recombinant TNC reversed the anti-vasospastic effects and protein expression changes by imatinib.

Second, two dosages (1 and 10 μg) of TNC were administered intracisternally to healthy rats, and the effects were evaluated by neurobehavioral tests and India-ink angiography at 24, 48, and 72 hours after the administration. Western blotting and immunohistochemistry were performed to explore the underlying mechanisms on constricted cerebral arteries after 24 hours. The effects of toll-like receptor 4 (TLR4) antagonists (LPS-RS), c-Jun N-terminal kinase (JNK), p38 inhibitors (SP600125 and SB203580) and recombinant OPN on TNC-induced vasoconstriction were evaluated at 24 hours. Higher dosages of TNC induced more severe cerebral arterial constriction, which continued for more than 72 hours. TNC administration also upregulated TLR4, and activated JNK and p38 in the smooth muscle cell layer of the constricted cerebral artery. LPS-RS blocked TNC-induced TLR4 upregulation, JNK and p38 activation, and vasoconstrictive effects. SP600125 and SB203580 abolished TNC-induced TLR4 upregulation and vasoconstrictive effects. In addition, recombinant TNC-induced prolonged contractions of rat basilar arteries were reversed by recombinant OPN, although recombinant OPN itself had no effect on the vessel diameter.

Third, imatinib (50mg/kg body weight) was administered intraperitoneally to rats undergoing SAH by endovascular perforation, and the effects were evaluated by neurobehavioral tests, India-ink angiography, Western blotting, terminal deoxynucleotidyl transferase-mediated uridine 5-triphosphate-biotin nick end-labeling staining and immunohistochemistry. Recombinant TNC was administered intracisternally to imatinib-treated SAH rats, and the effects were also evaluated. Imatinib significantly prevented cerebral vasospasm at both 24 and 72 hours post-SAH, but improved neurobehavioral tests only at 24 hours. SAH caused PDGFR activation, mitogen-activated protein kinases activation, TNC upregulation and caspase-dependent neuronal apoptosis in the cerebral cortex, all of which were significantly suppressed by imatinib treatment at 24 hours, but not at 72 hours post-SAH. Recombinant TNC reversed the anti-apoptotic effects and protein expression changes in brain by imatinib.

Discussion & Conclusion

We demonstrated for the first time that treatment by imatinib mesylate, an inhibitor of the tyrosine kinases of platelet-derived growth factor receptors, prevented cerebral vasospasm after subarachnoid hemorrhage at least partly via inhibiting the upregulation of TNC. We also demonstrated for the first time that TNC induced prolonged cerebral arterial constriction via TLR4 and activation of JNK and p38 in healthy rats. TNC may be involved in the pathogenesis of chronic vasoconstrictive diseases, such as cerebral vasospasm, and may provide a novel therapeutic approach to treat such diseases. Further investigations may prove that TNC provides a novel therapeutic approach against cerebral vasospasm.

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一般の皆様へ

脳動脈瘤破裂によるくも膜下出血は、現在でも死亡率は約 50%と高く、しかも生存者の 30% 以上に身体的障害を残す重篤な脳血管障害です。くも膜下出血患者の予後を改善するためには、2 次的に生じる脳血管攣縮や脳損傷を克服する必要があります。本研究では、炎症性サイトカインの 1 つである血小板由来増殖因子と近年注目されているマトリックス細胞蛋白と呼ばれる特殊な蛋白の 1 つであるテネイシン C が、くも膜下出血後に生じる脳血管攣縮や脳損傷の発生に関与していることを明らかにしました。今後、更に研究を進め、これらの成果をくも膜下出血に対する新たな治療法の開発につなげたいと考えています。

Centriole biogenesis and Chromosomal instability

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Abstract

Formation of a new centriole next to each existing one is fundamental for centrosome duplication and thus for genome integrity. Recent studies indeed highlighted that centriole amplification can be an origin of genome instability in tumor formation. We demonstrated that MCPH7/STIL is essential for centriole formation and also that increased expression levels of STIL promote formation of multiple new centrioles per pre-existing one. Therefore, regulated STIL levels and/or function ensure the “only-one rule” of centriole formation. In order to identify proteins which modulate STIL function, we employed the combined approach using MS-based proteomic analysis and a functional RNAi screening in human cells. As a result, we identified a tumor suppressor with a RNA-binding motif as a STIL-interacting protein that has a role in limiting the number of centrioles.

Key Words : Chromosomal instability, Centrosome, Centriole

Introduction

The mechanisms of centrosome duplication have been a long-standing mystery in biology. Like the genetic material, centrosome duplication must occur once per cell cycle, such that the two resulting centrosomes assemble a bipolar spindle during mitosis. Aberration in the number of centrosomes is implicated in genome instability and tumor formation. The centrosome is composed of a pair of centrioles surrounded by pericentriolar material (PCM). Centrioles are also essential for generating cilia and flagella across eukaryotic evolution. Despite being crucial for genome integrity and many biological processes, the mechanisms controlling centriole copy number remain incompletely understood.

Results

Centrioles are barrel-shaped microtubule-containing structures characterized by a universal 9-fold radial symmetry that they also impart to cilia and flagella. In most species, the centriole is organized around a cartwheel that comprises a central hub from which 9 spokes emanate outwards towards the microtubules. The molecular mechanisms and structural principles governing centriole assembly remain poorly understood.

To elucidate how the onset of centriole formation takes place in higher eukaryotes with a particular emphasis on SAS-6, a protein required for centriole formation from worm to human, we used a combination of multi-disciplinary approaches including cell biology, biochemistry, genetics, biophysics and structural biology (1-3). Our findings revealed how SAS-6 is targeted to the assembly

site of centrioles and timely modified (2), and further demonstrated that oligomerization of SAS-6 homodimers is at the root of the 9-fold symmetry of the cartwheel and thus of centrioles (3). These works represented the first molecular and structural insights into the mechanisms governing this fundamental process. Even though we demonstrated that SAS-6 self-assembly is a key step to initiate centriole formation, we hypothesized that something as upstream should facilitate this process based on the fact that SAS-6 self-assembly alone does not seem to be sufficient to spontaneously form central part of cartwheel.

To identify such upstream regulator, we first analyzed the MCPH7 protein STIL, which has been suggested to be related to the centriole proteins SAS-5 in *C. elegans* and Ana2 in *Drosophila*, both of which are essential for centriole formation. Although whether STIL is required for centriole formation in human cells has not been addressed, we found that siRNA-mediated depletion of STIL results in a failure of centriole formation, with mitotic cells harboring usually ≤2 Centrin-3 (a centriole marker) positive foci instead of the usual four, as is the case for cells depleted of SAS-6 (1). A similar failure of centriole formation was observed using two distinct siRNAs directed against STIL. We conclude that STIL is required for centriole formation in human cells. This also lends support to the notion that SAS-5/Ana2/STIL is an evolutionarily conserved module critical for centriole formation. Importantly in addition, we found that centriolar localization of STIL, at least in part, is critical for SAS-6 loading to centrioles, suggesting that STIL could facilitate self-assembly of SAS-6 at the assembly site of a new centriole. Indeed, overexpression of STIL led to the simultaneous formation of multiple new centrioles through SAS-6 recruitment at the assembly site, indicating that STIL is a critical regulator to control the copy number of new centrioles. Furthermore, we demonstrated that some STIL-depleted cells assemble an asymmetric bipolar spindle in which less pronounced astral microtubules emanate from one spindle pole (1). Importantly, we found in addition that whereas spindle position is normal in those STIL-depleted cells with a symmetric bipolar spindle, spindle position is randomized in the subset of cells with asymmetric spindles. Therefore, STIL is also critical for proper spindle positioning as well as centriole formation in human cells.

Next, we set out to decipher the mechanisms limiting the number of newly formed centrioles and maintaining genome integrity. We reasoned that such suppressors for centriole amplification would physically interact with and directly control critical centriolar regulators/components such as SAS-6 and STIL. Accordingly, we employed the combined approach using MS-based proteomic analysis and a functional RNAi screening in human cells, which led to the identification of RBM14, a tumor suppressor, as a STIL-interacting protein that has a role in limiting the number of centrioles. Depletion of this protein by siRNA in human cells induces dramatic amplification of centriolar intermediates through STIL function, and also results in multipolar spindle formation as a consequence. We are currently focusing on the underlying molecular mechanisms in more detail.

Discussion & Conclusion

Overall, we identified STIL as a novel regulator for centriole formation. STIL seems to have a critical role in the initiation of centriole formation most likely through the recruitment of SAS-6 at the assembly site of an emerging centriole. Furthermore, our data suggest that the expression levels of STIL and its interactor, RBM14, regulate the copy number of centrioles. It will be therefore tempting to investigate how they interact and mutually modulate their function/expression to properly coordinate this process.

We expect that our findings will make unique contributions to our understanding of centrosome duplication. Cancer cells often exhibit aberrations in centrosome number, which could be a cause of tumorigenesis. Furthermore, dysfunction of centrosomes is associated with human neurodevelopmental disorder and infertility. Therefore, centrosome duplication offers important therapeutic and diagnostic opportunities in oncology and stem cell biology.

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一般の皆様へ

近年、中心小体の過剰複製と細胞がん化の関与が指摘されており、実際に複数のがん抑制遺伝子が中心小体複製に関与することが報告されています。よって、中心小体複製の素過程における知見は細胞がん化メカニズムの解明にも寄与することが期待されています。また、今後の抗癌剤の標的探索という観点からも有益であると考えられます。なぜなら、現在までに微小管重合形成を阻害する抗癌剤は多数開発されており、今後も細胞分裂に必須である中心小体複製、紡錘体形成を標的にした有効な抗癌剤の開発が期待されているからです。さらには、本研究により、中心小体形成不全が原因と推定される遺伝病、繊毛病、男性不妊症など種々の疾病の原因解明に向けて基礎的な知見が提供されることが期待されます。

The role of specific cell adhesion molecules in dopaminergic innervation of striatal neurons

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Abstract

The main pathological feature of Parkinson disease is a selective degeneration of the nigrostriatal dopaminergic pathway. However, the precise mechanisms by which dopaminergic axons innervate striatal neurons are still unclear. In this study, we demonstrated that integrin α5β1 expressing in dopaminergic neurons participate in dopaminergic innervation of striatal neurons. This finding is beneficial for cell transplantation of dopaminergic neurons into the striatum of patients with Parkinson disease.

Key Words : dopaminergic innervation; integrin; Parkinson disease; embryonic stem cell

Introduction

The main pathological feature of Parkinson disease is a selective degeneration of the nigrostriatal dopaminergic pathway, and regeneration of the neuroprojection is one of the promising therapeutic approaches. However, the precise mechanisms by which dopaminergic axons innervate striatal neurons are still unclear. Integrins, heterodimers of α and β subunits that function as cell adhesion molecules, play an important role in embryonic development of central nervous system. In this study, we investigated the involvement of integrins in dopaminergic innervation of striatal neurons.

Results

We established a novel method to evaluate dopaminergic innervation of striatal neurons by using primary dissociated cells (Wakita et al., 2010). Mecencephalic and striatal cells were dissected from rat embryos, and then a mecencephalic cell region was formed adjacent to a striatal cell region on a plastic coverslip. After paired-culturing for 11 days, dopaminergic neurons in the mesencephalic cell region extended their axons to the striatal cell region. Immunofluorescent microscopy showed that dopaminergic axons elongated along striatal neurons and formed synapses with striatal neurons.

Integrin β1 subunit plays a central role in the function of integrin. Neutralizing integrin β1 antibody suppressed dopaminergic innervation of the striatal neurons. Next, we examined the role of RGD-binding integrins in dopaminergic innervation of striatal neurons. Inhibition of RGD-binding integrins by the small peptide RGDS and an integrin α5β1/αvβ3-blocking peptide ATN-161 (Ac-PHSCN-NH2) suppressed dopaminergic neurite outgrowth to the striatal cell region. To identify the integrin α subunit, we examined the effect of selective integrin-blocking peptides and neutralizing integrin α antibody. As a result, an integrin α5β1-blocking peptide A5-1 (VILVIF) and neutralizing

integrin α5 antibody attenuated the dopaminergic innervation of striatal neurons, while an integrin αvβ3-blocking peptide cyclo-RGDfV had no effect.

Integrin α5 and β1 subunits were expressed at the axonal growth cones and somas of dopaminergic neurons. In addition, dopaminergic axonal outgrowth was enhanced by coating with fibronectin, a ligand of integrin α5β1. To confirm that the integrin α5 subunit expressing in dopaminergic neurons participate in dopaminergic innervation of striatal neurons, we employed RNAi to knockdown of integrin α5 in mesencephalic cells. First, we prepared lentiviral vector expressing short hairpin RNA (shRNA) designed against rat integrin α5. As determined by immunoblot analysis, the shRNA decreased expression of endogenous α5 integrin to almost 40%. By infection of mesecepalic cells with lentiviral integrin α5 shRNA, the dopaminergic innervation of striatal neurons was siginificantly suppressed.

To explore a ligand of integrin α5β1, we focused on chondroitin sulfate and L1 cell adhesion molecule. It has been reported that chondroitin sulfate is expressed in and around the dopaminergic axons (Charvet et al., 1998). However, treatment with chondroitinase ABC that degrades chondroitin sulfate did not affect the dopaminergic innervation. The L1 cell adhesion molecule can bind with integrin α5β1, and promotes axon growth and cell migration (Thelen et al., 2002). Coating with L1 protein tended to enhance dopaminergic axonal outgrowth. With respect to a ligand of integrin α5β1, further studies are required.

Last, we produced mouse integrin α5-overexpressing embryonic stem cells for cell transplantation in Parkinson disease model mice. Integrin α5 cDNA was prepared from mouse whole brain. Integrin α5 cDNA was then subcloned into viral vector. Mouse embryonic stem cells infected by retroviral vector exhibited no fluorecense of marker protein because of gene silencing. Embryonic stem cells infected by lentiviral vector exhibited faint fluorescence, but the fluorescence intensity was increased during neural differentiation. At presence, we are examining the effect of overexpression of integrin α5 on dopaminergic differentiation. In the next experiment, we will transplant embryonic stem cell-derived integrin α5-overexpressing dopaminergic neuons into Parkinson disease model mice.

Discussion & Conclusion

In this study, we established an in vitro model for the quantitative analysis of the dopaminergic innervations of striatal neurons using primary dissociated neruons. Pharmacological analysis revealed that dopaminergic axons innervate striatal neurons through integrin α5β1. Selective knockdown of integrin α5 in mesencephalic cells with use of lentiviral vector demonstrated that integrin α5β1 expressing in dopaminergic neurons participate in dopaminergic innervation of striatal neurons. This result suggests that integrin α5-overexpressing dopaminergic neurons promote the striatal innervation. Furthermore, we produced mouse integrin α5-overexpressing embryonic stem cells. Transplantion of embryonic stem cell-derived integrin α5-overexpressing dopaminergic neuons into Parkinson disease model mice is expected to ameliorate motor dysfunction. In conclusion, our findings are beneficial for cell transplantation of dopaminergic neurons into the striatum of patients with Parkinson disease.

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一般の皆様へ

黒質 - 線条体ドパミン神経が変性するパーキンソン病において、ドパミン神経細胞を線条体に細胞移植しようと試みられている。しかし、ドパミン神経細胞が線条体神経細胞と相互作用し神経突起を伸ばす機序については明らかになっていない。本研究では、ドパミン神経細胞に発現する細胞接着因子であるインテグリン α5β1 がその相互作用に重要であることを見出した。本研究の成果は、将来的に、パーキンソン病患者に対するドパミン神経細胞移植において、線条体の広範囲にドパミン神経突起を伸長させ効率的に神経支配する目的で活用されることが期待できる。

In vivo imaging reveals molecular mechanisms of adult common diseases based on chronic inflammation

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Abstract

To elucidate the underlying mechanisms of adult common diseases based on chronic inflammation including metabolic and thrombotic disease, it is vital to examine the multi-cellular kinetics in living animals.

Therefore, we developed in vivo imaging technique based on single- and multi-photon microscopy, and we assessed dynamic immune and inflammatory cellular interplay in diseased conditions.

Key Words : In vivo imaging, inflammation, adult common diseases, metabolic syndrome

Introduction

Obesity associates with higher risks for various diseases, including atherosclerotic cardiovascular disease, type 2 diabetes and cancer. Moreover, recent studies have shown that obesity induces chronic inflammation within visceral adipose tissue, which appears to lead to metabolic abnormalities and inflammation in distant tissues. Inflammation is considered to play a pivotal role in the development of metabolic diseases.

Results

CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity

Recently, we found that large numbers of CD8+ effector T cells infiltrated epididymal adipose tissue in obese mice, whereas the numbers of CD4+ helper and regulatory T cells were diminished. The infiltration of CD8+ T cells preceded the accumulation of macrophages during diet-induced-obesity. Immunological and genetic depletion of CD8+ T cells reduced inflammatory (M1) macrophage infiltration and adipose tissue inflammation, and ameliorated systemic insulin resistance. The transfer of CD8+ T cells to CD8-deficient mice enhanced inflammation in obese adipose tissue, demonstrating the importance for CD8+ T cells in obese conditions. Coculture and other in vitro experiments revealed major interactions between CD8+ T cells, macrophages, and hypertrophied adipocytes. Several in vitro studies have shown that obese adipose tissue directly activates CD8+ T cells, which subsequently promotes recruitment and activation of macrophages, thereby initiating and maintaining the inflammatory cascades in obese adipose tissue. Infiltration of CD8+ T cells is, therefore, essential for the initiation and development of adipose inflammation.

Platelet Kinetics in Thrombus Formation, and Inflammatory Status

It is well known that platelets play an important role in inflammatory diseases, such as atherosclerosis. However, the molecular and cellular mechanisms of platelet kinetics in vivo are unknown, because experimental methodology to analyze kinetics of individual platelets in living animals is not well established. We have already established an in vivo visualization system, which enabled us to analyze thrombus formation at the individual platelet level. We can analyze platelet kinetics in vessels of various sizes, including large-sized arteries, veins, and small capillaries. In unstimulated conditions, single platelet moved in a "stop-and-go" manner along the vessel wall and interacted with endothelial cells. By inducing the production of ROS (reactive oxygen species) by laser injury, we can trigger thrombus formation in vessels of all sized including small capillaries and the carotid arteries and observe the kinetics of individual platelets. We could evaluate not only thrombus formation but also platelet functions at the individual platelet level. After laser injury, platelets started to adhere to the vascular wall, and aggregated. The thrombus caused decreased blood flow and the vessel was subsequently completely occluded. Using this technique, we elucidated that Lnk (adapter protein) regulates integrin signaling leading to stabilization of developing thrombus in vivo. We established the efficient culture system of human iPS-derived platelets, and we confirmed the functional role. These artificial platelets can circulate, and contribute to their thrombus formation in vivo, indicating the clinical usefulness considering the cell therapy for future.

In addition, we elucidated the contribution of inflammatory cytokines, ROS, and integrin signaling to our thrombosis models. Therefore, our imaging technique had very broad applications, such as evaluation of anti-platelet drugs in vivo, stem cell-derived platelet function in vivo, and the effect of genetic mutations on platelet functions.

Discussion & Conclusion

Cellular interrelationships associated with chronic inflammatory disease

Cellular reactions in inflammation are particularly dynamic and involve multiple cell types. Consequently, analysis of the multi-cellular dynamic processes involved in inflammation by in vivo imaging using intact tissues in living animals offers a powerful tool. Our results demonstrated the advantages and potential of our imaging technique to analyze multi-cellular interactions in inflammation in vivo. Using our new imaging technique to analyze the complex cellular interactions within obese adipose tissue, we were able to show that visceral adipose tissue obesity is an inflammatory disease. Our technique could be used to evaluate potential therapeutic interventions against inflammation in obesity. Clearly, further studies are needed to identify what initiates the cascade of inflammatory responses in obesity. To address these important questions, it will be necessary to combine in vivo visualization technologies with genetic and pharmacological interventions.

In summary, using our imaging system can be a powerful tool to analyze the inflammatory

conditions including metabolic syndrome and thrombosis. We clarified the mechanism by which thrombi are rapidly formed by discoid platelets on undisputed endothelium. The initial platelet aggregation subsequently leads to irreversible integrin- and actin-dependent thrombus development. Inflammatory cytokine signaling in endothelial cells (ECs) played pivotal role in discoid platelet aggregations in vivo. In addition, Our imaging revealed close spatial and temporal interrelationships between angiogenesis and adipogenesis in obese adipose. In addition, increased inflammatory cell interactions in obese adipose were visualized and we found that large numbers of CD8+ effector T cells infiltrated into obese adipose, and these cells were essential for the initiation and development of adipose inflammation.

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一般の皆様へ

生活習慣病がどうしておきるのか、どうやって治療をしたらいいのか、そのためには、からだのなかで何が起きているかをみる技術はとても有用です。私は、光を用いてカラダの中を直接みて、肥満や血管の傷害がどのように影響を及ぼすかを、検討してきました。免疫・炎症といった過程が「見る」ことにより、「わかり」ます。

The structural study of the human centromeric chromatin

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Abstract

The centromere functions as a chromosome site for kinetochore assembly. However, architecture of the centromeric chromatin has not been understood. CENP-B is known to bind to the CENP-B box DNA sequence, which is located in the centromeric chromatin region containing histone H3 variant CENP-A. However, how CENP-B efficiently binds to the CENP-A nucleosome is not known. In the present study, we tested the CENP-B binding to the CENP-A nucleosome, and found that Nap1 (nucleosome assembly protein 1) significantly stimulates the CENP-B binding to the CENP-A nucleosome in vitro.

Key Words : CENP-A, CENP-B, Nap1, centromere, nucleosome

Introduction

The centromere is the chromosome region for kinetochore assembly. CENP-A is the centromere-specific histone H3 variant that is widely conserved among eukaryotes, and is an essential component for the formation of functional kinetochores (1-6). On the other hand, CENP-B is the centromeric protein that specifically binds to a 17 base-pair DNA sequence, known as the CENP-B box (7). The CENP-B binding to the CENP-A nucleosome may be important for formation of the fundamental architecture of the centromeric chromatin. However, the mechanisms of efficient CENP-B binding to the CENP-A nucleosome remain unclear.

Results

CENP-B is the only sequence-specific DNA-binding protein found in the centromere-specific proteins. The CENP-B function in the centromere formation has remain elusive, but the CENP-B binding mechanism to chromatinized DNA may be important for understanding the centromeric chromatin architecture. To clarify the CENP-B binding mechanism, we performed the *in vitro* CENP-B binding assay with the CENP-A nucleosome. To do so, we purified the human histones (H2A, H2B, CENP-A, and H4) and the DNA-binding domain of CENP-B (CENP-B DBD), as recombinant proteins. CENP-B DBD contained the N-terminal amino acid residues 1-129 of human CENP-B. We first reconstituted CENP-A nucleosome with a 192 base-pair DNA, in which a CENP-B box sequence was located at the entry/exit regions of the nucleosome. We then tested CENP-B binding to the CENP-A nucleosome by an electrophoretic mobility shift assay (EMSA), using a polyacrylamide gel. The CENP-B DBD was incubated with the reconstituted CENP-A nucleosome under physiological salt conditions. In this assay, we detected only a small amount of

the specific complex with the CENP-B DBD bound to the CENP-B box sequence of the CENP-A nucleosome. This result indicated that CENP-B non-specifically interacts with the nucleosomal DNA, and forms aggregates, which may be inactive for functional centromeric chromatin formation. However, we found that the CENP-B DBD properly bound to the CENP-A nucleosome, and the specific complexes were formed in the presence of Nap1, an acidic histone chaperone, which reportedly inhibits non-specific histone binding to DNA. Since another histone chaperone sNASP did not promote the CENP-B binding, this CENP-B DBD assembly activity may be specific for Nap1. Furthermore, we found that incubation with excess Nap1 dissociates the CENP-B DBD from specific complexes with the CENP-A nucleosomes. Eviction of the CENP-B DBD from the specific complex was not observed in the presence of excess amounts of sNASP. We next tested whether the Nap1-mediated CENP-B assembly may occur on the canonical nucleosome containing histone H3, instead of CENP-A. We then found specific CENP-B assembly was efficiently observed in the canonical H3 nucleosome containing the CENP-B box within it. We therefore conclude that Nap1, but not sNASP, promotes the specific binding of CENP-B to the CENP-B box in a nucleosomal context by suppressing non-specific aggregate formation. We further investigated whether Nap1 can regulate the association of CENP-B with chromosomes *in vivo*. Consistent with *in vitro* results, we found that the interaction between Nap1 and CENP-B was detected by immunoprecipitation. To test whether Nap1 evicts CENP-B non-specifically bound to chromatin *in vivo*, we established the CENP-B eviction assay using the Nap1 tethering to a chromosome and CENP-B overexpression. For this purpose, we construct the mutant CENP-B box that abolishes the specific CENP-B binding. A chromatin-immunoprecipitation (ChIP) analysis showed that the non-specific binding of CENP-B to the mutant CENP-B box was increased under conditions where CENP-B was overexpressed. We then found that co-expressed Nap1 with CENP-B significantly reduced the non-specific CENP-B binding to the mutant CENP-B box selectively, whereas no significant reduction of the specific CENP-B binding to the host centromere with canonical CENP-B boxes was observed under the same conditions. We therefore conclude that the selective inhibition of non-specific CENP-B-DNA binding by Nap1 may regulate proper CENP-B binding to chromosomes *in vivo*.

Discussion & Conclusion

It has been reported that Nap1 eliminates non-nucleosomal histone-DNA interactions, and promotes correct nucleosome assembly *in vitro* (8). In the present study, we found that human Nap1 significantly inhibits non-specific CENP-B binding to the CENP-A or canonical H3 nucleosomes *in vitro*. In addition, Nap1 also promotes eviction of the CENP-B improperly bound to chromatin *in vivo*. Nap1 is an acidic histone chaperon that may simply bind electrostatically to the basic CENP-B DBD, and non-specifically inhibit the CENP-B-DNA interaction. However, we do not think this is the case, because another acidic histone chaperone, sNASP, affected neither specific nor non-specific CENP-B binding to nucleosomes *in vitro* and chromosomes *in vivo*. These studies have led us to propose a novel Nap1 function: the regulation of specific CENP-B loading at centromeric

chromatin by the inhibition of non-specific CENP-B binding to other chromosome loci. Thus, Nap1 may indirectly promote the specific assembly of CENP-A nucleosomes, a key structural element of active centromeres (9).

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一般の皆様へ

本研究の目的は、セントロメア領域の構築原理を解明することである。生物は、細胞の増殖と分裂を繰り返して生命活動を維持しており、細胞分裂時に遺伝情報を担うゲノム DNA を正確に継承する必要がある。この機構が破綻するとダウン症やガン化に関与することが示されており、正確な遺伝子の維持・継承機構の解明は非常に重要である。これまでに、セントロメアとよばれる特異的な領域が、ゲノム DNA の含まれる染色体の均等分配に重要であることが示されており、セントロメアの構築原理を明らかにすることは非常に重要である。本事業の助成によって得られた研究成果から、セントロメア構築原理の一端に重要な知見を与えることができた。

An intrinsic molecular mechanism governing the development of root nodule primordia

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Abstract

Nodulation is a form of *de novo* organogenesis that occurs mainly in legumes. In the present study, we investigated the detailed patterns of auxin response during nodule development in *L. japonicus*. In addition, we characterized a novel nodulation-deficient mutant, *tricot*. Taken together, our analyses will contribute further to our understanding of the genetic mechanism regulating nodule development.

Key Words : Auxin, Nodulation, Root nodule symbiosis

Introduction

During the course of plant evolution, mainly leguminous plants have acquired the ability to form de novo structures called root nodules. Two classic phytohormones, cytokinin and auxin, play essential roles in diverse aspects of cell proliferation and differentiation. Although recent genetic studies have established how activation of cytokinin signaling is crucial to the control of cortical cell differentiation, the physiological pathways through which auxin might act in nodule development are poorly characterized. Moreover, our understanding of molecular genetic mechanism of nodulation is still limited by the low number of nodulation-related genes that have been identified.

Results

During early nodule development, the host plant root is infected by soil bacteria collectively called rhizobia that induce dedifferentiation of some cortical cells; these cortical cells then proliferate to form the symbiotic root nodule primordium.

In the present study, we investigated the detailed patterns of auxin response during nodule development in *Lotus japonicus*. In order to determine the precise distribution of auxin response during nodule development in *L. japonicus*, we created stable transgenic plants that expressed a GFP and nuclear localization signal (NLS) fusion protein (GFP-NLS) under the control of *DR5*, which is a highly active synthetic auxin responsive element. Our analyses showed that auxin response predominantly occurred in dividing cortical cells and that cytokinin signaling and NODULE INCEPTION, a key transcription factor in nodule development, positively regulated this response. Additionally, we found that auxin response was inhibited by a systemic negative regulatory mechanism called autoregulation of nodulation (AON). The basis of AON is systemic long-distance signaling between root and shoot. In *L. japonicus*, *HYPERNODULATION ABERRANT ROOT*

FORMATION 1 (*HAR1*), which encodes a leucine-rich repeat receptor-like kinase, is hypothesized to function in shoots where it recognizes and responds to root-derived signals involved in the negative regulation of nodulation. Here, we showed that the *har1* mutant has increased and more widespread response of auxin in cortical cells than the WT. This indicates that auxin response prior to cortical cell division is controlled by the AON mechanism (including HAR1) and it determines not only the auxin response level but also the site of auxin response. Analysis of the constitutive activation of *LjCLE-RS* genes, which encode putative root-derived signals that function in AON, in combination with determination of auxin response patterns in proliferating cortical cells, indicated that activation of *LjCLE-RS* genes blocked the progress of further cortical cell division, probably through controlling auxin response (Suzaki et al., 2012).

Recent studies using *spontaneous nodule formation 2* (*snf2*) mutant line of *L. japonicus* have shown that activation of cytokinin signaling is crucial to the control of nodule organogenesis (Tirichine et al., 2007). The *snf2* phenotype was demonstrated to result from a gain-of-function mutation of the *LOTUS HISTIDINE KINASE 1* (*LHK1*) gene that encodes a putative cytokinin receptor. The mutation triggers formation of nodule-like structures (defined as spontaneous nodules) in the absence of rhizobia, due to the constitutive activation of LHK1. Here we focused on the downstream part of the cytokinin signaling pathway. We identified a mutant *tricot* (*tco*) in a screen for mutants that could suppress the *snf2* phenotype. The *tco* mutation prevented *snf2*-dependent spontaneous nodule formation, suggesting that *TCO* positively regulates nodule organogenesis in the downstream part of the cytokinin signaling pathway. Nodule development and rhizobial infection process were also impaired in the *tco* mutant. Additionally, auxin reporter analysis showed there are some defects relevant to auxin regulation in the *tco* mutant. The shoot phenotype of the *tco* mutant also indicated that *TCO* is involved in the maintenance of the shoot apical meristem (SAM). We used map-based cloning and found that *TCO* encodes a putative glutamate carboxypeptidase with possible orthology to *Arabidopsis* ALTERED MERISTEM PROGRAM 1. An expression analysis showed that *TCO* is expressed in meristematic regions of nodules and in the SAM (Suzaki et al., 2013).

Discussion & Conclusion

In comparison with cytokinin, relatively little is known of the role of auxin in nodule development. In the present study, however, we have uncovered the site of auxin action during nodule development. In addition, our data provide evidence for the existence of a novel fine-tuning mechanism that controls nodule development in a cortical cell stage-dependent manner. Our analyses of *tricot* mutant have not only identified a novel gene for regulation of nodule organogenesis but also provided significant additional evidence for a common genetic regulatory mechanism in nodulation and shoot apical meristem formation. This new data will contribute further to our understanding of the evolution and genetic basis of nodulation.

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一般の皆様へ

ダイズやエンドウなどのマメ科植物に代表される一部の植物は、根に「根粒」と呼ばれるこぶ状の器官をつくる能力を獲得することによって、土の中にいるバクテリアの一種である根粒菌と共生していることが知られています。根粒内部では、植物は根粒菌が固定した大気中の窒素を栄養源として受け取ることができます。本研究では植物ホルモンの１つであるオーキシンと根粒形成の制御関係を明らかにし、さらに根粒形成を制御する新たな遺伝子を単離しました。得られた知見は基礎的なものですが、将来的にはマメ科以外の有用な作物に応用することで、窒素肥料いらずの低化学肥料農業の実現や栄養の少ない土地での農業展開への貢献が期待されます。

Identification and characterization of novel skeletal muscle-derived angiogenesis growth factor

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Abstract

It has been proposed that skeletal muscle secretes factors, referred to as “myokines,” that influence the behavior of remote cells. Based upon microarray analysis, we have identified 5 novel muscle-secreted factors (MSFs), encoding secreted proteins, that are upregulated during Akt-mediated muscle growth. The present research performed in vivo and in vitro assays to assess the angiogenesis regulatory properties of these MSFs. We have shown that MSF1 and 5 play a role in promoting revascularization under conditions of ischemic stress. MSF1 and 5 expressions in muscle were up-regulated by Akt1 transgene activation during muscle hypertrophy and by ischemic injury. Administration of MSF1 and 5 improved revascularization in ischemic limbs of wild type mice.

Key Words : angiogenesis, growth factor, skeletal muscle, hypertrophy

Introduction

It has been proposed that skeletal muscle secretes factors, referred to as “myokines,” that influence the behavior of neighboring or remote cells [1)]. We have reported several myokines regulated by Akt signaling. Myogenic Akt signaling controls both fiber hypertrophy and VEGF synthesis [2)]. In addition to VEGF, it is likely that other as-yet-unidentified angiogenesis regulators contribute to the increase in capillary density in response to Akt1 induction in skeletal muscle. Based upon microarray analysis, we have identified 5 novel MSFs, encoding secreted proteins, that are upregulated during Akt-mediated muscle growth. The present research performed in vivo and in vitro assays to assess the angiogenesis regulatory properties of these MSFs.

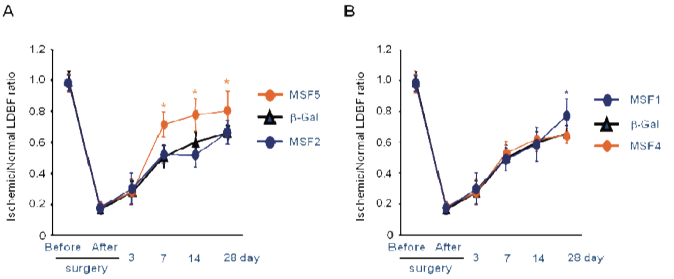
Results

In vivo evaluation of MSF angiogenic properties

Angiogenic properties of MSFs were evaluated. As shown in Figure 1A, ischemic/normal LDBF ratio was significantly increased 7 days after MSF 5 overexpression and improvement of blood flow recovery was persisted at each of the subsequent time points (14 and 28 days). As shown in Figure 1B, adeno-MSF1-treated mice showed a modest but statistically significant increase in flow recovery at 28 days after hind limb surgery. On the other hand, overexpression of MSF2, 3 or 4 in skeletal muscle had no effect on blood flow recovery in this model. These results suggest that MSF 1 and 5, but not MSF 2, 3, and 4 functions as an angiogenesis-regulatory protein.

To examine the extent of angiogenesis at the microcirculatory level, CD31 staining was performed. As shown in Fig 1C, capillary density was significantly increased in mice adductor muscle transferred with adeno-MSF 5 on 14 days after operation. These results suggest that MSF 5 functions as a pro-angiogenic factor.

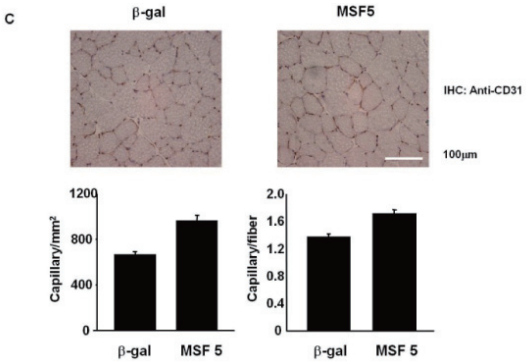
Figure 1



Gene transfer of MSF1 and 5 promotes angiogenesis in ischemic hind limbs. Quantitative analysis of the ischemic/nonischemic LDBF ratio in the MSF1, 2, 4, 5 and β-Gal-treated(control) mice.

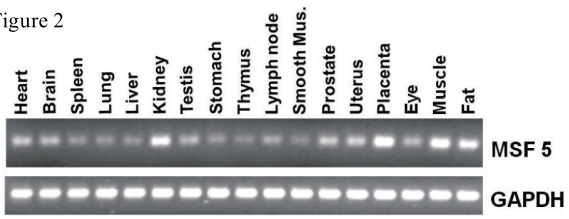
Characterization of angiogenic MSF expression

Based upon the results of in vivo angiogenesis assay, MSFs that show in vivo angiogenic properties were examined to understand their expression properties. mRNA expression profile of MSF 5 in adult mouse tissue was analyzed by semi-quantitative RT-PCR. As shown in Figure 2, MSF 5 mRNA was abundantly expressed in skeletal muscle, kidney and placenta, and moderately expressed in adipose tissue, suggesting that skeletal muscle is one of the major sources of MSF5. Expression profile of MSF 1 in adult mouse tissue is under investigation.



MSF 5 increases capillary density in WT mice.

Figure 2



Expression profile of MSF 5 in adult mouse tissue.
cDNAs from different mouse tissue was synthesized and analyzed by RT-PCR using MSF 5-specific primer pairs.

Expression profile of MSF 1 and 5 during physiological muscle growth and wasting was also evaluated. RNA was isolated from gastrocnemius muscle in various rodent models, including forced treadmill exercise test (Acute Ex), skeletal muscle atrophy model (denervation, starvation, acute uncontrolled diabetes (DM), chronic renal failure (CRF), tumor-bearing (Tumor)). The levels of mRNA expression were evaluated by quantitative real-time PCR (Table 1). mRNA expression of MSF 1and 5 were 6.8- and 1.9-fold, respectively, up-regulated in gastrocnemius muscle from skeletal muscle-specific Akt1 transgenic mice compared with that from control mice. MSF 1 mRNA expression was modestly up-regulated in tumor-bearing model, and down regulated in starvation model. Transcript expression of MSF 5 was up-regulated in denervation model, and decreased in acute exercise and another atrophy model.

Table 1
Summary of mRNA expression of MSFs in gastrocnemius muscle during physiological muscle growth and wasting.

		Atrophy model					Skeletal muscle
	Hindlimb ischemia	Denervation	Starvation	DM	CRF	Tumor	Akt1 TG
MSF 1	2.07	0.88	0.76	0.95	1.07	1.72	6.8
MSF 5	2.69	1.73	0.6	0.68	0.73	0.78	1.9

Although MSF 1and 5 are commonly up-regulated by Akt1, their expression profile in various physiological conditions seems to be different.Gene expression of these MSFs in functional overloaded skeletal muscle and skeletal muscle from myostatin deficient mouse, that shows

excessive muscle growth, is under investigation.

For protein detection, I have produced anti-peptide antibody against MSF 5. Peptide sequence corresponding to immunogenic regions of MSF 5 was determined by the prediction software (Accelrys Inc.). Adenoviral vector expressing Flag-tagged MSF 5 was transfected into 293T cells. After 2 days, the cell pellets and media fractions were collected and analyzed by western blot using anti-SNFL and anti-FLAG antibodies. MSF 5 protein was detected both in the cell lysates and in the media by anti-FLAG antibody; however, anti-SNFL antibody recognized only MSF 5 protein form cell lysates, but not culture media. It is possible that peptides sequence recognized by anti-SNFL antibody is modified during protein secretion. Therefore, I am producing another antibody that recognizes different peptide sequence from anti-SNFL antibody. Production of anti-peptide antibodies to MSF 1 is under processing.

Discussion & Conclusion

It has been proposed that skeletal muscle secretes factors, referred to as "myokines," that influence the behavior of neighboring or remote cells [1]. The present research shows that MSF1 and 5 play a role in promoting revascularization under conditions of ischemic stress. MSF1 and 5 expressions in muscle were up-regulated by Akt1 transgene activation during muscle hypertrophy and by ischemic injury. Administration of MSF1 and 5 improved revascularization in ischemic limbs of wild type mice.

Using this inducible transgenic system, we recently demonstrated that Akt1-mediated skeletal muscle growth attenuates cardiac remodeling after MI, and is associated with an increased capillary density in the heart [3]. We found that the improvement in left ventricular remodeling resulting from muscle growth accompanied by an increase in capillary density in the heart. We found that levels of a number of angiogenic growth factors are increased in serum following transgene activation including VEGF-A, SDF-1, FGF-1 and FGF2. This increase in angiogenic growth factor production was associated with an increase in the activating phosphorylation of eNOS at Ser1177 in heart. Because eNOS is pro-angiogenic, these data are consistent with the increase in myocardial capillary density in response to Akt1 induction in skeletal muscle.

In conclusion, the proposed study identified novel angiogenic factor that may have utility for therapeutic angiogenesis.

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一般の皆様へ

慢性心不全患者は高頻度に骨格筋委縮を呈し、それが独立した予後不良因子となることが知られています。一方、骨格筋量の維持・増加を目的とした運動療法は心不全患者における QOL や予後の改善に寄与することが報告されていますが、その有用性の機序は良くわかっていません。近年、運動により骨格筋から様々な因子が分泌され、遠隔臓器の機能制御に寄与することが明らかになってきました。私たちは、任意の時期に骨格筋肥大を誘導できるマウスを作成し、骨格筋の肥大が血管新生因子の発現亢進を介して心筋梗塞後のリモデリングを抑制することを昨年報告しました。本研究では新規の血管新生因子候補をいくつか同定することができました。本研究で得られた成果は心血管疾患患者に対して、筋力トレーニングを主体とした運動療法を行うことの理論的根拠を与えるものとなりうると考えられます。

Molecular mechanism of self-renewal and proliferation of a hematopoietic cell due to HMGA2 expression

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Abstract

Myeloproliferative neoplasms (MPNs) are clonal hematological disorders characterized by proliferative hematopoiesis. Clonal expansion mechanism has not been fully understood in the MPNs. Here we study the role of *HMGA2* on pathogenesis of MPNs. We found that *HMGA2* expression seems like to contribute to proliferative hematopoiesis with conferring clonal growth advantage in association with some signaling pathways and *let-7* micro RNA that negatively regulates *HMGA2* expression.

Key Words : Hematopoietic stem cell, HMGA2, clonal expansion

Introduction

Myeloproliferative neoplasms (MPNs) are clonal hematological disorders characterized by proliferative hematopoiesis and sometimes develop secondary myelofibrosis (MF) or acute myeloid leukemia (AML). It remains largely unknown how an abnormal clone acquire a clonal growth advantage with accumulation of some genetic abnormalities that contributes to disease progression. In this study we focused on the role of HMGA2 in the pathogenesis of MPNs with the respect of clonal growth advantage.

Results

HMGA2 expression is negatively regulated by *let-7*-family micro RNAs. Either downregulation of *let-7* or removal of *let-7*-binding sites from *HMGA2* is thought to cause overexpression of *HMGA2*. Since overexpression of *HMGA2* has been reported in hematopoietic cells from patients with MPNs, we generated transgenic mice that overexpress *HMGA2* without *let-7*-binding sites (Δ*Hmga2* mice). Δ*Hmga2* mice showed not only proliferative hematopoiesis but also clonal growth advantage at the level of a hematopoietic stem cell (Ikeda K et al, 2011). Then we have been investigating molecular mechanism that causes abnormal hematopoiesis in the presence of HMGA2 overexpression using the Δ*Hmga2* mice. Young adult Δ*Hmga2* mice showed deregulated expression *let-7* in BM and spleen cells compared with wild-type (WT) mice. The difference between Δ*Hmga2* mice and *WT* mice were greater in spleen (Ikeda K et al, 2012). We have also studied the influence of aging in the background of *HMGA2* overexpression, because *HMGA2* overexpression may be associated with myelofibrosis, which often occurs after long course of other types of MPN such as polycythemia vera or essential thrombocytosis. Interestingly, althogh hematopoiesis of old Δ*Hmga2* mouse shows

a distinct feature from that of young Δ*Hmga2* mice, myelofibrosis has not been seen for up to 18 months until now (Ikeda K et al, manuscript in preparation). Moreover, hematopoiesis under *Hmga2* overexpression might contribute to some signaling pathways in the young and old hematopoiesis. We are currently performing experiments using hematopoietic cells from Δ*Hmga2* mice and cell lines expressing HMGA2 to confirm such novel findings.

Discussion & Conclusion

In this study, we have found that *HMGA2* overexpression alone can cause a proliferative hematopoiesis mimicking MPN, at least in part caused by activation of some signaling pathways involving JAK-STAT or PI3K-AKT. Although some mutations including the JAK2V617F, has been found in patients with MPNs, 30-40% patients do not show any known mutations that activate signaling pathway. Thus, HMGA2 overexpression is a candidate abnormality, which possibly causes proliferative hematopoiesis in MPNs. In the aged mice, *Hmga2* overexpression caused abnormal hematopoiesis without myelofibrosis, suggesting that long-term *Hmga2* overexpression can cause secondary hematopoietic abnormality in addition to proliferative hematopoiesis. Finally, *Hmga2* overexpression may further influence *let-7* expression in a tissue specific manner, which possibly contributes to such abnormal hematopoiesis. We believe we will clarify a detailed HMGA2-involved mechanism in the pathogenesis of MPNs and hematopoiesis in the near future.

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一般の皆様へ

骨髄増殖性腫瘍は成熟した血液が増加する疾患で、一部は予後の悪い骨髄線維症や急性白血病に移行します。このように病気が進行してしまう理由はよくわかっていない面が多く残されています。私たちは、HMGA2 とよばれる遺伝子が、この疾患の形成や進行に関係していると考えており、HMGA2 を増減させる因子や、逆に HMGA2 によって影響をうける物質の経路を調べています。このことが将来診断や治療の役に立つことを期待しています。

The biological and pathological significance of Stox1 transcriptional factor

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Abstract

Key Words : Diabetes, metabolic syndrome, kidney fibrosis

Introduction

Storkhead-box protein 1 (*Stox1*) was identified using a genome-wide screening methods and single nucleotide polymorphism (SNPs) analyses, as gene associated with pregnancy disorder (1-4). The function of *Stox1* is unknown. The *Stox1* protein contains a winged helix domain, related to FOX family, and is speculated to be a transcription factor. There are several transcriptional splice variants of human *Stox1* and most of these involve the exon 3. We made stox1 deficient mice (stox1$^{-/-}$) and analyzed; preliminary analysis reveals stox1 would be key transcriptional factor to modulate renin expression.

Results

Stox1 has been originally reported as a candidate gene associated with preeclampsia. Preeclampsia experienced women has been reported to develop metabolic, cardiovascular, and chronic kidney disease in her later life. Therefore we hypothesized that stox1 deficiency may be shared molecular mechanisms between preeclampsia and these health problem. To prove this hypothesis we performed experiment using stox1$^{-/-}$ mice, inducing type 1 or type2/metabolic syndrome models mice.

1) Type I diabetic mice (129sv background): we injected streptozotocin (50mg/kgBW) for 5 consequence days and confirmed type 1 diabetes in stox1$^{-/-}$ and control mice (both 129sv background). We found after 4 weeks, some of the diabetic stox1$^{-/-}$ mice (129sv) developed massive proteinuria and ascites whereas nothing happened in control diabetic mice. Blood pressure, blood sugar levels were not different between all the groups. Histological analysis reveals that diabetic stox1$^{-/-}$ mice displayed significant interstitial fibrosis when compared to control diabetic mice. Glomerular hypertrophy was not change in the groups but some mice exhibited massive mesangiolysis and increased deposition of mesangial matrix in diabetic stox1$^{-/-}$ mice. Urine albuminuria was significantly increased in diabetic stox1$^{-/-}$ mice when compared to control diabetic mice. Therefore basically we stox1$^{-/-}$ would be the gene responsible for diabetic nephropathy deterioration. Kidney renin is higher in diabetic stox1$^{-/-}$ mice.
2) Type I diabetic mice (c57Bl6 background): we tried to analyze often used diabetic mice, C57Bl6 background. We backcrossed stox1$^{-/-}$ mice (129sv background) with wild type C57bl6 mice for

8 generation to make stox1$^{-/-}$ mice (C57Bl6 background). We successfully made the mice and induced same experiment using STZ injection. Again there were no differences in blood pressure and blood sugar between diabetic stox1$^{-/-}$ mice or control diabetic mice. However we did find no difference in albuminuria, glomerulosclerosis, and tubulo-interstitial fibrosis in all groups analyzed. different from 129sv mice, kidney renin was no changed in C57Bl6 stox1$^{-/-}$ mice.

3) Metabolic syndrome mice: we analyzed metabolic syndrome mice by feeding high fat diet (30% fat in energy) using C57Bl6 mice (129sv background mice are high fat diet resistant it is reasonable to use C57Bl6 background). We found massive deposition of lipid droplet in control high fat fed mice but in stox1$^{-/-}$ high fat fed mice, there is obviously less lipid droplet in the tubule. Lipid droplet have shown to play protective roles in kidney damage and less drop let in stox1$^{-/-}$ mice may be pathological significance, even though we could not find significant histological evidence of kidney damage in this metabolic syndrome models.

Discussion & Conclusion

Our analysis revealed that stox1 could play protective roles in kidneys under diabetic conditions. Some interesting lines of evidence are upcoming such as strain specific sensitivity of stox1 deficiency in type 1 diabetic mice. Also in high fat fed mice lipid droplet formations are less in stox1 deficient mice compared with control mice with high fat diet. When thinking about the shared molecular mechanisms between preeclampsia and metabolic syndrome and diabetes, some possible connected mechanisms can be hypothesized from stox1 deficiency mice such as vascular defects, endothelial dysfunction and biochemical abnormality such as autophagy deficiency. Therefore stox1 deficiency could be involved in the organ dysfunction in diabetes or metabolic syndrome. We are now analyzing much detailed molecular mechanisms involvement including microRNA profilings.

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一般の皆様へ

糖尿病患者数は年々増加しており、予備軍を含めると日本では 2000 万人に達すると考えられます。糖尿病合併症症例も増加しており、その中でも糖尿病性腎症による透析導入は全体の 44% にものぼります。したがって、その対策は急務ですが、画期的治療薬はなく、新しい治療標的を見つける事が必要です。今回の検討では糖尿病と妊娠高血圧腎症の間にある共通点に注目し、妊娠高血圧腎症とかかわりが強いたんぱく質 stox1 に注目して検討しています。現在までに stox1 の働きが十分でないと糖尿病状態で腎臓を保護できない可能性が分かってきております。今後治療標的になりうるかを更に検討いたします。

Novel role of neuronal Ca^{2+} sensor-1 as a survival factor activated in cardiomyocytes under stress.

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Abstract

In this study, we assessed the role of neuronal Ca^{2+} sensor-1 (NCS-1) in cardiac survival under stress. We found that *NCS-1*$^{-/-}$ myocytes were more susceptible to various kinds of stressors, with having reduced Akt activity. Using RNA interference, we identified phosphatidylinositol 4-kinase (PI4K) as an NCS-1 target that promotes cell survival. NCS-1 accelerates phosphoinositide turnover and increases stimulation-elicited Ca^{2+} levels, which in turn activate PI3K/Akt pathway as well as mitochondrial ATP synthesis. These would promote cardiomyocyte survival under stress.

Key Words : Calcium sensor, heart, survival, stress

Introduction

Dysregulation of Ca^{2+} homeostasis in cardiomyocytes often results in heart failure. Identifying molecular targets that regulate cardiomyocyte survival is therefore of therapeutic importance. Neuronal Ca^{2+} sensor-1 (NCS-1) is a small EF-hand Ca^{2+}-binding protein that is important for neuronal functions. Using *NCS-1*$^{-/-}$ mice, we recently identified a cardiac function for NCS-1 as a positive regulator of Ca^{2+} signals in immature and diseased hearts[1]. Since the mortality rate in neonatal *NCS-1*$^{-/-}$ mice was high and we previously reported that NCS-1 is involved in neuronal survival[2], we hypothesized that NCS-1 may be cardioprotective, especially under stress.

Results

Cultured neonatal mouse ventricular myocytes (NMVMs) or whole hearts from the wild-type (WT) and *NCS-1*$^{-/-}$ groups were subjected to several kinds of stressors (serum/glucose depletion, oxidative stress, and ischemia-reperfusion injury), and the resistance to stress-induced toxicity was evaluated by measuring the time to cessation of spontaneous beating, LDH release, live/dead cell staining and infarct size.

Long-term culture in serum-free media resulted in decrease in the spontaneous beating rate of NMVMs much faster in *Ncs1*$^{-/-}$ group compared to WT group, and *Ncs1*$^{-/-}$ myocytes ultimately ceased their spontaneous beating and morphological change occurred after 1 week of treatment. Cell viability analysis by counting the condensed chromosome in nuclei using Hoechst 33258-staining revealed that more apoptosis was induced in *Ncs1*$^{-/-}$ myocytes than WT myocytes. Similar vulnerabilities were also detected in *Ncs1*$^{-/-}$ group when we used H_2O_2 and 2-deoxyglucose to deplete cellular glucose as stressors.

Such cytotoxicity was largely prevented by WT NCS-1 overexpression but not by the low Ca^{2+}-binding NCS-1 mutant E120Q, suggesting that NCS-1 protects cardiomyocytes from stress-induced cytotoxicity in a Ca^{2+}-dependent manner. In NMVMs, a major survival pathway—PI3K/Akt signaling—was significantly reduced in the *NCS-1*$^{-/-}$ group, demonstrating that NCS-1 is important for cardiomyocyte survival.

To clarify the molecular mechanism of NCS-1-mediated cardiomyocyte survival, we focused on phosphatidylinositol 4-kinase (PI4K), a molecule located upstream of the PI3K/Akt pathway, and known to interact with NCS-1 in neurons[3]. Immunoprecipitation and immunofluorescence analysis revealed that NCS-1 interacts and co-localized with PI4K in the heart. Furthermore, NCS-1 increased the amount of the activation product PI4*P*, suggesting that PI4K is indeed activated by NCS-1 in the heart. By measuring Kir3.1 K^+ current, which was used as a PI(4,5)P_2 sensor, we found that NCS-1 promotes the production of a downstream signal molecule PI(4,5)P_2 as well as PI4*P*. Furthermore, PI4K RNA silencing decreased cardiomyocyte resistance to stress-induced cytotoxicity as observed for *NCS-1*$^{-/-}$, indicating that PI4K is a target of NCS-1, which promotes cardiomyocyte survival under stress.

Phosphoinositide turnover affects receptor stimulation-elicited Ca^{2+} signaling, a process involved in various cellular responses, we next compared the intracellular Ca^{2+} levels between WT and *NCS-1*$^{-/-}$ myocytes before and after receptor stimulation. Receptor-stimulation-elicited intracellular Ca^{2+} increase was significantly diminished in *NCS-1*$^{-/-}$ myocytes under extracellular Ca^{2+}-free condition. Since increased Ca^{2+} signals are reported to be required for activation of PI3K/Akt pathway, we analyzed the stimulation-elicited translocation of EGFP-Akt-PH, which was used as an indicator of PI3K/AKt pathway activation. Compared to WT group, plasma membrane localization of EGFP-Akt-PH was much less in *NCS-1*$^{-/-}$ group after receptor-stimulation. Furthermore, because increase in Ca^{2+} signaling also affects mitochondrial ATP syntheses, we measured intracellular ATP levels. We found that ATP levels were also considerably lower in *NCS-1*$^{-/-}$ myocytes. These results are consistent with the notion that stress-induced survival signaling is less activated in *NCS-1*$^{-/-}$ myocytes.

We finally compared the susceptibility of whole hearts to ischemia-reperfusion injury using Langendorff apparatus. The infarct area were significantly larger in *NCS-1*$^{-/-}$ hearts than that of WT hearts (39% ± 3.5% and 22% ± 4.1% for *NCS-1*$^{-/-}$ and WT mice, respectively; n = 6 for each). As observed for NMVM, phosphorylation levels of Akt and its downstream molecules GSK3beta and mTOR were both largely decreased in KO hearts, demonstrating that NCS-1 plays an important role in stress-tolerance in whole heart.

Discussion & Conclusion

We found that NCS-1 promotes cardiomyocyte survival under stress: in *NCS-1*$^{-/-}$ hearts, Akt -a major survival factor- was less activated upon stimulation with stressors. The NCS-1 effects are partially mediated by the activation of PI4K, which accelerates phosphoinositide turnover. Although,

the level of PI(4,5)P_2 is reported to decrease in the animal model of heart failure possibly because of jeopardizing cardiac cell function[3], decrease in phosphoinositides does not necessarily lead to inhibited Akt activation[4]. We found that NCS-1 modestly increases receptor stimulation-elicited Ca^{2+} levels, which itself can activate PI3K/Akt pathway[5]. Increase in cytosolic Ca^{2+} level is also involved in mitochondrial ATP synthesis, and we indeed detected that both activation of PI3K/Akt pathway and cytosolic ATP levels were decreased in *NCS-1*$^{-/-}$ myocytes.

In conclusion, we identified a novel role of NCS-1 as a survival factor activated in cardiomyocytes under stress.

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一般の皆様へ

心筋梗塞、心不全などの心疾患は、現在、世界でも死因の上位を占める重篤な社会問題である。心筋細胞の生死がどのような経路で調節されるか理解することが心不全の予防に重要であるが、その全容は未だ明らかでない。本研究では、心筋細胞の生存に関わる因子を明らかにすることを目的とし、NCS-1 というタンパク質が重要な役割を担うことを見出した。NCS-1 は、筋収縮や細胞死などに重要な働きをもつ細胞内カルシウムを鋭敏に察知する、“ カルシウムセンサー ” と呼ばれる一群のタンパク質の1つである。ストレスにより細胞内カルシウム濃度が上昇すると、NCS-1 およびその関連分子が活性化され、最終的に心筋細胞の生存を促す経路が活性化させることがわかった。本研究をさらに深めることにより、各種心疾患の予防につながる可能性がある。

Paternal uniparental disomy 14 and related disorders: placental gene expression analyses and histological examinations

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Abstract

Placental study of two cases with UPD(14)pat, and a case with maternal microdeletion involving *DLK1*, DMRs and *MEG3* showed as follows: 1) In the placentas of UPD(14)pat revealing placentomegaly, the expression of *DLK1* and *RTL1* increased, even though in the placenta of a case with maternal microdeletion revealing placentomegaly, only the expression of *RTL1* increased. 2) Electron microscopic study showed hyperplasia of the endothelial cells and the pericytes in the placentas of UPD(14)pat. 3) Expression analysis using fresh placental samples of UPD(14)pat showed excessive *RTL1* expression because of a synergic effect between the biallelic activation of *RTL1* and loss of functional microRNA-containing *RTL1as* as a repressor for *RTL1*. These results indicate that excessive *RTL1* expression causes placentomegaly and *RTL1* expression is regulated through an RNAi mechanism.

Key Words : UPD(14)pat, placenta, RTL1, histology

Introduction

Although recent studies in patients with UPD(14)pat and other conditions affecting the chromosome 14q32.2 imprinted region have successfully identified underlying (epi)genetic factors for the development of UPD(14)pat phenotype, several matters including regulatory mechanism(s) for *RTL1* expression, imprinting status of *DIO3*, and placental histological characteristics remain to be elucidated.

Results

We first performed molecular studies using fresh placental samples from two patients with UPD(14)pat. After correction for the amount of expression positive cells, *RTL1* expression level was assessed to be ~5 times higher in the placental samples of the two patients than in the control placental samples, whereas *DIO3* expression level was evaluated to be similar between the placental samples of the two patients and the control placental samples. We next carried out histological studies using the above fresh placental samples and formalin-fixed and paraffin-embedded placental samples obtained from a patient with a maternally derived microdeletion involving *DLK1*, the IG-DMR, the *MEG3*-DMR, and *MEG3*. Terminal villi were associated with swollen vascular endothelial cells and hypertrophic pericytes, together with narrowed capillary lumens. DLK1, RTL1,

and DIO3 proteins were specifically identified in vascular endothelial cells and pericytes, and the degree of protein staining was well correlated with the expression dosage of corresponding genes.

Fig 1.

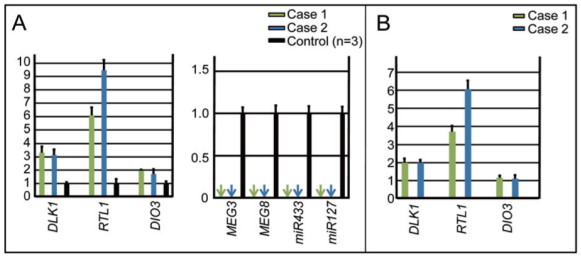

Fig 1. Quantitative real-time PCR analysis using placental samples. For a control, a pooled RNA sample consisting of an equal amount of total RNA extracted from three fresh control placentas was utilized.

A. Relative mRNA expression levels for *DLK1*, *RTL1*, and *DIO3* against *GAPDH* (mean ± SE) and lack of *MEGs* expression (indicated by arrows) (*mir433* and *miR127* are encoded by *RTL1as*) in the placental samples of cases 1 and 2.

B. Relative mRNA expression levels for *DLK1*, *RTL1*, and *DIO3* against *GAPDH* (mean ± SE), in the equal amount of expression positive placental cells (vascular endothelial cells and pericytes) of cases 1 and 2 (corrected for the difference in the relative proportion of expression positive cells between the placental samples of cases 1 and 2 and the control placental samples, on the assumption that the *DLK1* expression level is "simply doubled" in the expression positive placental cells of case 1 and 2).

Fig 2.

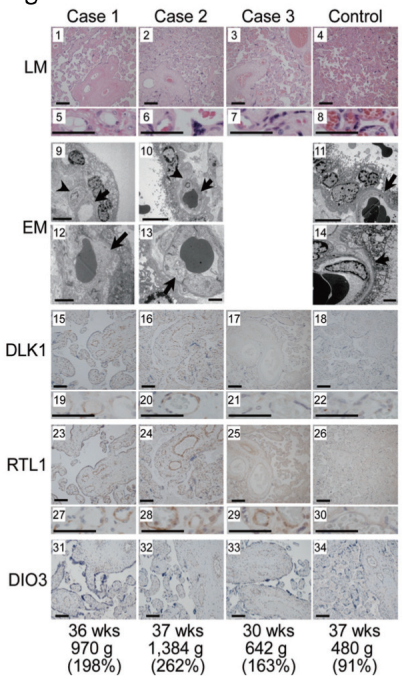

Fig 2. Histological examinations.
LM: light microscopic examinations; EM: electron microscopic examinations; DLK1, RTL1, and DIO3: immunohistochemical examinations for the corresponding proteins. The arrows and arrowheads in the EM findings indicate endothelial cells and pericytes, respectively.

Discussion & Conclusion

These results argue for *RTL1as*-encoded microRNAs functioning as a repressor for *RTL1* expression, and argue against *DIO3* being a paternally expressed gene. Furthermore, it is inferred that DLK1, RTL1, and DIO3 proteins, especially RTL1 protein, play a pivotal role in the development of vascular endothelial cells and pericytes.

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一般の皆様へ

14q32.2 領域にはインプリンティング遺伝子が多数存在し、14 番染色体父親性ダイソミー(UPD(14)pat) は、羊水過多、ベル型の小胸郭、腹壁異常、胎盤過形成を示す。インプリンティング遺伝子は胎盤・胎児の発育に重要な役割を果たし、マウスにおいて、本領域の父性発現遺伝子 *Rtl1* は胎盤の成長・機能に関与することが明らかとなっているが、ヒトでは検討されていない。我々は UPD(14)pat 表現型を示す患者の胎盤解析で、UPD(14)pat 胎盤での発現量は、正常に比較し、父性発現遺伝子の *RTL1* は 4-5 倍、*DLK1* の発現量は約 2 倍を示し、母性発現遺伝子である *MEG3, MEG8, snoRNAs, microRNAs* の発現は消失していた。DLK1 抗体、RTL1 抗体を用いた免疫染色では RTL1 については発現量が著しく増加していたが、胎盤過形成を認める微小欠失症例では DLK1 の発現は正常と同程度であった。以上の結果から胎盤過形成は *RTL1* の過剰発現によることがあきらかとなった。これまで *RTL1* の発現制御はマウスにおいて検討されており、母性発現遺伝子である *Rtl1as* 上の *microRNAs* が父性発現遺伝子の *Rtl1* の発現を trans に抑制していることが明らかとなっていたが、ヒトでは検討されていなかった。本研究で、upd(14)pat 胎盤において、RTL1 発現量が正常コントロールに比較し 4-5 倍程度発現していることが明らかとなった。これは、14 番染色体がともに父親に由来することから、*RTL1as* 上の *microRNA* の発現がなく、*RTL1* の発現抑制がなくなり、1 コピーあたりの *RTL1* の発現量が 2-2.5 倍となり、両アレルから 2 コピーの *RTL1* が発現し、4-5 倍の発現量になったと推測される。以上の結果はヒトにおける *RTL1* の発現制御が *RTL1as* 上の *microRNA* によって RNAi のメカニズムで行われていることを明らかにした初めての報告となった

Mechanism of dNTPs supply at beginning of DNA replication

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Abstract

DNA を複製する際に原料となる dNTPs は細胞周期の S 期に合成され S 期後期に最大濃度となる。このため S 期初期には DNA 合成局所特異的な dNTPs 供給機構が存在すると考えられ、その仕組みに付いて検討を行った。

Key Words：DNA 複製、dNTPs

Introduction

生物にとり DNA の安定性を維持することは、癌をはじめとする疾患を防ぐ為に重要である。DNA 損傷は DNA 複製過程において多数発生するため、DNA 複製を円滑に遂行することが安定生維持の基礎となる。最近の研究から、DNA 複製が行われる際にその原料となるデオキシリボ核酸 (dNTPs) が十分に供給されないと DNA に多くの損傷が発生し、発癌の原因となることが報告された (Bester AC *et al*. Cell. 2011)。

Results

dNTPs 濃度は細胞周期に依存して厳密にコントロールされており、S 期後期に濃度は最大となる。これは dNTPs 産生経路の律速酵素、リボヌクレオチドレダクターゼ (RNR) の調節サブユニットが S 期に発現してくるに伴い酵素活性を獲得、増大する為であることが明らかにされている。DNA 複製が開始する前段階、G1 期において dNTPs 濃度は低い状態で保たれている。一方、dNTPs を基質とし DNA ポリメラーゼにより触媒される、DNA 複製速度は S 期初期から後期にかけて基本的に一定であると考えられている。このことから申請者は DNA 複製開始時において、DNA ポリメラーゼに dNTPs を効率よく供給する機構が存在するものと予想した。この機構は発癌予防において重要な働きを担っていることが予想され、癌の発症リスクを減じる為の知見を与えると考えられる。

研究の方法としてはまず RNR が DNA 複製マシナリーに局在することを示すために PCNA と RNR の細胞内局在を免疫染色法により検討した。HeLa 細胞をコラーゲンコートしたカバースライド上において培養し、Toriton X 処理を行い可溶性画分を除去した。このクロマチン結合サンプルを RNR の触媒サブユニットである RRM1 と PCNA に対する抗体で免疫染色を行った。この結果 RRM1 と PCNA の共局在を確認することができた。

次に RNR が複製マシナリーに局在する分子機構を明らかにするために、DNA 複製過程に関わる分子との相互作用を検討した。293 細胞に HA タグを付けた RRM1 および調節サブユニット RRM2 を強制発現させ抗 HA 抗体で RNR 複合体を精製し LC/MS により RNR と相互作用する分子の同定を試みた。しかしながらこの解析によって RNR 複合体に含まれる DNA 複製関連因

子を同定することが出来なかった。

このため RNR と結合しうる分子のスクリーニングを既知の DNA 複製関連因子に絞り、それらに対する抗体をもちいて IP-Western blot を行った。この解析から DNA 複製前にライセンシングを行う pre-replicative complex の Cdc6 と MCM ヘリカーゼのサブユニット MCM6 を RNR と相互作用しうる分子として同定することが出来た。

RNR が Cdc6 および MCM6 と相互作用することから、RNR は Cdc6 依存的に DNA 複製起点にリクルートされ、ここで MCM6 と結合することで DNA 複製開始時に局所的に dNTPs を産生する機構が存在することが考えられた。この仮説を証明するために RNR の複製起点への局在を MCM4 origin における ChIP assay を行うことにより、RNR の DNA 複製起点への局在を検討した。この結果 RNR が MCM4 origin に局在していることが示された。

Discussion & Conclusion

RNR は dNTPs de novo 合成経路において NDPs から dNDPs への還元を触媒する、経路の律速酵素である。dNTPs は細胞増殖の際に染色体 DNA が複製されるための原料であり、哺乳動物細胞内においては細胞周期依存的に厳密にその濃度が制御されている。申請者は DNA 二重鎖切断修復時において dNTPs 合成が Tip60 依存的に損傷部位にリクルートされた RNR が行っていることを報告している（Niida et al. Genes & Development 2010）。本研究において RNR が DNA 複製起点に局在し、pre-RC complex の構成因子 CDC6 と G1/S 期特異的に結合することが見出された。このことは初期 DNA 合成開始時において DNA 複製部位に局所的な dNTPs 供給を行っていることを予想させる。DNA 合成が開始し複製マシナリーが鋳型 DNA 上を新規 DNA を合成しながら移動する時には MCM6 との結合を介し RNR も移動して行くのかもしれない。本研究をさらに進めるためには RNR と CDC6 および MCM6 との結合領域を決定し、その結合を妨げる様な点変異体を細胞内に導入した時の異常を測定することが重要であろうと考えられる。現在この点について検討を進めている。

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一般の皆様へ

遺伝情報がコードされている DNA の安定性を維持することは癌などの疾患を防ぐために非常に重要です。今回の研究では DNA 合成酵素を至適な条件下で機能させるために必要なデオキシリボヌクレオチド (dNTPs) を効率よく DNA 合成酵素に供給する仕組みについて研究を行いました。まだ全容は明らかとなっていませんが、とても重要な機構であると考えており研究を進めております。

Characterization of mammalian homologue of Unc119 as a tumor suppressor

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Abstract

Mammalian homolog of *Caenorhabditis elegans* Unc119 (Munc119) functions as a specific myristoyl receptor. Previous reports suggest its implication in the primary cilia formation. We identified Munc119 as a putative binding partner of RASSF6, which is a tumor suppressor and localized at the primary cilia in epithelial cells. In this study we revealed that Munc119 interacts with MDM2 and enhances its auto-ubiquitination and degradation to stabilize p53 and that its depletion impairs p53-mediated G1/S arrest, delays DNA repair after DNA damage and eventually results in polyploidy. These findings have revealed a novel function of Munc119 as a tumor suppressor.

Key Words : Cell cycle; p53; RASSF; tumor suppressor

Introduction

Unc119 was discovered in *Caenorhabditis elegans* (*C.elegans*) based on the recessive mutation affecting locomotion. Human homologue Munc119 was identified as a retina-specific gene 4 (HRG4). Munc119 expression is reduced in human colon, stomach, and breast cancers. Munc119 interacts with various proteins and plays versatile roles in the primary cilia formation, trafficking of transducinα, regulation of endocytosis, and activation of src family kinases. We identified Munc119 as a putative interactor with a tumor suppressor RASSF6 and analyzed the tumor suppressive role of Munc119 in this study.

Results

Mammalian homolog of Munc119 is a RASSF6 interactor.

RASSF6 expression is suppressed in human cancers and is known as a tumor suppressor. RASSF6 is closely related with the tumor suppressive Hippo pathway, but our previous findings suggest that it exerts a tumor suppressive role independently of the Hippo pathway. To clarify the molecular mechanism behind RASSF6-mediated tumor suppression we performed a yeast two-hybrid screening using RASSF6 as a bait on human kidney cDNA library and obtained Munc119 as a positive clone. We confirmed the co-immunoprecipitation of endogenous RASSF6 and Munc119 from rat liver and the co-localization of these proteins in rat kidney. As the *in silico* data support that Munc119 expression is reduced in human cancers, we tested the possibility that Munc119 also functions as a tumor suppressor.

Munc119 induces G1/S arrest in HeLa cells.

RASSF6 expression causes apoptosis and G1/S arrest in HeLa cells. In contrast Munc119 did not induce apoptosis. Munc119 coexpression and depletion did not influence RASSF6-induced apoptosis in HeLa cells. However Munc119 depletion enhanced cell proliferation and BrdU incorporation in HeLa cels. In the cell cycle analysis, Munc119 expression induced G1/S arrest, while its depletion promoted the cell cycle progression in HeLa cells.

Munc119 regulates cell cycle *via* p53.

We recently revealed that RASSF6-mediated apoptosis and cell cycle regulation depend on p53 (the paper under revision). Therefore we examined whether p53 is involved in Munc119-mediated cell cycle regulation. Munc119 expression did not induce G1/S arrest in p53-negative H1299 cells. Munc119 depletion increased BrdU incorporation in p53-positive U2OS cells but not in p53-negative SaOS2 cells. In Munc119-depleted HeLa cells, p53 degradation was facilitated. MG132 treatment recovered p53 protein expression. We confirmed in experiments using heterologous HEK293FT cells that Munc119 interacted with MDM2, the most important E3 ligase for p53, and enhanced auto-ubiquitination.

Munc119 depletion impairs DNA repair and causes polyploidy.

We tested the idea that Munc119 depletion impairs G1/S checkpoint and leads to the genomic instability. We treated HeLa cells with VP-16 to induce DNA damage and immunostained γ-H2AX as a marker of DNA damage. γ-H2AX was detected immediately after the VP-16 treatment but remarkably diminished at 24 h after VP-16 removal in the control cells. In contrast, γ-H2AX signals remained in the Munc119-depleted HeLa cells. Munc119 depletion induced polyploid cells in VP-16-treated HeLa cells.

Discussion & Conclusion

In this study we confirmed that Munc119 is a *bona fide* interactor of RASSF6. Unlike RASSF6, Munc119 does not cause apoptosis, but plays a role in the cell cycle regulation. Our data suggest that Munc119 interacts with MDM2 and regulates p53 protein expression. We propose a model that upon DNA damage Munc119 facilitates MDM2 auto-ubiquitination and stabilizes p53 to induce G1/S arrest. As Munc119 depletion impairs p53-mediated G1/S arrest and causes polyploidy, Munc119 could function as a tumor suppressor. It is highly important to test Munc119 expression in human cancer samples in the near future. We have recently found that RASSF6 exerts its tumor suppressive roles depending on p53. We used p53-compromized HeLa cells for most experiments of this study and cannot so far clarify whether and how Munc119 influences RASSF6-induced apoptosis and cell cycle regulation. However now that p53 is important for the function of Munc119 as well as that of RASSF6, we need to test how Munc119 works in p53-intact cells for further studies. It will be intriguing to examine whether the cell cycle regulations by Munc119 and RASSF6 correlate with the primary cilia formation.

一般の皆様へ

DNA の損傷や染色体分裂の異常は発がんやがんの悪性化に密接に関係しています。網膜変性症の原因遺伝子産物である Muncl19 が、このような遺伝子に異常を生じた細胞の分裂を抑制し、腫瘍抑制因子として働きうることを私たちは明らかにしています。生命の設計図のおさめられている染色体を守る仕組みの解明につながることが期待されます。

Epigenetic involvement in a mouse model of E-cadherin-p53-deficient diffuse-type gastric cancer

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Abstract

Diffuse-type gastric cancer (DGC) exhibits rapid disease progression and a poor patient prognosis. However, there is no optimal therapeutic strategy for DGC. We have reported an E-cadherin/p53 double conditional knockout (DCKO) mouse line that recapitulates human DGC histologically and molecularly (Shimada *et al*., 2012). Furthermore, we have established DGC cell lines from primary tumors and lymph node metastases of the DCKO mice. In this study we identified that epigenetic mechanism can play a significant role in diffuse-type gastric carcinogenesis, and demonstrated that some epigenetic drugs had tumor suppressor activity in vitro and in vivo using the mouse gastric cancer cell lines.

Key Words : Diffuse-type gastric cancer

Introduction

Although several types of anti-cancer drugs have been developed, none of them dramatically improve overall survival of cancer patients. This is because human tumor cell lines commonly used in laboratories bear little resemblance to primary cancer due to over passage, but xenografts of human cancer specimens are not appropriate for drug testing because of cost and effort. We have established a mouse model of DGC and cell lines from mouse DGC. Then, we clarified the mechanism of DGC and screened anti-cancer molecules using the low-passage mouse cancer cell lines, and then assessed the effectiveness of these candidates in vivo.

Results

Establishment of cell lines from DGC of the DCKO mice

We have established mouse gastric cancer cell lines (GC) from primary DGC and lymphatic dissemination of the DCKO mice. We also utilized mouse gastric epithelial cell lines (GE) derived from gastric mucosae of fetal *p53*-null mice (Fukamachi *et al*., 2004) as control. We examined biological traits of the GC cell lines comparing with those of the GE cell lines: 1) The former had 30-fold more sphere-forming capacity than the latter in serum-free condition using Ultra-Low Attachment Culture Dishes (Figure 1A). 2) When subcutaneously injected to immunocompromised mice with Matrigel, only 100 cells of the GC cell lines immediately induced large tumors, while even 10^5 cells of the GE cell lines did not (Figure 1B). 3) The GC cell lines were resistant to

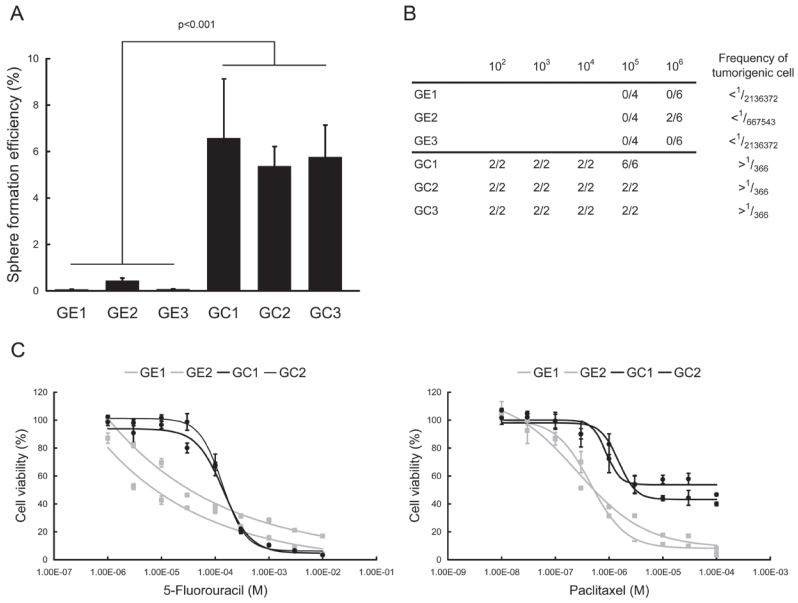


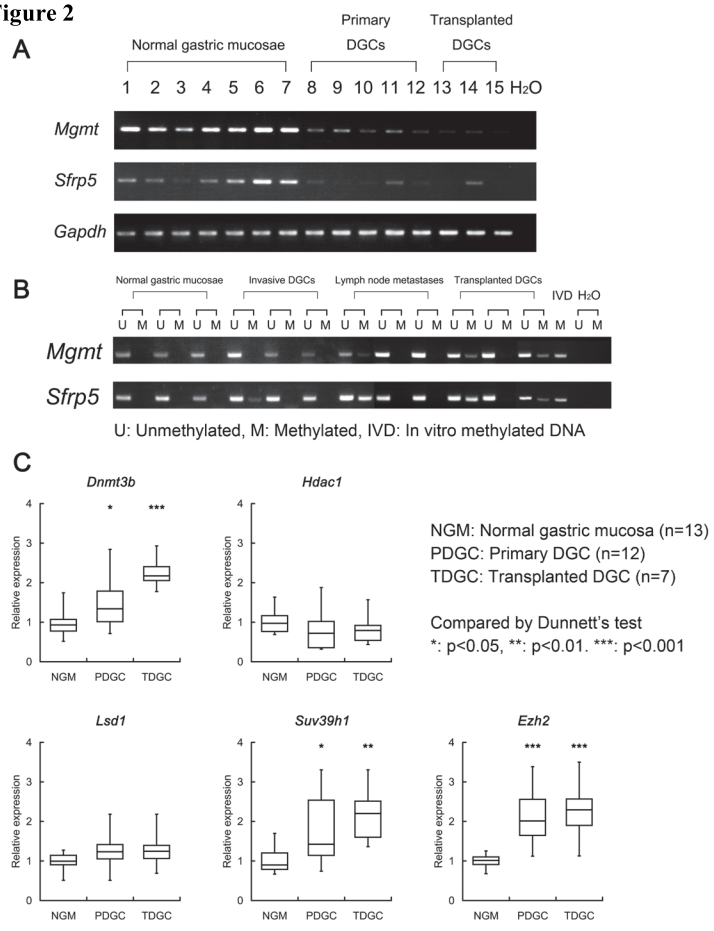
Figure 1

5-Fluorouracil and Paclitaxel, cytotoxic agents generally used in patients with advanced gastric cancer (Figure 1C).

Epigenetic involvement in DGC formation

To identify what molecular mechanism plays an important role in DGC formation of the DCKO mice, we performed microarray analysis of normal gastric mucosae and primary gastric cancer, and compared their gene expression patterns. The results indicated that the expression levels of frequently-methylated genes in several types of cancer including stomach cancer (Ohm *et al.*, 2007) were down-regulated whereas those of epigenetic modulators were up-regulated in mouse DGC. We conducted reverse-transcription polymerase chain reaction (RT-PCR) and methylation-specific PCR (MSP) of *Mgmt* and *Sfrp5*, reported as frequently-methylated genes, and validated down-regulated expression (Figure 2A) and DNA methylation (Figure 2B) of these two genes in mouse cancer lesions. Quantitative RT-PCR (qRT-PCR) also revealed overexpression of *Dnmt3b* (DNA methyltransferase), *Suv39h1* (H3K9 methyltransferase) and *Ezh2* (H3K27 methyltransferase)

Figure 2



in primary gastric cancers and transplanted tumors (Figure 2C). These data suggest that epigenetic alteration can be involved in diffuse-type gastric carcinogenesis.

Evaluation of epigenetic drugs in vitro

Next, we assessed tumor suppressor activity of epigenetic drugs, that is, 5-Aza-2'-deoxycytidine (AZA) as DNA methyltransferase inhibitor, Trichostatin A (TSA) and sodium butyrate (NaB) as histone deacetylase inhibitors, Tranylcypromine (TCP) as Lsd1 (H3K4 demethylase) inhibitor, Chaetocin as Suv39h1 inhibitor, and 3-Deazaneplanocin A (DZN) as Ezh2 inhibitor, using the GC and GE cell lines. The dose-response curves showed that AZA attenuated proliferation only in the GC cell lines (Figure 3A), and morphological changes of the mouse cancer cell lines to epithelial phenotype were observed by treatment of TSA, NaB and DZN, implying differentiation induction (Figure 3B). We also exposed the GC cell lines to the six epigenetic inhibitors described above in low dose (IC80) under sphere-forming conditions, because sphere-forming property of cancer cells correlates tumorigenicity in vivo in this experimental system, as often reported in other studies. Remarkably, TSA, NaB and Chaetocin blocked self-renewal of the GC cell lines almost completely (Figure 3C).

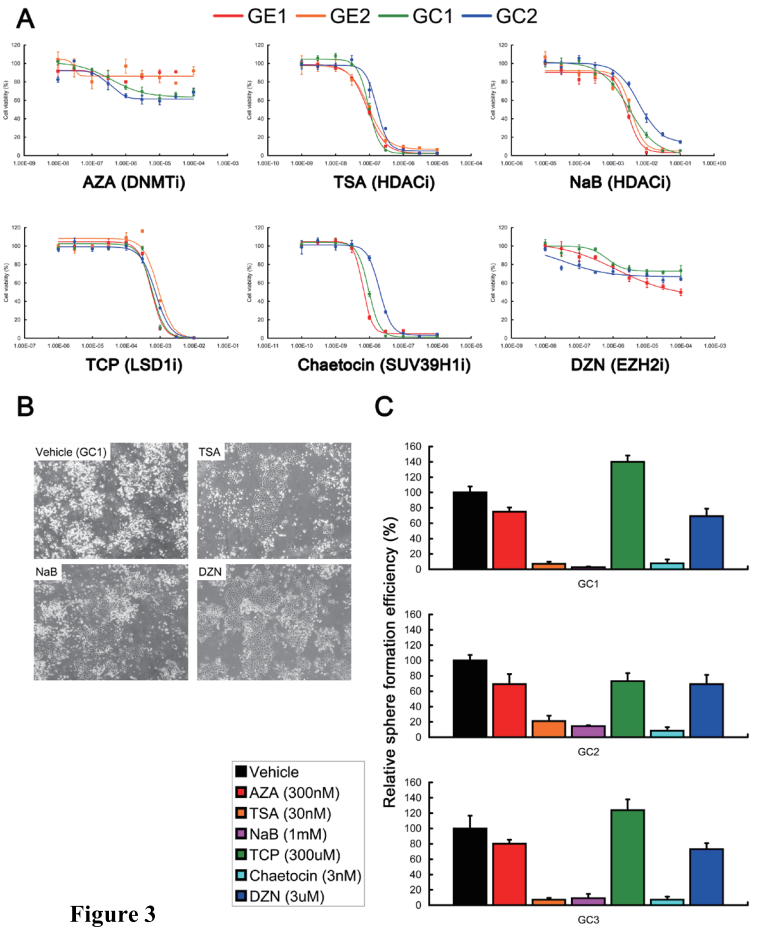


Figure 3

Evaluation of epigenetic drugs in vivo

Finally, two weeks after we subcutaneously transplanted 10^5 cells of GC1 in nude mice, we intraperitoneally administered vehicle (10% dimethyl sulfoxide), TSA (10 mg kg^{-1}), NaB (1200 mg kg^{-1}) and Chaetocin (0.5 mg kg^{-1}) to these DGC-bearing mice every day. The tumors of treated mice grew less rapidly than

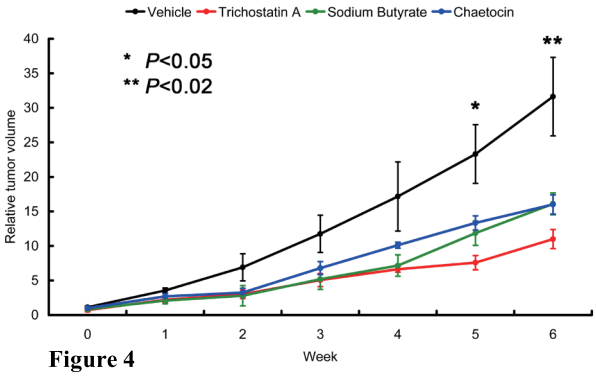


Figure 4

those of control mice, and their volumes were strikingly reduced five and six weeks after medical care started (Figure 4). We also found the same results in GC3 (data not shown).

Discussion & Conclusion

We succeeded in culturing mouse DGC in vitro, and the cell lines exhibited high sphere-forming and tumorigenic activity. The DCKO mice and their cancer cell lines provide us a relevant approach to research and development of DGC both in vivo and in vitro, respectively. Using this mouse-based system, we identified that epigenetics contributed to DGC formation, and demonstrated that epigenetic agents could be efficient in this preclinical trial.

Certainly, a critical problem still remains that only epigenetic inhibitors can not lead to complete remission of DGC in this report. We are now investigating gene expression profiles of GC and GE cell lines, and have detected some oncogenic pathways activated in DGC of the DCKO mice. Combining epigenetic agents with drugs targeted to such signaling pathways, we will achieve radical treatment of DGC not only in the DCKO mice but also in human beings.

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一般の皆様へ

胃がんは、依然として発症数と死亡数が多いがんの一つです。胃がんの中でも、スキルス胃がんに代表される未分化型胃がん（DGC）は、浸潤と転移が著しく、予後が悪いことが知られています。我々は世界で最初に DGC のマウスモデルを作製し、その胃がんから細胞株を樹立することにも成功しました。それらを利用して、DGC ではエピジェネティクスが重要な働きをしており、エピジェネティクス阻害薬が実験室レベルのみではなく、生体レベルでも DGC の治療に有効であることがわかりました。今後は、この実験系を利用して、更なるメカニズムの解明と臨床への早期応用を目指していきます。

Characterization and generation of organ-specific vascular cells from human iPS cells towards development of novel in vitro models for intractable vascular diseases

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Abstract

In this study, we aimed to characterize organ-specific vascular cells from cerebral artery, aorta, pulmonary artery and renal glomeruli in order to develop the directed differentiation methods from human iPS cells into the organ-specific vascular cell types. These methods could be used to establish novel in vitro disease models for intractable vascular disorders with patient-derived iPS cells.

Key Words : iPS cell, organ-specific vascular cell, in vitro model

Introduction

Among the most remarkable advances in iPS cell research is the embodiment of disease modeling using patient-derived iPS cells. It has been shown that the in vitro iPS model successfully recapitulates some aspects of intractable disorders, suggesting the feasibility that the iPS model can provide a platform for studies aiming at both understanding pathological mechanisms and discovering new drug compounds. To create novel iPS disease models for intractable vascular disorders, the specificity of vascular cells in each organ or tissue need to be characterized, and the directed differentiation protocols from human iPS cells into organ-specific vascular cell types are required.

Results

(1) Characterization of organ-specific vascular cells.

We first tried to isolate vascular endothelial cells from mouse brain, lung, aorta and kidney tissues. However, the tissue samples were so small and we found it very difficult to obtain enough amount of mRNA or protein from them. Then, we used the vascular samples removed from adult micro mini pigs. The vascular tissue samples including both endothelia and smooth muscle cells from pig aorta, pulmonary artery and renal artery were collected and examined by Mass Spectrometry. The results showed that these three samples shared the expression of many cytoskeleton proteins, such as smooth muscle actin, cardiac muscle actin, skeletal muscle actin and actinin, while several molecules specifically expressed in each of three vascular types were also identified, which could be used to characterize and distinguish the three vascular types. We are currently examining the detail of both temporal and spatial expression patters of the candidate molecules using embryonic and

adult mouse vascular samples. In addition, we are now trying to isolate only vascular endothelial cell samples from adult micro mini pigs to focus on the endothelial analyses.

(2) Development of directed differentiation methods from human iPS cells into organ-specific vascular cells.

We performed high-throughput chemical screening to identify potent chemical compounds that efficiently induce vascular endothelial cells from human iPS cells. To prepare the starting materials for the screen, we used the previously-reported differentiation protocol into vascular endothelia using two step treatment, 1st step with BIO (6-bromoindirubin-3'-oxime) and 2nd step with VEGF (vascular endothelial growth factor; Homma K. et al., 2010). We examined the effects of adding each of around 6,000 different chemicals into the 2nd step, in order to identify compounds that synergistically promote the differentiation into vascular endothelia with VEGF. Out of 6,000 chemicals examined, we found one hit compound that could increase the induction rate of vascular endothelia from human iPS cells from 10% up to 20%. We are optimizing the differentiation protocols for vascular endothelia using the hit compound, examining the mechanisms of action of the compound and characterizing the endothelial cells induced by the compound treatment.

(3) Derivation of patient-specific iPS cells from intractable vascular disorders.

We generated iPS cell lines from the patients with three intractable vascular disorders, intracranial aneurysms associated with autosomal dominant polycystic kidney disease (ADPKD), microscopic polyangiitis (MPA), and malignant hypertension, by reprogramming dermal fibroblasts or T cells with retrovirus or episomal vector method. We examined the following tests in each cell line derived from patients to select suitable iPS cell clones for further investigation, (1) silencing of transgene for reprogramming (2) pluripotent marker gene expression, such as OCT4, NANOG, SSEA3, SSEA4, TRA1-60 and TRA1-81 (3) multipotent differentiation potential into three embryonic germ layers using embryoid body (EB) and teratoma formation (4) karyotype analyses (5) transcriptome (6) methylation analyses (7) mutational analyses of causative genes. Then, we confirmed that these patient-derived iPS cell lines can be differentiated into both vascular endothelia and smooth muscle cells using the previously-reported vascular differentiation protocol (Homma K. et al., 2010).

Discussion & Conclusion

In Mass Spectrometry analyses of adult micro mini pig vascular samples, most of the identified molecules were cytoskeleton proteins which are normally expressed in muscular cells. We guessed that the majority of cells included in the vascular samples were smooth muscle cells rather than endothelia, and then smooth muscle-related genes were mainly identified. These results indicated that the purification of endothelial cells from the samples was required to characterize endothelial signatures of organ-specific vasculature. We are now isolating the endothelial cells by flow cytometry sorting using an endothelial cell surface marker, VE-cadherin. In conclusion, vascular cells may be different in protein expression patterns among the organs or tissues they are belonging to. In this study, we also identified the candidate chemical compound that promotes

the differentiation of human iPS cells into vascular endothelia, and succeeded in deriving patient-specific iPS cell lines from the three intractable vascular disorders.

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一般の皆様へ

本研究は、特定の臓器の血管にしか発生しない難治性の血管疾患に対する iPS 疾患モデルを作製するために、脳動脈、大動脈、肺動脈、腎動脈の血管細胞の遺伝子発現の特徴などを解明し、その情報を基にヒト iPS 細胞から各臓器特異的な血管細胞の作製法開発を目指すものである。

Fine mapping of autophagy-related proteins during autophagosome formation in *Saccharomyces cerevisiae*

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Abstract

Autophagy is a bulk degradation system mediated by biogenesis of autophagosomes under starvation conditions. Here, we visualized forming autophagosomes as cup-shaped structures using fluorescence microscopy by enlarging a selective cargo of autophagosomes, and finely mapped the localizations of autophagy-related (Atg) proteins. The results obtained by this observation suggest that Atg proteins play individual roles at spatially distinct localizations during autophagosome formation. These findings will facilitate detailed investigations of the function of each Atg protein during autophagosome formation.

Key Words : autophagy; autophagosome; autophagy-related (*ATG*) genes; endoplasmic reticulum exit sites (ERES); yeast

Introduction

Autophagy is a bulk degradation system widely conserved among eukaryotes from yeast to mammals. When eukaryotic cells are faced with nutrient starvation, they degrade cytoplasmic materials to obtain building blocks that help them adapt to severe environmental conditions. Currently, over 30 *ATG* genes have been identified as necessary for various types of autophagy in yeast. Among them, 18 *ATG* genes are essential for autophagosome formation upon starvation (Mizushima et al., 2011; Nakatogawa et al., 2009). These Atg proteins are involved in autophagosome formation as components of several discrete functional units, but their interrelationships are not yet well understood.

Results

Among Vps (vacuolar protein sorting) proteins, which are involved in sorting of Prc1 (carboxypeptidase Y; CPY) to the vacuole (Raymond et al., 1992), Vps15, Vps30, and Vps34 are essential for autophagosome formation as components of phosphatidylinositol (PtdIns) 3-kinase complex I (Kametaka et al., 1998; Kihara et al., 2001; Obara et al., 2008). We investigated the localizations of representative GFP-fused Atg and Vps proteins in GAC cells expressing 2×mCherry-Atg8 (Ch-Atg8), and finely mapped the localization of Atg/Vps proteins during autophagosome formation. Atg13 and Atg17, and PtdIns 3-kinase complex I were localized to a dot at the junction between the forming autophagosome and the vacuolar membrane, which we termed VICS. By contrast, Atg1, Atg8, and the Atg16–Atg12–Atg5 complex labeled the VICS and the forming

autophagosome. We measured the fluorescence intensity along the forming autophagosome and found that the profiles of these proteins and Ch-Atg8 overlapped extensively. The Atg2–Atg18 complex and Atg9 were distributed as two or three dots associated with the forming autophagosome; some of these dots were apparently distinct from the VICS. Time-lapse microscopy showed that Atg2-GFP was initially visualized as a dot and subsequently appeared as multiple dots at the edge of the forming autophagosome.

In mammalian cells, physical connections between forming autophagosomes and the endoplasmic reticulum (ER) have been observed by electron microscopy (Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009). We found that the forming autophagosome was localized in the close proximity to the ER. When we labeled the ER lumen with GFP-HDEL (Okamoto et al., 2006) and observed cells with three-dimensional fluorescence microscopy, we found that the forming autophagosome was associated with the ER at two or three sites. Despite close association of the forming autophagosome with the ER, neither ER transmembrane nor lumenal proteins were ever detected at the forming autophagosome.

ER exit sites (ERES), which are subdomains of the ER specifically responsible for the production of COPII vesicles, can be labeled by fluorescent protein-tagged Sec13 or Sec16 (Connerly et al., 2005). We visualized the localization of ERES in GAC cells by three-dimensional fluorescence microscopy, using Sec13-GFP as a marker, and found that the forming autophagosome was localized in the close proximity to ERES at two or three sites per cell. The same result was obtained when Sec16-GFP was used. Association of the forming autophagosome with ERES labeled with Sec16-GFP was clearly detected in 79% of the cells (38 cells were examined three-dimensionally). Sec16, which is essential for ER-to-Golgi transport, is thought to be involved in organization of ERES as a scaffold (Connerly et al., 2005). Previously, our group showed that a temperature-sensitive *sec16-2* mutant is defective in autophagy at the non-permissive temperature (Ishihara et al., 2001). These data suggest that ERES play important roles in autophagosome formation.

As mentioned above, forming autophagosomes are associated with the ER at two or three sites per cell. We also show that the Atg2-Atg18 complex forms two or three dots at the edge of the forming autophagosome. These observations prompted us to examine the spatial relationship between ERES and the Atg2-Atg18 complex. By three-dimensional fluorescence microscopy, we found that dots labeled with Atg18 along the GAC were adjacent to ERES.

Discussion & Conclusion

In this study, taking advantage of yeast genetics, we visualized forming autophagosomes by fluorescence microscopy and finely mapped the localizations of Atg proteins. We found that the Atg13, Atg17, and PtdIns 3-kinase complex I were localized to the VICS. Atg1, Atg8, and the Atg16–Atg12–Atg5 complex labeled the VICS and the forming autophagosome. We also found that the Atg2-Atg18 complex and Atg9 were localized to the edge of the forming autophagosome but not to the VICS. Moreover, we showed that the forming autophagosome is associated with the ER via

ERES. Taken together, our data show that Atg proteins play individual roles at spatially distinct sites during autophagosome formation. These results will facilitate investigations of each Atg protein's function in the near future.

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一般の皆様へ

単細胞生物である出芽酵母（パン酵母）も我々哺乳類も、遺伝情報を含む DNA が膜で区画化された真核生物と呼ばれる生き物です。こうした膜区画がどのような分子機構で形成されているかはほとんど分かっておらず、我々は栄養飢餓で誘導されるオートファゴソームと呼ばれる膜区画の形成過程に注目して解析を進めています。本研究は形成中のオートファゴソーム上でオートファゴソーム形成に関わるタンパク質の分布を詳細に明らかにした点で斬新であり、今後の個別のタンパク質の機能解析に向けての大きな手がかりとなるものです。

Synthesis of fatty acids having various carbon frameworks

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Abstract

New synthetic method of fatty acids using nickel butadiene system catalyzed cross-coupling reaction between alkyl groups was developed. Unsaturated fatty acid and its regioisomers can be prepared by iterative coupling using the catalytic system. The Ni catalyst was also effective for the introduction of secondary alkyl groups into C2 position of benzothiazoles via C-S bond cleavage.

Key Words : fatty acid, cross-coupling, Grignard reagent, alkyl halides, benzothiazole

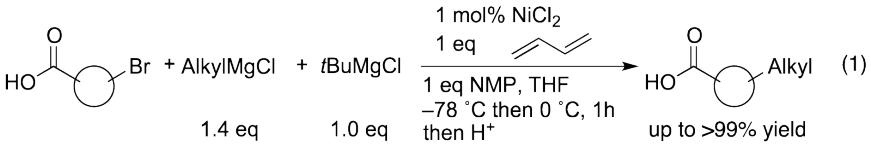
Introduction

Fatty acids are essential to natural life because they act as key components in our body and have various carbon chain frameworks, where unsaturated bond(s) as well as branched structure are often founds. Therefore, development of efficient synthetic method to construct carbon frameworks of natural and artificial fatty acids or their synthetic equivalents is important task.

Results

Transition metal-catalyzed cross-coupling reactions are one of the most important tools to construct new carbon skeletons from simple organic molecules in synthetic organic chemistry and now are widely used in various areas including functional materials, drug synthesis and so on. However, cross-coupling between two alkyl groups is rarely discussed compared to that of vinyl and aryl groups partly due to easy β-hydrogen elimination from alkylmetal intermediates.[1] We have developed transition metal-catalyzed cross-coupling reaction of alkyl halides with organometallic reagents in the presence of unsaturated hydrocarbon like 1,3-butadiene as an appropriate additive.[2]

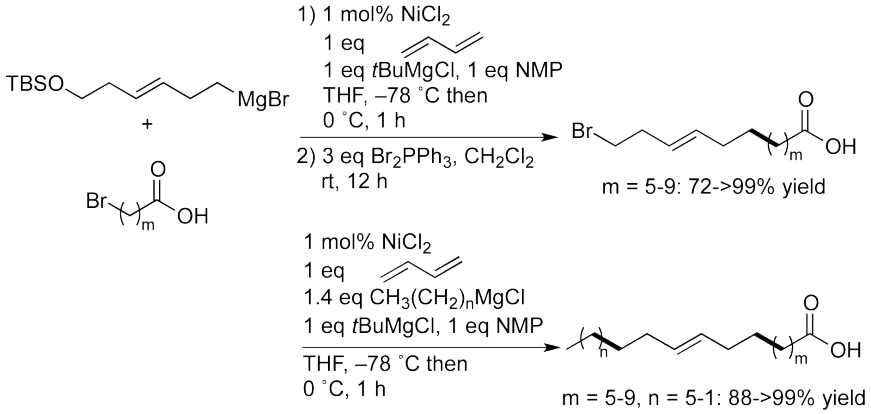
Because fatty acids consist of long saturated hydrocarbon chain, this C-C bond forming reaction can be assumed as an appropriate synthetic tool for constructing carbon frameworks of fatty acids though organometallic reagents as well as transition metal are known to be reactive toward carboxylic acid moiety in fatty acids, leading unwanted side-reactions. To overcome such drawback, we initiated our study on the synthesis of fatty acids using cross-coupling reaction by searching suitable conditions to achieve selective coupling reaction at C-halogen bond over carboxylic acid moiety and found that pre-treatment of bromoalkanoic acid with 1 equivalent of *t*-BuMgCl was effective to protect carboxylic acid moiety by forming magnesium carboxylate and cross-coupling reaction smoothly proceeded by catalytic amount of $NiCl_2$ in the presence of 1,3-butadiene as an additive as shown in eq 1. The present catalyst was effective for various bromoalkanoic acids as well as alkyl Grignard reagents and fatty acids having various carbon chains were obtained in good to excellent yields.[3]



By using the Ni-catalyzed cross-coupling reaction, we succeeded to prepare long unsaturated fatty acid, nervonic acid, from readily available oleic acid by reduction, bromination and cross-coupling sequence in good yield.

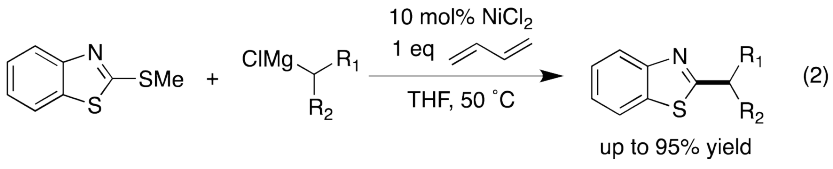
In addition, trans fatty acid, elaidic acid, and its regioisomers could be synthesized by iterative coupling as shown in scheme 1. It is well known that trans fatty acids increase the risk of coronary heart disease, however, toxic assessment of their regioisomers is not well explored due to the synthetic difficulty of such isomers. Therefore, the synthetic method to obtain these regioisomers in selective manner is highly desirable to evaluate their health risk.

For example, 6-bromohexanoic acid coupled with Grignard reagent having trans olefin and silyl ether moieties to give corresponding 12-bromo-9-dodecenoic acid in 72% yield after direct bromination of silyl group by Br_2PPh_3. Subsequent coupling of the 12-bromo-9-dodecenoic acid with hexyl Grignard reagent gave elaidic acid in 90% yield. Regioisomers of elaidic acid having trans olefin moiety at ω-5 to ω-8 positions were prepared by changing length of starting material, bromoalkanoic acid and Grignard reagent, in good to excellent yields as sole regioisomers.



Scheme 1 Synthesis of elaidic acid and its regioisomers

The Ni/butadiene system was also good catalyst for the coupling reaction of methylthiobenzothiazoles with secondary alkyl Grignard reagents through C-S bond cleavage, providing alkylated benzothiazoles in good yields (eq 2).[4] Interestingly, the cross-coupling did not proceed in the absence of 1,3-butadiene additive. Because thiazole derivatives having alkyl groups on C2 position are synthetic equivalents of fatty acids and often found in natural and synthetic compounds, the present catalyst provides a simple and efficient method for introducing an alkyl group in this position.



Discussion & Conclusion

The present Ni-catalyzed cross-coupling of bromoalkanoic acids and benzothiazoles with alkyl Grignard reagents provides useful synthetic tools to synthesize carboxylic acids and their synthetic equivalents having various carbon frameworks and will open up new fields of fatty acid chemistry in both biological and materials disciplines. The iterative coupling using Grignard reagents having siloxy group was found to be useful building block to construct various carbon frameworks. The alkylation of benzothiazoles using Ni-butadiene catalytic system was also developed, where congested secondary alkyl groups were efficiently introduced into C2 position of benzothiazoles through C-S bond cleavage. Further investigations on synthesis of biological active compounds using transition metal-catalyzed cross-coupling reaction are ongoing in our group.

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一般の皆様へ

脂肪酸は多彩な炭素骨格を有する化合物群であり、生体内をはじめとし幅広く存在しています。この脂肪酸類の炭素骨格を構築する手法の開発は、微量天然脂肪酸や非天然脂肪酸の生理活性等を評価する上で重要な研究課題です。我々は、カルボキシル基を有するアルキルハライド類とアルキルグリニャール試薬とのニッケル触媒によるクロスカップリング反応を利用し、一段階で様々な炭素骨格を有する脂肪酸類の合成に成功しました。また、炭素—硫黄結合の切断を伴ったクロスカップリング反応によりベンゾチアゾール類の 2 位に 2 級アルキル基を導入することにも成功した。

Analyses of structural dynamics of a G protein-coupled receptor using site-directed fluorescent labeling.

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Abstract

The effects of constitutively activating mutation on structural dynamics of opsin, a G protein-coupled receptor, were analyzed using site-directed fluorescence labeling techniques. Fluorescence spectroscopic analyses revealed that the mutation makes the receptor protein more flexible and more easily converted to the active state, which couples and activates G protein.

Key Words : GPCR, fluorescence labeling, membrane protein, protein dynamics

Introduction

Constitutively activating mutations (CAMs) have been found in various G protein-coupled receptors have been identified in various G protein-coupled receptors (GPCRs). These mutations elevate G protein activation efficiency of ligand-free GPCRs. Also, some of CAMs are known to be linked to genetic diseases. It is still unclear how CAMs makes ligand-free GPCRs can couple and active G protein in ligand-independent manner. In this study, effects of constitutively activating mutation on structural dynamics of opsin, the ligand-free form of light sensing GPCR rhodopsin, were analyzed using site-directed fluorescence labeling techniques.

Results

A fluorescence probe bimane was specifically attached on the cytoplasmic end of sixth transmembrane helix of opsin with or without a CAM. This is because the sixth transmembrane helix is known to move outward upon signal-induced activation of various GPCRs including rhodopsin. Respective labeled opsins were successfully purified in a detergent solution. Using these bimane labeled opsins, the fluorescence emission spectra were compared in the presence or absence of the CAM. The fluorescence spectrum was changed by the CAM, indicating that the CAM induces some conformational changes in the sixth transmembrane helix. Next, G protein mimetic peptide was added to the labeled opsins and the peptide-induced spectral shift was analyzed. In the case of normal opsin (without the CAM), addition of the peptide caused no changes, but the peptide significantly shifted the fluorescence spectra of the labeled opsin with the CAM. These results suggest that conformation of opsin is highly constrained to the inactive conformation, but the CAM converts the opsin conformation into the active conformation that can interact with G protein. Also, binding of the G protein mimetic induced additional conformational changes only in opsin with the CAM. These results indicate that conformational changes in the sixth helix are involved in not only

signal-induced activation but also mutation-induced activation of GPCRs.

In order to resolve the CAM-induced conformational changes in detail, the fluorescence quencher tryptophan was introduced on the fifth transmembrane helix of the labeled opsins. The changes in relative arrangement of the fifth and sixth helices are analyzed base on intra-molecular fluorescence quenching profiles. The analyses showed that the CAM caused rearrangement of the sixth transmembrane helix relative to the fifth one. This result confirmed that the CAM induces rearrangement of the sixth helix like photo-activation of rhodopsin. Next, energetics aspect of the CAM-induced conformational changes was analyzed. For this purpose, the dynamic quenching constant was measured at various temperatures. The dynamic quenching constant is calculated as [1/tw – 1/t0], in which tw is a fluorescence lifetime in the presence of quencher tryptophan and to is a fluorescence lifetime without tryptophan. Using the constant values measured at various temperatures, Arrhenius plot was constructed. The plot clearly showed that introduction of the CAM decreased the slopes. That means that the activation energy in the formation of dynamic quenching complex is decreased by the CAM. Since fluorophore and quencher were introduced on the sixth and fifth helices, respectively, this result strongly suggests that the CAM decreased energy barrier between inactive and active conformations of opsin. In other words, the CAM makes conformational changes of the helices in opsin more easily. In that way, the CAM affects structural dynamics of ligand-free opsin.

Taken together, the CAM in ligand-free opsin caused some conformational changes and affected the energetic aspect of conformational changes as well. Further analyses using various GPCRs and the mutants labeled with a fluorescent probe would reveal how CAMs affect molecular properties and structural features of GPCRs, resulting in signal-independent G protein activation. Furthermore, such analyses may provide new information to understand how some CAMs cause genetic diseases.

Discussion & Conclusion

This study clearly showed that fluorescence labeling study can assess how a CAM affect conformation(s) of a GPCR and its conformational dynamics. Also, fluorescence labeling methods used in this study can be easily applied for not only other GPCRs but also other membrane proteins. Recently, crystallographic studies of a wide variety of GPCRs have been developed dramatically, providing valuable many static structures of various GPCR conformations. Thus, dynamic aspect of the GPCRs should be studied and revealed. Combination of the crystal structures with structural dynamics analyses like this study will extend our understanding of structure-function relationships of GPCRs. In other words, in order to understand how GPCRs function, both static structures and dynamic aspects of the receptors should be analyzed.

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Koyanagi M, Takada E, Nagata T, Tsukamoto H and Terakita A.
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Tsukamoto H and Farrens DL.

一般の皆様へ

ヒトなどの動物を構成する細胞は細胞外からのシグナルを、何らかのメカニズムで受容して、そのシグナルをもとに生理活動を調節します。細胞外のシグナルを受け取る役割を果たす主要な部品（タンパク質）の一つが G タンパク質共役受容体 (GPCR と略されます) です。GPCR をコードする遺伝子に特定の変異が入ると、シグナルを受けていないのにもかかわらず、細胞内にシグナルを受容したという（間違った）情報を伝達してしまいます。今回私は GPCR に変異が入った時に、タンパク質のかたちが変わりやすくなることで、「間違った」情報伝達をしてしまうことを明らかにしました。

Regulation of the generation and maintenance of memory T cells in the intestine

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Abstract

We examined how gut-specific homeostatic proliferation of T cells contributes to mucosal memory T cells. This study revealed that the homeostatic proliferation of CD4 T cells in the mesenteric lymph nodes (MLNs) critically contributes to generating gut-tropic Th17 cells. In addition, Th17 generation in MLNs was markedly suppressed by blockade of OX40 signals in T cells. These results reveal that MLNs are critical in inducing gut-tropic effector memory Th17 cells through OX40 signaling.

Key Words : Mucosal immunity, homeostatic proliferation, Th17 cell, mesenteric lymph node,

Introduction

Since the systemic homeostatic proliferation of naïve T (T_N) cells supplies a memory-phenotype T cell pool, this system can be used to generate effector memory T (T_{EM}) cells, including Th17 cells. However, it is unclear whether the homeostatic proliferations may also occur in an "organ-specific" fashion such as in the intestine and MLNs. Therefore, we examined the homeostatic proliferation in MLNs and intestinal tissue, and demonstrate that the T_{EM}-phenotypic CD4 T cells accumulate in the host's small intestine through homeostatic proliferation.

Results

The small intestine harbors numerous $CD4^+$ effector memory T (T_{EM}) cells

Naive and central memory T cells are reported to circulate through secondary lymphoid tissues, while T_{EM} cells accumulate in extralymphoid organs, including the small intestine (1). To confirm this, we looked for naïve and memory $CD4^+$ T cell phenotypes. Of the $CD4^+$ T cells found in secondary lymphoid tissues such as the spleen, inguinal lymph nodes (ILNs), and MLNs, approximately 70% had a naïve T phenotype and 20–30% had a T_{EM} phenotype. In the lamina propria of the small intestine, 80–90% of $CD4^+$ T cells had a T_{EM} phenotype. These data confirm that T_{EM} cells preferentially accumulate in extralymphoid organs, including the small intestine.

Mesenteric lymph nodes (MLNs) but not Peyer's patches (PPs) are essential for generating $\alpha_4\beta_7^+$ gut-homing T cells

We examined $\alpha_4\beta_7$ levels on donor cells, derived from naïve CD4 T cells, undergoing homeostatic proliferation. After transfer with naïve CD4 donor T cells, some donor T cells acquired higher levels $\alpha_4\beta_7$ through homeostatic proliferation. In particular, fast-dividing cell populations from the small

intestine and MLNs contained larger proportions of $\alpha_4\beta_7^+$ donor cells than did those recovered from the spleen. On the other hand, slow-dividing population of the donor cells in these organs retained their initial, intermediate $\alpha_4\beta_7$ level. These results indicate that the $\alpha_4\beta_7^+$ phenotype develops during fast homeostatic proliferation.

To examine whether PPs and MLNs are required for generating $\alpha_4\beta_7^+$ gut-homing T cells, we determined the frequencies of $\alpha_4\beta_7^+$ donor cells in Peyer's patch-deficient (PPX) and mesenteric lymphadenectomized (MLX) mice. The $\alpha_4\beta_7^+$ proportion was unchanged in fast-dividing cells recovered from the small intestine or spleen of PPX mice but the proportion was significantly reduced in MLX mice. These findings suggest that MLNs but not PPs are essential for generating $\alpha_4\beta_7^+$ cells through fast cell division.

OX40 signaling is critical for fast proliferation in MLNs

The *in vivo* blockade of T cell costimulatory signals mediated through OX40, which belongs to the TNF receptor superfamily, improves experimental colitis by suppressing the homeostatic proliferation of pathogenic $CD4^+$ T cells (2). Therefore, we examined OX40 expressed on donor T cells in MLNs during homeostatic proliferation, and found OX40 on fast- but not slow-proliferating cells. To clarify the role of OX40 in fast proliferation, we treated recipient mice with an inhibitory anti-OX40L mAb and monitored donor cell proliferation in MLNs. Treatment with the anti-OX40L mAb significantly inhibited the generation of fast-proliferating $\alpha_4\beta_7^+$ cells in MLNs, without affecting slow cell division. Treatment with the anti-OX40L mAb also strongly reduced the total, fast-proliferating, and $\alpha_4\beta_7^+$ donor T cell populations in the small intestine. This indicates that OX40 costimulation is required for generating gut-tropic, fast-dividing cells in MLNs.

Gut-tropic Th17 cells arise in MLNs during homeostatic proliferation

The $CD4^+$ T cell population in the small intestine includes a significant number of Th17 cells (3). Therefore, we measured IFN-γ and IL-17A cytokine levels in donor cells recovered from the small intestine, spleen, and MLNs. Neither cytokine was produced by the slow-dividing population, while the fast-dividing population expressed both. When gating on the fast-dividing population, an IFN-γ profile was dominant in the spleen, but in the intestine IL-17A-producing cells greatly outnumbered IFN-γ-producing ones. In MLNs, IFN-γ and IL-17A were detected at equal levels. These results indicate that Th17 cells are generated through fast proliferation in an organ-specific manner, and that gut-specific Th17 cells may arise in MLNs or the small intestine. Furthermore, removing MLNs from recipient mice significantly reduced the $IL\text{-}17A^+$ but not the $IFN\text{-}\gamma^+$ population in the small intestine. Collectively, these results demonstrate that MLNs are essential for the generation of gut-tropic Th17 cells through fast homeostatic proliferation.

Discussion & Conclusion

Our results clearly demonstrate that gut-specific fast homeostatic proliferation plays a critical role in inducing gut-homing $\alpha_4\beta_7^+$ $IL\text{-}17A^+$ T cells, which is dependent on OX40 signals. Although the systemic inhibition of OX40 signals, either in OX40L-KO mice or by treatment with an anti-

OX40L mAb, drastically ameliorates several types of IBDs and GVHD, our study revealed that the OX40-OX40L interactions necessary for generating intestinal Th17 cells may occur in the MLNs. Therefore, it may be possible to develop therapeutic strategies for IBDs and GVHD by controlling the OX40-OX40L interactions in MLNs. In addition, the finding that the role of MLNs in developing gut-homing T_{EM} cells, including Th17 cells, is distinct from that of the PPs or spleen may contribute to a deeper understanding of intestinal mucosal immunity.

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一般の皆様へ

免疫反応の司令塔であるヘルパーT 細胞の制御機構の解明は、アレルギーや免疫疾患の原因解明や治療法の開発に直結する重要な研究です。私たちは、腸管粘膜免疫を司る Th17 型と呼ばれるヘルパーT 細胞が作られる場所が腸間膜リンパ節である可能性を見いだしました。さらに、Th17 型ヘルパーT 細胞が作られる過程で OX40 受容体からのシグナルが必須であることを発見しました。腸間膜リンパ節の T 細胞やそこに存在する OX40 受容体の働きの仕組みを解明することで、近い将来には、食物アレルギーや炎症性腸疾患の治療法の開発が期待されます。

Studies on the regulatory mechanisms underlying cell cycle-dependent centrosome replication

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Abstract

The guanine nucleotide exchange factor ARHGEF10 negatively regulates centrosome duplication through the activation of the small GTPase RhoA. In this study, we investigated the mechanisms for this novel signalling pathway. Furthermore, the role of ARHGEF10 in malignant progression of human cancer was explored.

Key Words : centrosome; GTP-binding protein; guanine nucleotide exchange factor

Introduction

The centrosome serves as the major microtubule-organizing center in mammalian cells. Aberrations in centrosome numbers, which frequently occur in aggressive human tumors, represent a crucial cause of missegregation of chromosomes, leading to a phenotype termed genetic instability. Therefore, the duplication cycle of the centrosome must be rigorously controlled in the cell. In this study, we attempted to clarify the detailed mechanism through a novel signaling pathway involving the small GTPase RhoA.

Results

A single centrosome consists of a pair of centrioles surrounded by the amorphous pericentriolar material. Centrioles are cylindrical structures built of nine sets of triplet microtubules, and lie at right angles to each other and in close proximity at one end. The pericentriolar material harbors a number of protein complexes, including the γ-tubulin ring complex, which acts as a template for microtubule nucleation. During M phase of the cell cycle, two centrosomes orchestrate the assembly of bipolar mitotic spindles, which is prerequisite for accurate chromosome segregation.

The signal transducing small GTPase RhoA has been implicated in a variety of cellular responses in mammalian cells. Upstream of RhoA, diverse Dbl family guanine nucleotide exchange factors (GEFs) act as signal-specific regulators of RhoA depending on the cellular context. Tandem catalytic DH and regulatory PH domains are conserved in all Dbl family members. The Dbl family GEF ARHGEF10 was originally identified as the product of the gene associated with slowed nerve-conduction velocities of peripheral nerves. However, underlying molecular mechanisms remain elusive.

In our effort to clarify the biological function of this poorly characterized GEF, we found a novel signaling pathway that negatively regulates centrosome duplication during cell cycle progression.

RhoA, but not other Rho family small GTPases examined, was biochemically identified as a substrate of ARHGEF10. ARHGEF10 was localized in the centrosome in adenocarcinoma HeLa cells in both G1/S and M phases. Knockdown of ARHGEF10 expression by RNA interference resulted in multipolar spindle formation in M phase. An intact centrosome composed of two centrioles and the pericentriolar material was found in each spindle pole. Partial, but not full, knockdown of RhoA by RNA interference elicited similar phenotypes. Aberrant mitotic spindle formation following ARHGEF10 knockdown was rescued by ectopic expression of constitutively activated RhoA. Thus, it is likely that RhoA is a downstream target of ARHGEF10 in this signaling pathway.

We further isolated the kinesin-like motor protein KIF3B as a binding partner of ARHGEF10. Knockdown of KIF3B also caused multipolar spindle phenotypes. The supernumerary centrosome phenotype was also observed in S phase-arrested osteosarcoma U2OS cells when the expression of ARHGEF10, RhoA or KIF3B was abrogated by RNA interference.

Multinucleated cells were not increased upon ARHGEF10 knockdown. Inhibition of the RhoA effector kinase ROCK by its specific pharmacological inhibitor Y-27632, in contrast, induced not only multipolar spindle formation, but also multinucleation. These results suggest that unregulated centrosome duplication rather than aberration in cytokinesis may be responsible for ARHGEF10 knockdown-dependent multipolar spindle formation.

Given that more than two intact centrosomes appear in G2/M phase, excess duplication may occur due to aberrant disengagement of the newly synthesized (daughter) centriole in S phase. To obtain further evidence supporting this possibility, we tried to quantify doublet (engaged) and singlet (disengaged) centrioles in S phase. Counterstaining of centrioles by anti-centrin and anti-C-Nap1 antibodies is expected to give us the engagement status of the centriole (Tsou and Stearns *Nature* **442**, 947 (2006)). We are already successful in staining centrin in synchronized cells, but the conditions for the staining of C-Nap remain to be established. We, in fact, tried several different purchased anti-C-Nap antibodies at various concentrations, and only one antibody is promising. Currently, we are trying to describe the precise mechanisms for ARHGEF10-RhoA mediated regulation of centrosome duplication.

Discussion & Conclusion

ARHGEF10 is a RhoA GEF, which is localized in the centrosome in both G1/S and M phases. ARHGEF10 regulates mitotic spindle formation through the action of RhoA. ARHGEF10-regulaed RhoA may be implicated in the control of centrosome duplication rather than cytokinesis. The motor protein KIF3B is a binding partner of ARHGEF10, a subset of which is co-localized with ARHGEF10 in the centrosome. Thus, KIF3B may regulate ARHGEF10 upstream of RhoA, being involved in the regulation of centrosome duplication. Recently, mutations in the *ARHGEF10* gene was found in various types of human cancer cells. Dysregulated centrosome duplication and cell division in cells lacking ARHGEF10 activity may be a cause of malignant progression of human cancer.

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一般の皆様へ

本研究では、中心体の過剰な複製を抑制することにより、中心体複製と細胞分裂の同調を保証するという、我々が見出した全く新しい制御機構の全容を解明するとともに、その異常による細胞の異数化とヒト癌の悪性化との関連性を明らかにすることを目的としている。本研究において中心体の複製回数を厳密に制御するメカニズムの詳細が明らかにされれば、細胞機能の基本原理の一端が解明されるという意味で学術的にインパクトが大きい。さらに、癌の悪性化を誘導する新規のメカニズムの解明となるとともに、この過程が癌治療の新規の標的となることが期待される。

microRNA-mediated regulation of vasculogenesis

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Abstract

Secreted factors with potent morphogenetic activity are key elements of cellular communication, and precise regulation of their expression is required to elicit appropriate physiological responses. MicroRNAs (miRNAs) are versatile post-transcriptional modulators of gene expression. We found that muscle-specific miRNAs miR-1/206 negatively regulate angiogenesis during zebrafish development. miR-1/206 directly regulate the levels of Vascular endothelial growth factor A (VegfA) in muscle, controlling the strength of angiogenic signaling to the endothelium. Our findings uncover a novel function for miR-1/206 in the control of developmental angiogenesis through the regulation of VegfA, and identify a key role for miRNAs as regulators of cross-tissue signaling.
Key Words : vasculogenesis, zebrafish, microRNA, VEGF

Introduction

Secreted signaling molecules are powerful regulators of development that are able to elicit behaviors such as proliferation, migration and differentiation in a concentration-dependent manner. One developmental context in which these signaling molecules play an important role is the formation of the vascular network. Secreted growth factors such as vascular endothelial growth factor (VEGF) function at different levels during the establishment and remodeling of the vasculature.

Results

miR-1 and miR-206 are evolutionarily conserved, have common expression patterns in the muscle, and share a large part of their sequence (1). To investigate whether miRNAs regulate the cross-talk between the muscle and surrounding tissues during developmental angiogenesis, we analyzed the effect of blocking miR-1 and miR- 206 function on the vasculature during zebrafish embryogenesis. Embryos in which miR-1/206 was inhibited display larger intersegmental vessels (ISVs), accompanied by a significant increase in the number of endothelial cells. Consistent with the expression of miR-1/206 in the muscle, mosaic analysis supports a non-cell-autonomous effect of miR-1/206 in the vasculature. These results support a role for miR-1/206 in the regulation of a pro-angiogenic factor that is non-autonomous to endothelial cells.

Next, we analyzed the 3'UTRs of angiogenic genes for putative miR-1/206 target sites. We found that zebrafish *vegfaa* contains three miR-1/206 target sites in its 3'UTR. To determine whether miR-1/206 regulate the 3'UTR of *vegfaa*, we analyzed the expression of a firefly luciferase reporter

containing the *vegfaa* 3'UTR, relative to a *Renilla* luciferase control, in the presence or absence of miR-1/206 duplex. The luciferase activity of the wild-type *vegfaa* reporter was significantly repressed by miR-1/206, and the regulation of the *vegfaa* reporter depended on the presence of miR-1/206 target sites. Furthermore, we directly visualized the levels of endogenous *vegfaa* mRNA by in situ hybridization. We found that *vegfaa* levels in the somites were upregulated in embryos in which miR-1/206 was inhibited, consistent with the loss of a negative regulatory factor. Together, these results indicate that miR-1/206 can regulate *vegfaa* expression during development. We reasoned that if the increase in angiogenesis observed in miR-1/206 inhibition is due to VegfAa misregulation, reducing the levels of VegfAa signaling would rescue this phenotype. To test this possibility, we first employed SU5416, a selective inhibitor of the VEGF receptor Kdrl (also known as VEGFR2, Flk1), to block Vegf signaling (2). We found that increasing concentrations of SU5416 rescued the angiogenesis phenotype of miR-1/206 inhibition. Second, reducing the levels of VegfAa with a translation-blocking antisense oligo nucleotides rescued the miR-1/206 KD vascular phenotype. Together, these results indicate that the phenotype observed when blocking miR-1/206 function is dependent on VegfAa activity, consistent with the proposed role of miR-1/206 in the regulation of *vegfaa* during angiogenesis.

miRNAs are widespread regulators of gene expression, and it is predicted that each miRNA has the potential to regulate several hundred target mRNAs (3). To directly address the interaction of miR-1/206 with *vegfaa* in vivo, we employed target protectors (TPs), which are antisense oligo nucleotides complementary to the miRNA target sites in a 3'UTR(4). Indeed, protection of the miR-1/206 target sites in *vegfaa* had a pro-angiogenic effect, resulting in ISV with elevated numbers of endothelial cells compared with controls. These results indicate (1) that despite the large number of miR-1/206 targets, the miR-1/206 inhibition phenotype is at least in part a result of altered *vegfaa* regulation and (2) that miR-1/206 directly regulate the levels of *vegfaa* to modulate angiogenesis during development.

Discussion & Conclusion

Our study shows that miR-1/206 negatively regulate angiogenesis during zebrafish development. Because miRNAs have hundreds of putative targets, it is challenging to determine the physiological function of individual miRNA-target interactions. Our findings identify a novel regulatory layer that modulates the cross-talk between muscle and vasculature during developmental angiogenesis.

VegfA plays a central role in the stimulation and control of angiogenesis, requiring it be tightly regulated during embryonic development and homeostasis. However, less is known about the regulatory systems that control Vegf production during development. Our findings indicate that *vegfaa* is targeted by miR-1/206 in the muscle to modulate the expression of the ligand, allowing the organism to regulate this signaling pathway.

The identification of miR-1/206-mediated regulation of Vegf might be significant beyond developmental angiogenesis. As in developmental angiogenesis, VEGF signaling is an essential

component of the angiogenic response of a wide variety of tumors, making it a prime clinical target for the development of therapeutic agents. Future studies will be required to understand whether the regulation of VEGF by miRNAs plays an important role in angiogenesis during human disease and cancer.

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一般の皆様へ

高度に複雑化した器官の形作りには、細胞同志がコミュニケーションを取り合うことが重要です。今回私たちは複雑に入り組んだ血管ネットワークの構築に着目し、小型淡水魚ゼブラフィッシュを用いてその制御機構を解析しました。その結果、マイクロ RNA と呼ばれる小さな RNA 分子の一つである miR-1/206 が、骨格筋から発せられる血管内皮細胞増殖因子 A（VegfA）の量を調節する役割をはたしていることを明らかにしました。この知見は基礎生物学のみならず、受傷後の血管網の再構築やがん細胞による血管誘導現象の解明などの医学分野にも波及効果が期待されます。

The role of molecular motor for regulating T cell activation

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Abstract

When T cells are activated, T cell receptors (TCRs) make clusters and move toward center of the T cell-antigen presenting cell (APC) interface. In this study, I tried to reveal the mechanism of TCR clustering and movement by protein identification and fluorescence imaging, and found very unique new structure for T cell receptor clustering. The structure contains general cytoskeletal-regulating molecules, adhesion molecules and TCR signaling molecules. Analyzing this structure will explain how the TCR clustering and movement analogously regulate TCR signaling.

Key Words : T cell receptor, cell signaling, dynein, cytoskeleton

Introduction

When T cells recognize a peptide- major histocompatibility complex on APC, dozens of T cell receptor microclusters (TCR-MCs), which contains several tens of receptors, kinases and adaptors, are generated and start T cell activation. TCR-MCs move to the center of the T cell-APC interface to be endocytosed and terminate activation. We have demonstrated that the TCR-MCs movement depends on dynein motor and microtubules, and the movement negatively regulates T cell activation. However, we have not revealed molecular mechanism of TCR clustering and movement.

Results

To reveal the molecular mechanism of TCR clustering and movement, I used immuno-precipitaion and protein identification by mass spectroscopy.

Firstly, to know how TCR are connected to dynein motor, I planned double precipitation of tagged-TCR and tagged-dynein. Because It has already known that TCR recruit so many molecules, so I wanted to concentrate the molecules between TCR and dynein by secondary precipitation by dynein. I used His-tag, FLAG-tag, GFP-tag for TCR component CD3ε chain, CD3ζ chain or dynein light chain. However unfortunately, I failed in double precipitation because every tag had defects in expression, adsorption or elution.

Next I performed simple dynein light chain (LC-8) -precipitation. GFP-tagged dynein light chain expressed in T cell hybridoma. The cells were activated by antigen presenting cells or kept resting on ice and lysed with 1% triton-100 solutions and precipitated by GFP antibody labeled with magnet. The precipitants were examined on two-dimensional protein gel electrophoresis. Comparing the silver protein staining patterns with or without T cell activation, I fortunately found several spots that were detected only on T cell activated sample. Next I identified the spots by mass spectroscopy

of out-sourcing service.

The identified molecules were very exciting. Although I cannot declare the molecular names at this moment, I will report them shortly in my next paper. I confirmed that some of the molecules were also co-precipitated with TCR component CD3ε chain. I further confirmed the localization of the molecules by using lipid bilayer system. Fortunately, the molecules formed clusters at TCR-MCs and moved together toward center.

On the other hand, I analyzed TCR and dynein at lower antigen doses. Because, when the stimulation was weak, not only the size and number but also the movement of TCR-MC were different, so I thought the mechanism of TCR-MCs movement is different. I checked the association and co-localization of TCR-MCs and dynein complexes. T cells were activated by antigen presenting cells loaded 10nM or 1μM antigen peptide and lysed for anti-CD3ε precipitation. I confirmed co-precipitation of dynein complex proteins in each dose of stimulation. On the planer bilayer, co-localization of TCR-MCs and dynein complexes were observed. Therefore even with weak stimulation, dynein was recruited to TCR-MCs. I also checked the association under microtubule-inhibitors, which inhibit tubulin polymerization and disappears tubular structures. The co-precipitation and co-localization still observed under treatment of microtubule-inhibitors. These results suggested the association of the dynein complex and the TCR complex is independent of microtubules.

I examined some molecules important for TCR signaling under weak stimulation. I wanted to know the behavior of signaling molecules. Famous essential kinase ZAP70, which binds to TCR component CD3ζ chain, makes co-localizing clusters at TCR-MCs, and the number and movement of ZAP70 clusters were quite similar to TCR-MC. The recruitment and clustering of ZAP70 will be totally depends on TCR-mediated signals and the movement will depend on dynein. On the other hand, the adaptor protein SLP76, which is essential for T cell activation, induces clusters independently of TCR-MCs, particularly by weak stimulation. Further, they seemed to be dissociated from TCR-MCs on the way to leach to cSMAC. Therefore, the regulation of SLP76 clustering and movement appears to be different from TCR-MCs and ZAP70.

Interestingly, I recently found that the SLP76 clusters at lower dose accompany the molecules identified as T cell activation-dependently dynein-precipitated protein.

Discussion & Conclusion

The molecules identified as T cell activation-dependently dynein binding protein in T cell were very famous proteins. I think that because they have so well known functions nobody have analyzed their functions on TCR-MCs. In addition, I found they were recruited at SLP76 clusters under weak stimulation. The SLP76 cluster seems to be independent from TCR clusters. I am thinking about the possibility that it is a structure to evoke antigen-independent or self-recognizing T cell activation. Hereafter, I will show the composition of the SLP76 and identified proteins clusters and relationship between dynein complex. I will finally clarify the biological significance of the clusters.

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一般の皆様へ

本研究では、ワクチンによる免疫記憶形成などを司るTリンパ球が、細胞表面に発現した受容体を介して、体内に侵入した外敵を識別する仕組みを解析しています。蛍光顕微鏡を用いた画像解析で、反応した受容体の集合体がダイナミックに動いて免疫反応の規模を調節する様子を追いながら、その動きに影響を与える分子や因子を特定しています。本研究の結果には、リンパ球が自己に対して免疫反応を開始してしまう自己免疫疾患の発生機序の解明などが期待されます。

Generation of Batf reporter/conditional knock-out mice for elucidation of Batf function during in vivo immune responses

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Abstract

We have newly generated Batf conditional knock out mice which allow us to analyze cell-type specific role of Batf. The mice have GFP reporter which is inserted into floxed allele such that we were able to look at expression of BATF-GFP fusion protein in activated T cells and B cells. By using this new mouse strain, we are going to investigate the function of Batf in memory T or B cells.
Key Words : T-cell, B-cell, transcription factor, BATF

Introduction

We have previously discovered the transcription factor BATF as a crucial factor for in vivo antibody response. BATF is essential for not only B cell class-switching but also development of follicular helper T cells which are the helper T cell subsets required for germinal center formation. Given the multi-function of BATF in lymphocytes, BATF conditional knock-out mice were required to elucidate cell-type specific function of BATF in vivo.

Results

We generated Batf floxed/GFP Knock-In mice. In these mice, exon 3 of Batf gene is flanked by loxp sequences. Also, GFP is inserted into the stop codon in exon3, so that BATF-GFP fusion protein should be expressed from the mutated allele. First, we looked at GFP expression in activated T cells or B cells in vitro. Naïve T cells or B cells did not express GFP. However, upon stimulation with anti-CD3, purified CD4 or CD8 T cells expressed GFP. Similarly, purified B cells became GFP positive after culture with LPS or anti-IgM. GFP expression was nicely correlated with Batf mRNA expression, suggesting that GFP knock-In strategy worked well. Next we looked at GFP expression in T or B cells in vivo after immunization with soluble protein antigens. At 7 days after immunization, robust BATF-GFP expression was observed in follicular helper T cells, which are $CXCR5^{hi}PD1^{hi}$ cells. $CXCR5^{int}$ T cells showed modest levels of BATF-GFP. After 1 month, survived memory T cells expressed BATF-GFP at low level. By contrast, BATF-GFP expression in B cells at any time points was quite low. At day7, where most of the antigen-specific B cells showed germinal center B cell phenotype, BATF-GFP expression was neglectable. Memory B cells did not express BATF-GFP either. It might be possible that pattern of BATF expression was different between T cell and B cells. In T cells, it appeared that BATF was kept expressed from effector to memory response. In contrast, BATF expression might be very transient in B cells, which made detection of BATF-

GFP in B cells difficult.

We have crossed Batf floxed/reporter mice with several Cre-expressing strains, such as CD4-cre, Mb1-cre, or ERT2-cre. We are now able to analyze T- or B cell specific function of BATF in vivo. Also, we are going to delete BATF in inducible manner at given time point to look at the function of BATF in memory T cell survival or maintenance.

Discussion & Conclusion

Having established Batf reporter/conditional knock-out mice, we are now able to track BATF-GFP expression in T-cell or B cells in vivo during immune responses. Notably, analysis of BATF-GFP expression in vivo suggested the difference in regulation of BATF expression between T cells and B cells. BATF was thought to function in effector T cells. However, our analysis clearly showed that memory T cells also expressed BATF, suggesting the possibility that BATF regulates survival or function of memory T cells. We are going to examine the role of BATF in memory T cells using Batf fl/fl x ERT2-cre mice.

一般の皆様へ

転写因子 BATF は抗体産生応答を制御する重要な因子である。本研究により BATF が抗体産生応答に加えて、記憶免疫応答をも制御する可能性が示唆された。したがって BATF の機能を研究し、人為的にその機能を制御することでより良いワクチン開発の指針が得られる可能性がある。

A study on the molecular mechanisms of mitochondrial regulation by the genes responsible for early-onset familial forms of Parkinson's disease

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Abstract

Mitochondrial quality control is an important issue in higher animals that depend on aerobic respiration. A series of elegant studies has demonstrated that two Parkinson's disease (PD)-associated genes, *PINK1* and *parkin*, are involved in the maintenance of healthy mitochondria. Parkin, in cooperation with PINK1, specifically recognizes damaged mitochondria with reduced mitochondrial membrane potential ($\Delta\psi m$), rapidly isolates these mitochondria from the mitochondrial network and eliminates these organelles through the ubiquitin-proteasome and autophagy pathways. Our studies, combined with *Drosophila* genetics and cell biology, contribute to an understanding of the molecular mechanisms of PINK1- and Parkin-mediated mitochondrial regulation and the pathological mechanisms explaining how defects in the PINK1-Parkin pathway lead to neurodegeneration in PD.

Key Words : Parkinson's disease, ubiquitin ligase, kinase

Introduction

Mitochondrial dysregulation is now implicated in various human diseases, including cancer, diabetes, myopathy and neurodegeneration. The pathological hallmark of the second most common neurodegenerative disorder, Parkinson's disease (PD), is the progressive degeneration of dopaminergic neurons in the midbrain. Although mitochondrial dysfunction has long been believed to be closely associated with the etiology of PD, there was no direct evidence for the association. A series of studies on two PD-associated genes, *PINK1* and *parkin*, has now shown that these two genes work in a coordinated manner in mitochondrial maintenance, including the mitophagy of damaged mitochondria. This finding strongly implies that the dysregulation of mitochondria is one of the major causative or contributing factors in the etiology of PD.

Results

The loss of PINK1 or Parkin in *Drosophila* causes mitochondrial degeneration in the thorax muscles, which results in abnormal wing posture. Using this wing phenotype, we screened modifier genes for the PINK1/Parkin phenotype, which revealed two pathways involved in PINK1-Parkin signaling: namely, a pathway for mitochondrial motility and the target of rapamycin complex 2

(TORC2)- nuclear Dbf2-related (NDR1) pathway.

Miro is an outer mitochondrial protein that binds to microtubule-associated transport complexes, such as the kinesin motor in conjunction with Milton. Knockdown of Miro alleviated the mitochondrial degeneration caused by a loss of PINK1. We found that Miro is one of the substrates of the Parkin ubiquitin ligase in mitophagy and that Miro's ubiquitination and subsequent degradation by Parkin in damaged mitochondria is an important process that prevents damaged mitochondria from moving to the axonal terminus (ref. 3). Nearly simultaneously, Wang *et al.* reported that Miro directly phosphorylated at Ser156 by PINK1 upon mitophagy is recognized by Parkin as a degradation substrate (Wang *et al.*, Cell 2011). However, we disproved this idea, reporting that Miro is not directly phosphorylated by PINK1 *in vitro* and that the Miro Ser156Ala phospho-mutant is equally degraded by Parkin *in vivo.*

TORC2, which is a highly conserved kinase complex, is known to regulate actin cytoskeleton signaling. We found that the genes that encode TORC2 components, such as rictor and sin1, improve the PINK1 wing phenotype. Further genetic interaction studies revealed that Tricornered/NDR, a kinase downstream of TORC2, behaves as a regulator of the downstream PINK1 pathway, likely independently of Parkin (ref. 1).

Parkin translocates to the mitochondria upon the loss of mitochondrial membrane potential ($\Delta\Psi m$) in a PINK1 kinase activity-dependent manner. However, little is known about how PINK1 activation recruits Parkin to damaged mitochondria. We attempted to monitor and compare the phosphorylation status of Parkin in wild-type and *PINK1*-deficient cells, thereby excluding the possibility of phosphorylation by kinases other than PINK1. We found that Parkin is phosphorylated at Ser65 of its ubiquitin-like (Ubl) domain in a PINK1-dependent manner. Furthermore, we showed that this phosphorylation event is implicated in the regulation of the mitochondrial translocation of Parkin and the subsequent degradation of mitochondrial surface proteins during mitophagy (ref. 2). However, Parkin mutants lacking mitochondrial translocation activity were also phosphorylated upon PINK1 activation, suggesting that Ser65 phosphorylation is not sufficient for the mitochondrial translocation of Parkin and raising the possibility that the presence of another cytosolic kinase(s) regulated by PINK1 (Fig 1).

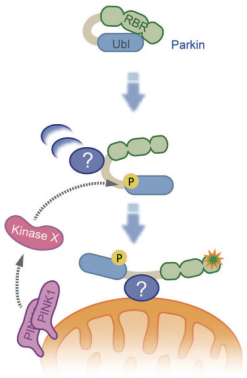

Fig. 1 Model for Parkin translocation and activation. The Parkin Ubl domain masks C-terminal RING-IBR-RING (RBR) domains for E3 activity. A Parkin phosphorylation event at Ser65 (P), combined with unknown factor(s) (?), stimulates the mitochondrial translocation of Parkin, releasing the RBR domains from autoinhibition by the Ubl domain.

Discussion & Conclusions

Two gene products implicated in early-onset PD, PINK1 and Parkin, have recently been implicated in mitochondrial quality control. PINK1- and Parkin-mediated mitophagy includes two major components: commitment to elimination by PINK1 and Parkin and the execution of elimination by autophagy. Although canonical autophagy components appear to be involved in the autophagy stage, at least under experimental conditions, the molecular mechanisms by which PINK1 and Parkin signal

the commitment to elimination remain largely unclear. This concept is an important issue, and our future studies may lead to the identification of new molecules associated with neurodegenerative disorders and an understanding of the etiology of idiopathic PD and *PINK1*- and *parkin*-linked PD.

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一般の皆様へ

本研究は、パーキンソン病原因遺伝子の機能を解明し、パーキンソン病の予防や治療に貢献することを究極の目的としています。若年性パーキンソン病の原因遺伝子 Parkin と PINK1 は、細胞活動に必要なエネルギーを産生するミトコンドリアの品質管理に関与することが明らかになってきました。ミトコンドリアは細胞にとって必須の器官でありながら、一方でエネルギー産生時に加齢や老化につながる活性酸素種を発生させることから、適切な監視下で働かせることが必要です。この監視（品質管理）が Parkin と PINK1 の役割であることが、私たちを含む多くの研究で分かってきました。

Fundamental study for long intergenic RNA (linc RNA) and epigenetic regulation by tumor suppressor p53 using ES/iPS cells to control cancer stem cells

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Abstract

The tumor suppressor p53 has been shown to control energy balance and metabolic homeostasis, concerning its role in Warburg effect and cancer stemness. In this context, we have attempted to isolate and characterize p53 chromatin complexes, and to perform genome-wide screening of RNA-seq and ChIP-seq using tumor and ES/iPS cells. We found that p53 exerts multiple functions with a complexity of crosstalk to intra-nuclear structure and transcriptional regulation for a wide variety of transcripts including linc RNAs in senescent and ES/iPS cells, linking to the molecular mechanism of cancer stemm cells.

Key Words : Tumor suppressor p53, non-coding RNA, epigenetics, stem cell

Introduction

Recent several lines of evidences have indicated that aging process is subjected to regulatory network and the nuclear reprogramming to generate induced pluripotent stemm cells can be achieved by introducing certain transcriptional factors, OCT3/4, SOX2, KLF4 and Myc so-called “Yamanaka f factors”. It is now well appreciated that these include the nutrient-sencing pathways (such as insulin and AKT/mTOR signals), and transcriptional and chromatin regulations (Sirtuins etc) with profound consequences. Importantly as well, it is also evident that key molecules including tumor suppressor p53 that regulate cellular senescence and reprogramming process are critically involved in the pathogenesis of cancer, cardiovascular disease and metabolic disorders, a testament to importance and crosstalk of transcriptional and epigenetic regulators in the four inevitables in our life. Here, to approach this molecular mechanism, we have performed to isolate and characterize the p53 chromatin complexes using multifaceted biochemical methodologies coupling the ChIP assay, size-fractionation by sucrose gradient ultracentrifugation and LC-MS/MS analysis and to clarify the gene expression profile including long intergenic RNA (linc RNA) and epigenetic status using RNA-sequence and ChIP-sequence in cancer cells and ES/iPS cells.

Results

Tumor suppressor p53 receives multiple forms and diverse range of stress signals such as DNA damage, oxidative or metabolic stress, and then initiates different cellular outcomes including cell-cycle arrest, apoptosis, and/or cellular senescence. Recent several lines of evidence have

suggested that p53 pathway is linked to reprogramming process by pluripotent factors and oncogenic signals and p53 systematically regulates intracellular metabolic pathways to preserve anti-oxidant and bioenergetic function. To elucidate the p53 mechanisms, we have attempted to isolate and characterize p53 chromatin complexes *in vivo* by manipulating biochemical techniques and mass-spectrometry, and to perform genome-wide screening of RNA-seq and ChIP-seq using tumor and non-tumor cells including senescent cells and ES/iPS cells. We found several chromatin regulators and transcriptional coactivators such as CAS/CSE1 (chromosome segregation factor), Sp110 (component of PML nuclear body protein forming a multi-protein complex) and zinc finger proteins in association with p53 chromatin complexes. Given that they are components of intra-nuclear structures and have functional domain such as bromodomain and PHD zinc finger motif that potentially function as "reader" for histone codes in epigenetic and chromatin-mediated transcriptional regulation, p53 is bona-fide a epigenetic regulators to be "the guardian of the genome" and "the cellular gatekeeper". Further, genome-wide analyses revealed that p53 can actively and/or repressively control cell cycle regulators, metabolic regulators, senescence associated secretary proteins (SASP) and linc RNAs in cancer, senescent and ES/iPS cells. Indeed, ChIP-sequence and RNA-sequencing revealed that p53 controls glutamine metabolism through GLS2, a key enzyme to produce glutamate, and thereby a regulator of glutathione synthesis and energy production via α–ketoglutarate in cancer and iPS/ES cells. The expression of GLS2, mainly localized in mitochondria, was induced in response to DNA damage in tumor and non-tumor cells. Biochemical and metabolomics analysis revealed that p53-GLS2 regulates intracellular metabolic pathways to control ROS and energy supply through glutamine metabolism. Consistently, *in vitro and vivo study* including mouse xenograft model displayed that GLS2 overexpression inhibited the tumor formation as well as cancer cell invasion via glutamate-dependent GSH synthetic pathway. Further, immunohistological analysis displayed that the GLS2 expression significantly increased in the liver of obesity or NASH patients, whereas it decreased in HCC. Importantly as well, GEO database analysis showed that GLS2 expression is correlated to the prognosis of breast cancer and HCC patients, indicating its potential role for tumor suppression. When we examined what kind of intracellular signaling or upstream pathway are significantly regulated in ES/iPS cells using KEGG pathway analysis, we found that pluripotent markers such as OCT4, NANOG, KLF2 and SOX2, oncogenic genes such as c-Myc and n-Myc, cell proliferation related genes such as E2F1 are significantly upregulated as expectedly. Interestingly as well, master regulators such as SREBP1 and 2 or repressor for cellular senescence are elevated. In addition, we found that mitochondria of ES/iPS cells are immature and TCA cycles are suppressed strongly, suggesting that ES/iPS cells are like as cancer cells in terms of the aberrant glucose metabolism known as "Warburg effect". However, ES/iPS cells are quite different from cancer cells in ROS regulation, in which ES/iPS cells control the low levels of ROS to maintain the pluripotency and stemmness through p53-GLS2 system. Further, we found the ES/iPS or senescence cell specific linc RNAs are regulated in both p53-dependent and –independent manner, possibly linking to the epigenetic gene profile.

Discussion & Conclusion

Thus, our study suggests that p53 exerts multiple functions with a complexity of crosstalk to intra-nuclear structure and transcriptional regulation for a wide variety of transcripts including linc RNAs in senescent and ES/iPS cells, linking to not only the molecular mechanism of cancer stemm cells, but also life-style related disease and age-related disease such as diabetes and cardiovascular disorders.

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一般の皆様へ

発がんの鍵を握る「がん幹細胞」という概念が注目を集めたのは、京都大学の山中伸弥博士がiPS 細胞の作製に成功した時期にほぼ近い。がんを治療しても再発や転移を起こしたり、抗がん剤が効かなくなるといった治療抵抗性の原因は、がん幹細胞にあるという概念である。がん幹細胞のメカニズムを遺伝子レベルで解明しようにも数が少なくて難しいことから、がん幹細胞に近い性質をもつ ES/iPS 細胞を用いて共通性と非共通性のメカニズムや分子機構を明らかにすることで、がん幹細胞研究に応用できると着想したのが本研究の目的です。最先端技術を用いた解析から、「ゲノムの守護神」として知られる癌抑制遺伝子 p53 の研究に基づいて、ES/iPS 細胞とがん幹細胞に共通したエピゲノム分子基盤及びがん化メカニズムの一旦が明らかとなり、将来的ながん幹細胞標的治療に応用することが期待される。

Drugs discovery for CKD patients using transporter manipulation

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Abstract

The promoter region of SLCO4C1 gene has several GATA motifs, and indoxyl sulfate up-regulated GATA3 mRNA and subsequently down-regulated SLCO4C1 mRNA. Overexpression of GATA3 significantly reduced SLCO4C1 expression, and silencing of GATA3 increased SLCO4C1 expression vice versa. Administration of indoxyl sulfate in rats reduced renal expression of slco4c1 and under this condition, plasma level of guanidinosuccinate, one of the preferable substrates of slco4c1, was significantly increased without changing plasma creatinine. Furthermore, in 5/6 nephrectomized rats, treatment with oral adsorbent AST-120 significantly decreased plasma indoxyl sulfate level and conversely increased the expression of slco4c1, following the reduction of plasma level of guanidinosuccinate. These data suggest that the removal of indoxyl sulfate and blocking its signal pathway may help to restore the SLCO4C1-mediated renal excretion of uremic toxins in CKD.

Key Words : SLCO4C1, transporter, GATA, indoxyl sulfate

Introduction

Chronic kidney disease (CKD) is a global health problem that carries a substantial risk for cardiovascular morbidity and death. With the progression of CKD, various uremic toxins accumulate, subsequently causing renal damage and hypertension. Recently, we have revealed that human kidney-specific organic anion transporter SLCO4C1 excretes uremic toxins, and the up-regulation of SLCO4C1 resulted in the reduction of blood pressure and renal inflammation in a CKD model. However, the expression levels of SLCO4C1 is down-regulated in the renal failure. The down-regulation mechanism of SLCO4C1 in the renal failure has not been well elucidated.

Results

The accumulated uremic toxins inhibit the expression of various renal transporters and this inhibition further reduces renal function and subsequently causes the accumulation of uremic toxins. However, the precise mechanism has been poorly understood. Here we report that indoxyl sulfate, a uremic toxin, directly suppresses the renal-specific organic anion transporter SLCO4C1 expression through GATA transcription factor. The GATA3 mRNA was increased by indoxyl sulfate in contrast to SLCO4C1 mRNA. A specific GATA-inhibitor K-7174 completely canceled the inhibitory effects of indoxyl sulfate. In addition, over-expression of GATA3 significantly inhibited SLCO4C1 and knockdown of GATA3 increased SLCO4C1 conversely. In indoxyl sulfate-treated

rats, renal expression of slco4c1 was decreased. Under this condition, plasma guanidinosuccinate, which is a favorable substrate for slco4c1, was significantly increased without changing plasma creatinine. In 5/6 nephrectomized rats, treatment with oral absorbent AST-120 significantly decreased the plasma indoxyl sulfate level and conversely increased the renal expression of slco4c1, resulting the reduction of plasma guanidinosuccinate concentration.

Furthermore, indoxyl sulfate also suppressed erythropoietin production in erythropoietin producing cells through induction of GATA2 and GATA3.

These data suggest that indoxyl sulfate enhances the expression of GATA factors and causes the accumulation of uremic toxins and anemia in CKD status. Therefore, the removal of indoxyl sulfate may help to restore the SLCO4C1-mediated renal excretion of uremic toxins and anemia.

Discussion & Conclusion

It is suggested that IS plays a key role of the formation of the “vicious cycle” between the accumulation of uremic toxins and renal damage in CKD. Therefore, the removal of IS and blocking its signaling pathway should be an effective strategy to restore the SLCO4C1-mediated renal excretion of uremic toxins.

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一般の皆様へ

慢性腎臓病においてトランスポーターの発現や機能低下が腎不全を発症する契機本研究ではヒトの尿毒症物質を腎臓からくみ出す役割を果たすトランスポーターである SLCO4C1 の発現を増強することで尿毒症物質の排泄が促進されることを明らかにし新たな CKD 治療戦略となる可能性を報告した。

Molecular mechanism of semaphorin receptor activation implicated in axon-pathfinding

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Abstract

Plexin is the cell-surface receptor on neuron and binds with the axon guidance cue semaphorin to induce axon repulsion. We here established the overproduction system of the extracellular domain of plexin to analyze its three-dimensional structure.

Key Words : Axon Guidance, Cell-surface receptor, Structural Biology, X-ray Crystallography

Introduction

During development of the central nervous system, neurons extend elongated fibers, called axons, to form precise connections with their target neurons. Plexin is the cell-surface receptor on neuron and binds with the axon guidance cue termed as semaphorin to mediate the signal transduction inducing axon repulsion. To analyze the molecular mechanism by which plexin is activated by semaphorin, we here attempted to overproduce the extracellular domain of plexin for structure determination through X-ray crystallography.

Results

Most of the plexin family members contain ten modules, a sema domain, three Plexin-Semaphorin-Integrin (PSI) domains and six immunoglobulin-like domains, in the extracellular region. In this study, we first attempted to produce the extracellular domain of plexin as a soluble protein towards the structure determination at an atomic level. For that purpose, we designed several kinds of constructs of the extracellular domain.

Many of extracellular proteins and extracellular domains of membrane proteins are, in general, glycosylated and possess disulfide-bonds. Such proteins are difficult to produce in conventional overproduction systems using *E. coli.* In this study, we therefore utilized a mammalian expression system to produce recombinant protein of high quality. Specifically, we used HEK293S GnT1(-), a mutant cell line of HEK293 that is defective in glycosylation processing, as the expression host to reduce the glycosylation heterogeneity. It has been established that proteins produced from this cell line possess high-mannose-type *N*-glycosylations and are suitable for crystallization. In fact, we succeeded in determining several crystal structures of the extracellular proteins or the extracellular domains of cell-surface receptors that are associated with development of the central nervous system.

For stable expression, we transfected the HEK293S GnT1(-) cell with the plasmids encoding several

kinds of the partial fragments of the plexin extracellular domain. The transfectants were plated on 96-well plates and selected for resistance to antibiotics. The clone showing the highest secretion level was cultured in the high-density cell-culture system BelloCell® (CESCO). With this system, we obtained about 2L culture supernatant containing the protein of interest within two weeks.

Since we produced the proteins with a polyhistidine tag, we first apply the culture supernatant to the ion-chelated chromatography resin for purification. Subsequently, we treated the eluted protein with protease to cleave off the tag sequence. Furthermore, we purified the protein by using size-exclusion chromatography.

Results

Finally, we concentrated the eluate by ultrafiltration and used it for crystallization. With this procedure, we successfully purified milligram quantity of the target protein from about 1 L of the culture supernatant.

The initial crystallization screens were conducted using commercial screening kits. 0.1 μL of the protein solution and the reagents, respectively, were dispensed into 96-well plates using the robotic crystallization system HYDRA II Plus One (Matrix Technologies Corporation) and equilibrated against 80 μL of the reservoir at 20°C by the sitting-drop vapor-diffusion method. Optimization of the crystallization conditions was performed by the hanging-drop vapor-diffusion method using 24-well plate where 0.5 μL of the protein solutions and the reagents, respectively, were mixed and equilibrated against 500 μL of the reservoir. After we obtained crystals, we performed X-ray diffraction analysis at the synchrotron radiation facility Photon Factory (KEK, Tsukuba, Japan). For data collection, crystals were quickly soaked in the cryoprotectant and flash-frozen in liquid nitrogen. Data were collected with the CCD detector and processed with the program HKL2000. Since the resolution of X-ray diffraction was very low, we were not able to solve the crystal structure. We, however, could determine the crystallographic parameters such as the space group and cell constants. The crystallographic analysis suggested that the protein crystallized in this study is relatively flexible.

Discussion & Conclusion

We here established the overproduction systems for the extracellular domain of plexin. In addition, we succeeded in purifying and crystallizing one of the overproduced proteins. However, diffraction quality of the crystal was not sufficient and the structure determination has not been completed so far. Since the protein that was crystallized is heavily glycosylated, we will probably have to eliminate the glycosylations by treating with glycosidase or introducing point mutations to the consensus sequences, which will reduce the flexibility and surface entropy of the protein. Information about the entire structure of plexin and its mobility will contribute to understanding of the structural change of plexin during the activation process.

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一般の皆様へ

ヒトの体を構成する細胞の表面には、外界からのシグナルを受け取るための多種多様な受容体蛋白質が存在しています。我々は、そのような受容体の中でも、特に神経の発生において重要な役割を担っているプレキシンという受容体に興味を持ち、その立体的な形をX線結晶解析という手法で明らかにする研究に取り組んでいます。プレキシンがシグナルを受け取る時にどのような形をとっているかが明らかになれば、それを標的とした薬剤のデザインが可能になると期待されます。

Promoter-based super improvement of gene expression in an antibody production

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Abstract

Recombinant antibodies have become fundamental in biomedical research where they are used in numerous therapeutic and diagnostic applications. For this reason there is an increasing demand for an economical production system for antibodies in mammalian cells. In this study, we performed to enhance expression of a cargo antibody gene by modifying promoter. A newly designed-plasmid vector showed 10 to 100 fold higher expression than that attained by commercially available vectors. This vector certainly improved the antibody production in HEK293 cells. This innovative vector shows a great promise for productive application of recombinant antibodies.

Key Words : Plasmid, Gene expression, Antibody

Introduction

The production of recombinant antibodies for use in therapeutic and diagnostic applications, attracts growing interest in the development of techniques that allows ever increasing large-scale production of the antibodies. For an exploitation of the recombinant antibodies, the main problem is low level of expression in animal cell culture leading to high cost of antibody production. Of course, during the last decade several approaches have been attempted to optimize these *in vitro* processes with the aim of improving production yield. One of the main factors that limit the efficiency of recombinant antibody production is insufficient activity of promoters loaded on plasmid vectors. Therefore, in this study, we attempted to improve vector promoter with structural modification of the plasmid vector. The exploited vector brought significantly higher expression of the antibody used in this study than that attained by commercially available vectors.

Results

1. Design of vector: We developed a novel promoter system, designed CMViR-C. The CMViR is composed of the human cytomegarovirus early promoter (CMV), the same virus-derived intron A (i), and the R segmemnt with part of the U5 sequence of the long terminal repeat of human T-cell leukemia virus type 1 (R), and the connected promoter CMViR is inserted at 5' head of the inserted cDNA. On the other hand, C from the CMViR-C shows CMV enhancer (C), which is located to 3' end of the inserted cDNA-polyA sequence. The designed vector, named pCMViR-C, causes very strong expression for various kinds of cargo cDNAs in many different mammalian cell types. In this study, the designed plasmid was employed and tried to express recombinant antibody. Human

IgG antibody gene is composed of two subunits, Heavy chain (HC) and Light chain (LC). To force an expression simultaneously HC and LC genes, we used BiP IRES (internal ribosomal entry site) sequence as the best one among the examined IRES sequences. As an objective IgG antibody of this study, we used the HC and LC genes derived from the anti-myc tag IgG antibody (clone 9E10, ATCC) and inserted them to the designed vector under the BiP IRES-mediated connection.

2. Evaluation of the pCMViR-C vector for antibody expression in transient transfection: The vector was assessed for the expression of cargo antibody genes, HC and LC, in transient transfections using HEK293 cells. The vector brought around 10 fold higher expression than that attained by commercially available vectors loaded CMV and CAG promoter, respectively.

3. Evaluation of the pCMViR-C vector for antibody expression in stable transfection: The designed vector with antibody genes was then employed for stable transfection. In antibiotics-selected pools, the stable transformants in mixed culture unexpectedly gave lower expression for the antibody production than that induced by the transient transfection. Even after the single cell cloning, however, clonal productivities of the antibody in positive clones searched did not show any appreciable enhancing-improvement of the expression. These results suggest that the vector favors as circular structure for the high expression rather than the linear structure of the plasmid after the incorporation into chromosome.

4. Functional improvement of the pCMViR-C vector to stable expression: To make it better the plasmid for stable expression, EBNA1-OriP system derived from EBV-based episomal vector was further employed. The system induces sustained localization of the vector in the nucleus and replicates it with keeping circular structure in human cells. The newly designed vector, pCMViR-C-EBO, was assessed for the antibody expression in HEK293 cells, resulting in similar high expression in transient transfection as compared with the former pCMViR-C vector. In addition, the expression was kept even after the establishment of stable clones.

5. Function of the produced antibody: Finally, to determine whether the expressed anti-myc tag IgG retains the same binding ability as compared with a commercially available one (clone 9E10), a Western blot of serially diluted purified IgG expressed from HEK293 cell cultures was carried out against a reference protein, GFP fused with myc-epitope tag. We confirmed that a similar detection was possible for the antibody preparation, HEK293 expressed IgG, in comparison to the company-derived one.

Results

This study validated a newly designed vector, pCMViR-C, that robustly enhances various kinds of gene expression in mammalian cells. The advanced expression system should be a valuable tool for recombinant antibody production. We therefore studied whether this system could be also working on a large scale of antibody production. The antibody IgG gene is composed of two component genes, HC and LC, so that HC and LC genes were connected by BiP IRES sequence, which causes simultaneous expression of them in the same vector. The exploited construct actually

showed dramatically high expression of the individual two genes, finally leading to produce the matured recombinant IgG protein with high productivity in transiently transfected HEK293 cells. However, this vector was not suitable for making stable transformants by reason of yet unidentified mechanism, so that we attempted to improve the vector adapting stable expression. The decreased expression in stable transformants might be reasoned by vector structure, since the incorporated vector on chromosome exists as a linear structure. According to the idea, we employed EBV-based episomal self-replication system. The promoter-EBNA1 and OriP sequence units were inserted to pCMViR-C, eventually producing the improved vector, pCMViR-C-EBO. When utilized the vector for stable expression, the isolated stable clone did not show any appreciable change about the production as compared with the former vector in transient expression as well as long-term stable expression. From these results, we strongly indicate that the innovative construct allows great promise for economical production of the antibodies utilized in therapeutic and diagnostic applications.

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一般の皆様へ

近年、がんやリウマチの治療薬として抗体医薬の市場は急速に拡大している。抗体医薬のもっとも大きな課題は、製造コストが高いことにある。コストを下げるために、哺乳動物高発現ベクターの開発等が求められている。抗体医薬の抗体は、目的の抗体遺伝子搭載発現ベクターを導入した動物細胞を大量培養することにより製造されているが、従来の発現ベクターを用いた場合は、導入された抗体遺伝子が抗体を生産しない場合や、培養途中で生産しなくなってしまうことが多く、抗体を安定的に高効率で生産する動物細胞を取得するには多大な時間を要していた。代表者が独自に開発した当プラスミドベクターは、本研究成果より、上記問題を克服し、抗体医療における様々な抗体や生理活性タンパク質を大量調製するための哺乳動物産生系に新たな光明をもたらすことが期待された。

Analysis of a novel mechanism of tumorigenesis involving cMyc proto-oncogene product

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Abstract

Aberrant activation of Wnt/β-catenin signaling has been implicated in colon cancer formation (1). The proto-oncogene cMYC has been identified as a target of the Wnt/β-catenin pathway in colorectal cancer cells. Here we report the identification of novel Wnt/cMyc target long non-coding RNA (lncRNA), termed ncRNA-X. We also show that ncRNA-X is required for the tumorigenicity of colorectal cancer cells. These findings highlight a key role for ncRNA-X in cMyc-driven transformation.

Key Words : cMyc, Wnt signal, long non-coding RNA (lncRNA)

Introduction

cMyc is among the most commonly over-expressed genes in many types of human cancers. cMYC encodes a basic helix–loop–helix/leucine zipper (bHLH/LZ) transcription factor that regulates cell growth, cell proliferation, cell cycle, and apoptosis (2). Although deregulation of cMYC contributes to tumorigenesis, the mechanism of cMyc-mediated cell transformation remains elusive.

Results

1) Identification of Wnt/cMyc target lncRNA

To obtain new insights into the function of cMyc, we attempted to identify target genes of cMyc and to analyze the role of target genes in cMyc-driven tumorigenesis. In order to identify novel cMyc transactivated lncRNAs, high throughput RNA sequencing (RNA-seq) was performed on RNA from HT29 cells following knockdown of cMyc using a lentivirus expressing an shRNA against cMyc, and on RNA from cells infected with control lentivirus. We found that 120 lncRNAs showed a statistically significant down-regulation of their expression levels following knockdown of cMyc in HT29 cells. qRT-PCR analysis revealed that knockdown of β-catenin led to reduce the expression of 7 (out of 120) candidate target genes. Moreover, to explore that cMyc transactivates these genes directly, we performed chromatin immunoprecipitation (ChIP) assays on HT29 cells using anti-cMyc antibody. We detected cMyc binding to the promoter region of ncRNA-X, which contains consensus cMyc/MAX binding sites. Taken together, these results suggest that Wnt/cMyc signaling pathway regulates ncRNA-X expression.

2) Analysis of ncRNA-X expression level in colon cancer tissues

Quantitative analysis of ncRNA-X and cMyc expression in human colon cancerous and

corresponding non-cancerous tissues by real-time RT–PCR. ncRNA-X was highly expressed in most of the colorectal tumors examined, whereas it was expressed at low levels in most of the corresponding non-cancerous tissues. Furthermore, the expression of ncRNA-X is significantly correlated with the expression of cMyc and Axin2, which is a well-established general target gene of Wnt signaling. Thus, ncRNA-X expression is aberrantly enhanced in most human colorectal tumors.

3) ncRNA-X is required for the tumorigenesis of colorectal tumor cells

To clarify the importance of ncRNA-X in colorectal tumorigenesis, we infected colon cancer HT29 cells with a lentivirus expressing an shRNA against ncRNA-X. When the stably expressing cells were transplanted into nude mice, cell growth was significantly retarded compared to cells infected with control lentivirus. Furthermore, the suppressive effects of ncRNA-X knockdown on tumorigenesis were comparable to those of cMyc or β-catenin knockdown. In addition, knockdown of ncRNA-X in HT29 cells led to significant decrease in cell proliferation under adherent cell culture conditions. These results indicate that the Wnt/cMyc-ncRNA-X pathway may play an important role in colorectal tumorigenesis.

4) Identification of proteins that are associated with ncRNA-X

We next wanted to investigate the mechanism by which ncRNA-X facilitates tumor formation. Thus, we attempted to identify proteins that are associated with ncRNA-X by an RNA-pulldown experiment. We incubated in vitro-synthesized biotinylated ncRNA-X and antisense ncRNA-X transcripts (negative control) with whole cell lysates and isolated co-precipitated proteins with streptavidin beads. We resolved the RNA-associated proteins on a SDS-PAGE gel, cut out two bands specific to ncRNA-X, and are now subject them to mass spectrometry.

Discussion and Conclusion

It has been shown that cMYC is a target gene of Wnt/β-catenin signaling and plays important roles in tumor formation and progression. We have demonstrated here that Wnt/cMyc signaling pathway regulates ncRNA-X expression in colorectal tumor cells. Furthermore, we have shown that ncRNA-X is required for the tumorigenicity of colorectal cancer cells. In addition, we also found that ncRNA-X expression is aberrantly enhanced in most human colorectal tumors. Thus, the Wnt/cMyc-ncRNA-X pathway may be critical for colorectal tumorigenesis. We envision that the cMyc-ncRNA-X pathway could be a promising target for the therapy of colorectal tumors.

References

1. Clevers H and Nusse R: Wnt/β-catenin signaling and disease. *Cell*, **149**, 1192-205 (2012).
2. Meyer N and Penn LZ: Reflecting on 25 years with MYC. *Nat Rev Cancer*, **8**, 976-990 (2008).

一般の皆様へ

Wnt シグナル経路の異常亢進は大腸癌細胞に於いて最も一般的にみられる特徴です。Wnt シグナル経路の標的因子 c-Myc が癌発症に深く関与していることは種々の研究成果から十分に確立しているにも関わらず、c-Myc が介在する癌発症機構は良く分かっていません。さらに、長鎖 ncRNA に関する研究は始まったばかりであり、その生理機能が明らかになっているものは極僅かです。その為、c-Myc の標的因子として同定した長鎖 ncRNA を手掛かりとして、Wnt シグナル経路の異常亢進による大腸癌発症機構を分子レベルで明らかにしようとする本研究は、新たな創薬ターゲットの提示や癌の病理・病態の理解に貢献できるだけでなく、ncRNA の分野における新たな分子基盤の構築としても意義深いものであると考えています。

III.

Reports from the Recipients of Grants for International Meetings

The 8th Annual Conference of OOTR

1. Representative Person
   Masakazu Toi
   OOTR (Organisation for Oncology and Translational Research), President

2. Opening period and Place
   20th (Fri.) - 21st (Sat.) April, 2012
   The Westin Miyako Hotel, Kyoto

3. Number of participants / Number of participating countries and areas
   165 people, 22 countries, 5 areas

4. Total cost
   JPY16,600,000.-

5. Main use of subsidy
   Programme and Guidance Making expenses

6. Result and Impression
   OOTR the 8th Annual Conference concluded successfully. Important lectures were given by world experts in oncology using leading-edge innovative technologies to progress research into cancer prevention and treatment. Many current topics regarding cancer treatment were discussed leading to new ideas for future investigation and as targets for possible clinical trials. Attendees left feeling they had gained valuable insight into new methods of cancer treatment and their general impression was that the conference achieved and exceeded all their expectations.

7. Additional description
   We would like to take this opportunity to thank Novartis Foundation for their most generous support of OOTR the 8th Annual Conference. Without their generosity it would not have been possible to achieve such a great success.

The 22nd CDB Meeting "RNA Sciences in Cell and Developmental Biology II"

1. Representative Person
   Toshifumi Inada
   Professor, Tohoku University, Graduate School of Pharmaceutical Sciences

2. Opening period and Place
   Jun 11 (Mon) - 13 (Wed), 2012
   RIKEN Center for Developmental Biology, Hyogo, Japan

3. Number of participants / Number of participating countries and areas
   Number of participants: 149 (abroad 31 / domestic 118)
   Number of participating countries and areas: 12 countries (Japan, Taiwan, USA, Singapore, Australia, China, Canada, Austria, Spain, UK, France, Germany)

4. Total cost
   JPY 5,514,119.-

5. Main use of subsidy
   Printing of abstract books (200 copies)

6. Result and Impression
   The 22nd CDB Meeting "RNA Sciences in Cell and Developmental Biology II" brought together nearly 149 scientists in Japan to exchange ideas on how RNAs are transcribed, processed, localized, exported to cytoplasm, and subjected to quality control, and diseases caused by defects in mRNA processing. The conference was held in June 2011 in Kobe, and consisted of 1 keynote, 11 sessions with total of 30 oral presentations, including 18 invited speakers. In addition, 60 posters were presented and 12 of them were selected for short oral presentations. The three days conference in which 149 attendants representing 12 countries got together turned out to be very successful in terms of high quality in science as well as of deepness of interaction among young scientists and invited speakers. The conference has greatly contributed to an advance of RNA research both in Japan and the world.

7. Additional description
   The organizing committees greatly appreciate The Novartis Foundation for the support.

20th International Symposium on Molecular Cell Biology of Macrophages

1. Representative Person
   Shigeo Koyasu, D.Sc.
   Department of Microbiology and Immunology, Keio University School of Medicine

2. Opening period and Place
   June 15th and 16th, 2012
   Sanjo Conference Hall, The University of Tokyo, Tokyo

3. Number of participants / Number of participating countries and areas
   130 participants from 9 countries (Austria, Brazil, China, France, Holland, Japan, UK, USA)

4. Total cost
   JPY6,040,000.-

5. Main use of subsidy
   Invited speaker's airfare, the venue and a meeting program.

6. Result and Impression
   Forty papers (19 oral and 21 poster presentations) were presented and discussion was very active. In particular, a lot of unpublished data were presented in the session for innate lymphocytes, inducing very active discussions. Recent studies have revealed multiple innate lymphocyte subsets, which correspond to helper T cell subsets. Those include Th1 cytokine producing NK and NK-like cells, Th2 cytokine producing natural helper cells and Th17 cytokine producing lymphoid tissue inducer cells. The interaction between macrophages and these innate lymphocytes seems to play important roles in controlling macrophage activities.

7. Additional description
   Among posters presented by young researchers including graduate students and postdocs, three posters were chosen for the best poster awards.

Plant and Microbes Adaptations to the Cold 2012: Toward risk assessment and management of sustainable agriculture in the cool and cold regions

1. Representative Person
   Naoyuki Matsumoto (Hokkaido University)
   Ryozo Imai (NARO Hokkaido Agricultural Research Center)
   (Co-Chair)

2. Opening period and Place
   June 24-28，2012
   Conference Center, Hokkaido University (Sapporo, Japan)

3. Number of participants / Number of participating countries and areas
   102/14 (Japan, U.K., Sweden, Italy, Hungary, France, Canada, Iceland, Korea, Germany, U.S.A., Finland, Russia, Norway)

4. Total cost
   JPY5,370,000.-

5. Main use of subsidy
   Travel and accommodation expenses for foreign invited speakers

6. Result and Impression
   The conference was started by the Keynote lecture “Global change in winter climate and agricultural sustainability” followed by 7 scientific sessions of 41 oral presentations and 42 poster presentations, a special session for which a farmer from Canada and a representative of farmers’ association from Tokachi area in Hokkaido of Japan are invited as panelists to report the forefront of winter climate change, and finished by the wrap up discussion. Presentations indicated the rapid progress in researches on plant-microbe interactions under low temperature and the future direction: the trend of the understanding the cold acclimation of plants and microbes is moving into the “fine tuning” network of cold response genes from major cold regulated genes; molecular genetical approaches are becoming a powerful tool to accelerate breeding cold hardy and/or snow mold resistant crops by creating molecular markers and new breeding strategy; climate changes caused by global warming are requiring new strategies for sustainable agricultural production, such as the control of late frost damage induced by loss of winter hardiness ascribed to early spring. In general, discussions during formal and informal sessions were quite fruitful and helpful for future collaborative works.

7. Additional description
   The proceedings of the conference will appear as “Plant and Microbe Adaptations to Cold in Changing World” (Publisher: Springer, NY, USA) in June 2013.

The 18th Young Asian Biochemical Engineers' Community (YABEC)

1. Representative Person
   Takeshi Omasa
   Chairman, Organizing Committee of YABEC 2012
   Professor, The University of Tokushima
   Guest Processor, Osaka University

2. Opening period and Place
   From 26 th October 2012 to 28th October 2012 at the University of Tokushima, Tokushima, JAPAN

3. Number of participants / Number of participating countries and areas
   173 participants including 80 participants from China, Taiwan, and Korea. The participants' nationalities are Japanese, Chinese, Taiwanese, Korean, Vietnamese, and Indian.

4. Total cost
   JPY5,500,992.-

5. Main use of subsidy
   Travel and accommodation costs for participants from abroad

6. Result and Impression
   Since the first YABEC symposium was held in Seoul in 1995, this is the 18th symposium and this symposium series has developed into a key academic event among young Asian Biochemical Engineers. This symposium is 5th YABEC symposium in Japan and first symposium in local city. Tokushima city is located on eastside of Shikoku Island and delta area of Yoshino River. This YABEC 2012 is the second YABEC symposium which is held under the auspices of AFOB. This meeting was very successful with total 4 plenary lectures, 16 oral presentations and 135 poster presentations. Total 117 Ph.D scientists and 56 students attended. Most participants had very nice impression on the organization of the meeting. All participants also enjoyed food and culture, Awa-odori in Tokushima.

7. Additional description
   The particular session, "Biofun" was held at this meeting. The purpose of this session is to enjoy the biotechnology. The interesting presentation entitled "DNA and music", "Paintings and biotechnology" were presented in this section and all participants enjoyed these presentation.

The 8th 3R Symposium
(International Symposium on DNA Replication, Recombination and Repair)

1. Representative Person
   Kaoru Sugasawa
   Professor, Biosignal Research Center, Organization of Advanced Science and Technology, Kobe University

2. Opening period and Place
   November 25th (Sun) - 28th (Wed), 2012
   Awaji Yumebutai International Conference Center

3. Number of participants / Number of participating countries and areas
   184 participants / 16 countries and areas

4. Total cost
   JPY12,350,000.-

5. Main use of subsidy
   Printing - abstract books
   Meeting expenses - the equipment fee

6. Result and Impression
   The 3R Symposia have been aimed to discuss and better understand basic molecular mechanisms and biological relevance of DNA replication, recombination and repair (collectively called "3R"), which are fundamental functions for organisms to regulate stability and diversity of the genetic information.In this 8th Symposium, implications of the 3R in human diseases were a bit more consciously regarded than the previous ones, thereby subtitled: "Molecular mechanism and pathology of the 3R".
   As the leading scientists from the related fields, 19 speakers were invited from abroad and 20 from Japan.In addition, 18 speakers were selected from abstracts submitted to give oral presentation.In general, scientific quality of their talks was very high, stimulating active discussion throughout the meeting.In poster sessions, as many as 116 posters were presented, and it was quite impressive that many participants were absorbed in intense discussion, forgetting about the time.Overall, this meeting was filled with free and pleasant atmosphere, so that it was very successful.

7. Additional description
   Especially to encourage young postdocs and students, 3 or 4 posters were chosen for each session and the presenters were invited to short talks.Although this was a new attempt for this symposium, it was highly evaluated by many participants and thus significant from the viewpoint of nurturing young scientists.

26th Grant Report (FY2012)

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 2012 are as follows.

26th Novartis Research Grant:	40 Researchers (JPY 1 mil.),	Subtotal JPY 40 mil.
Research Meeting Grant:	6 Meetings (JPY 0.4 mil.),	Subtotal JPY 2.4 mil.
		Total JPY 42.4 mil.

26th Novartis Research Grant (FY2012)

The grant is to aim supporting creative research in Japan in the field of bio, life science and relevant chemistry. The 40 grantees are as follows.

#	Name	Institution	Title	Research Project
1	Rikinari Hanayama	Osaka University, Immunology Frontier Research Center	Associate Professor	Inflammation caused by secreted lysosomal enzymes
2	Satoru Yokoyama	Institute of Natural Medicine, Toyama University	Assistant Professor	RAC1 inhibitor is an anti-tumor metastasis drug?
3	Koichiro Nishino	University of Miyazaki, Veterinary Biochemistry and Molecular Biology	Associate Professor	Analysis of induction mechanism of human TERT gene expression in human pluripotent stem cells.
4	Atsushi Kuhara	Konan University, Faculty of Science and Engineering	Junior Associate Professor	Molecular mechanism of temperature response through secretory signaling
5	Kohichi Matsunaga	Institute for Molecular and Cellular Regulation, Gunma University	Assistant Professor	Role of Rab GTPases in the intracellular logistics of insulin granule
6	Shuji Yamashita	Tohoku University, Graduate School of Science	Assistant Professor	Chemical Biology Based on the Unnatural Steroidal Compounds
7	Yuji Hara	Tokyo Women's Medical University, Institute for Integrated Medical Sciences	Associate Professor	Molecular mechanisms underlying the membrane repair pathway in skeletal muscle
8	Akira Nakamura	RIKEN Center for Developmental Biology	Laboratory Head	Transcriptional quiescence for the establishment of germ cell fate in Drosophila
9	Shuh-ichi Nishikawa	Niigata University, Faculty of Science	Professor	Regulation of plasma membrane receptor-like kinase functions by endoplasmic reticulum quality control in plant cells
10	Katsuaki Hoshino	Faculty of Medicine, Kagawa University	Professor	Development of a novel therapeutic approach for the treatment of autoimmune diseases by antibodies against plasmacytoid dendritic cells.
11	Kyoko Hida	Hokkaido University, Graduate School of Dental Medicine	Associate Professor	Prevention tumor metastasis by targeting tumor endothelial cells
12	Yuko Seko	National Rehabilitation Center for Persons with Disabilities	Section Chief	In vitro derivation from human somatic cells to finally differentiated photoreceptors; generating complete disc structures of rods and cones
13	Mamoru Sugita	Nagoya University, Center for Gene Research	Professor	Identification of cytidine deaminase activity for elucidating the molecular mechanism of plant organelle RNA editing
14	Yutaro Obara	Yamagata University, School of Medicine	Associate Professor	Clarification of novel mechanism of catecholamine biosynthesis by ERK5
15	Shin-ichi Horike	Kanazawa University, Advanced Science Research Center	Associate Professor	Assessing the biological significance and function of neuron-specific imprinted genes in brain plasticity

#	Name	Institution	Title	Research Project
16	Masami Yamada	Osaka-City University, Graduate School of Medicine	Associate Professor	Intracellular logistics regulated by LIS1 and lissencephaly
17	Takatsune Shimizu	Faculty of Pharmaceutical Sciences, Hoshi University	Associate Professor	Elucidation of molecular functions of Imp3 in osteosarcoma progression, and development of novel anti-Imp3 targeted therapeutic approach
18	Mitsuhiro Arisawa	Hokkaido University, Faculty of Pharmaceutical Sciences	Associate Professor	Development of metal nanoparticles for organic synthesis aiming a substitution and reduction of rear metals
19	Richard Wong	Institute of Science and Engineering, Kanazawa University	Professor	Epigenetic regulation by nuclear pore complex in cancer progression.
20	Tomonao Inobe	Frontier Research Core for Life Sciences, University of Toyama	Assistant Professor	Artificial Regulation of Protein Degradation by the Proteasome
21	Hiroyuki Tezuka	Tokyo Medical and Dental University	Assistant Professor	Regulatory mechanism of mucosal healing by DNA methylation
22	Shin-ichiro Kitajiri	Kyoto University Hospital	Assistant Professor	Actin Cytoskeleton Structure Regulation by TRIOBP, a novel gene responsible to human deafness
23	Yuko Ozaki	Research Institute for Radiation Biology & Medicine, Hiroshima University	Postdoctoral Research Associate	Study of chromosome instability caused by the impairment of centrosome maturation
24	Hiroyasu Nakano	Juntendo University, Graduate School of Medicine	Associate Professor	Elucidation of the mechanisms by which impaired clearance of apoptotic cells results in severe inflammation
25	Hidenori Tabata	Institute for Developmental Research, Aichi Human Service Center	Section Head	Molecular mechanisms for the production and maintenance of proliferative cells in the subventricular zone
26	Kazuhide Asakawa	National Institute of Genetics, Department of Developmental Genetics	Assistant Professor	Role of the VIPLa-mediated glycoprotein trafficking in locomotion in zebrafish
27	Takayoshi Suzuki	Kyoto Prefectural University of Medicine, Department of Chemistry	Professor	Explorative study on lysine demethylase inhibitors by rational drug design
28	Akiko Taguchi	Faculty of Medicine, University of Miyazaki	Associate Professor	Molecular mechanism of aging-like effect by diabetes
29	Kiyotsugu Yoshida	Jikei University, School of Medicine	Professor	Comprehensive proteomicanalysis of molecules that are targeted for cancer prognosis or therapy
30	Hideki Nishitoh	Department of Medical Sciences, University of Miyazaki	Professor	Molecular mechanism of Derlin family-mediated novel endoplasmic reticulum quality control system
31	Ryosuke Matsubara	Kobe University, Graduate School of Science	Associate Professor	Development of NO donor drug based on furoxan structure
32	Hiroshi Watanabe	Niigata University, Medical and Dental Hospital	Assistant Professor	Development of Novel Genetic Screening Assay using Next-Generation Sequencer and Clinical Application of Personalized Medicine in Inherited Arrhythmia Syndromes
33	Hidemi Misawa	Keio University, Faculty of Pharmacy	Professor	Research on a novel neuroprotective/ anti-inflammatory prototoxin targeting to nicotinic acetylcholine receptor
34	Yutaka Kodama	Center for Bioscience Research and Education, Utsunomiya University	Assistant Professor	Development of an orange-colored bimolecular fluorescence complementation (BiFC) assay
35	Akira Kikuchi	Graduate School of Medicine, Osaka University	Professor	Regulation of secretion of Wnt proteins by post-translational modifications in polarized epithelial cells
36	Seijiro Hosokawa	Waseda University, Faculty of Science and Engineering	Associate Professor	Synthetic Studies on the virulent cell wall lipid of Mycobacterium tuberculosis
37	Mitsuru Kitamura	Kyushu Institute of Technology, Graduate School of Engineering	Associate Professor	Synthesis of anticancer anthracycline antibiotics having aza-spiro structure
38	Yasuharu Kanki	Research Center for Advanced Science and Technology, The University of Tokyo	Project Researcher	Determination about the essential factors in vascular endothelial cell differentiation

#	Name	Institution	Title	Research Project
39	Kazuaki Yoshioka	Kanazawa University, School of Medicine	Assistant Professor	Essential roles of class II PI3-kinase C2α in vascular formation and homeostasis.
40	Koichi Matsuo	School of Medicine, Keio University	Professor	Mechanisms of Eph family receptor-mediated regulation of bone mineralization

FY 2012 Research Meeting Grant

The grant is to aim supporting international research meetings in Japan in the field of bio, life science and relevant chemistry. The 6 grantees are as follows.

#	Meeting	Date (Place)	Representative	
			Institution / Title	Name
1	8th Annual Conference of OOTR (Organization for Oncology and Translational Research)	2012.4.20 ～ 21 (Kyoto)	(NPO) OOTR / President	Masakazu Toi
2	RNA Sciences in Cell and Developmental Biology 2012	2012.6.11 ～ 13 (Kobe)	Graduate School of Pharmaceutical Sciences, Tohoku University / Professor	Toshifumi Inada
3	20th International Symposium on Molecular Cell Biology of Macrophages	2012.6.15 ～ 16 (Tokyo)	Keio University School of Medicine / Professor	Shigeo Koyasu
4	Plant and Microbe Adaptation to the Cold 2012	2012.6.24 ～ 28 (Sapporo)	Hokkaido Agricultural Research Center / Research Director	Norio Iriki
5	The 18th Symposium of Young Asian Biochemical Engineers' Community	2012.10.26 ～ 28 (Tokushima)	Institute of Technology and Science, University of Tokushima / Professor	Takeshi Omasa
6	The 8th 3R Symposium (International Symposium on DNA Replication, Recombination and Repair)	2012.11.19 ～ 22 (Awaji)	Biosignal Research Center, Kobe University / Professor	Kaoru Sugasawa

第 26 期（2012 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。2012 年度は、下記の総額 4,240 万円の助成事業を実施しました。

第 26 回ノバルティス研究奨励金　40 件（1 件 100 万円）4,000 万円
研究集会助成　6 件（1 件 40 万円）　240 万円
総額 4,240 万円

第 26 回ノバルティス研究奨励金（2012 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。2012 年度は 40 件の助成を行いました。

（受付順・敬称略、所属・職位は申請時、贈呈額は 1 件 100 万円）

No	氏名	所属	職位	研究課題
1	華山　力成	大阪大学免疫学フロンティア研究センター	特任准教授	リソソーム酵素の放出による炎症の発症機構
2	横山　悟	富山大学和漢医薬学総合研究所	助教	Rac1 阻害剤は“抗がん転移剤”になりうるのか?
3	西野 光一郎	宮崎大学農学部	准教授	ヒト多能性幹細胞における TERT 遺伝子の発現獲得機構の解析
4	久原　篤	甲南大学理工学部	講師	内分泌シグナルを介した温度応答の分子機構
5	松永　耕一	群馬大学生体調節研究所	助教	複数の Rab GTPase によるインスリン顆粒の細胞内物流システム
6	山下　修治	東北大学大学院理学研究科	助教	非天然ステロイドを基盤とする有機分子ツールの創製と生体機能制御
7	原　雄二	東京女子医科大学統合医科学研究所	テニュアトラック准教授	骨格筋における形質膜修復機構の分子基盤
8	中村　輝	理化学研究所発生・再生科学総合研究センター	特別主管研究員	ショウジョウバエ生殖細胞形成におけるゲノムワイドな転写抑制とその意義
9	西川　周一	新潟大学理学部	教授	小胞体品質管理による植物細胞膜受容体様キナーゼの機能発現機構
10	星野　克明	香川大学医学部	教授	形質細胞様樹状細胞を標的とした自己免疫疾患制御手段の開発
11	樋田　京子	北海道大学大学院歯学研究科	特任准教授	腫瘍血管内皮細胞を標的としたがんの転移制御
12	世古　裕子	国立障害者リハビリテーションセンター研究所	研究室長	ヒト体細胞から網膜視細胞への in vitro における最終分化―外節のある杆体と錐体の作製―
13	杉田　護	名古屋大学遺伝子実験施設	教授	植物オルガネラの RNA 編集の分子機構解明に向けたシチジンデアミナーゼ活性の同定
14	小原 祐太郎	山形大学医学部	准教授	ERK5 による新しいカテコールアミン類の生合成促進機構の解明
15	堀家　慎一	金沢大学学際科学実験センター	准教授	高次な精神発達制御を司るゲノムインプリンティング遺伝子の生物学的意義と機能の解明

No	氏名	所属	職位	研究課題
16	山田　雅巳	大阪市立大学大学院 医学研究科	准教授	LIS1 による細胞内ロジスティクスと滑脳症発症の関係
17	清水　孝恒	星薬科大学薬学部	准教授	骨肉腫悪性化に関わる RNA 結合タンパク Imp3 の機能解明及び分子標的療法の開発
18	有澤　光弘	北海道大学大学院薬学研究院	准教授	希少元素代替・削減型有機合成用金属ナノパーティクル触媒の開発
19	ウォング リチャード	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	伊野部 智由	富山大学先端ライフサイエンス拠点	特命助教	プロテアソームによる蛋白質分解の人工制御
21	手塚　裕之	東京医科歯科大学難治疾患研究所	助教	DNA メチル化による粘膜治癒機構の解明
22	北尻 真一郎	京都大学医学部附属病院	助教	ヒト難聴から同定された新規分子 TRIOBP による、アクチン束化様式と聴覚制御
23	尾崎　佑子	広島大学原爆放射線医科学研究所	博士研究員	中心体成熟不全によるがん細胞の染色体不安定性惹起メカニズムの解明
24	中野　裕康	順天堂大学医学部	准教授	死細胞貪食破綻による炎症誘導のメカニズムの解明
25	田畑　秀典	愛知県心身障害者コロニー発達障害研究所	室長	脳室下帯分裂細胞の産生と維持の分子機構
26	浅川　和秀	大学共同利用機関法人情報・システム研究機構国立遺伝学研究所	助教	ロコモーションに必要な糖タンパク質輸送系の解明
27	鈴木　孝禎	京都府立医科大学大学院医学研究科	教授	合理的設計によるリシン脱メチル化酵素阻害薬の創製研究
28	田口　明子	宮崎大学医学部	准教授	糖尿病による老化類似作用の分子機構の解明
29	吉田　清嗣	東京慈恵会医科大学	教授	癌の診断・治療の標的となる分子同定を目指した網羅的プロテオーム解析
30	西頭　英起	宮崎大学医学部	教授	Derlin ファミリーを介した新規小胞体品質管理機構の解明
31	松原　亮介	神戸大学大学院理学研究科	准教授	フロキサン構造を有する NO ドナー医薬品の開発
32	渡部　裕	新潟大学医歯学総合病院	助教	次世代シークエンサーを応用した致死性不整脈症候群の新たな遺伝子検索アッセイの構築と個別化治療の確立
33	三澤 日出巳	慶應義塾大学薬学部	教授	ニコチン受容体をターゲットとする新たな神経保護・抗炎症プロトキシンの研究
34	児玉　豊	宇都宮大学バイオサイエンス教育研究センター	助教	橙色二分子蛍光補完法の開発
35	菊池　章	大阪大学大学院医学系研究科	教授	Wnt タンパク質の翻訳後修飾と極性化上皮細胞における分泌制御
36	細川 誠二郎	早稲田大学大学院先進理工学研究科	准教授	化学ワクチンを指向した結核菌の毒性細胞壁成分の合成研究
37	北村　充	九州工業大学大学院工学研究院	准教授	アザスピロ構造を有するアントラサイクリン系抗がん抗生物質の合成
38	神吉　康晴	東京大学先端科学技術研究センター	特任研究員	血管内皮細胞分化決定因子の同定
39	吉岡　和晃	金沢大学医薬保健研究域医学系	助教	クラスⅡ型 PI3 キナーゼ C2 αによる血管形成・健全性維持の機能解明
40	松尾　光一	慶應義塾大学医学部	教授	Eph ファミリー受容体による骨ミネラリゼーションの制御機構

2012 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。2012 年度は 6 件の助成を行いました。

（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 8 回 OOTR 年次学会	2012.4.20-21 （京都）	特定非営利活動法人 OOTR・理事長	戸井　雅和
2	細胞と分化生物学における RNA 研究会	2012.6.11-13 （神戸）	東北大学大学院 薬学研究科・教授	稲田　利文
3	第 20 回マクロファージ分子細胞生物学国際シンポジウム	2012.6.15-16 （東京）	慶應義塾大学 医学部・教授	小安　重夫
4	植物と微生物の低温適応 2012	2012.6.24-28 （札幌）	農業・食品産業技術総合研究機構 北海道農業研究センター・研究領域長	入来　規雄
5	第 18 回生物化学工学アジア若手研究者の集い YABEC2012	2012.10.26-28 （徳島）	徳島大学大学院 ソシオテクノサイエンス研究部・教授	大政　健史
6	第 8 回国際 3R シンポジウム	2012.11.19-22 （淡路）	神戸大学 バイオシグナル研究センター・教授	菅澤　薫

Balance Sheet

As of March 31, 2013

(Unit: JP Yen)

Account	Amount
I Assets	
1. Current Assets	
Total Current Assets	49,067,338
2. Fixed Assets (Long-term)	
(1) Basic Fund	
Basic Fund Total	1,100,000,000
(2) Other Long-term Assets	
Other Long-term Assets Total	59,861,958
Long-term Assets Total	1,159,861,958
Assets Total	1,208,929,296
II Liabilities	
1. Current Liabilities	
Current Liabilities Total	40,054,374
Liabilities Total	40,054,374
III Equity (Net Assets)	
1. Designated Net Assets	
Designated Net Assets Total	1,000,000,000
(Amount appropriating to Basic Fund)	(1,000,000,000)
2. General Net Assets	168,874,922
(Amount appropriating to Basic Fund)	(100,000,000)
Equity Total (Net Assets)	1,168,874,922
Liabilities & Equity Total	1,208,929,296

Movement of Net Assets

April 1st, 2012 - March 31, 2013

(Unit: JP Yen)

Account	Amount
I General Net Assets Changes	
1. Ordinary Income & Expenditure	
(1) Ordinary Income	
Interest from Basic Fund	20,583,328
Donation	40,050,000
Other Income	334,524
Ordinary Income Total	60,967,852
(2) Ordinary Expenditure	
Project Expenses	52,966,501
Grant Expense	42,400,000
Novartis Research Grant	40,000,000
Research Meeting Grant	2,400,000
Administrative Expense	4,351,506
Ordinary Expenditure Total	57,318,007
Ordinary Balance of Current Period	3,649,845
2. Nonrecurring Profit & Loss	
Nonrecurring Balance of Current Period	0
General Net Assets Balance of Current Period	3,649,845
General Net Assets Ending Balance	168,874,922
II Designated Net Assets Changes	
Designated Net Assets Change	0
Designated Net Assets Ending Balance	1,000,000,000
III Net Assets Balance Ending Balance	1,168,874,922

第 26 期（2012 年度）財務報告

貸 借 対 照 表

2013 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	49,067,338
2．固定資産	
(1) 基本財産	
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	59,861,958
固定資産合計	1,159,861,958
資産合計	1,208,929,296
Ⅱ 負債の部	
1．流動負債	
流動負債合計	40,054,374
負債合計	40,054,374
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	168,874,922
(うち基本財産への充当額)	(100,000,000)
正味財産合計	1,168,874,922
負債及び正味財産合計	1,208,929,296

正味財産増減計算書

2012 年 4 月 1 日～ 2013 年 3 月 31 日

（単位：円）

科　　目	決 算 額
Ⅰ 一般正味財産増減の部	
1．経常増減の部	
(1) 経常収益	
基本財産運用益	20,583,328
受取寄付金	40,050,000
雑収益	334,524
経常収益計	60,967,852
(2) 経常費用	
事業費	52,966,501
支払助成金	42,400,000
ノバルティス研究奨励金	40,000,000
研究集会助成金	2,400,000
管理費	4,351,506
経常費用計	57,318,007
当期経常増減額	3,649,845
2．経常外増減の部	
(1) 経常外収益	
経常外収益計	0
(2) 経常外費用	
経常外費用計	0
当期経常外増減額	0
当期一般正味財産増減額	3,649,845
一般正味財産期末残高	168,874,922
Ⅱ 指定正味財産増減の部	
当期指定正味財産増減額	0
指定正味財産期末残高	1,000,000,000
Ⅲ 正味財産期末残高	1,168,874,922

List of Board Members

[Board of Trustees] As of Sep. 1, 2013

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	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	Member of the Japan Academy, Emer. Prof., Univ. of Tokyo
Councilor	Norio AKAIKE	Director, Kumamoto Health Science Univ., Emer. Prof., Kyushu Univ.
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	Hiroyuki KAWASHIMA	Former Prof., Grad. School of Medical & Dental Sciences, Niigata Univ.
	Shigeo KOYASU	Acting Director, RIKEN Center for Integrative Medical Sciences
	Tadanori MAYUMI	Emer. Prof., Osaka Univ.
	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)
	Yoshiyasu NINOMIYA	President, Novartis Pharma K.K.
	Masakatsu SHIBASAKI	Director, Microbial Chemistry Research Center, Microbial Chemistry Research Foundation
	Takao SHIMIZU	Director-General, MD, Research Institute, National Center for Global Health and Medicine
	Toshifumi WATANABE	Corporate Officer, Senior Science Advisor, Novartis Pharma K.K.

[Grantee Selection Committee]

Post	Name	Title
Chairman	Hiroaki SASAI	Prof., Inst. of Scientific & Industrial Research, Osaka Univ.
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
	Shigeyuki KAWANO	Prof., Grad. School of Frontier Sciences, Univ. of Tokyo
	Takeo KISHIMOTO	Visiting Prof., Science & Education Center, Ochanomizu 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 Medicine, Univ. of Tokyo
	Hiroyuki TSUTSUI	Prof., MD, School of Medicine, Hokkaido Univ.
	Hiroyuki NAKAMURA	Prof., Faculty of Science, Gakushuin Univ.
	Junichi NABEKURA	Prof., MD, National Inst. for Physiological Sciences
	Masanori HATAKEYAMA	Prof., MD, Grad. School of Medicine, Univ. of Tokyo
	Tomoko BETSUYAKU	Prof., MD, School of Medicine, Keio Univ.
	Tetsuji MIURA	Prof., MD, Sapporo Medical Univ.
	Masabumi MINAMI	Prof., Grad. School of Pharmaceutical Sciences, Hokkaido Univ.
	Takashi MINEGISHI	Prof., MD, Grad. School of Medicine, Gunma Univ.
	Toyoaki MUROHARA	Prof., MD, Grad. School of Medicine, Nagoya Univ.
	Masato YASUI	Prof., MD, School of Medicine, Keio Univ.

公益財団法人ノバルティス科学振興財団

役 員 名 簿

2013 年 9 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長･教授 慶應義塾大学名誉教授	2012年4月1日	非常勤
理　　事	浅 野　茂 隆	神戸大学大学院医学系研究科客員教授 東京大学名誉教授	2012年4月1日	非常勤
	石 川　裕 子	ノバルティスホールディングジャパン(株)代表取締役社長、ノバルティス ファーマ(株)取締役副社長	2012年4月1日	非常勤
	遠 藤　政 夫	山形大学名誉教授	2012年4月1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	2012年4月1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

2013 年 9 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	日本学士院会員 東京大学名誉教授	2012年4月1日	非常勤
評 議 員	赤池 紀扶	熊本保健科学大学理事・研究顧問 九州大学名誉教授	2012年4月1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	2012年4月1日	非常勤
	小安 重夫	理化学研究所 統合生命医科学研究センター長代行	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	2012年4月1日	非常勤
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	眞弓 忠範	大阪大学名誉教授	2012年4月1日	非常勤
	二之宮 義泰	ノバルティス ファーマ㈱代表取締役社長	2013年7月4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	2012年4月1日	非常勤
	渡邉 敏文	ノバルティス ファーマ㈱ 執行役員シニアサイエンスアドバイザー	2012年4月1日	非常勤

選考委員名簿

2013 年 9 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	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
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
	河野 重行	東京大学大学院新領域創成科学研究科教授	2013年6月14日	非常勤
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	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
	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
	中村 浩之	学習院大学理学部教授	2013年6月14日	非常勤
	鍋倉 淳一	自然科学研究機構生理学研究所教授	2013年6月14日	非常勤
	畠山 昌則	東京大学大学院医学系研究科教授	2013年6月14日	非常勤
	別役 智子	慶應義塾大学医学部教授	2013年6月14日	非常勤
	三浦 哲嗣	札幌医科大学医学部教授	2013年6月14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
	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
	安井 正人	慶應大学医学部教授	2013年6月14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。

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詳しくは、財団事務局までお問合せ下さい。
（電話：03-5464-1460、E メール：novartisfound.japan@novartis.com）

事務局より

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事務局は、今後とも財団の設立目的である学術の進展に寄与するべく、公益事業の遂行に邁進する所存です。

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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