別紙１

研究報告書表紙

厚生労働科学研究費補助金

障害者対策総合研究事業

エピジェネティクス解析に基づいた網膜硝子体疾患に対する病態解明と発症予防および治療法の開発

平成２５年度　総括研究報告書

研究代表者　三村　達哉

平成２６（２０１４）年　４月

別紙２

研究報告書目次

目　次

Ⅰ．総括研究報告
エピジェネティクス解析に基づいた網膜硝子体疾患に対する病態解明と
発症予防および治療法の開発 ―――――――――― 1
三村　達哉

II．研究成果の刊行に関する一覧表 ―――――――――― 4

III．研究成果の刊行物・別刷 ―――――――――― 6

別添３

Ⅰ．総括研究報告

厚生労働科学研究費補助金（障害者対策総合研究事業）
（総括）研究報告書

エピジェネティクス解析に基づいた網膜硝子体疾患に対する病態解明と発症予防および治療法の開発

研究代表者　三村達哉　東京女子医科大学東医療センター眼科学教室　講師

研究要旨
エピジェネティクスとは、DNA の塩基配列の変化なしに、遺伝的しかも可逆的に遺伝子機能の発現が変化する現象で、DNA メチル化やヒストン修飾が関与する。癌をはじめあらゆる疾患におけるエピジェネティクス異常が、病態に関与し、診断や治療の標的となることが明らかになりつつあるが、感覚器疾患との関わりについてはまったく知られていない。本研究では、眼感覚器疾患の中で視機能に直接に影響を与える網膜硝子体疾患とエピジェネティクス異常の関係に焦点をあてて研究を行う。眼内での DNA、ヒストン、クロマチンのメチル化異常を調べることにより、原因不明であった眼疾患の病態を明らかにするとともに、特定の部位のメチレイションを抑制することにより、疾患の予防および、治療をすることを最終目標とする。

A．研究目的：

本研究では、眼内組織の老化のメカニズムを明らかにするために、近年 DNA の塩基配列に変化なしに遺伝的しかも可逆的に遺伝子機能に変化を及ぼすことが明らかになったエピジェネティクスの観点から、網膜硝子体疾患とエピジェネティクス異常の関係を調べることを目的とする。

B．研究方法：

研究計画および方法

本研究は眼感覚器の中心となる網膜視機能障害に焦点をあて、網膜硝子体疾患における各種サイトカインとエピジェネティクスとの関係を調べることを目的としている。研究の対象は糖尿病網膜症、網膜静脈閉塞症、加齢性黄斑変性症に伴う黄斑浮腫などに対し手術を施行した患者である。硝子体手術を必要とする患者に同意を得たのち、前房水、硝子体液、血液、内境界膜を採取する。硝子体手術を必要とした黄斑円孔および黄斑上膜の症例をコントロールとする。

Ⅰ 研究デザイン

①症例対照研究、②前向きコホート研究、対象：黄斑浮腫患者、非黄斑浮腫患者（黄斑上膜、黄斑円孔）、症例数：criteria を満たす年間手術数は約 160 例で、そのうち年間 100 例を目標に研究にエントリーする。

（平成 23 年-24 年度）研究開始 2 年間は患者のエントリーならびに術前術後の網膜視機能の評価、サンプルの採取を行う。視力検査、細隙灯顕微鏡検査、眼底検査、蛍光眼底造影、光干渉断層計、網膜感度検査および網膜電図などを行い、各種網脈絡膜疾患に伴う網膜視機能評価を行う。

（平成 24-25 年度）

サンプルの採取およびサイトカイン濃度の測定

①症例対照研究黄斑浮腫患者群と非黄斑浮腫患者群との間における前房水、硝子体・血液中サイトカイン濃度、黄斑部血流速度の比較、組織学的研究、眼内液サイトカイン濃度およびと黄斑部血流速度との相関解析。測定候補サイトカインは VEGF, VEGF receptor-2 (VEGFR-2), Erythropoietin (EPO), ICAM-1, IL-6, PEDF , Vascular endothelial adhesion molecule-1 (VCAM-1) , Monocyte chmoattractant protein-1 (MCP-1), Stromal-derived factor-1 (SDF-1)。

②眼内サンプル中の細胞の DNA のエピジェネティクス変化を調べ、メチル化を調べる。

（倫理面への配慮）

すべての研究は虎の門病院、東京大学、東京女子医科大学の倫理委員会の承認を得て行う。治療開発を前提とした研究であり、動物実験、臨床試験を行う予定のため、倫理委員会の指針、動物実験の対する指針、および研究に関与するあらゆる倫理指針を遵守する。動物の取り扱いは、苦痛を伴うものは必ず全身麻酔下に行い、両眼が失われる可能性のある場合は片眼のみに処置を行う。全ての実験において動物は the Association for Research in Vision and Ophthalmology の規約および、実験動物の飼養及び保管等に関する基準(総理府)に従って扱う。人を扱う研究では，ヘルシンキ宣言（世界医師会総会 World Medical Assembly)の勧告に従って行う。また遺伝子解析はヒトゲノム・遺伝子解析研究に関する倫理指針（文部科学省、厚生労働省、経済産業省）を遵守する。具体的には以下のように行う。患者を対象とする臨床試験においては十分な説明をした後、文書による同意を得てから行う（インフォームド・コンセント）。

C．研究結果：

本年度は次の結果を得た。①硝子体サンプル中の全遺伝子のメチル化率は網膜症＋＞＞網膜症－であった。②全遺伝子のうち、メチル化率の高い遺伝子上位５種は血管新生/血管内皮増殖の転写因子と低酸素誘導性の転写因子であった。③眼内（前房水）中の抗老化遺伝子であるサーチュイン濃度は加齢黄斑変性のある患者では黄斑変性のない患者よりも低下していた。④加齢黄斑変性患者の尿中乳酸塩濃度は基準値に対し平均 872.9%増加し、尿中ピルビン酸塩濃度は基準値に対し平均 223.9%増加していた。ミトコンドリア機能を反映する乳酸塩／ピルビン酸塩比は基準値に対して 418.3%増加していた。

D．考察：

(1) メチル化解析により眼内遺伝子のメチル化が網膜症の病態に関与していると考えられる。
(2) 血管維持に必要な転写因子のメチル化が、網膜血管障害発症に関与している可能性がある。
(3) 加齢黄斑変性患者で、解糖系と TCA サイクルの中間体であるピルビン酸の増加と、ビルビン酸の還元で産生する乳酸塩が尿中に増加しており、乳酸塩／ピルビン酸塩比が高いことは解糖系における ATP 産生の低下が加齢に影響していると考えられる。

E．結論

眼内の血管維持に必要な転写遺伝子のメチル化が網膜血管障害ならびに眼加齢に関与していると考えらえた。

F．健康危険情報

本研究の結果により、健康に及ぼす危険事項は確認できなかった。

G．研究発表

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H．知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他・賞罰

平成２５年９月　三村達哉　第 28 回基礎医学医療研究助成

別添５

研究成果の刊行に関する一覧表

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III. 研究成果の刊行物・別刷

代表的なレビュー論文1報のみ添付する

SciMedCentral

JSM Ophthalmology

Review Article

Retinal Neuroprotective Effect of Sirtuins

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Keywords
- Review
- SIRT1
- Retina
- Resveratrol

Abstract

In this paper, we review current knowledge about the retinal neuroprotective effect of sirtuins. The sirtuins are highly conserved nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylases that are involved in mammalian diseases of aging. The human genome encodes seven different sirtuins (SIRT1-7). SIRT1 is localized in the nucleus and cytoplasm of the cells making up all normal ocular structures, including the retina. Age-related macular degeneration (AMD) is a typical age-related condition due to the lifelong accumulation of molecular damage caused by reactive oxygen species (ROS). SIRT1 can decrease ROS levels and promotes cell survival under oxidative stress. Upregulation of SIRT1 has a protective effect against retinal degeneration in animal models. Resveratrol is a polyphenolic SIRT1 activator that has been shown to increase the lifespan and to protect various organs against aging, including oxidative stress-induced retinal damage. Anti-aging therapy with resveratrol could be an attractive treatment option for age-related macular degeneration.

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Retinal Neuroprotective Effect of Sirtuins

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INTRODUCTION

istone deacetylases (HDACs) are enzymes that deacetylate histones, but also act on certain non-histone substrates. Class III HDACs, which are known as sirtuins, catalyze deacetylation of the acetyl-lysine residues of histones using nicotinamide adenine dinucleotide (NAD+) as a cofactor. Silent information regulator 2 (Sir2) was the first gene of the sirtuin family to be discovered. Sir2 shows a high level of evolutionary conservation and is an important regulator of senescence, cell differentiation, stress tolerance, metabolism, and cancer in several organisms. Sirtuins have been suggested to have a role in aging [1,2], calorie restriction [1-10], and inflammation. Overexpression of Sir2 prolongs the lifespan of various organisms, whereas deletion or mutation of Sir2 leads to a shorter lifespan [11-13]. Seven human Sir2 homologues (sirtuins) have been identified to date, and these are designated as SIRT1 to SIRT7 [14-15]. Sirtuins are also important in preventing age-related ocular diseases [16]. In this review, we focus on the retinal neuroprotective effect of sirtuins.

ENZYMATIC ACTIVITY OF SIRTUINS

Sirtuins carry out deacetylation via a two-step reaction that consumes NAD+ and releases nicotinamide (NAM), O-acetyl-adenosine diphosphate (ADP) -ribose (AADPR), and the deacetylated substrate [17-21]. Sirtuin activity is regulated by the intracellular [NAD]/[NADH] ratio and responds to changes of cellular metabolism [22-25]. NAD+ is an activator of sirtuins, while nicotinamide and NADH are inhibitors. Sirtuins can catalyze deacetylation or ADP-ribosylation reactions, with both of these reactions involving cleavage of NAD+ as a cofactor and the production of nicotinamide (NAM). Five sirtuins (SIRT1, SIRT2, SIRT3, SIRT5, and SIRT7) catalyze deacetylation of the lysine residues of their target proteins, using NAD+ as cofactor and releasing nicotinamide along with the production of 2'-O-acetyl-ADP ribose [25-27]. In contrast, SirT4 and SirT6 catalyze ADP-ribosylation which involves transfer of an ADP-ribosyl moiety to the substrate [26,28].

Sirtuins have a highly conserved core domain that contains a catalytic domain and an NAD+-binding site [29]. Human SirT2 is composed of two globular domains, one of which is large, while the other is small. The large domain contains an inverted classical open α/β Rossmann-fold, six β-strands that form a parallel β-sheet, and six -helices, while the small domain is composed of a helical module and a zinc-binding module. The active site is located at the interface between the large and small domains, along with a binding site for NAD+. The NAD+-binding pocket can be divided into three spatially distinct regions, which are the A site showing affinity for adenine–ribose, the B site with affinity for nicotinamide–ribose, and the C site that binds NAD+. In the presence of an acetyl-lysine substrate, the NAD+-bound B site undergoes a conformational change that brings nicotinamide into proximity with the C site so that it can be cleaved. The ADP

ribose product then returns to the B site, allowing deacetylation to occur. The C site is the binding site for free nicotinamide. At high concentrations, nicotinamide can occupy the site and block the conformational change of NAD+ [30].

RETINAL DISTRIBUTION AND ROLE OF SIRTUINS

Jaliffa and associates investigated the localization of SIRT1 in adult mouse eyes by in situ hybridization They found SIRT1 in the nucleus and cytoplasm of cells from all normal ocular structures, including the cornea, lens, iris, ciliary body, and retina. They reported that SIRT1 mRNA was detected in the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) of the mouse retina by *in situ* hybridization (Figure 1) [31]. However, there has been no report about detection of SIRT1 in the human eye.

SIRT1-deficient mice are smaller than normal at birth and usually die during the early postnatal period [32]. Even if these mice survive, they exhibit infertility and often have a shorter snout than normal mice [32]. Additionally, all SIRT1-deficient mice fail to open one or both eyes [32]. To date, only a few studies have assessed the ocular role of SIRT1, including its relationship with the development of cataract [33,34], retinal degeneration [31,35-41], optic neuritis [42], and uveitis [43]. The most important ocular role of SIRT1 may be protecting the retina and optic nerve against degeneration.

AMD AND OXIDATIVE STRESS

Free radicals are atoms or molecules with at least one unpaired electron in an outer shell and these radicals are known to play an important role in the pathogenesis of cellular aging. In most biological structures, free radical damage is closely associated with oxidative damage, with antioxidants being particularly important for diminishing the cumulative effect of oxidative damage over the long lifespan of humans by passivating free radicals. AMD is a typical age-related condition, which is considered to arise from aging and the lifelong accumulation of molecular damage caused by reactive oxygen species (ROS) [44]. In the retina, ROS (including free radicals) cause damage that leads to apoptotic cell death, dysfunction of the retinal pigment epithelial cells, accumulation of lipofuscin, formation of drusen, and impairment of Bruch's membrane, and ROS-related damage is considered to be responsible for the pathological changes of AMD [45].

The Age-Related Eye Disease Study demonstrated that oxidative stress can promote the development of AMD, while antioxidants and zinc supplements delay the progression of AMD and loss of vision [46]. Oxidatively modified proteins have been detected in drusen by proteomic analysis [47]. SIRT1 decreases ROS levels and promotes cell survival under oxidative stress [48]. Thus, SIRT1 may prevent ROS-dependent apoptosis of retinal neurons under oxidative stress [49]

NEUROPROTECTIVE EFFECT OF SIRT1 IN THE RETINA

The retina is part of the nervous system. Various factors (including aging, UV radiation, and oxidative stress) can induce permanent damage to the retinal architecture [44], while SIRT1

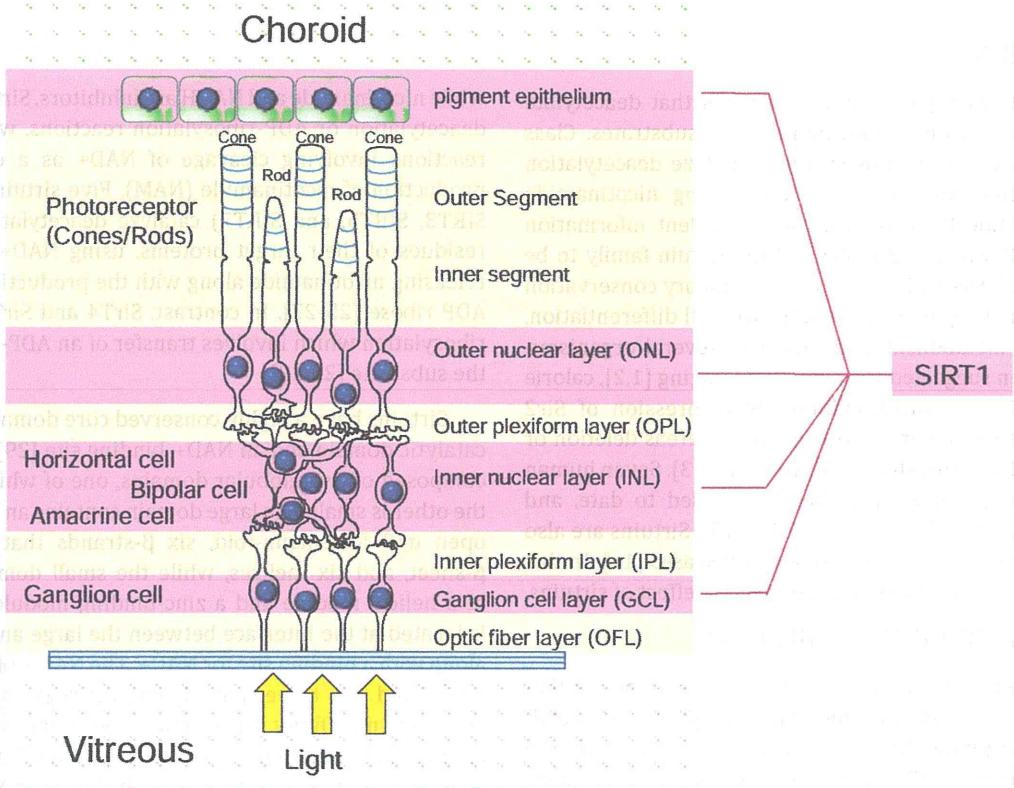

Figure 1 Localization of SIRT1 in the mouse retina (Jaliffa 2009). SIRT1 is expressed in the retinal pigment epithelium (RPE), outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL).

appears to have a neuroprotective effect on the retina. SIRT1 is localized in most layers of the normal mouse retina (including the ONL, INL, GCL, and RPE) [31]. In SIRT1-deficient adult mice, multiple retinal cell layers are significantly thinner than in normal mouse eyes, while the inner and outer nuclear layers are disorganized [35]. The inner and outer photoreceptor cell segments are also difficult to detect in SIRT1-deficient adult mice, indicating that SIRT1 has an important role in ocular morphogenesis [35].

Several experimental studies have demonstrated a protective effect of SIRT1 against retinal and optic nerve damage. For example, intravitreal injection of SIRT1 activators prevents RGC loss in a dose-dependent manner by stimulating SIRT1 enzymatic activity in mice with optic neuritis [42]. This neuroprotective effect is blocked by sirtinol, a SIRT1 inhibitor [42]. Absence of E2fs, the transcription factor for SIRT1, causes downregulation of the p53 deacetylase activity of SIRT1, resulting in p53 hyperacetylation and an increase of apoptosis in the mouse retina [37]. Transfer of the SIRT1 gene with Oct 4 prevents retinal cell loss and improves electroretinographic responses in rats with retinal phototoxicity [40]. Furthermore, upregulation of SIRT1 by resveratrol protects cultured retinal cells from antibody-induced apoptotic death [36]. Resveratrol is a natural polyphenol found in red grapes and red wine that has been shown to enhance SIRT1 activity [10,25,50] (Figure 2), and it also has a protective effect against phototoxic degeneration of the mouse retina *in vivo* [38]. These findings suggest that SIRT1 can provide protection against diseases caused by oxidative stress-induced retinal damage, such as AMD, while anti-aging therapy with resveratrol could be a potential treatment for retinal damage.

SUMMARY

We reviewed the influence of sirtuins on retinal aging and degeneration. Some clinical trials of SIRT1 activators have already been started for a variety of diseases, including cardiovascular disease, cancer, diabetes, and Alzheimer's disease. However, many uncertainties remain, especially concerning the preventative effect of SIRT1 on AMD. SIRT1 activators such as resveratrol, rather than SIRT1 itself, may be candidate drugs for AMD.

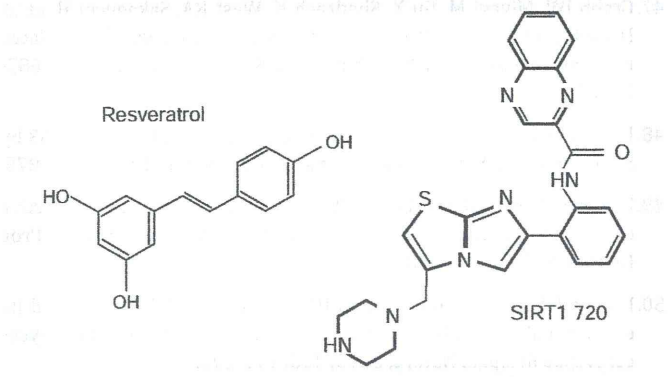

Figure 2 Chemical structure of two SIRT1 activators. Resveratrol is a polyphenolic compound found in grapes and wine, which is known as an activator of SIRT1. The experimental drug SIRT1720 is low molecular weight SIRT1 activator that is 1,000 times more potent than resveratrol.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and for writing this paper.

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