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学位論文題名

Suppressive effects of naturally-derived and novel synthetic substances on ocular inflammation

（天然由来および新規化合物の眼炎症抑制効果）

学位論文内容の要旨

[Background and Objectives]

Control of intraocular inflammation is important theme of modern ophthalmology. With anatomical specificity of the eye even mild inflammatory response in the confined environment of ocular globe may cause major damage and cause permanent vision loss. We used several approaches including use of antioxidant (Astaxantin), NF-κB inhibitors (IMD0354, IMD1041) and molecular chaperones (HSP70) in mouse UVB keratatis and rat endotoxin induced uveitis models.

[Methods and results]

Amelioration of ultraviolet-induced photokeratitis treated with astaxanthin eye drops in mice.

Astaxanthin (AST), 3,30-dihydroxy-b,b-carotene-4,40-dione, a carotenoid without vitamin A activity, has potential clinical applications due to its higher antioxidant activity than β-carotene and α-tocopherol. Acute ultraviolet (UV) exposure causes photokeratitis and induces various inflammatory changes in the cornea. In the present study, we examined whether topical administration of AST has therapeutic effects on UV-photokeratitis in mice.

Six- to 8-week-old C57BL/6 male mice were used. C57BL/6 male mice were administered AST in instillation form with following concentration: 1 mg/ml, 0.1 mg/ml, and 0.01 mg/ml to right eyes, left eyes were instilled with vehicle alone. After the instillation, the mice were irradiated with UVB at the dose of 400 mJ/cm^2 under anesthesia. Eyeballs were collected 24 hours after irradiation, and stained with H&E and TUNEL. As in vitro study, NIH-3T3 cells were cultured with AST. Cytotoxicity was quantified with LDH assay.

UVB irradiation caused disruption of the corneal basement membrane and thinning of the corneal epithelium; however, the epithelium was well preserved after irradiation in AST-treated corneas. The corneal epithelium thickness was 35.75±1.7, 29.75±1.7 and 8.5±2.8 μm in mice treated with 1, 0.1 and 0.01 mg/ml of AST, respectively. The mean corneal epithelial thickness was 4.75±4.6 in untreated eyes after irradiation. Non-irradiated corneal epithelium was 38.25±2.5 μm thick. Apoptotic cells were counted as 2.75±3.7, 2.25±2.8, 19.0±3.2, and 23.0±5.3 in eyes treated with 1, 0.1, 0.01, and 0 mg/ml of AST respectively. Significantly fewer apoptotic cells were observed in AST-treated UV-irradiated mice than controls ($p<0.01$). In vitro study showed lesser cytotoxicity of NIH-3T3 cells in AST-treated cultures after UVB-irradiation. The percentages of mean cytotoxicity after irradiation were 23.0±5.3%, 59.25±5.3%, 77.75±7.6 %, and 86.75±4.3% in wells added 1, 0.1, 0.01, and 0 mg/ml of AST, respectively.

In the current study, we showed that AST has the protective effect regarding UVB irradiation in vivo and in vitro. AST might be a promising naturally-derived material protecting ocular surface from the toxicity of ultraviolet.

Amelioration of endotoxin-induced uveitis treated with IMD-0354 NF-κB inhibitor in rats.

Endotoxin-induced uveitis (EIU) is an animal model for acute ocular inflammation. There are several substances that play major roles in the development of inflammatory changes in EIU including TNF-α, Interleukin (IL)-1β IL-6 and others. This inflammatory cytokines trigger the degradation of IκB by activating IκB kinases (IKKs). Released NF-κB subsequently translocates to the nucleus, where it expresses its proinflamatory function. IMD-0354 decrease NF-κB activation by inhibition of IKK. In this study, we examined whether administration of IMD-0354 has therapeutic effects on EIU in rats.

Six-week-old male Lewis rats were used. EIU was induced by subcutaneous injections of 200 μg of LPS from *Escherichia Coli* that had been diluted in 0.1 ml of phosphate buffered saline. IMD-0354 was administered intraperitoneally 30, 10, 3 or 0 mg/kg suspended in 1.0 ml of 0.5% CMC sodium. Naïve rats were used as control. The rats were euthanized 24 hours after LPS injection, and the aqueous humor was collected, the total protein concentration in the aqueous humor samples was measured with a Qbit protein Assay kit. For cell counting, the aqueous humor was suspended in Turk stain solution, and the cells were counted using a hemocytometer under light microscopy. Some eyes in each group were fixed with PFA 4% via intracardial injection and stained with anti-NF-κB antibodies.

The total protein concentration of aqueous humor was 92.5±5.3, 101.5±11.7, 112.6±3.2 and 117.3±3.0 in rats treated with 30, 10, 3 or 0 mg/kg BW of IMD-0354, respectively. Naïve rats mean protein concentration was 21.5±4.7, it was significantly lower in IMD-0354 30 mg/kg BW ($p<0.01$) and 10 mg/kg BW ($p<0.01$) treated groups.

The number of inflammatory cells in aqueous humor was 46.4±16.8, 68.25±30.1, 128.41±54.9, and 133.3±44.0x10^4 in rats treated with 30, 10, 3 or 0 mg/kg BW of IMD-0354, respectively. There were no inflammatory cells detected in Naïve eyes.

Multiple NF-κB positive nuclei was detected in untreated eyes 249.00±27.8, There were significantly less cells detected in IMD-0354 30 mg/kg BW ($p<0.01$) 146.6±3.0 No NF-κB positive cells were detected in Naïve slides. These results suggest that IMD-0354 reduce intraocular inflammation in rat EIU by inhibition of IKK and reduced expression of NF-κB.

Induction of heat shock protein 70 ameliorates ultraviolet-induced photokeratitis in mice.

Acute ultraviolet (UV) B exposure causes photokeratitis and induces apoptosis in corneal cells. Geranylgeranylacetone (GGA) is an acyclic polyisoprenoid that induces the expression of heat shock protein (HSP)-70, a soluble intracellular protein expressed in various tissues, including the eyes. The HSPs mainly function as intracellular chaperones, protecting cells against various stress conditions. In the present study, we examined whether the induction of HSP70 has therapeutic effects on UV-photokeratitis in mice. C57BL/6 female mice were divided into four groups, GGA-treated (500 mg/kg/mouse) and UVB-exposed (400mJ/cm^2), GGA-untreated and UVB-exposed (400mJ/cm2), GGA-treated (500 mg/kg/mouse) but not UVB-exposed, and naïve controls. Eyeballs were collected 24 hours after irradiation, and corneas were stained with H&E, TUNEL, anti-HSP70 antibody, and Phospho-(serine/threonine) Akt substrate antibody. The irradiated corneal epithelium was significantly thicker in the eyes of mice treated with GGA as compared with those given vehicle alone ($P<0.01$). Significantly fewer TUNEL-positive cells were observed in the eyes of GGA-treated mice than in controls after irradiation ($P<0.01$). Corneal HSP70 levels were significantly elevated in the corneas of mice treated with GGA ($P<0.05$). Phospho-(Ser/Ther) Akt substrate expression was increased in corneas after irradiation when they were treated with GGA. GGA treatment induced HSP70 and ameliorated UV-induced corneal damage through the activation of the Akt signal.

[Conclusions]

We identified several natural and synthetic substances that reduce intraocular inflammation in UVB corneal damage model and rat EIU model. This work let to two publications on Mol Vis scientific journal. Discovered anti-inflammatory features of subscribed substances may lead to good targets to develop new drugs and supplements.

学位論文審査の要旨

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学位論文題名

Suppressive effects of naturally-derived and novel synthetic substances on ocular inflammation

(天然由来および新規化合物の眼炎症抑制効果)

本研究で、申請者は天然由来および新規化合物を用いてそれらの眼炎症抑制効果を検討した。

まず、申請者はアスタキサンチン (AST) に関する研究をおこなった。AST は β-カロテンやリコピンなどと同じカロテノイドの一種であり、甲殻類の殻やそれらを餌とするマダイ等の体表、サケ科魚類の筋肉の赤色部分などに含まれている。また、AST は強力な抗酸化作用を有し、同時に紫外線等から生体を防御する因子として機能することが知られ、眼科領域においては AST は光障害から目を保護することが報告されている。この研究ではマウスを用いて急性の紫外線角膜障害に対する AST の効果を検討した。その結果、AST の眼局所投与は急性紫外線角膜障害を軽症化することが明らかとなった。

次に、申請者は新規分子標的化合物 IMD-0354 に注目して研究をおこなった。Nuclear factor (NF)-κB は免疫反応の中心的役割を果たす転写因子の一つであり、生体内でアポトーシスや細胞増殖、炎症、分化などを制御する重要な因子である。新規分子標的化合物 IMD-0354 (N-(3,5-bis-trifluoromethyl-phenyl)-5-chloro-2-hydroxy-benzamide)は、IκB キナーゼ (IKK) β のリン酸化を特異的に阻害する。エンドトキシン誘発ぶどう膜炎 (EIU) はリポ多糖 (LPS) 投与により惹起される非特異的急性ぶどう膜炎モデルであり、NF-κB が亢進することが知られているため、この研究ではラット EIU モデルを用いて新規分子標的化合物 IMD-0354 の NF-κB/IKKβ経路阻害による炎症軽減効果を検討した。その結果、特異的 IKKβ阻害薬 IMD-0354 は急性ぶどう膜炎を安全かつ強力に抑制することが明らかとなった。

さらに、GGA (geranylgeranylaceton) のマウス急性紫外線角膜炎モデルに対する効果を検討した。GGA は分子シャペロンである熱ショック蛋白 (HSP) 70 を誘導することが知られている。また、さまざまなストレスに対してストレス耐性、細胞保護効果を示すことが報告されている。この研究ではマウス急性紫外線角膜障害モデルに対する GGA 全身投与の効果について検討した結果、GGA

投与により角膜での HSP70 の発現量が増加し、急性紫外線角膜障害が軽症化することが明らかとなった。また、そのメカニズムとして角膜上皮細胞質内で細胞生存シグナル Akt のリン酸化が誘導されていることがわかった。

質疑応答では主査と副査から以下のような質問があり、申請者はおおむね適切に回答した。

1. AST の研究で眼局所投与を選んだ理由

これまでの AST の研究報告はすべて経口摂取であり、我々の過去のぶどう膜炎モデルやヒト眼精疲労試験も経口摂取であった。しかし、今回検討した急性紫外線角膜障害モデルは病変が眼表面でかつ急性病変であるため、ドラッグデリバリーとして点眼が有利であると思い至った。また、これまで眼局所投与の報告は全くないため、新規性も高いと考え眼局所投与を選択した。

2. AST は紫外線曝露後でも角膜保護効果があるか

発表スライドには結果を示さなかったが、紫外線曝露直後に点眼しても AST は角膜障害を軽減した。学位申請論文には当該結果を記載した。

3. AST とプレドニゾロンを比較しなかった理由について

我々の研究室では以前、急性ぶどう膜炎モデルを用いて AST とデキサメサゾンの効果を比較検討した。今回の急性紫外線角膜障害モデルは紫外線エネルギーによる角膜上皮への直接障害が主であり、さらに炎症が障害を重症化させるモデルであること、AST を点眼で投与する実験であることから、全身投与で用いるプレドニゾロンは対照薬剤として不適切と考えた。今回の検討では、溶媒投与群を対照としたが、将来ステロイド薬との比較を本モデルでも検討したいと考えている。

4. HSP が細胞死を抑制するメカニズムについて

GGA は転写因子 HSF-1 を単量体から三量体に変換、HSF-1 はプロモーター領域に結合して HSP70 産生を誘導することが知られている。今回の検討においては、HSP70 は細胞生存シグナル Akt リン酸化を誘導し、caspase 9 を抑制することで急性紫外線角膜障害モデルの炎症と細胞死を抑制すると考えられた。

これら３報の検討結果は、国際英文誌 Mol Vis と Int J Mol Sci に既に３本の原著論文として掲載されるとともに、音羽賞ならびに日本眼科学会総会座長賞を受賞するなど高く評価され、今後の急性眼炎症に対する安全で有効な候補物質となることが期待される。

審査員一同はこれらの成果を高く評価し、大学院課程における研鑽や取得単位なども併せ申請者が博士（医学）の学位を受けるのに充分な資格を有するものと判定した。