Research Article

Asthma Promotes Choroidal Neovascularization via the Transforming Growth Factor beta1/Smad Signalling Pathway in a Mouse Model

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Short Title: Asthma Promotes Choroidal Neovascularization

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Abstract

Introduction: The association between age-related macular degeneration (AMD) and asthma is controversial. Transforming growth factor beta (TGF-β), which plays a critical role in asthma, has been extensively studied with regard to its function in choroidal neovascularization (CNV). In the present study, we aimed to investigate the role of TGF-β and the possible mechanism of CNV formation complicated with asthma and to explore the effect of a TGF-β inhibitor on CNV development in asthma mouse models.

Methods: Laser-induced CNV and ovalbumin-induced asthma mouse models were divided into five groups: control group, acute asthma group, chronic asthma group, inhibitor-treated acute asthma group, and inhibitor-treated chronic asthma group. The gene expression patterns of angiogenic cytokines, vascular endothelial growth factor (VEGF) receptors and inflammasomes in the control group, acute asthma group and chronic asthma group were detected using a QuantiGene Plex 6.0 Reagent System. Fundus fluorescein angiography (FFA) and histology of CNV lesions stained with haematoxylin-eosin (HE) were performed to evaluate CNV formation. Quantitative real-time PCR and western blotting were used to assess TGF-β1, TGF-β2, and VEGF expression and Smad2/3, AKT, p38 MAPK, and ERK1/2 signal transduction and phosphorylation in retinal and choroidal tissue from each group.

Results: In this study, we verified that laser treatment led to more CNV and vascular leakage in asthmatic mice than that in control mice. The changes were particularly notable in the chronic asthma group. The respective TGF-β1, VEGF, and phosphorylated Smad2/3 (p-Smad2/3) mRNA and protein levels in retinal and choroidal tissue were significantly upregulated in both the acute and chronic asthma groups. After injection of a TGF-β inhibitor, a distinct decline in VEGF, TGF-β1, and p-Smad2/3 protein and mRNA levels was observed, and the mean CNV area also decreased.

Conclusion: We provide new evidence that asthma could be a risk factor for CNV development via the TGF-β1/Smad signalling pathway. A TGF-β inhibitor can be applied as a useful, adjunctive therapeutic strategy for preventing CNV formation in asthmatic patients.
Introduction

Age-related macular degeneration is a common, chronic, progressive degenerative disorder of the macula that affects older individuals and features loss of central vision as a result of abnormalities in the photoreceptor/retinal pigment epithelium/Bruch’s membrane/choroidal complex[1]. Two forms of AMD are recognized: nonexudative (dry) and exudative (wet) types[2]. Choroidal neovascularization (CNV) is a severe complication of wet AMD and is known as the major cause of sudden and disabling loss of central vision in wet AMD. The risk factors implicated in CNV include personal and environmental factors (e.g., age, sex, race/ethnicity, heredity, smoking, sunlight exposure, nutritional factors and alcohol consumption)[3]. However, the molecular mechanisms involved in CNV remain largely unknown. Over the past decade, accumulated evidence has implicated the complement cascade and immune mechanisms in CNV and has led to a general consensus that inflammation is a key driver of CNV development, which could disrupt the barrier of Bruch’s membrane, leading to growth of CNV from the choroid into the subRPE (retinal pigment epithelium) and subretinal spaces and is thought to be the pathological basis[4].

Asthma, a common chronic lung disease, is defined by airway inflammation, hyperresponsiveness, heightened mucus production and airway remodelling[5]. The pathological changes in airway remodelling include epithelial damage, subepithelial fibrosis, mucus gland hyperplasia, increased smooth muscle wall, and vascular hyperplasia[6]. According to global statistics, approximately 235–330 million people have been affected by asthma, and 250–345 thousand people die every year due to asthma[7]. The mechanisms underlying both CNV and asthma development are similar; however, the relationship between CNV and asthma is still controversial. Some studies have reported a promotive effect of asthma in CNV occurrence via complement factor 3 (C3), while others have shown the opposite results[8-10]. Previous studies have reported that TGF-β expression is increased in bronchial specimens from asthmatic patients[11, 12]. Ojiaku et al. established that TGF-β
enhances airway smooth muscle proliferation and ECM deposition via fibroblast activation, eventually leading to structural alterations in the airway[13]. Likewise, several studies support a promotive role of TGF-β in neovascular age-related macular degeneration (nAMD). The proangiogenic function of TGF-β in nAMD could be direct, through stimulation of choroidal EC proliferation, or indirect, through induction of VEGF-A secretion by RPE cells or promotion of macrophage-mediated inflammation[14]. A TGF-β inhibitor administered via intravitreal injection and/or intravenous injection may abate CNV development[15].

Considering the crucial roles of TGF-β in both asthma and CNV, we hypothesize that TGF-β may promote the effect of asthma in CNV susceptibility, and therefore, selective blockade of TGF-β might abate CNV development in the setting of asthma. Thus, in the present study, we aimed to investigate the possible effect and mechanisms of TGF-β with regard to the relationship between CNV and asthma. The encouraging results of our study provide a new strategy for treating CNV in asthmatic patients.

Materials and Methods

Mice

All animal experiments were performed in strict accordance with the Association for Research in Vision and Ophthalmic and Vision Resolution (ARVO) for the Use of Animals in Ophthalmic and Vision Research and with the guidelines provided by the Animal Care Use Committee of Peking University (Beijing, China). Specific, pathogen-free male C57BL/6J mice (6-8 weeks old, 20-25 g) were purchased from the Laboratory Animal Center, Peking University People’s Hospital. Animal care and experiments were conducted under institutional guidelines, and food and tap water were provided ad libitum.

Study Groups

C57BL/6J mice were randomly divided into five groups and treated as follows. Control group: Mice in this group were sensitized on day 0 via intraperitoneal injection of 500 μL sterile physiological saline instead of ovalbumin. From day 14 to
17, the mice were exposed to physiological saline aerosol. On day 18, laser photocoagulation was conducted, and the mice in this group received an intraperitoneal injection of 0.5 ml saline.

Acute asthma group: Mice were sensitized and challenged with ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO, USA) on day 0 via intraperitoneal injection of 10 μg OVA emulsified in 1 mg of Imject Alum (Pierce, Rockford, IL USA) in a total volume of 500 μL. Mice were challenged for 30 minutes by OVA aerosol (1% weight/volume diluted in sterile physiological saline) nebulized with an ultrasonic nebulizer (PARI BOY, PARI GmbH, Sternberg, Germany) on days 14-17[16]. On day 18, when the acute asthma model had been established, laser photocoagulation was conducted, and the mice in this group received an intraperitoneal injection of 0.5 ml saline.

Chronic asthma group: Mice were sensitized on days 0 and 14 via intraperitoneal injection of 10 μg OVA emulsified in 1 mg of Imject Alum (Pierce, Rockford, IL USA) in a total volume of 500 μL. From day 21, mice were challenged for 30 minutes 3 days/week for 6 weeks by OVA aerosol (1% weight/volume diluted in sterile physiological saline) nebulized with an ultrasonic nebulizer[16]. On day 64, when the chronic asthma model had been established, the mice in this group received laser photocoagulation and intraperitoneal injection of 0.5 ml saline.

Inhibitor-treated acute asthma group: Primarily, the procedures employed to establish an acute asthma model were identical to those described above. However, on day 18, when the acute asthma model had been established, laser photocoagulation was performed, and mice in this group were injected in the abdominal cavity with 0.5 g/L SB431542 solution (Sigma Aldrich, St. Louis, MO, USA, 10 mg/kg, 0.25 mg/0.5 ml per mouse)[17].

Inhibitor-treated chronic asthma group: Primarily, the procedures employed to establish a chronic asthma model were identical to those described above. However, on day 64, when the chronic asthma model had been established, laser photocoagulation was performed, and the mice in this group were injected in the abdominal cavity with 0.5 g/L SB431542 solution (10 mg/kg, 0.25 mg/0.5 ml per mouse).
Mouse Model of CNV

The mice in each group were fully anaesthetized with an intraperitoneal injection of ketamine (80 mg/kg)-xylazine (10 mg/kg), the pupils were dilated with 1% tropicamide. CNV lesions were induced by laser photocoagulation (532 nm, 150 mW, 50 ms, 50 µm) (Coherent 130SL, Coherent, Santa Clara, CA, USA) performed when the asthma model had already been established. Lasers were conducted on one eye of each mouse. Five laser spots were placed concentrically around the optic disc. The morphologic end point of laser injury was the appearance of a cavitation bubble, which is thought to be correlated with disruption of Bruch’s membrane. Laser combined with hemorrhage or subretinal bleeding at the time of laser application were excluded.

Fluorescein Angiography

Fourteen days after photocoagulation, CNV leakage areas in laser lesions were evaluated using fluorescein angiography (FA) with a Phoenix Micron IV Retinal Imaging Microscope (Phoenix, Pleasanton, CA, USA) according to the manufacturer’s illustrations. All mice were anaesthetized with an intraperitoneal injection of ketamine (80 mg/kg)-xylazine (10 mg/kg). FA was performed immediately after intraperitoneal injection of 0.03 ml fluorescein sodium (FLUORESCITE Injection; Alcon, Fort Worth, TX, USA). Both early-phase (1 minute after injection) and late-phase (5 minutes after injection) fundus angiograms were collected to observe fluorescence leakage. Late-phase fundus angiograms were used for analysis. Images were analysed using ImageJ software (http://imagej.nih.gov/ij/; provided to the public by the National Institutes of Health, Bethesda, MD, USA).

Histological Analysis of Mouse Lungs

To verify whether the asthmatic mouse models were successfully established in this study, we conducted pulmonary histopathology in the control group, acute asthma group and chronic asthma group. Fourteen days after photocoagulation, mice were euthanized by inhalation of CO2 gas after FA was conducted. The left lung lobe
from each animal was isolated and fixed in 10% (v/v) neutral buffered formalin. The specimens were dehydrated, embedded in paraffin, and cut into sections (5 mm thickness) that were then stained with haematoxylin/eosin (H&E) (Beyotime Biotechnology). The inflammation in lung tissues was microscopically determined by the degree of cell infiltration around the basal membrane of bronchi or vessels, which was graded on a scale from 1 to 4. In brief, a value of 1 was assigned for occasional cuffing with inflammatory cells, a value of 2 was assigned for a thin layer (two to three cells thick) of inflammatory cells, a value of 3 was assigned when bronchi or vessels were surrounded by a thick layer of four to five inflammatory cells, and a value of 4 was assigned when bronchi or vessels were surrounded by a layer of more than five inflammatory cells [18].

**Gene Expression of Chemokines in Acute asthma, Chronic asthma and Control groups using a QuantiGene Plex 6.0 Reagent System**

Fourteen days after fluorescein angiography, 3 mice (3 laser-treated eyes) in the acute asthma, chronic asthma and control groups were used for QuantiGene analysis. Target-specific RNA molecules in C57BL/6J mice were detected using the QuantiGene Plex 6.0 reagent system according to the manufacturer’s protocol (Affymetrix, Fremont, CA). Briefly, RNA from retina-choroid layer lysates was captured with fluorescence microspheres. Signals of cascade amplification were detected using Luminex 100 xMAP technology and Bio-Plex 5.0 software (Bio-Rad Laboratories, Hercules, CA). The geometric means of the expression of two housekeeping genes, PPIB (NM_022536) and HPRT1 (NM_012583), were used for normalization. Fold-changes were determined by the relative ratios among the normalized values in the acute asthma group, chronic asthma group and control group. For one measurement, retina-choroid layer samples from 3 mice were combined, and the experiments were repeated 3 different times.

**Western Blotting**

Fourteen days after laser photocoagulation, immediately following euthanasia of the mice, 6 laser-treated eyes from mice in each group were enucleated and
dissected on ice, carefully isolating the retinal and choroidal tissue. Retina-choroid complexes were solubilized and homogenized in RIPA lysis buffer (P0013B, Beyotime Institute of Biotechnology), which was combined with 1% phenylmethanesulfonyl fluoride (ST506, Beyotime Institute of Biotechnology). After 30 minutes on ice, the tissues were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were used for protein concentration using bicinchoninic acid (BCA, P0010S, Beyotime) protein assay. Equal amounts of protein were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The proteins were visualized via enhanced chemiluminescence western blot (W-B) detection reagents (WBKLS0100, Millipore). Band densities were tested with antibodies against TGF-β1 (ab92486, Abcam, USA), TGF-β2 (ab36495, Abcam, USA), VEGF (ab46154, Abcam, USA), smad2/3 (#5678, CST), phosphorylated smad2/3 (p-smad2/3) (#8828, CST), AKT (#4691, CST), phosphorylated AKT (p-AKT) (1:1000, #4060, CST), p38 mitogen-activated protein kinase (p38 MAPK) (1:1000, #8690, CST), p38 MAPK (#8690, CST), phosphorylated p38 MAPK (p-p38 MAPK) (#4511, CST), ERK1/2 (#9102; CST), and phosphorylated ERK1/2 (p-ERK1/2) (#4370, CST), followed by incubation with a horseradish peroxidase (HRP)-conjugated goat antibody against rabbit IgG (#7074, Cell Signaling Technology, USA). For sequential blotting with additional antibodies, the membranes were stripped with a restorative western blot stripping buffer and reprobed with the indicated antibodies. Western blot analyses were repeated three times, and qualitatively similar results were obtained.

RNA Extraction and Relative Quantitative Real-Time PCR

Fourteen days after laser photocoagulation, retinal and choroidal tissues from 5 laser-treated mice in each group were lysed in Trizol (Invitrogen, Carlsbad, CA, USA), and RNA was extracted according to the manufacturer’s instructions. Reverse transcriptase reactions were performed using a RevertAid First Strand cDNA Synthesis Kit with oligo-dT primer (Fermentas, Pittsburgh, PA, USA). Real-time PCR reactions were performed with SYBR Green PCR mix (Thermo, Pittsburgh, PA, USA) using an ABI7300 real-time PCR system (Applied Biosystems, Life Technologies, Foster City, CA, USA). Real-time PCR primers targeting the following genes were used:
TGF-β1, forward 5’- AACAATCTGGCGTTACCTT-3’ and reverse 5’-GCTAATGTTGCTCCTCTAC-3’; TGF-β2, forward 5’-GCTAATGTTGGCCCTCCTAC-3’ and reverse 5’-GGACTTTGGTGAGTGTGC-3’; VEGF, forward 5’-TTAGGATAGCTGGGTGTTGAGTAG-3’; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5’-GGTACCTCTTGACGACGAACA-3’ and reverse 5’-GAGTCCACTGGCGTCTTCAC-3’. Relative multiples of changes in mRNA expression were determined by calculating fold change $= 2^{\Delta\Delta ct}$. Each experiment was repeated five times.

**Histological Analysis of CNV**

Fourteen days after laser photocoagulation, eyeballs from 5 laser-treated eyes were removed from mice in each group and fixed in 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer for 24 hours. After removal of the anterior segments, the posterior eyecups were embedded in paraffin. Sagittal sections of 8 mm were cut through the centre of the eye at the site of laser photocoagulation. The sections were stained with H&E and assessed using light microscopy (LEICA DFC 300FX, Leica, Solms, Germany). A computer-assisted image analysis system was used to estimate neovascularization based on the B/C ratio (B represents the thickness between the bottom of the pigmented choroidal layer and the top of the neovascular membrane, and C represents the thickness of the intact pigmented choroid next to the lesion) [9-10]. Measurements were performed on four sections from each laser photocoagulation site.

**Statistical Analysis**

Data analysis was performed using the following statistical software: Prism 8 (GraphPad Software, Inc., San Diego, CA) and SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA). The data are presented as the mean±standard error. Statistical significance was determined using either one-way analysis of variance or a Student’s t-test, depending on the experimental group analysed. P values of less than 0.05 were considered to indicate statistical significance.
Results

**Increased Inflammation in Lungs of Mice Sensitized and Challenged with OVA**

To evaluate whether the mouse models could successfully reflect asthmatic status, histopathological changes in lung tissues from mice in the control, acute asthma and chronic asthma groups were observed using H&E staining. The histological analysis was performed on samples from 10 mice of each group. Compared with lungs from control group mice treated with sterile physiological saline only, the lungs of mice sensitized and challenged with OVA showed an asthma-like pathology, such as significantly increased inflammatory cell infiltration, pulmonary oedema, thickened walls of bronchial epithelium and congested blood vessels. The inflammatory score of lung tissues was significantly increased in the OVA-sensitized/challenged group compared with the control group. The chronic asthma group demonstrated more severe pulmonary inflammation than the acute asthma group (Fig. 1).

**Upregulation of Angiogenic factors and Inflammasomes in Acute and Chronic Asthma Mice**

To verify our hypothesis that TGF-β may play a role in the development of CNV in asthmatic patients, we established animal models representing asthma and CNV. Moreover, we further divided the asthmatic models into acute and chronic groups to determine whether CNV susceptibility was consistent with asthma severity.

In the present study, the expression levels of several angiogenic cytokines, VEGF receptors and inflammasomes were detected using the QuantiGene Plex 6.0 reagent system, and the results are shown in Fig. 2. Three mice (3 laser-treated eyes) in the control, acute asthma and chronic asthma groups were analysed. In the QuantiGene assay, multiple factors were upregulated, including VEGFA (vascular endothelial growth factor alpha), TGF-β1, MAPK3 (mitogen-activated protein kinase 3), PYCARD (human cell apoptosis-associated speck-like protein), and IL18 (interleukin 18), especially in the chronic asthma group (Fig. 2). The VEGF receptors FLT (Ims-related tyrosine kinase 1, VEFGR1) and KDR (kinase insert domain receptor, VEGFR2) did not
show any significant changes. There were no significant changes in TGF-β2 between the asthma groups and the control group.

**The Two Types of Asthma Mice Treated with Laser Showed More Fluorescein Leakage from Laser-Induced CNV and More CNV Formation on Histological Analysis than Mice without Asthma**

The results of our QuantiGene assay confirmed that the asthma mouse models treated with a laser exhibited VEGF upregulation. Thus, we conducted fluorescein angiography to determine whether mice with asthma were more likely to develop CNV. Each group consisted of 10 mice (10 laser-treated eyes). FA was performed 14 days after laser-induced CNV, and hyperfluorescence leakage was observed at the lesion sites in the mice with asthma. Moreover, the fluorescein leakage was more significant in the chronic asthma group. There was a total of 49 CNVs in the 10 mice with chronic asthma, while the number of CNV patches in the acute asthma group was 44 and in control group was 37 (Fig. 3).

Histopathology performed on hematoxylin and eosin stains paraffin cross-sections also confirmed that there was increased invasion in the retina around the laser scars in mice with acute and chronic asthma compared with mice in the control group. Each group contained 5 mice (5 laser-treated eyes). There were significant differences in the B/C ratio between the two asthma groups and the control group (Fig. 4). The above results suggested that the chronic mouse model was more likely to form CNV than the acute asthma group, which illustrated morphologically that more serious asthma conditions seemed to have a greater chance of developing CNV.

**TGF-β1 Overexpression in Asthma Groups and Western Blot Analysis of Pathway Molecules in the Two Asthma Groups and Control Group**

We investigated the possible mechanism underlying the observation that mice with asthma were more likely to develop CNV. Quantitative real-time PCR (QRT-PCR) and western blot analysis were performed to assess the respective mRNA and protein levels of TGF-β1, TGF-β2, and VEGF and the expression and phosphorylation of the signal transduction molecules Smad2/3, AKT, p38MAPK, ERK1/2 and their
phosphorylation in the acute asthma mice, the chronic asthma mice and the control mice. β-Actin served as an internal loading control. Five mice (5 laser-treated eyes) in the control, acute asthma and chronic asthma groups were involved in the QRT-PCR analysis. Six mice (6 laser-treated eyes) in each group were involved in the western blot analysis. Our results showed that TGF-β1 and VEGF expression levels were significantly increased in the retinal and choroid tissue of mice with asthma compared with control mice; in particular, mice with chronic asthma expressed much more TGF-β1 and VEGF protein (Fig. 5). There was no significant difference in TGF-β2 expression among acute asthma mice, chronic asthma mice and control mice. The expression levels of proteins downstream of TGF-β1, Smad2/3 and P-Smad2/3 were significantly increased in the asthma groups (Fig. 6). However, other pathway molecules, such as AKT, p38 MAPK, and ERK1/2, and their phosphorylation status remained at the same levels in these three groups (Fig. 6). These results suggest that the TGF-β1/Smad signalling pathway might be involved in the overexpression of VEGF following laser-induced CNV in asthma.

**TGF-β1 Inhibitor Attenuated the Formation of Laser-induced CNV Promoted by Asthma**

To test whether a TGF-β1 inhibitor can suppress CNV development in the presence of asthma, SB431542 or saline was intraperitoneally injected on the same day of laser photocoagulation. FA and histopathology were performed to evaluate the possible effect. Ten mice (10 laser-treated eyes) in the control, inhibitor-treated acute asthma and inhibitor-treated chronic asthma groups were assessed in the FA analysis. Five mice (laser-treated eyes) in each group were assessed in the histological analysis. In the SN431542-treated groups, regardless of the acute asthma or chronic asthma group, the leakage was significantly reduced compared with that in the control group (Fig. 7). There was a total of 24 CNVs in the inhibitor-treated acute asthma group, 26 in the inhibitor-treated chronic asthma group, and 37 in the control group. Histopathological results showed that the CNV area in eyes treated with SB431542 was significantly less than that seen in saline-treated control eyes (Fig. 8), which indicated that SB431542 could inhibit the formation of laser-induced CNV in mice by
means of intraperitoneal injection to control asthma. However, the inhibition did not appear to be different between the two types of asthma groups.

**VEGF and TGF-β1/Smad Signalling Pathway Were Down-regulated in Retinal and Choroidal Tissue from SB431542-Treated Asthma Group Mice**

We observed that CNV formation was attenuated by the TGF-β1 inhibitor SB431542. To further investigate the possible mechanism underlying our results, quantitative real-time PCR and western blotting were conducted to evaluate the respective mRNA and protein levels of VEGF and TGF-β1 and the expression and phosphorylation of the signal transduction molecules Smad 2/3, AKT, p38 MAPK, and ERK1/2, with β-actin serving as the internal loading control. Five mice (5 laser-treated eyes) in each group (control group, acute asthma group, chronic asthma group, inhibitor-treated acute asthma group, inhibitor-treated chronic asthma group) were assessed in the QRT-PCR analysis. Six mice (6 laser-treated eyes) in each group were assessed in the western blot analysis. We found that TGF-β1 expression was downregulated in the retinal and choroidal tissue from mice in the two inhibitor-treated asthma groups and that the low TGF-β1 expression in CNV induced downregulation of VEGF (Fig. 9). The expression and phosphorylation of the signalling proteins Smad 2/3, AKT, p38 MAPK, and ERK1/2 were detected, and only the Smad 2/3 and p-Smad2/3 levels decreased after inhibitor application, whereas AKT, p38 MAPK, and ERK1/2 expression and phosphorylation remained at nearly the same levels among the groups (Fig. 10).

**Discussion**

CNV is a complicated process that develops in several retinal diseases, such as AMD, pathological myopia, and traumatic choroidal laceration[19]. The mechanisms underlying the formation and progression of pathogenetic CNV have not yet been fully elucidated. Experimental and clinical studies have revealed that a dynamic balance between positive and negative regulators is a key step[20]. Several angiogenic factors, such as VEGF, platelet-derived growth factor (PDGF), angiopoietin, stromal derived factor (SDF)-1, b-fibroblast growth factor (FGF),
pigment epithelium-derived factor (PEDF) and thrombospondin-1, have been confirmed to be positive regulators, whereas the negative regulators of CNV include pigment epithelium-derived factor (PEDF), tissue inhibitors of matrix metalloproteinases (TIMPs), and endostatin[21, 22]. Among positive regulators, vascular endothelial growth factor-A (VEGF-A) plays a central role in promoting CNV, and intravitreal injection of anti-VEGF-A agents is the current treatment of choice for nAMD. Unfortunately, most patients require frequent, repeated injections and regular long-term follow-up, and a significant percentage of patients show resistance to anti-VEGF-A therapies[23]. Thus, there is a need for investigation of alternative targets and intervention strategies in addition to VEGF and inflammatory cytokines. Among all these factors, TGF-β1 is a potential target for treating CNV[15].

Similar to CNV, although advances have been made in treatment and therapeutic guidelines over the past decades, asthma remains a worldwide health problem, with a high burden of disability and health costs[24]. Airway remodelling is a multifaced process that encompasses several structural changes in the airway wall, including basement membrane thickening, extracellular matrix deposition, and airway smooth muscle cell hypertrophy and hyperplasia[25, 26]. TGF-β1, a key mediator of fibrotic responses, is increased in asthma and drives airway remodelling by inducing the expression of ECM proteins. Furthermore, TGF-β1 secretion is related to increased ECM protein production, leading to enhanced proliferation[27]. Previous studies have demonstrated that allergic airway inflammation can be prevented or ameliorated by suppressing the TGF-β1/Smad signalling pathway[28-31]. Moreover, TGF-β1 has been confirmed to modulate the expression of various growth factors, such as VEGF[32].

Currently, the controversy regarding the relationship between CNV and asthma is fierce. The results reported in different studies are quite distinct. Shalev et al. found that a history of asthma was a risk factor for CNV[33]. However, Jie Jin Wang and Ronald Klein were unable to replicate Shalev’s findings[34, 8]. A previous study reported that C3 may play a fundamental role in CNV associated with asthma and suggested that systemic inflammation was the leading cause of this relationship[10]. Melgert et al. found that increased M2 macrophage polarization, which leads to
release of greater amounts of TGF-β, and activation was present in asthma and suggested that it plays an important role in allergic asthma[35]. Similarly, Apte et al. confirmed that choroidal neovascularization develops following laser treatment of the posterior pole of murine eyes and is accompanied by massive macrophage infiltration [36]. Furthermore, in old mice, Long V et al. found that M2 macrophages play a key role in intraocular tumour development, similar to their role in choroidal angiogenesis[37]. Thus, given the common immune-inflammatory property and the special function of TGF-β1 in both asthma and CNV, we established animal models of asthma and CNV, in which we tested and verified that TGF-β1 in the retina/choroid complex was much more highly expressed in asthmatic mice than in control mice, demonstrating a relationship between asthma and CNV. We observed that local expression of VEGF in the retina/choroid complex in asthmatic groups, especially in the chronic asthmatic group, was upregulated compared with that in the non-asthmatic group. Administration of the TGF-β1 inhibitor in the asthmatic groups at the onset of angiogenesis potently attenuated CNV lesion size, which was associated with the levels of Smad2/3 phosphorylation and VEGF expression. Using the mixed animal model, our study uncovered for the first time the important function of the TGF-β1/Smad signalling pathway in the development of CNV in an asthmatic animal model. Meanwhile, our results suggest that TGF-β1/Smad signalling pathway inhibition may have potential as a novel therapeutic for nAMD complicated with asthma, which differs from the current treatment for CNV lesions.

TGF-β has been identified as a powerful pleiotropic growth factor that regulates cell growth, differentiation, apoptosis, and motility; extracellular matrix production; cellular immune responses during angiogenesis; wound healing; joint inflammation; tumour growth and metastasis; and immunoregulation[38-40]. The TGF-β superfamily, which includes TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3), activins, inhibins, growth factors, and differentiation factors, was reported to be involved in the initiation, maintenance, and resolution of inflammatory responses[41, 42]. The role of TGF-β in the pathogenesis of nAMD remains somewhat controversial[43]. Both pro- and antiangiogenic activities have been suggested for TGF-β signalling in the development and progression of nAMD[32]. Immunofluorescence staining and
western blot analysis demonstrated that TGF-β1 protein expression is upregulated during LI-CNV development in mice[44, 4], and both TGF-β1 and TGF-β2 mRNA levels (the latter being expressed more prominently) were upregulated after laser treatment, especially in the endothelium of neovascular regions[42]. Furthermore, VEGF-A expression and CNV formation in the RPEBC complex was found to be reduced after intraperitoneal injection of LY2157299 (an ALK5 and TβRII inhibitory compound) or intravitreal injection of decorin (a TGF-β binding and inhibitory protein)[4]. However, there is evidence of an antiangiogenic function of TGF-β in nAMD. Andreas Ohlmann confirmed that ocular overexpression of active TGF-β1 induced atrophy of the choriocapillaris without any sign of CNV in a transgenic mouse model[45]. Anca Sindrilaru reported that induced conditional deletion of TβRII in the entire eye or in the vascular endothelium of the eye, but not in RPE, caused increased retinal expression of VEGF-A, CNV development, and induction of other phenotypic characteristics of nAMD[46]. In a rat model mimicking early AMD stages, intravitreal injection of human recombinant TGF-β1 prevented retinal insult induced by intravitreal injection of amyloid-beta 1–40 fragments, a constituent of drusen[47]. However, for asthma, a previous study found that TGF-β1, which is highly expressed in lung tissue, was significantly higher in serum from asthma patients than in serum from normal people[48]. In addition, the elevated level of serum TGF-β1 was consistent with the severity of asthma[49]. This may be an explanation of why TGF-β1 rather than TGF-β2 was notably upregulated in asthma groups in our study. Additionally, we found that high TGF-β1 expression upregulated the expression of VEGF in RPE of the mouse CNV model with acute and chronic asthma compared with the simple CNV model, which could explain the relationship between the two diseases and the possible mechanism. Thus, our findings provide additional evidence for TGF-β1-mediated promotion of CNV development. Monitoring the level of ocular TGF-β1 in patients suffering from both nAMD and asthma might have a substantial clinical impact and offer a potential therapeutic target.

Downstream TGF-β signalling occurs through canonical and noncanonical pathways. The canonical pathway, which is also called the Smad pathway, involves three signalling components (type I (RI), type II (RII), and type III (RIII)). After RIII
binds to TGF-β, RII is recruited and then phosphorylates RI. Subsequently, RI phosphorylates Smad2 and Smad3, which form a heteromeric complex with Smad4. This complex translocates to the nucleus, binds to DNA, and regulates the transcription of many genes[13]. TGF-β receptors also signal through multiple noncanonical pathways, including the JNK/p38 MAPK, ERK1/2, and PI3K/Akt pathways[46]. Thus, we further explored the possible pathways through which TGF-β promotes neovascularization in the setting of asthma. Our current results showed that the TGF-β1/Smad pathway functions as an angiogenesis promoter in CNV development and that injection of the TGF-β inhibitor SB431542 resulted in significant inhibition of phosphorylated Smad 2/3. SB431542 is a TGF-β1 receptor kinase inhibitor that interrupts activation of downstream signalling pathways. SB431542 has previously been reported by Tanaka et al. to reduce an in vivo antitumour immune response associated with TGF-β activity[50]. Ke et al. revealed that SB431542 could reverse the promotive effect of CSE on the invasiveness of lung cancer cells by inhibiting the activity of TGF-β1/Smad2 pathways and decreasing MMP3 expression[51]. Our findings showed that the suppression of phosphorylated Smad 2/3 was consistent with the significant decrease in the protein expression levels of proangiogenic molecules, including TGF-β1 and VEGF. This supports the hypothesis that SB431542 suppresses CNV development in asthmatic patients via the TGF-β1/Smad pathway and indicates that the possible effect of TGF-β is more likely to involve TGF-β1 activity rather than TGF-β2 activity, which stimulates VEGF overexpression by the RPE in asthmatic mice.

In summary, we verified that asthma could promote progression of CNV lesions in a laser-induced mouse model though the TGF-β1/Smad pathway. The TGF-β1-specific inhibitor SB431542 may offer a useful therapeutic strategy for preventing CNV, which is associated with downregulation of VEGF, TGF-β1 and phosphorylated Smad 2/3. However, we did not investigate whether asthmatic mice without laser treatment already differed in the evaluated factors, and there are no results on this aspect in previous studies. Accordingly, the mechanisms that underlie the promotive effects of asthma in CNV progression need to be explored in more detail. Although distinct mechanisms have been proposed in different studies, based on the present study, we
have good reason to believe that the future of CNV management in patients with asthma may require combined therapies with multiple drugs acting on different mediators, such as VEGF, the complement system, and TGF-β1. Anti-TGF-β1 inhibitors should be given more attention in CNV treatment.

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Statement of Ethics

All animal experiments were performed in strict accordance with the Association for Research in Vision and Ophthalmic and Vision Resolution (ARVO) for the Use of Animals in Ophthalmic and Vision Research and to the guidelines provided by the Animal Care Use Committee of Peking University (Beijing, China).

Disclosure Statement

The authors declare no competing interests.

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Author Contributions

Conceived and designed the experiments: F.Y., Y.S., L.H., and X.L. Performed the experiments: F.Y. and Y.S. Analyzed the data: F.Y., Y.S., Y.B., S.L., and L.H. Contributed reagents/materials/analysis tools: F.Y., Y.S., Y.B., S.L., H.X. Wrote the paper: F.Y., Y.S., L.H., and X.L.
References


Figure Legends

Fig. 1. Histopathological changes in pulmonary tissues from mice in control, acute asthma and chronic asthma groups (200X). a: Control group; b: acute asthma group; c: chronic asthma group; d: results and statistical analysis of inflammatory scores in each group. Lungs in the asthma groups showed significantly increased inflammatory cell infiltration compared with those in the control group. The chronic asthma group demonstrated more severe pulmonary inflammation than the acute asthma group. The data are presented as the means ± SEM. Ten mice were analysed in each group. **p<0.01, ***p<0.001 vs control group; ##p<0.01 vs acute asthma group.

Fig. 2. mRNA expression of angiogenic cytokines, VEGF receptors and inflammasomes in the control, acute asthma and chronic asthma groups. A QuantiGene assay was used to determine the mRNA levels in laser-treated retina-choroid complexes with or without asthma. Fold-changes were determined by the relative ratios of (a) TGF-β1, VEGF, TGF-β2, MAPK1, MAPK3, and MAPK8; (b) PGF, KDR, FLT1, HGF, HIF1A, and CXCL12; (c) IL1beta, IL18, IL18BP, and IL33; and (d) PYCARD, CASP1, CARD6, IFNG, NOD2, NLRP3, and NLRP1A between the normalized values of the two types of asthma groups and the control group. Three mice (3 laser-treated eyes) were analysed in each group. Values are expressed as the means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Fig. 3. FA assessment of CNV leakage. Angiographic analysis of CNV leakage 14 days after laser photocoagulation in mice in the acute asthma, chronic asthma and control groups. a: Control group; b: acute asthma group; c: chronic asthma group; d: results and statistical analysis of the CNV leakage determined by FA. The data are presented as the means ± SEM. Each group consisted of 10 mice (10 laser-treated eyes). **p<0.01 vs control group; #p<0.05 vs acute asthma group.

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