Ferulic acid attenuates microglia-mediated neuroinflammation in retinal degeneration

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Abstract

Background: To use a rd10 mouse model as a means of exploring the impact of Ferulic acid (FA) on microglia-mediated neuroinflammation as well as associated retinal degeneration.

Methods: Rd10 mice received different concentrations of FA treatment every day from postnatal day (P)4 to P24. At P25, mice visual function were detected by electroretinogram, then retinae were collected for further investigation. Retinal microglia activation state and relevant cytokines were evaluated by qPCR, Western blot and immunofluorescent staining. The retinal structure was assessed by HE Staining.

Results: 50mg/Kg FA supplement exhibited optimal protection against retinal degeneration, with treated mice exhibiting more photoreceptor nuclei as well as significant wave amplitude amplification in electroretinograms. FA suppressed microglia activation both in vivo and in vitro, inhibited pro-inflammatory factors Tnfα, IL1β, Ccl2 expression in rd10 retinae. Furthermore, FA suppressed the activation of STAT1 and subsequently IRF8 expression, potentially highlighting a role for these pathways in FA-mediated immunomodulatory activity.

Conclusions: Attenuating neuroinflammation by FA may be beneficial to retard retinal degeneration.

Background

Retinal degeneration diseases are conditions wherein progressive neuronal loss is responsible for symptom onset, with the most common form being retinitis pigmentosa (RP) as it is the leading cause of inherited retinal degeneration-associated blindness, affecting approximately 1.5 million people worldwide[1]. With the advent of next generation sequencing and recent advances in gene therapy, the identified mutant genes responsible for RP show signs of a growth spurt[2]. Gene therapy for RP is limited by the heterogeneous genetic basis for this disease, highlighting the importance of developing therapies that function independently of mutation status[3]. The drugs targeting the broad pathological processes which are common to all kinds of RP with various mutations may work in this complex disorder[4].

Neuroinflammation is now widely recognized to participate in many chronic neurodegenerative diseases, including multiple sclerosis, Alzheimer’s disease, and Parkinson’s disease, and attenuating
neuroinflammation is an effective therapeutic approach for these diseases[5]. There is increasing evidence that microglia, the resident immune cells in retina, would induce immune responses and create a chronic inflammatory environment in retina of RP patients[6]. The anterior vitreous cavity of RP patients was found to contain many inflammatory cells, and the levels of various pro-inflammatory chemokines and cytokines were up-regulation in the aqueous humor and vitreous fluid of individuals with this disease[7], suggesting a role for such inflammation in the process of retinal degeneration. The microglia-initiated neuroinflammation is thus considered to be primary hallmark of RP.

Ferulic acid (FA), phenol compound present in the wall of plants, is a major active ingredient in some traditional Chinese medicine(TCM), such as ferula asafetida, angelica, ligusticum wallichii, which always used to improve microcirculation of ischemic diseases in TCM prescription[8, 9]. Nowadays, a growing body of research demonstrated that FA suppressed detrimental immunoreactions under various conditions. It is currently available and has a good application prospect for treating Alzheimer disease(AD), based on its potent immunomodulatory property[10, 11]. In an ovalbumin-driven model of respiratory allergies, FA administration was shown to dampen Th2-mediated immunity[12]. These research results indicated that FA may be a novel immunosuppressive agent. As microglia-mediated neuroinflammation is believed to be one of the key processes responsible for neurodegeneration in RP and related diseases, immunosuppression targeting microglia is promising for treating these diseases. We therefore speculated that FA may be capable of slowing or arresting the retinal degeneration process owing to its ability to suppress microglia-mediated neuroinflammation.

In our study, the property of FA immunomodulatory on the pathological process of retinal degeneration was carried out in the rd10 murine RP model system. The results showed that FA suppressed microglia transforming into a reactive phenotype and rescued the degenerative retina in rd10 mice. The underlying mechanism may be that FA suppressed the expression of interferon regulatory factor 8(IRE8), a key factor promoting microglia activation, thus reduced the production of inflammatory cytokines.

Methods

**Rd10 Mice and FA Treatment**
Rd10 mice (The Jackson Laboratory) were housed in a specific pathogen-free facility in Animal Laboratories of Yantai Yuhuangding Hospital. The ARVO Statement for Use of Animals in Ophthalmic and Vision Research was adhered to for all animal studies. For treatment, rd10 animals received intragastric administration of 25mg/kg, 50mg/kg and 100mg/kg FA (1270311, sigma) every day from postnatal day (P)4 to P24. Animals were sacrificed via sodium pentobarbital injection (200mg/kg; i.p.) at P25 and eyeballs were enucleated for further investigation.

**Electroretinogram (ERG) Recordings**

Before the ERG recordings, mice received dark adaptation overnight. Mice were anesthetized with intraperitoneal pentobarbital sodium (50mg/kg) prior to pupil dilation using 1% tropicamide. ERGs were recorded by a Ganzfeld stimulator (Roland Consult, Germany) that generated and controlled the light stimuli. Scotopic ERG were recorded with a single flash of 1.3ms duration with intensity of -1.52, -0.52, 0.48 and 1.0 log cd s/m². A total of 5 responses per intensity were averaged together for each flash stimulus. Intraperitoneal sodium pentobarbital (200mg/kg) was then used to sacrifice mice. Amplitudes of the major ERG components (a- and b-wave) were measured (RETI system software) using automated and manual methods.

**Hematoxylin & Eosin Staining**

Eyeballs were fixed in formalin overnight prior to paraffin embedding and 3μm section preparation. Before staining, xylene was used for deparaffinization, and sections were rehydrated using an ethanol gradient prior to PBS washing. And then stained with hematoxylin and eosin. A microscope (Leica DM4000, Germany) was used to analyze retinal histology and to count outer nuclear layer nuclei.

**Cell Culture and FA Treatment**

BV2 murine microglial cells (Kunming Institute of Zoology) were cultured as previously described. In brief, cells were maintained in DMEM (High glucose) containing 10% FBS and penicillin/streptomycin. FA (1 mg/mL in PBS) was used to pretreat microglia for 2 h and then stimulated with LPS (50ng/ml; L6529, Sigma). After 24 h, cells were collected for downstream analyses.

**Immunofluorescence Staining**

For retinal wholemounts, eyes were immersed in 4% paraformaldehyde (PFA) fixative for 30 minutes,
retinal cups were separated carefully from eyeballs. Both retinal wholemounts and cell slides were
stained using primary and secondary antibodies, washed extensively and retina flat-mounted. Primary
antibodies: anti-iba1 (019-19471, Wako Chemicals), anti-iNOS (sc-7271, Santa Cruz). Secondary
antibodies: donkey anti-rabbit Alexa Fluor 488IgG H&L, donkey anti-goat Alexa Fluor 555IgG H&L. The
retinal wholemounts and cell slides were visualized via confocal microscopy (Carl Zeiss LSM710,
German).

**RNA Sequence**

RNA was isolated from retinæ using TRizol Reagent (Invitrogen), and a Bioanalyzer 2100(Agilent) was
used to gauge the quality of the resultant nucleic acid. RNA preparation, library construction, and
sequenceing was conducted using a BGISEQ-500 instrument at the Beijing Genomics Institute (BGI,
Shenzhen, China).

**RT-PCR**

Total RNA from rd10 retina were isolated using the RNAiso Plus kit (TAKARA Bio inc, Japan), and the
Reverse Transcriptase Superscript II Kit (TAKARA Bio inc, Japan) was then used to prepare cDNA
following the instructions. Real time PCR was performed in 20μL reaction system, containing10μL of
2×SYBR Premix Ex Taq, 2μL of cDNA, and 10μmol/L of the primer pairs. Thermocycler settings were:
95°C for 30s; 40 cycles of 95°C for 5s, 60°C for 34s.

**Western Blot**

RIPA buffer (Biocolors, Shanghai, China) containing dissolved protease and phosphatase inhibitor mini
tablets (Thermo Fisher Scientific, MA, USA) was used to lyse homogenized retinal tissue. Samples
were then spun for 10 minutes at 10,000 rpm, after which a BCA assay was used for protein
quantification. Equivalent protein amounts were utilized for western blotting. The primary antibodies
were incubated overnight include anti-STAT1, anti-pSTAT1(14994S, 7649S, CST), anti-IRF8, β-actin
(ab28696, ab28696, Abcam, Cambridge, MA). After washed with PBST, the membranes were probed
with HRP-linked secondary antibodies (1:2000) for 1 h at room temperature.

**Statistics**

Each experiment including immunostaining, qPCR and western blotting was replicated 3 times. All
quantitative data was analyzed using 2-tailed Student t test or one-way ANOVA by SPSS 21.0. Data are means ± standard error of the mean (SEM). P<0.05 was the significance threshold.

Results

**FA Could Ameliorate Retinal Degeneration in Rd10 Mice.**

FA has been showed some efficacy in brain disorders and neurodegenerative diseases. As nervous tissue, retina is the continuation of brain, we thus evaluated the effect of FA on retinal degeneration in rd10 mice. Because the photoreceptor death peaking around P25[13], we treated the rd10 mice with different doses of FA intragastric administration(25mg/kg, 50mg/kg, and 100mg/kg) every day from P4 to P24. HE staining was performed on retinal sections to evaluate the retinal structure at P25. As shown in Fig.1B, after treatment with PBS, there was only one row in outer nuclear layer(ONL) of rd10 mice at P25. Notably, rd10 mice under 25mg/kg/d, 50mg/kg/d or 100mg/kg/d FA treatment showed significant protective effect, about two, four, three rows in ONL respectively(Fig.1CDE). We also calculated the number of nucleus in ONL, as shown in Fig.1F, the nuclei amount were 63.32±3.34, 95.20±3.53, 92.46±3.74 per 500μm in 25mg/kg, 50mg/kg, and 100mg/kg FA-treated group, while 47.23±3.34 in PBS-treated group. Together this shows that FA can prevent retinal degeneration in rd10 mice, and 50mg/kg/d may be optimal dose which we used for further investigation.

**FA Supplement Improved Retinal Function in Rd10 Mice.**

Next, we accessed the retinal function of rd10 mice by ERG at P25. Average b-wave amplitudes for each group of rd10 mice were analyzed at light intensities of -1.52, -0.52, 0.48 and 1.0 log cd s/m², respectively (Fig.2A). As shown in Fig.2B,b-wave amplitudes were 50.34±2.53μV, 85.65±4.54μV, 102.42±3.28μV, 164.44±3.17μV under a variety of scotopic testing conditions in the FA-treated group, while 14.52±1.43μV, 30.26±2.3μV, 52.45±1.08μV, 56.47±3.27μV in PBS-treated group, confirmed FA can protect retinal function in rd10 mice.

**FA Suppressed Microglia Activation and Retinal Inflammation in Rd10 Mice.**

Microglia are the primary form of retinal immune cell, and play a role in the degenerative process via regulating neuroinflammation. As expected, we noticed that Iba-1⁺ microglia activated in rd10 reti
at P25, just like an amoeba with swollen body and stubby branches, while ramified-resting microglia looking like an octopus with small body and dancing tentacles in C57 mice (Fig. 3A). After FA treatment, the number of IBA1+ positive microglia was significantly decreased in rd10 retinae at P25 (Fig. 3A). Naturally, FA suppressed the mRNA expression of chemokines and inflammatory cytokines, such as Tnfα, IL1β, Ccl2 by inhibiting microglia activation (Fig. 3B). These results suggested that FA could alleviate the microglia inflammatory response during retinal degeneration.

**FA Suppressed Microglia Activation in Cultured BV2 Cells under LPS Insults.**

To explore the effect of FA on microglia activation in vitro, BV2 cells were treated with LPS, which is a classical agonist to trigger microglia. Using immunofluorescence, we found a lot more iNOS+ (a inflammatory marker) BV2 cells in LPS treatment group, indicating the BV2 cells transforming into a reactive phenotype. After FA treatment, the number of iNOS+ BV2 cells was significantly decreased (Fig. 4). These data suggested that FA could suppress microglia activation in vitro.

**FA Modulated IRF8 Activation and Phosphorylation of STAT1 in Microglia in Rd10 Mice.**

Rd10 retina RNA-seq analysis was conducted to explore the underlying mechanism of FA suppressing microglia-mediated inflammation. IRF family, STAT1 family, PPAR, AP1, NFκb, HIF1α are accepted transcription factors which may regulating microglia polarization, IRF8 mRNA expression decreased most obviously in rd10 retina after FA treatment in this study (Fig. 5). We next measured IRF8 and STAT1 signaling via western bloting, revealing that IRF8 expression on protein level was significantly suppressed in the FA-treated rd10 retina. STAT1 phosphorylation was also reduced following FA treatment, and this could lead to reduced IRF8 expression (Fig. 6A). Furthermore, FA administration inhibited IRF8 expression and phosphorylation of STAT1 in LPS stimulation BV2 cells (Fig. 6B). FA may therefore act via regulating STAT1 activation and IRF8 expression.

**Discussion**

The progression of RP is largely driven by neuroinflammatory processes. Herein, we demonstrated the efficacy of FA, Chinese herbal monomer showing immunomodulatory potential. Specifically, we demonstrated in a murine rd10 model that FA was able to suppress neuroinflammation, thereby slowing the associated degenerative progression of this disease. Together, these findings highlight...
the potential value of using FA or similar immunomodulatory treatments as a means of slowing retinal degeneration in RP patients.

FA has been recognized as an important chemical structure serving several biological activities, including anti-inflammatory, antioxidant, antiviral, antiallergic, antimicrobial, antithrombotic, anticarcinogenic, and hepatoprotective actions, directly or indirectly[14]. Among these characteristics, there is growing attention that FA suppress inflammation through regulating immune response[15-17]. FA can modulate immune activity in many cell types. For example, Cho et al. found that sustained FA treatment of mice can suppress Aβ-induced astrocyte activation, thereby preventing the associated inflammatory cytokine and free radical production that can drive AD-associated inflammation[18]. FA treatment attenuated dextran-sulfate-sodium-induced colitis in the model mice and induced Treg differentiation[19]. FA can also suppress LPS-mediated IL-1β and IL-6 secretion from macrophages[20]. In this study, we demonstrated that FA could suppressed microglia activation and reduced the expression of IL-1β, IL-6 and CCL2 in rd10 mice.

Microglia are the only immune cell type that normally reside in the retina in significant numbers. In the retina of humans suffering from RP, rod apoptotic death is associated with a migration of these microglia from the inner to the outer retina. By P16 in the murine rd10 RP model system, the infiltration of activated microglia into the subretinal space can be detected even though the apoptotic death of photoreceptors is only evident at P19, indicating that these microglia themselves are capable of driving the apoptosis of these cells[21]. Microglia activation participate in inflammation by secreting inflammatory cytokines and chemokines accelerating photoreceptor apoptosis, including TNF-α, IL-1β, IL-6, CCL2 and so on[22]. Furthermore, these cells can also phagocytose both dead apoptotic and live stressed photoreceptor cells, aggravating retinal degeneration[23-25].

Activation-induced gene expression in microglia is tightly regulated by many transcription factors[26]. IRF family proteins are thought to be essential regulators of immune cell activation and responsiveness[27]. IRF8 is almost exclusively expressed in myeloid and lymphoid cells of the immune system, with retinal IRF8 being found only in microglia[28], which have features in common with cells of the myeloid lineage. Changes in retinal IRF8 levels are therefore thought to affect
changes in microglial activity and/or infiltration. IRF8 is known to regulate the expression of IFN-β, IL-12, iNOS, and related genes [29, 30]. In this study, high IRF8 was observed in rd10 retina. In vitro, we found lipopolysaccharide insults induced a marked elevation of IRF8 in BV2 cells, associating with more iNOS+ microglia, suggesting a key role for IRF8 in microglia activation. Strikingly, FA reduced IRF8 and p-STAT1 levels, supporting the concept that FA suppressed microglia activation partly through regulating IRF8 expression. Additional study of the mechanistic basis for microglial activation and the regulatory role of FA in this process has the potential to offer further insight into the process and prevention of neuroinflammation, in addition to increasing the therapeutic utility of FA as an agent used for treating neurodegenerative diseases.

Conclusion

In conclusion, our findings herein suggest that treatment with FA is sufficient to suppress microglial activation in a murine model of RP, thereby markedly attenuating associated neurodegeneration and associated disease progression. At a mechanistic level, the efficacy of FA seems to be at least partially linked to its ability to suppress STAT1 activation and IRF8 expression. These results thus highlight the immunomodulatory and anti-inflammatory properties of FA, suggesting that it or derivatives thereof may have value as a means of treating patients suffering from retinal degeneration.

List Of Abbreviations

RP: Retinitis pigmentosa; FA: Ferulic acid; IRF8: interferon regulatory factor 8; TCM: Chinese medicine

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the animal ethical committee of the YantaiYuhuangding Hospital, and performed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals.

Consent for Publication

Not applicable.

Availability of data and material

The datasets used and analyzed during the current study available from the corresponding author on
reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

XWS, PS and YBL contributed to study design, data analysis. XWS and LML prepared manuscript. XWS, PS and PFS performed experiments. All authors have read and approved the submission of this manuscript.

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Figures
Figure 1

Histologic analysis of retinal thickness after FA treatment. A. Full view of the retinal mid-peripheral area following H&E staining. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. B-E. FA-treated rd10 mice exhibited increases in the thickness of the ONL (scale bar, 50μm). F. Statistical analysis of nuclei amount in the ONL revealed that FA supplement delayed the retinal degeneration in rd10 mice (***p<0.001, n=6 eyes).
FA preserves retinal function in rd10 mice. A. At P25, single-flash ERG recordings at -1.52, -0.52, 0.48 and 1.0 log cd s/m² light intensities were used to measure murine retinal functionality. Compared with PBS-treated rd10 mice, FA-treated mice showed significantly amplifying waves under multiple different testing conditions. B. Statistical analysis of b waves in PBS- and FA-treated rd10 mice (**p<0.001, n=9 mice).
FA suppressed microglia activation and inflammation in the context of retinal degeneration.

A. In retinal whole mounts, numbers of iba1+ activated microglia were significantly decreased in FA-treated group (***p<0.001, n=6 eyes; scale bar, 50μm). B. The mRNA level of TNFα, IL1β, CCL2 were suppressed after FA treatment (**p<0.01, ***p<0.001, n=6 eyes).
FA suppressed LPS-induced microglia activation in vitro. Immuno-fluorescence staining showed numerous iNOS+ microglia in the cultured BV2 cells under LPS stimulation, while no microglia activation in PBS-control. After FA treatment, the number of iNOS+ activated microglia reduced obviously (Scale bar, 40μm).
Figure 5

Rd10 retina RNA-seq analysis. After FA administration, many transcription factors showed differential expression in rd10 retina, mRNA expression fold change=$\log_2(rd10+FA/rd10)$ (n=3).

Figure 6

FA modulated STAT1 phosphorylation and IRF8 activation a in vivo and vitro. FA-treated retina from rd10 mice exhibited reduced STAT1 signaling and IRF8 expression by Western blotting. In vitro, LPS up-regulated the expression of IRF8 and STAT1 phosphorylation in BV2 cells, while FA treatment reduced both STAT1 activation and IRF8 expression levels in these cells.
Supplementary Files

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