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Interleukin-4 and melatonin ameliorate high glucose and interleukin-1β stimulated inflammatory reaction in human retinal endothelial cells and retinal pigment epithelial cells

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Purpose: We aimed to evaluate the effects of two immune regulatory factors, interleukin-4 (IL-4) and melatonin, on several inflammatory mediators that are involved in inflammation and angiogenesis in diabetic retinopathy (DR), in high glucose or interleukin-1β (IL-1β) induced primary human retinal endothelial cells (RECs) and human retinal pigment epithelial (RPE) cells.

Methods: Human RECs and RPE cells were cultured in 30 mM D-glucose or 10 ng/ml IL-1β, with or without the presence of 40 ng/ml IL-4 or 100 μM melatonin. The mRNA and protein levels of vascular endothelial growth factor (VEGF), intercellular cell adhesion molecule-1 (ICAM-1), matrix metalloproteinases 2 (MMP2), and matrix metalloproteinases 9 (MMP9) were measured using real-time PCR and enzyme-linked immunosorbent assay (ELISA), respectively.

Results: High glucose and IL-1β induced the expression of VEGF, ICAM-1, MMP2, and MMP9 in human RECs and RPE cells. IL-4 and melatonin downregulated the expression of VEGF, ICAM-1, MMP2, and MMP9 induced by high glucose and IL-1β.

Conclusions: Our results demonstrated that IL-4 and melatonin inhibited inflammation and angiogenesis triggered by high glucose and IL-1β, which suggests that these immune regulatory factors may be of potential therapeutic value in DR.

The balance between anti-inflammatory and inflammatory factors is disrupted in diabetes, which leads to overexpression of vascular endothelial growth factor (VEGF), inflammatory mediator recruitment and aberrant chronic inflammatory responses in the retina. Consequently, aberrant inflammatory responses in the retina cause increased vascular permeability, capillary non-perfusion, neurodegeneration and neovascularization, which are characteristics of diabetic retinopathy (DR) [1]. Inhibition of the molecules involved in these processes can reverse the development of DR.

Interleukin-4 (IL-4), a member of the interleukin family originally identified in the immune system, is an anti-inflammatory cytokine that is typically associated with regulating inflammation in the brain [2]. IL-4 has also been considered an anti-angiogenesis factor especially in the inflammatory milieu [3]. In addition, we have previously observed that IL-4 was differentially expressed in diabetic patients with DR and without based on a cDNA microarray [4]. We speculate that IL-4 serves as a protective factor of DR due to the potential anti-inflammation and anti-angiogenesis function. Melatonin regulates the expression of cytokines in immunocompetent cells in the immune system. Experimental and clinical data have demonstrated that melatonin can improve the clinical course of inflammation-related diseases by inhibiting the expression of adhesion molecules and proinflammatory cytokines [5]. Several other studies have also suggested melatonin and IL-4 interact closely [6-8].

Hyperglycemia is one of the most important initiators in DR [1]. As a major proinflammatory cytokine secreted by lymphocytes and macrophages, interleukin-1β (IL-1β) can trigger and amplify inflammation during DR progression [9]. In this study, we simulated diabetic circumstances with D-glucose and IL-1β supplements to investigate the effects of IL-4 and melatonin on several inflammatory mediators in human retinal endothelial cells (RECs) and retinal pigment epithelial (RPE) cells that consist of the retinal inner barrier and outer barrier, respectively. Our study aimed to evaluate the potential protective effects of IL-4 and melatonin on the retina under diabetic conditions in vitro.

METHODS

Cell culture: All experiments were conducted according to the tenets of the Declaration of Helsinki for research involving human subjects and approved by the Ethics Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University,

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Table 1. Primers used in gene expression analysis.

Gene (human)	Forward primers	Reverse primers	Product length (bp)
VEGF	AAGGAGGAGGGCAGAATCAT	ATCTGCATGGTGATGTTGGA	226
ICAM-1	CAGAGGTTGAACCCCACAGT	CCTCTGGCTTCGTCAGAATC	196
MMP2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC	112
MMP9	TTGACAGCGACAAGAAGTGG	GCCATTCACGTCGTCCTTAT	179
β-actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	250

Abbreviations: vascular endothelial growth factor (VEGF), intercellular cell adhesion molecule-1 (ICAM-1), matrix metalloproteinases 2 (MMP2), matrix metalloproteinases 9 (MMP9).

Guangzhou, China. Primary cultured human RECs and RPE cells were prepared from eyes (obtained from the Zhongshan Ophthalmic Center Eye Bank) as previously described [10,11]. Briefly, the eyes were cut circumferentially 3 mm posterior to the limbus and retinas were harvested. Retinas were then minced gently, digested in 2% trypsin for 20 min followed by 0.1% collagenase for 20 min at 37 °C. The homogenate was subjected to centrifugation and the pellet was resuspended and grown in fibronectin-coated flasks and maintained in human endothelial-serum free medium (HE-SFM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 5 ng/ml recombinant human β-endothelial cell growth factor (β-ECGF; R&D Systems, Minneapolis, MN), and 1% insulin-transferrin-seleniumin (ITS; Gibco). After the vitreous and the retina were removed, the RPE cells were mechanically harvested, separated by digestion with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) and then maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin sulfate (100 mg/ml) and amphotericin B (2.5 mg/ml; Gibco) and were characterized by the typical hexagon shape with epitheloid morphology and pigment granules. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. RECs at passages 3–5 and RPE cells from passages 6-8 were used in all experiments.

Cell treatments: Cells were seeded in six-well plates. After the cells reached subconfluence, the medium was replaced with HE-SFM or DMEM that contained 1% serum and the cells were incubated for 24 h for synchronization. Cells were then incubated in 30 mM D-glucose (for 48 h) or 10 ng/ml IL-1β (for 24 h) with or without the presence of 40 ng/ml human recombinant IL-4 or 100 μM melatonin. At the end of the incubation, the cells were processed for the following PCR and enzyme-linked immunosorbent assay (ELISA) analysis. The experiment was repeated at least three times.

Evaluation of gene expression using quantitative real-time PCR: Total RNA was extracted from cultured RECs and RPE cells using TRIzol (Invitrogen Life Technologies, Grand Island, NY) and was reverse transcribed with the TaKaRa First Strand Synthesis kit (TaKaRa, Dalian, China). Quantitative real-time PCR (qPCR) was conducted on an ABI Prism 7000 system with a SYBR Green PCR kit (TaKaRa) by denaturing at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C, annealing at 60 °C, and extension at 72 °C for 10 s, respectively. One μg of each RNA and 2 μl of each cDNA were used to measure the expression of the target genes. The primers used are summarized in Table 1.

Evaluation of protein levels using ELISA: At the end of the incubation, supernatants of the cells were harvested to analyze cytokine secretions using a double monoclonal antibodies sandwich ELISA assay kit. Human VEGF, intercellular adhesion molecule 1 (ICAM-1) and matrix metalloproteinases 2 (MMP2) ELISA kits were purchased from ExCellBiology (Shanghai, China), and a human matrix metalloproteinases 9 (MMP9) ELISA kit was purchased from NeoBioScience (Shenzhen, China).

Statistical analysis: Results are presented as mean±standard deviation (SD). The two-tailed Student *t* test was used to compare differences between two groups. $p<0.05$ was considered statistically significant.

RESULTS

IL-4 and melatonin downregulated the expression of VEGF in diabetic condition: The mRNA and protein levels of VEGF in the human RECs and RPE cells incubated with 30 mM D-glucose (for 48 h) or 10 ng/ml IL-1β (for 24 h) were up-regulated two- to four-folds. To evaluate the potential ability of IL-4 and melatonin to inhibit VEGF-related pathological processes including inflammation, blood–retinal barrier (BRB) breakdown and angiogenesis in DR, we added 40 ng/ml human recombinant IL-4 or 100 μM melatonin to the culture medium to test whether they could regulate VEGF

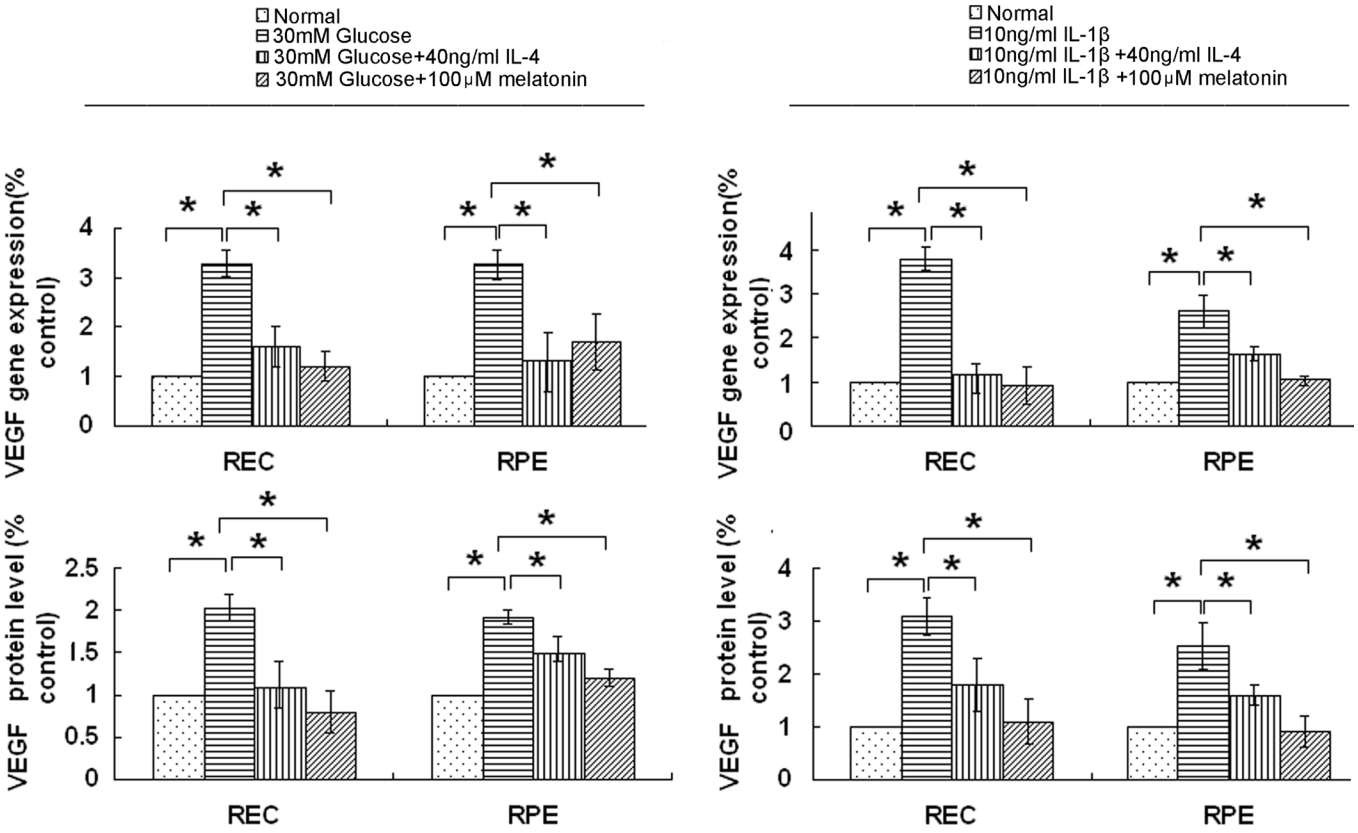

Figure 1. Interleukin-4 (IL-4) and melatonin downregulated the expression of vascular endothelial growth factor (VEGF). Human retinal endothelial cells (RECs) and retinal pigment epithelial (RPE) cells were cultured to a state of subconfluency and then maintained in human endothelial-serum free medium (HE-SFM) or Dulbecco's Modified Eagle Medium (DMEM) that contained 1% serum for 24 h for synchronization. The cells were then exposed to D-glucose (30 mM; 48 h incubation) or interleukin-1β (IL-1β; 10 ng/ml; 24 h incubation) in the presence or absence of IL-4 (40 ng/ml) or melatonin (100 μM). Total RNA was extracted, and the supernatants were harvested. VEGF expression was analyzed using quantitative real-time PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The data are expressed as the mean±standard deviation (SD; n = 4; *p<0.05 versus the control group).

expression. The real-time quantitative PCR and ELISA results demonstrated that the VEGF expression induced by D-glucose or IL-1β was significantly suppressed by IL-4 and melatonin at the mRNA and protein levels (Figure 1).

The expression of ICAM-1 was upregulated under high glucose and IL-1β stimulation but significantly inhibited by IL-4 and melatonin: ICAM-1 is an adhesion molecule and increased expression of ICAM-1 in the serum and vitreous humor of patients and animals with DR has been observed. We first tested the expression of ICAM-1 in RECs and RPE cells incubated with D-glucose and IL-1β in vitro. After the cells were treated with 30 mM D-glucose (for 48 h) or 10 ng/ml IL-1β (for 24 h), the mRNA and protein levels of ICAM-1 expression were significantly higher than those in the control cells. We then added IL-4 or melatonin to the media to investigate the effects on the expression of ICAM-1. We found that the mRNA and protein levels of ICAM-1 induced by high glucose or IL-1β in both types of cells were substantially suppressed by IL-4 and melatonin treatment (Figure 2).

IL-4 and melatonin downregulated the expression of MMP2 and MMP9 induced by high glucose or IL-1β: The expression of MMP2 and MMP9 was induced by high glucose or IL-1β in the RECs and RPE cells in our study. Since MMPs play critical roles in inflammation, extracellular matrix remodeling and proliferative membrane formation in DR, we further investigated whether the MMP2 and MMP9 expression induced by high glucose or IL-1β could be ameliorated by IL-4 and melatonin. Our results demonstrated that IL-4 and melatonin significantly reduced the mRNA and protein levels of MMP2 and MMP9 that were upregulated by D-glucose or IL-1β (Figure 3).

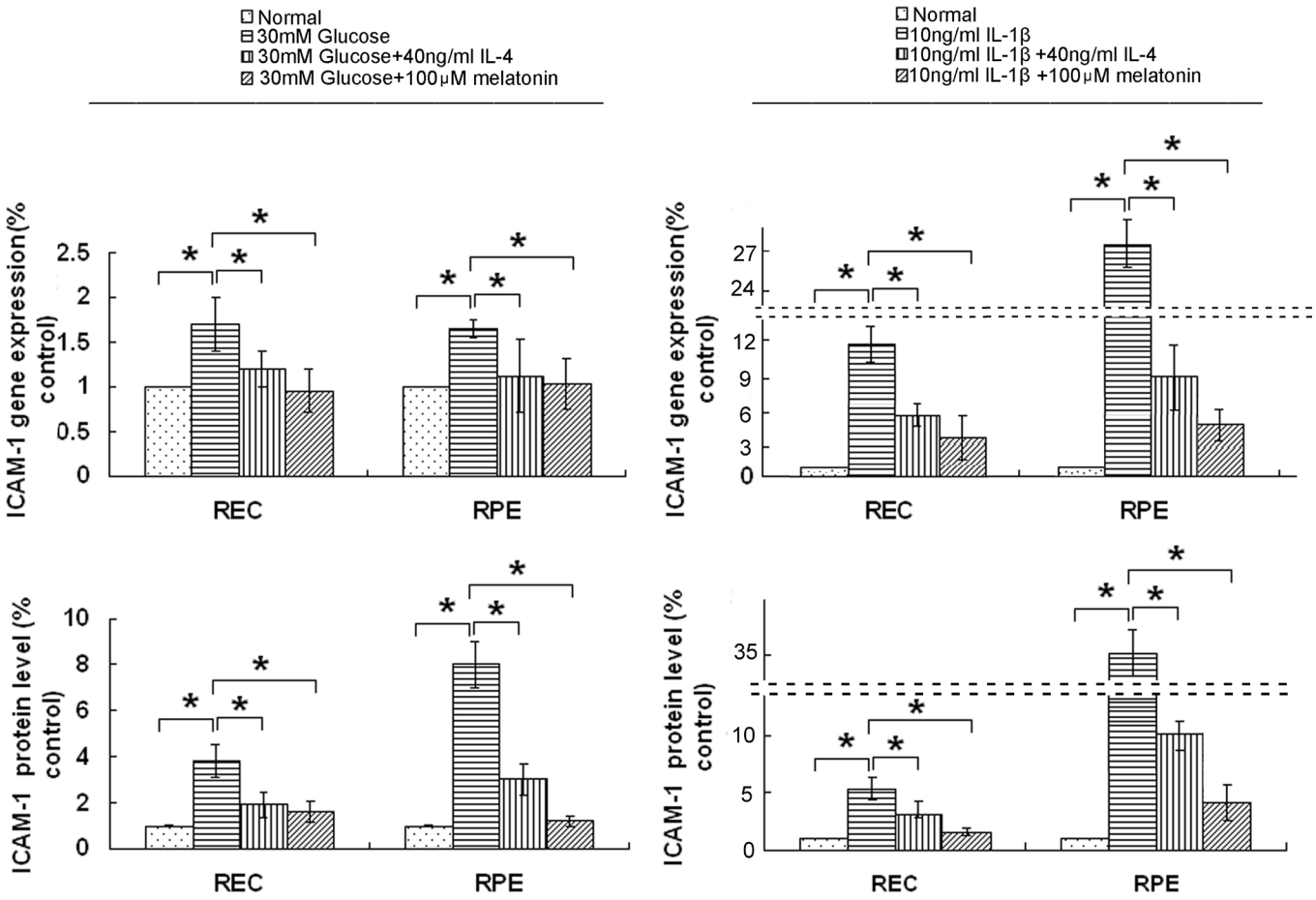

Figure 2. Interleukin-4 (IL-4) and melatonin downregulated the expression of intercellular cell adhesion molecule-1 (ICAM-1) induced with D-glucose or interleukin-1β. After D-glucose (30 mM; 48 h incubation) or interleukin-1β (IL-1β; 10 ng/ml; 24 h incubation) induction with or without IL-4 (40 ng/ml) or melatonin (100 μM), total RNA was extracted from the human retinal endothelial cells (RECs) and retinal pigment epithelial (RPE) cells, and the supernatants were harvested for quantitative real-time PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA) to measure the levels of ICAM-1 mRNA and protein, respectively. The mRNA and protein levels of ICAM-1 increased significantly after induction with D-glucose or IL-1β, but were significantly downregulated by IL-4 and melatonin. The data are expressed as the mean± standard deviation (SD; n = 4; *p<0.05 versus the control group).

DISCUSSION

In the present study, we observed that IL-4 and melatonin inhibited the expression of several cytokines, including VEGF, ICAM-1, MMP2, and MMP9, induced by high glucose and IL-1β in human RECs and RPE cells. These results suggest that these immune regulatory factors may protect the retina with their anti-inflammatory abilities.

Inflammation typically has beneficial effects if it is acute. However, if inflammation persists or is frequent and becomes chronic inflammation, it will have adverse effects and result in inflammatory damage. Previous studies have demonstrated the critical roles of inflammation in the development of early and advanced DR [12]. Various biochemical and physiologic alterations that are consistent with inflammation have been found in the serum, retina or vitreous of diabetic animals and patients [13-19]. Gene profiling analysis of diabetic retinas from rodents was consistent with the results of inflammatory response [20]. In addition, inhibition of these inflammatory reactions could reduce the development of the characteristic lesions of retinopathy in animals, suggesting that these inflammatory changes are important in the development of DR [21].

Hyperglycemia is a proinflammatory environment. Incubation of retinal cells with high glucose caused upregulation of several proinflammatory mediators [22]. In addition, diabetes-like retinopathy could be induced by long-term experimental hyperglycemia [12]. IL-1β is a major proinflammatory cytokine secreted by lymphocytes and macrophages and is the main trigger of the inflammatory

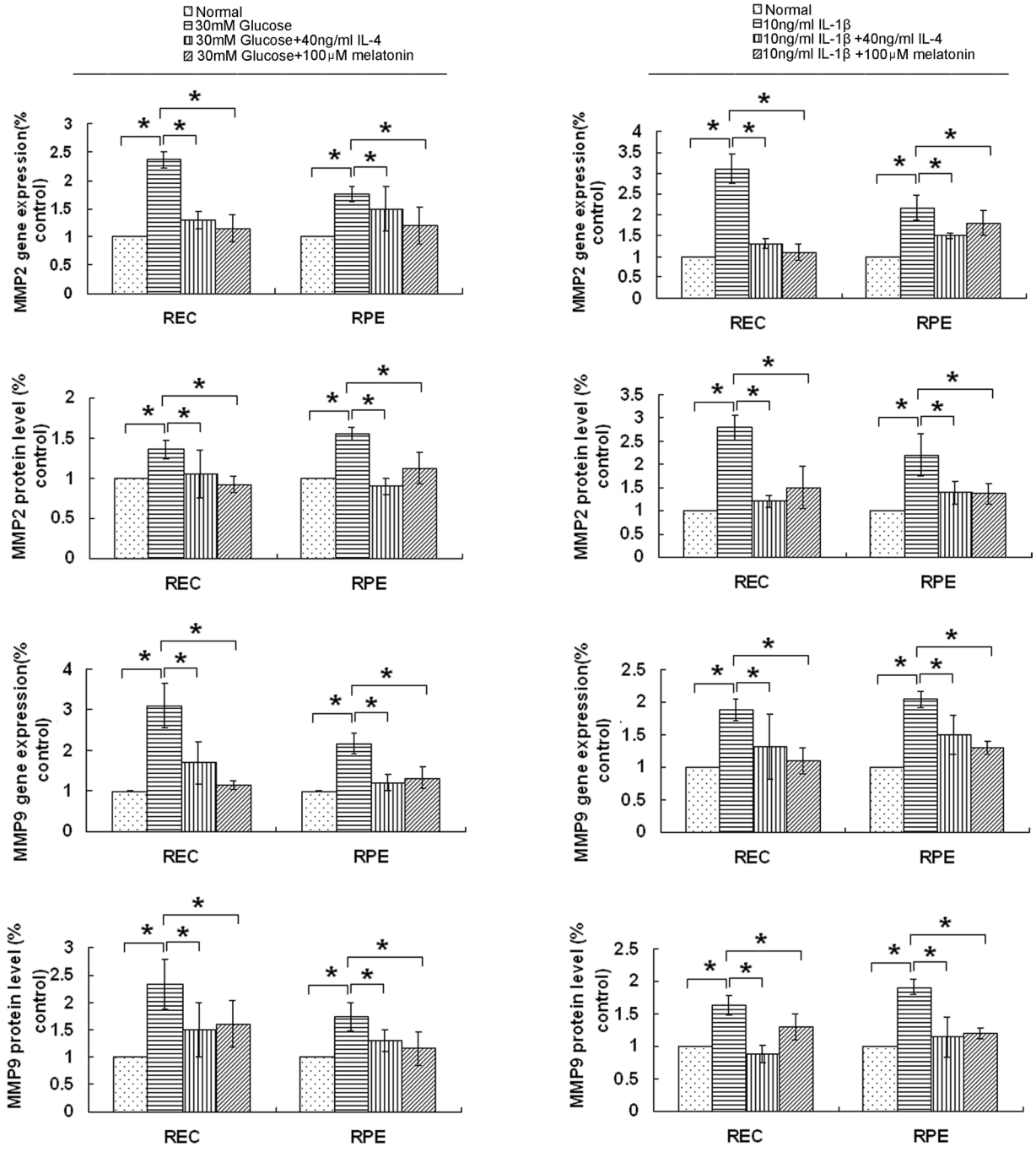

Figure 3. Interleukin-4 (IL-4) and melatonin downregulated the expression of matrix metalloproteinases 2 (MMP2) and matrix metalloproteinases 9 (MMP9). Human retinal endothelial cells (RECs) and retinal pigment epithelial (RPE) cells were cultured for 24 h (interleukin-1β [IL-1β] stimulation) or 48 h (D-glucose stimulation) with or without IL-4 (40 ng/ml) or melatonin (100 μM). The mRNA and protein levels of the MMP2 and MMP9 genes were analyzed with quantitative real-time PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The expression of MMP2 and MMP9 was significantly decreased by IL-4 and melatonin in human RECs and RPE cells induced by D-glucose or IL-1β. The data are expressed as the mean±standard deviation (SD; n = 3; *p<0.05 versus the control group).

cascade. High-glucose-treated retinal endothelial cells showed an increased level of IL-1β. Incubation of retinal cells with recombinant active IL-1β could also lead to the downstream inflammatory cascade, therefore suggesting an interaction effect between IL-1β and hyperglycemia and a continuous feedback loop [23]. Yang et al. also investigated the interaction between hyperglycemia and IL-1β, and their results suggested that interrupting the vicious circle could limit the progression of DR [9]. Based on previous studies that revealed the triggering and maintaining roles of hyperglycemia and IL-1β in DR, in our study, we used high glucose and human recombinant IL-1β supplements to induce hyperglycemia and inflammatory circumstances of DR and to observe their effects on the target cells and whether they could be blocked with IL-4 and melatonin.

The inner barrier of the retina formed by the tight junctional complexes between RECs acts as the first barrier and the first victim in vessel lesions [24]. RPE cells lying in the interface between the neural retina and the choriocapillaris form the outer blood–retinal barrier. RECs and RPE cells can secrete a wide range of cytokines in vivo and serve as a rational cellular targets for anti-inflammatory agents [22,24,25].

Several inflammatory molecules are involved in DR. In this study, we selected several representative and critical inflammatory factors to analyze. VEGF, first described as "vascular permeability factor", enhances vascular permeability and endothelial cell migration and proliferation during angiogenesis. VEGF plays a critical role in the pathogenesis of DR at several levels and blockade of endogenous VEGF is associated with anatomic and functional improvements in the affected eyes [26]. ICAM-1 is an adhesion molecule. White blood cells bind to ICAM-1 on the surface of endothelial cells in a multistep process, leading to adherence of the blood cells to the endothelial wall, a characteristic of inflammation [27]. ICAM-1 has been reported to be upregulated by several stimulators, including VEGF, PARP activation and oxidative stress, which are all related to DR [28]. Studies have documented the important proinflammatory roles of MMPs, including MMP2 and MMP9, in the retinas of diabetic animals [29,30]. Although the underlying mechanisms have not yet been fully elucidated, data from numerous studies have suggested that MMPs prompt penetration of inflammatory cells by disrupting the blood–retinal barrier, a hallmark of early changes in DR [31]. Increased levels of MMP2 and MMP9 have been found in angiogenesis [32] and vitreous proliferative membranes in DR [33,34].

Traditional genetic studies on human diseases have focused on identifying genes that predispose to particular diseases. However, a relatively unexplored, but important, area of study is investigating the role of genetic factors in maintaining healthy human condition [35]. This has driven our group to search for resistance genes based on genome-wide profiling in diabetic patients with and without DR. Our results have demonstrated that IL-4 was differentially expressed between diabetic patients with and without DR and is a protective factor in DR [4]. IL-4 is a multifunctional cytokine produced by CD4/Th2 cells that can influence chemokine productions [36]. In addition, IL-4 was reported to have anti-angiogenesis activity by blocking corneal neovascularization induced by basic fibroblast growth factor and protecting rat rod photoreceptors from thapsigargin-induced cell death [37,38]. Based on our previous studies, our results in this study confirmed our speculation that IL-4 is an anti-inflammation factor in the retina and suppresses inflammatory processes in DR.

Melatonin is a multitasking molecule, and several groups have shown that melatonin reversed chronic and acute inflammation [39]. Accumulating evidence suggests that melatonin plays important roles in retinal physiology and pathophysiology, but the underlying mechanisms have not been fully defined [40]. Melatonin has also been reported to have protective effects on the retina and retinal cells [41-44]; however, the anti-inflammatory potential in retinal cells has rarely been investigated. Consistent with the anti-inflammation role, our results showed that melatonin effectively downregulated the production of targeted cytokines in DR-related stimuli, suggesting that melatonin can block retinal inflammation associated with DR.

Taken together, the present study suggests that IL-4 and melatonin are involved in inflammation in DR and may have significant therapeutic function for diabetic complications by inhibiting the expression of cytokines induced by IL-1β and high glucose and therefore ameliorating inflammation in DR. The regulation mechanism of IL-4 and melatonin remains to be further illustrated, but our results suggest that IL-4 and melatonin application may be an efficient method for inhibiting the development of DR.

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