·Basic Research·

Repression of retinal microvascular endothelial cells by transthyretin under simulated diabetic retinopathy conditions

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Abstract

• AIM: To investigate biological effects of transthyretin (TTR) on the development of neovascularization under simulated diabetic retinopathy (DR) condition associated with high glucose and hypoxia.

• METHODS: Human retinal microvascular endothelial cells (hRECs) were cultured in normal and simulated DR environments with high glucose and hypoxia. The normal serum glucose concentration is approximately 5.5 mmol/L; thus, hyperglycemia was simulated with 25 mmol/L glucose, while hypoxia was induced using 200 μmol/L $CoCl_2$. The influence of TTR on hRECs and human retinal pigment epithelial cells (hRPECs) was determined by incubating the cells with 4 μmol/L TTR in normal and abnormal media. A co-culture system was then employed to evaluate the effects of hRPECs on hRECs.

• RESULTS: Decreased hRECs and hRPECs were observed under abnormal conditions, including high-glucose and hypoxic media. In addition, hRECs were significantly inhibited by 4 μmol/L exogenous TTR during hyperglycemic culture. During co-culture, hRPECs inhibited hRECs in both the normal and abnormal environments.

• CONCLUSION: hREC growth is inhibited by exogenous TTR under simulated DR environments with high-glucose and hypoxic, particularly in the medium containing 25 mmol/L glucose. hRPECs, which manufacture TTR in the eye, also represses hRECs in the same environment. TTR is predicted to inhibit the proliferation of hRECs and neovascularization.

• **KEYWORDS:** transthyretin; diabetic retinal; human retinal microvascular endothelial cells; human retinal pigment epithelial cells; hyperglycemia; hypoxia

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INTRODUCTION

Diabetes is a chronic disease characterized by hyperglycemia and diabetic retinopathy (DR). DR is considered to be the most frequent microvascular complication of diabetes and is a common cause of moderate and severe vision loss [1-2]. In diabetes, retinal abnormalities clinically characterized by microaneurysms, hemorrhages, lipid exudates, macular edema, capillary occlusion, cotton-wool spots, and neovascularization (NV) can be identified as DR [3-4]. The development of DR is directly associated with the duration of diabetes and severity of hyperglycemia. As previously reported, ischemic retinal hypoxia playsavital role in the molecular pathogenesis of retinal NV, and the expression of a subunit of hypoxia-inducible factor (HIF)-1, HIF-1-α, can be significantly increased[5-6]. In a mouse model of ischemic retinopathy, excess vascular endothelial growth factor (VEGF) has been related to an increased HIF-1-α level[7]. Other HIF-1-regulated genes and products, including placental growth factor, platelet-derived growth factor-B, and stromal-derived growth factor (SDF)-1, have also been reported. Placental growth factor is a member of the VEGF gene family and binds with VEGF receptor-1 to recruit bone marrow-derived cells. These factors could stimulate the development of retinal NV [8]. In transgenic mice, retina-specific expression of platelet-derived growth factor-B has been shown to lead to severe NV and retinal detachment[9-10]. CXCR4 is the receptor for SDF-1 and reduces retinal NV in ischemic retinas; however, CXCR4 is inhibited when SDF-1 is overexpressed, thus promoting retinal NV [11].

Transthyretin (TTR) is a 55 kDa homotetramer that is found in serum and cerebrospinal fluid. In the eye, TTR is synthesized and secreted by human retinal pigment epithelial cells (hRPECs) [12]. It has also been identified as a fluid carrier for thyroxine and retinol [13-14]. According to previous reports, TTR mutants can down-regulate pro-angiogenic genes in human umbilical vein endothelial cells, thereby inducing apoptosis and inhibiting migration [15]. Individuals

with type 1 diabetes show low serum TTR levels, and the protein is found mainly as a monomer; whereas in type 2 diabetes, TTR is detected at normal concentrations [16]. Significantly increased TTR serum levels have been detected in patients with severe myopia compared with healthy controls [17]; higher proportions of misfolded TTR with abnormal secondary structures were also found in the vitreous of said patients, causing TTR to lose their natural bio-functions [18-19]. However, the effects of TTR on human retinal microvascular endothelial cells (hRECs) and on the development of NV in DR remain unclear.

In this study, hRECs were cultured with exogenous TTR or with expressing cells (hRPECs) to evaluate the effects of TTR on the growth of hRECs under normal and simulated DR conditions.

MATERIALS AND METHODS

Materials This study applied the principles of the Declaration of Helsinki for the use of human subjects and was approved by the Ethics Committee of Nanjing Medical University.

hRECs and hRPECs were purchased from Shanghai Bioleaf Biotech Co., Ltd. (China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and phosphate-buffered saline were obtained from Life Technologies (USA). $CoCl_2$ and glucose were purchased from Sigma. Human TTR and a human TTR ELISA kit were supplied by Sino Biological Inc. (China) and Abnova (USA), respectively. Other reagents and chemicals were obtained from local companies and were of analytical grade or better.

Culture of Human Retinal Microvascular Endothelial Cells and Human Retinal Pigment Epithelial Cells Under Normal and Simulated Diabetic Retinopathy Conditions hRECs and hRPECs from passage 3 were used in the following experiments. The cells were cultured under normal and simulated DR conditions as described previously [20-22]. Media were prepared as shown in Table 1. Under normal culture conditions, cells were grown at 37℃ using a Series 8000 WJ cell incubator (Thermo Scientific, USA) in DMEM containing 5.5 mmol/L glucose and 10% fetal bovine serum. A total of 25 mmol/L glucose was used in DMEM to simulate DR[20], and hypoxia was induced using 200 μmol/L $CoCl_2$ [21-22]. Cells were cultured for 72h, and the cell index was detected using the xCELLigence real-time cell analysis (RTCA) MP system (Roche). The xCELLigence RTCA system is an established electronic cell sensor array. This system uses microelectronic biosensor technology that is verified for real-time, label-free, dynamic, and non-offensive monitoring of cellular features. The relative cell index was automatically calculated and read based on the number, morphology, adhesion, and size of cells.

Effects of Transthyretin on the Growth of Human Retinal Microvascular Endothelial Cells and Human Retinal Pigment Epithelial Cells TTR mutants can inhibit the growth of human umbilical vein endothelial cells in liver disease by regulating pro-angiogenic genes. hRECs and hRPECs from passage 6 were cultured under normal and abnormal conditions, and approximately 4 μmol/L TTR was thenadded to the mediain accordance with a previously described protocol [15]. Media were prepared as shown in Table 2. The cells were grown for 72h, and the cell index was detected usingthe xCELLigence RTCA MP system.

Table 1 The medium for hRECs and hRPECs

Medium	DMEM	FBS (%)	Glucose (mmol/L)	$CoCl_2$ (μmol/L)
LG	+	10	5.5	-
LG+hypoxia	+	10	5.5	200
HG	+	10	25	-
HG+hypoxia	+	10	25	200

LG: Low-glucose; LG+hypoxia: Low-glucose with hypoxia; HG: High-glucose; HG+hypoxia: High-glucose with hypoxia; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum.

Table 2 The medium containing TTR for hRECs and hRPECs

Medium	DMEM	FBS (%)	Glucose (mmol/L)	$CoCl_2$ (μmol/L)	TTR (μmol/L)
LG	+	10	5.5	-	-
LG+TTR	+	10	5.5	-	4
LG+hypoxia	+	10	5.5	200	-
LG+hypoxia+TTR	+	10	5.5	200	4
HG	+	10	25	-	-
HG+TTR	+	10	25	-	4
HG+hypoxia	+	10	25	200	-
HG+hypoxia+TTR	+	10	25	200	4

LG: Low-glucose; LG+TTR: Low-glucose with TTR; LG+hypoxia: Low-glucose with hypoxia; LG+hypoxia+TTR: Low-glucose with hypoxia and TTR; HG: High-glucose; HG+TTR: High-glucose with TTR; HG+hypoxia: High-glucose with hypoxia; HG+hypoxia+TTR: High-glucose with hypoxia and TTR; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; TTR: Transthyretin.

Co −culture of Human Retinal Microvascular Endothelial Cells and Human Retinal Pigment Epithelial Cells hRECs and hRPECs from passage 9 were used in the Transwell co-culture system. TTR has been shown to be synthesized and secreted by hRPECs in the eye [12]. The expression of TTR in hRPECs was detected using a human TTR ELISA kit (Abnova). hRPECs were cultured in normal low-glucose (LG) medium for 24, 48 and 72h. After this, hRPECs were collected, lysed in radio immunoprecipitation assay lysis buffer containing 1 mmol/L) phenylmethylsulfonyl fluoride (PMSF), incubated in ice-water for 10min, and centrifuged at 10000 g for 5min. Supernatants were applied to the ELISA in accordance with the manufacturer's instructions.

A transwell co-culture was subsequently performed, with hRPECs used as the source of TTR. About 50 μL of hRECs (approximately 8000 cells/50 μL) in DMEM was added to an E-plate. About 50 μL of hRPECs (approximately 2000 cells/50 μL) in DMEM was also added to the inserts in the CCD receiver plate. After overnight growth at 37℃, the inserts were placed into the E-plates. The cells were then

Table 3 The index of hRECs under natural and abnormal conditions

Parameters	4h	8h	16h	24h	36h	48h	60h	72h
LG	1.835±0.061	2.222±0.107	3.284±0.181	4.834±0.272	8.206±0.739	11.406±1.341	13.057±1.581	14.299±1.804
LG+hypoxia	1.506±0.059	1.658±0.129	2.261±0.194	3.074±0.206	4.610±0.361	7.040±0.635	9.135±0.867	9.341±0.748
HG	1.480±0.071	1.816±0.835	2.829±0.136	3.977±0.181	5.269±0.155	6.220±0.172	7.183±0.273	8.281±0.349
HG+hypoxia	1.305±0.065	1.546±0.780	2.021±0.058	2.622±0.366	3.377±0.075	4.246±0.135	4.866±0.171	5.096±0.193
^{a}P	0.008	0.018	0.007	0.003	0.000	0.000	0.001	0.000
^{c}P	0.008	0.037	0.147	0.079	0.000	0.000	0.000	0.000
^{e}P	0.162	0.059	0.003	0.005	0.000	0.017	0.020	0.006

^{a}LG *vs* LG+hypoxia; ^{c}LG *vs* HG; ^{e}HG *vs* HG+hypoxia.

Table 4 The index of hRPECs under natural and abnormal conditions

Parameters	4h	8h	16h	24h	36h	48h	60h	72h
LG	1.086±0.011	1.332±0.008	2.013±0.400	3.451±0.132	6.875±0.353	11.312±0.772	14.381±0.938	15.065±1.118
LG+hypoxia	1.304±0.009	1.250±0.020	1.734±0.494	2.306±0.113	3.770±0.391	6.080±0.807	7.985±1.018	8.872±0.903
HG	1.099±0.048	1.178±0.069	1.427±0.116	2.167±0.248	3.693±0.520	5.998±1.131	9.497±1.683	12.946±1.916
HG+hypoxia	1.380±0.062	1.417±0.061	1.440±0.491	1.857±0.076	2.581±0.190	3.552±0.294	4.776±0.420	5.911±0.431
^{a}P	0.000	0.035	0.005	0.000	0.000	0.002	0.002	0.004
^{c}P	0.752	0.204	0.013	0.011	0.004	0.007	0.071	0.771
^{d}P	0.000	0.048	1.000	0.669	0.244	0.190	0.107	0.059

^{a}LG *vs* LG+hypoxia; ^{c}LG *vs* HG; ^{d}HG *vs* HG+hypoxia.

co-cultured in high-glucose (HG) and HG plus hypoxia (HG+hypoxia) media (Table 2) for 48h. The cell index was determined using the xCELLigence RTCA MP system.

Statistical Analysis Origin 9.1 software (OriginLab, USA) was used in data processing, and statistical significance was determined using the t-test and analysis of variance. $P<0.05$ was considered statistically significant.

RESULTS

Growth of Human Retinal Microvascular Endothelial Cells and Human Retinal Pigment Epithelial Cells hRECs-P3 and hRPECs-P3 were grown to approximately 80%-90% confluence. These cells were then washed twice with phosphate-buffered salineand treated with 0.05% trypsin for 2-3min. Trypsin was neutralized after adding LG DMEM. A total of 3000 cells per well were cultured on plates in LG medium after an overnight incubation. The attached pericytes were washed twice and incubated with 200 μL fresh DMEM, including LG, LG with hypoxia (LG+hypoxia), HG, and HG+hypoxia media (Table 1). The cells were cultured for 72h, and the plates were scanned using the xCELLigence RTCA MP system for 5min each. The relative cell index was automatically calculated and read based on the number, morphology, adhesion, and size of cells.

As shown in Figure 1 and Table 3, the growth of hRECs was much higher in LG than in LG+hypoxia or HG media ($P<0.05$). The growth of hRECs was further decreased in HG+hypoxia medium ($P<0.05$).

In the hRPEC culture (Figure 2 and Table 4), the growth of hRPECs was also higher in LG than in LG+hypoxia or HG media during almost the whole process ($P<0.05$). However, the effect of the HG+hypoxia medium was not significant ($P>0.05$).

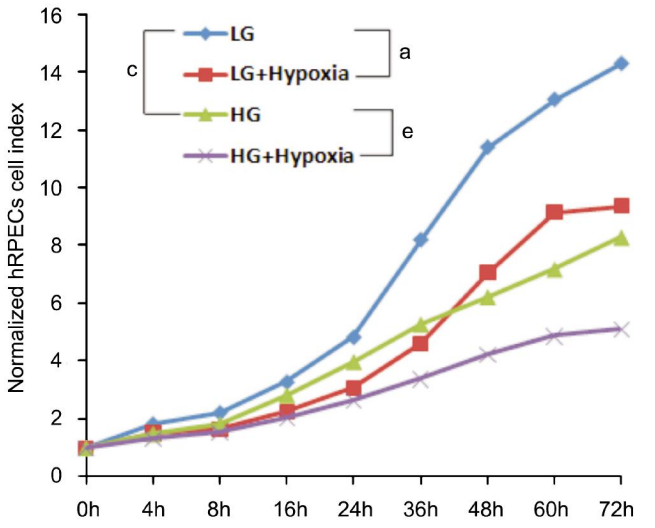

Figure 1 The growth of hRECs under natural and abnormal conditions hRECs were grown under natural and simulated DR conditions. LG: Low-glucose; LG+hypoxia: Low-glucose with hypoxia; HG: High-glucose; HG+hypoxia: High-glucose with hypoxia. ^{a}LG *vs* LG+hypoxia, ^{c}LG *vs* HG, ^{e}HG *vs* HG+hypoxia. High glucose level (25 mmol/L) and hypoxia induced with 200 μmol/L $CoCl_2$ could significantly repressed the growth of hRECs ($^{a}P<0.05$, $^{c}P<0.05$, $^{e}P<0.05$).

Effects of Transthyretin on Cell Growth hRECs-P6 and hRPECs-P6 cells were trypsinized for 2-3min, and LG DMEM was added to neutralize trypsin. The cells were harvested, spun down, and washed several times with the medium, and 3000/well cells were cultured on plates. After

Table 5 The index of hPECs under high glucose conditions with TTR

Parameters	4h	8h	16h	24h	36h	48h	72h
HG	1.047±0.022	1.134±0.023	1.454±0.034	1.874±0.035	2.576±0.048	3.217±0.090	3.513±0.145
HG+TTR	1.119±0.008	1.255±0.042	1.546±0.112	1.795±0.152	1.950±0.134	2.058±0.129	1.974±0.097
HG+hypoxia	1.107±0.021	1.143±0.034	1.362±0.083	1.477±0.129	1.595±0.197	1.635±0.263	1.391±0.275
HG+hypoxia+TTR	1.181±0.025	0.948±0.019	0.836±0.022	0.832±0.023	0.781±0.039	0.744±0.043	0.488±0.056
^{e}P	0.039	0.008	0.478	0.731	0.056	0.007	0.002
^{g}P	0.030	0.030	0.000	0.009	0.015	0.031	0.001

^{e}HG *vs* HG+TTR; ^{g}HG+hypoxia *vs* HG+hypoxia+TTR.

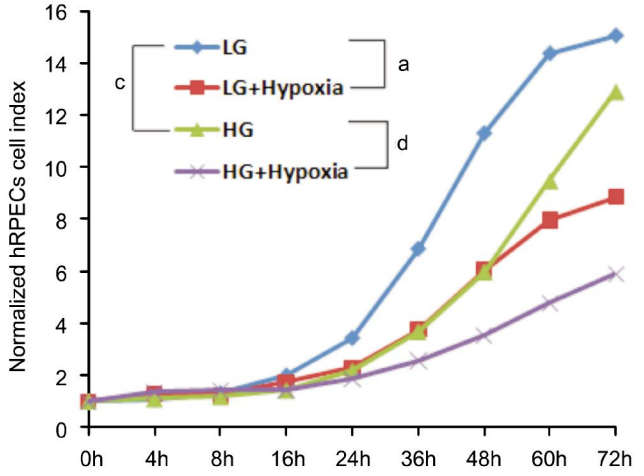

Figure 2 The growth of hRPECs under natural and abnormal conditions hRPECs were cultured under natural and simulated DR conditions. LG: Low-glucose; LG+hypoxia: Low-glucose with hypoxia; HG: High-glucose; HG+hypoxia: High-glucose with hypoxia. ^{a}LG *vs* LG+hypoxia, ^{c}LG *vs* HG, ^{d}HG *vs* HG+hypoxia. The growth of hRECs in LG was higher than that in HG or LG+hypoxia medium during almost the whole process ($^{a,c}P<0.05$). But the effect of hypoxia in HG medium was not significant ($^{d}P>0.05$).

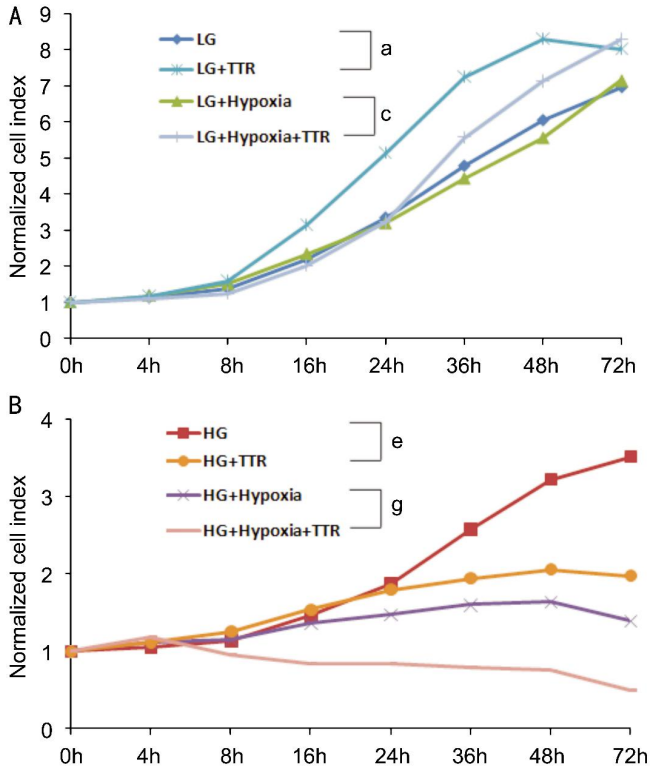

Figure 3 The effect of TTR on the growth of hRECs hRECs were cultured under natural and simulated DR conditions with 4 μmol/L TTR. LG: Low-glucose; LG+TTR: Low-glucose with TTR; LG+hypoxia: Low-glucose with hypoxia; LG+hypoxia+TTR: Low-glucose with hypoxia and TTR; HG: High-glucose; HG+TTR: High-glucose with TTR; HG+hypoxia: High-glucose with hypoxia; HG+hypoxia+TTR: High-glucose with hypoxia and TTR. ^{a}LG *vs* LG+TTR, ^{c}LG+hypoxia *vs* LG+hypoxia+TTR, ^{e}HG *vs* HG+TTR, ^{g}HG+hypoxia *vs* HG+hypoxia+TTR. A: In low glucose media, TTR slightly enhanced the growth of hRECs ($^{a,c}P<0.05$); B: Only in high glucose media, could TTR inhibit the growth of hRECs ($^{e,g}P<0.05$).

an overnight incubation, the attached pericytes were washed twice and cultured with 200 μL of LG, LG+hypoxia, HG, and HG+hypoxia media. Human TTR (4 μmol/L) was also added to the media (Table 2). The relative cell index was read using the xCELLigence RTCA MP system for 5min each.

In all HG cultures (Figure 3B, Table 5), including those under the $CoCl_2$-induced hypoxic conditions, TTR significantly decreased the growth of hRECs ($P<0.05$). By contrast, in all LG cultures (Figure 3A, Table 6), including those in LG and LG+hypoxia media, TTR slightly enhanced the growth of hRECs ($P<0.05$).

TTR slightly promoted hRPEC cell growth under normal and simulated DR conditions (Figure 4), but the differences were not always significant (Tables 7, 8).

Co –culture of Human Retinal Microvascular Endothelial Cells and Human Retinal Pigment Epithelial Cells In the eye, TTR is expressed by the retinal pigment epithelium. In this study, hRPECs were therefore employed as the TTR producer in the Transwell co-culture system.

TTR expression in hRPECs-P9 was detected by ELISA. As shown in Figure 5, TTR expression in the cells reached its peak at around 48h.

hRECs-P9 and hRPECs-P9 were washed and trypsinized when 80%-90% confluency was reached. Trypsin was then neutralized using the LG medium. Subsequently, 8000 hRECs and 2000 hRPECs in the LG medium were added to the E-plate and inserts of the CCD receiver plate, respectively. After an overnight incubation at 37℃ , the

Table 6 The index of hPECs under low glucose conditions with TTR

Parameters	4h	8h	16h	24h	36h	48h	72h
LG	1.129±0.025	1.382±0.088	2.202±0.164	3.347±0.275	4.785±0.459	6.055±0.387	6.975±0.249
LG+TTR	1.160±0.015	1.589±0.050	3.137±0.220	5.133±0.497	7.261±0.532	8.300±0.362	8.020±0.324
LG+hypoxia	1.189±0.018	1.511±0.041	2.343±0.105	3.198±0.121	4.445±0.281	5.572±0.364	7.167±0.373
LG+hypoxia+TTR	1.107±0.018	1.233±0.020	2.007±0.570	3.236±0.192	5.586±0.230	7.141±0.209	8.317±0.393
[a]*P*	0.260	0.000	0.000	0.000	0.000	0.000	0.000
[c]*P*	0.014	0.009	0.010	0.871	0.001	0.001	0.002

[a]LG *vs* LG+TTR; [c]LG+hypoxia *vs* LG+hypoxia+TTR.

Table 7 The index of hRPECs under low glucose conditions with TTR

Parameters	4h	8h	16h	24h	36h	48h	72h
LG	1.094±0.020	1.171±0.039	1.347±0.081	1.539±0.123	1.871±0.262	2.331±0.481	3.990±1.317
LG+TTR	1.178±0.018	1.390±0.046	1.687±0.100	1.968±0.140	2.353±0.180	2.858±0.244	4.751±0.596
LG+hypoxia	1.022±0.011	1.056±0.012	1.182±0.020	1.344±0.023	1.651±0.097	2.103±0.251	2.674±0.439
LG+hypoxia+TTR	1.115±0.013	1.253±0.034	1.555±0.080	1.873±0.106	2.224±0.167	2.512±0.244	2.944±0.365
[a]*P*	0.009	0.001	0.011	0.043	0.21	0.38	0.621
[b]*P*	0.039	0.049	0.049	0.053	0.068	0.272	0.598

[a]LG *vs* LG+TTR; [b]LG+hypoxia *vs* LG+hypoxia+TTR.

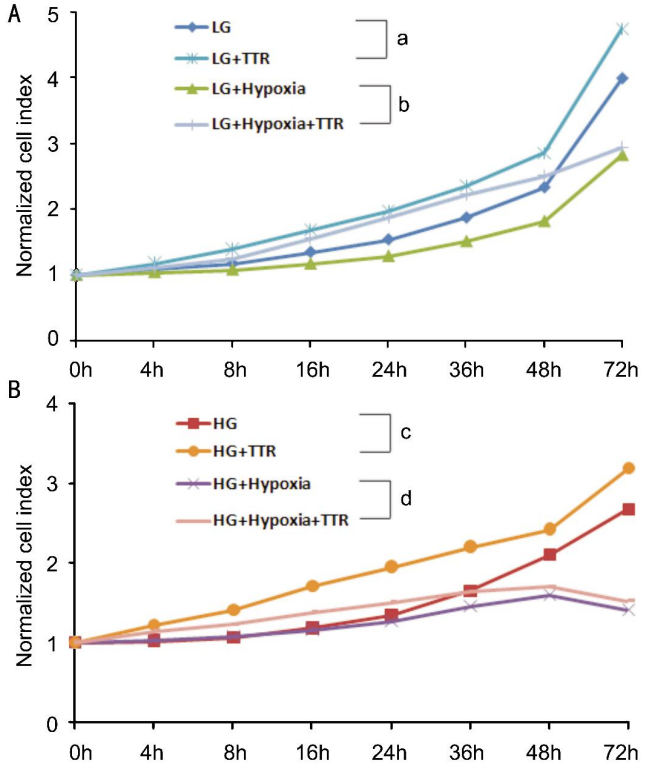

Figure 4 The effect of TTR on the growth of hRPECs hRPECs were cultured under natural and simulated DR conditions with 4 μmol/L TTR. LG: Low-glucose; LG+TTR: Low-glucose with TTR; LG+hypoxia: Low-glucose with hypoxia; LG+hypoxia+TTR: Low-glucose with hypoxia and TTR; HG: High-glucose; HG+TTR: High-glucose with TTR; HG+hypoxia: High-glucose with hypoxia; HG+hypoxia+TTR: High-glucose with hypoxia and TTR. [a]LG *vs* LG+TTR, [b]LG+hypoxia *vs* LG+hypoxia+TTR, [c]HG *vs* HG+TTR, [d]HG+hypoxia *vs* HG+hypoxia+TTR. TTR slightly enhanced the growth of hRPECs under all culture conditions, but the differences were not always significant (a, b, c, d).

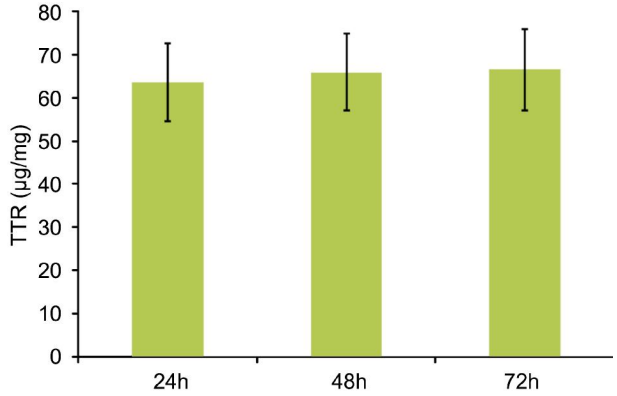

Figure 5 The expression of TTR in hRPECs The TTR contents in hRECs were detected by ELISA assay after the cells were cultured in natural medium for 24, 48 and 72h, respectively. It revealed that the expression of TTR reached the peak at approximately 48h and was then remained quite stable.

inserts were placed into the E-plates, and the two cell types were co-cultured in normal, HG, LG+hypoxia, and HG+hypoxia media (Table 1). The relative cell index was read using the xCELLigence RTCA MP system.

In co-culture, hRECs cultured alone were used asthe control. As shown in Figure 6, Tables 9, 10, hRECs growth was significantly inhibited by the co-cultured hRPECs under normal and simulated DR conditions (P<0.05).

DISCUSSION

As previously reported, retinal NV is closely associated with high levels of glucose in DR development, as well as with ischemic retinal hypoxia. HIF-1-α is induced by hypoxia and plays a vital role in the development of NV [5-6] by regulating other significant factors for vascular endothelial growth, angiogenesis, and apoptosis [7-11]. NV in DR has been studied for decades; however, TTR has seldom been described in

Table 8 The index of hRPECs under high glucose conditions with TTR

Parameters	4h	8h	16h	24h	36h	48h	72h
HG	1.045±0.016	1.084±0.015	1.173±0.018	1.290±0.013	1.513±0.054	1.822±0.120	2.831±0.294
HG+TTR	1.216±0.017	1.407±0.030	1.702±0.055	1.949±.0.063	2.199±0.058	2.420±0.066	3.182±0.085
HG+hypoxia	1.031±0.017	1.073±0.021	1.162±0.032	1.264±0.062	1.456±0.111	1.595±0.156	1.407±0.231
HG+hypoxia+TTR	1.139±0.019	1.229±0.049	1.376±0.100	1.503±0.147	1.642±0.193	1.697±0.220	1.517±0.190
^{c}P	0.002	0.002	0.004	0.007	0.016	0.055	0.342
^{d}P	0.001	0.005	0.021	0.038	0.096	0.297	0.445

^{c}HG *vs* HG+TTR; ^{d}HG+hypoxia *vs* HG+hypoxia+TTR.

Table 9 The index of hPECs under low glucose conditions with hRPECs

Parameters	4h	8h	16h	24h	36h	48h
HREC-LG	1.932±0.082	2.513±0.112	3.444±0.126	4.002±0.169	4.623±0.347	5.085±0.446
HREC&HRPEC-LG	1.409±0.052	1.852±0.085	2.483±0.222	2.946±0.239	3.126±0.184	3.228±0.186
HREC-LG+Hypoxia	1.764±0.052	2.230±0.059	3.385±0.017	3.961±0.136	3.977±0.286	4.055±0.306
HREC&HRPEC-LG+hypoxia	1.270±0.016	1.304±0.016	1.589±0.047	1.867±0.090	1.856±0.207	1.952±0.216
^{a}P	0.000	0.019	0.038	0.049	0.026	0.045
^{c}P	0.000	0.034	0.000	0.006	0.028	0.038

^{a}hREC-LG *vs* hREC&hRPEC-LG; ^{c}hREC-LG+hypoxia *vs* hREC&hRPEC-LG+hypoxia.

Table 10 The index of hPECs under high glucose conditions with hRPECs

Parameters	4h	8h	16h	24h	36h	48h
HREC-HG	1.775±0.123	2.206±0.094	2.604±0.256	2.910±0.219	3.247±0.375	3.614±0.398
HREC&HRPEC-HG	1.341±0.049	1.655±0.074	1.710±0.107	1.877±0.160	2.013±0.230	2.069±0.262
HREC-HG+hypoxia	1.669±0.040	2.010±0.061	2.471±0.148	2.463±0.220	2.306±0.312	2.141±0.262
HREC&HRPEC-HG+hypoxia	1.181±0.085	1.186±0.092	1.198±0.090	1.265±0.110	1.247±0.109	1.268±0.114
^{e}P	0.000	0.047	0.045	0.049	0.048	0.047
^{g}P	0.000	0.007	0.001	0.048	0.042	0.046

^{e}HREC-HG *vs* hREC&hRPEC-HG; ^{g}hREC-LG+hypoxia *vs* hREC&hRPEC-HG+hypoxia.

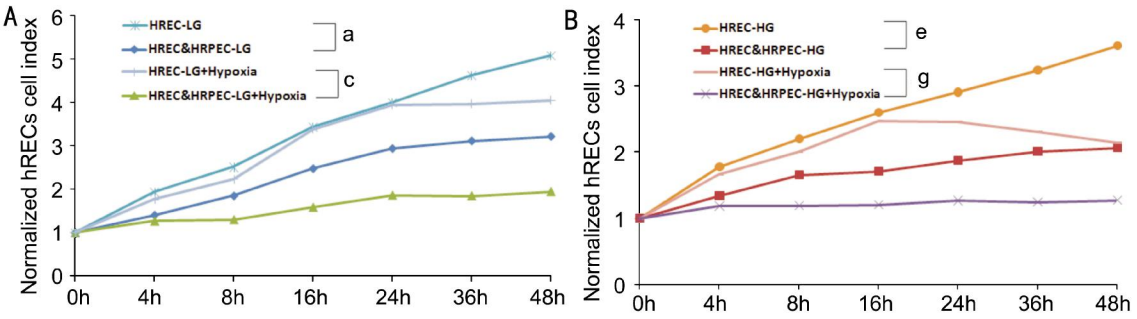

Figure 6 The effect of hRPECs on hRECs in co−culture system In the trans-well co-culture system, hRECs was cultured with hRPECs under both natural and abnormal DR conditions. hREC-LG: hRECs in low glucose; hREC&hRPEC-LG: hRECs co-cultured with hRPECs in low glucose; hREC-LG+hypoxia: hRECs in low glucose and hypoxia; hREC&hRPEC-LG+hypoxia: hRECs co-cultured with hRPECs in low glucose and hypoxia; hREC-HG: hRECs in high glucose; hREC&hRPEC-HG: hRECs co-cultured with hRPECs in high glucose; hREC-HG+hypoxia: hRECs in high glucose and hypoxia; hREC&hRPEC-HG+hypoxia: hRECs co-cultured with hRPECs in high glucose and hypoxia. ^{a}hREC-LG *vs* hREC&hRPEC-LG, ^{c}hREC-LG+hypoxia *vs* hREC&hRPEC-LG+hypoxia, ^{e}HREC-HG *vs* hREC&hRPEC-HG, ^{g}hREC-LG+hypoxia *vs* hREC&hRPEC-HG+hypoxia. hRPECs significantly repressed the growth of hRECs in low glucose (A) and high glucose (B) media ($^{a,c,e,g}P < 0.05$).

this field. TTR has recently been reported to affect the growth, migration, and apoptosis of human umbilical vein endothelial cells by regulating the expression of protein factors in NV; however, this study was only performed under normal conditions[15].

The effect of TTR on the development of NV in DR environments remains unclear. In the current study, DR environments were simulated, and exogenous TTR and hRPECs (the producer of TTR in the eye) were employed to affect hREC growth. In HG and HG+hypoxia media, hREC growth was inhibited by 4 μmol/L exogenous TTR. Incontrast, hREC growth was slightly increased by TTR in LG and LG+hypoxia media. These resultssuggest that in the process of DR, TTR affects the development of NV only

under hyperglycemic conditions, and hypoxia does not induce the bio-function of TTR. In addition, in co-culture, hRPECs inhibited the growth of hRECs in both HG and LG media. TTR was secreted by hRPECs in the eye, and ELISA showed that the expression was stable for 726h; thus, hREC repression under LG conditions could be attributed to some unknown mechanisms. Therefore, quantitative reverse transcription polymerase chain reaction (qRT-PCR) should be employed in further investigations to determine the levels of key genes in DR NV and reveal the as-yet unknown mechanisms behind this condition.

In several clinical investigations into severe myopia, individuals with diabetes and myopia have been found to be less likely than those without myopia to have DR [23-24]. Higher concentrations of TTR were detected in the serum and vitreous of individuals with severe myopia, and the presence of some abnormal TTRs with misfolded structures was confirmed. Lower TTR concentrations havealso been detected in the vitreous of patients with DR [17-19]. These findings agree with the experimental results ofthe current study, potentially revealing that diabetic patients with myopia are less likely suffer from DR.

The results of the present study suggest that TTR might repress hREC growth and that HG, but not hypoxia, is an important factor. Further investigations are necessary to determine whether TTR can affect migration and tube-forming processes in DR. qRT-PCR should be employed in further studies to detect the levels of key genesin important pathways for the development of NV in DR.

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