

Rhein enhances the cytotoxicity of effector lymphocytes in colon cancer under hypoxic conditions

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Received May 10, 2018; Accepted September 6, 2018

DOI: 10.3892/etm.2018.6855

Abstract. The immunosuppressive tumor microenvironment limits the application of adoptive immunotherapy for solid tumors. Hypoxia is closely associated with the formation of the immunosuppressive tumor microenvironment. Hypoxia-inducible factor-1 (HIF-1) is an oxygen-sensitive transcriptional activator that drives the transcription of several immunosuppressive molecules. In addition, previous studies confirmed that rhein downregulated the expression of HIF-1 α , a subunit of HIF-1, in pancreatic cancer cells. The present study established correlations between mRNA expression levels of HIF-1 α and six immunosuppressive molecules in colorectal cancer (CRC) tissue samples. This study examined the effect of rhein on the expression levels of HIF-1 α and six immunosuppressive molecules in CRC cell lines under hypoxic conditions by western blot analysis and reverse transcription-quantitative polymerase chain reaction. This study demonstrated that rhein downregulated the expression of HIF-1 α and immunosuppressive molecules in CRC cells under hypoxic conditions. In addition, the present study analyzed the cytotoxicity of peripheral blood lymphocytes *in vitro* using a non-toxic cytotoxicity assay. This study demonstrated that *in vitro*, rhein enhanced the cytotoxicity of effector lymphocytes toward tumor cells under hypoxic conditions, and therefore rhein may be used in combination with effector lymphocytes for the treatment of CRC.

Introduction

The success of CD19-targeted chimeric antigen receptor (CAR) T-cells in hematopoietic malignancies has led to further research surrounding the use of adoptive immunotherapy in the treatment of solid tumors (1). Carcinoembryonic antigen (CEA)-targeted CAR T-cells have previously been used to treat liver and peritoneal metastases of colorectal cancer (CRC) (2-4). However, the application of CAR T-cells for the treatment of solid tumors is limited due to the inherent immunosuppressive tumor microenvironment (5).

Currently, several studies have demonstrated that when tumor cells are exposed to hypoxic conditions, immunosuppressive molecules including programmed cell death ligand-1 (PD-L1), vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), galectin-1, interleukin (IL)-10 and transforming growth factor-1 (TGF- β 1) are upregulated (6-10). These immunosuppressive factors serve an important role in the immunosuppressive tumor microenvironment inducing T-cell apoptosis, suppressing the maturation of dendritic cells and influencing the differentiation of immune cells (11). In addition, hypoxia-inducible factor-1 (HIF-1) is an important oxygen-sensitive response factor that drives the transcription of several immunosuppressive molecules, which include PD-L1, VEGF, COX-2 and galectin-1 (6,11).

Rhein is a lipophilic anthraquinone mainly extracted from rhizomes of several traditional medicinal plants (12). A previous study revealed that rhein decreased the level of HIF-1 α (a subunit of HIF-1) in tumor cells under hypoxic conditions (13). The present study identified correlations between HIF-1 α and the immunosuppressive molecules in tissue samples from patients with CRC. This study also demonstrated that rhein induces an immunoregulatory effect, downregulating the expression of HIF-1 α and immunosuppressive molecules in CRC cell lines under hypoxic conditions. In addition, rhein enhanced the cytotoxicity of effector lymphocytes toward CRC tumor cells under hypoxic conditions. It may be hypothesized that rhein downregulates the expression of immunosuppressive molecules by inhibiting the expression of HIF-1 α under hypoxic conditions, and as a result this may enhance the cytotoxicity of effector lymphocytes.

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Key words: rhein, hypoxia, effector lymphocyte, immunosuppressive tumor microenvironment, hypoxia-inducible factor-1, colorectal cancer

Materials and methods

Patient samples. Fresh frozen tissue samples from 12 patients (8 males and 4 females aged from 49-83 years old) with CRC were provided by the Department of Colorectal Surgery, Tianjin Union Medical Center (Tianjin, China). All the tissue samples were collected from patients pathologically diagnosed with CRC between January and March 2018, and the characteristics of each patient are summarized in Table I. This study was approved by the Tianjin Nankai Hospital ethics commission (Tianjin, China; approval no. NKYY_YX_IRB_044_01).

Cell culture. The human CRC cell lines, HT29, HCT116, Colo205 and SW620 were obtained from the Tianjin Institute of Integrative Medicine for Acute Abdominal Diseases, Tianjin Nankai Hospital and the 293T cell line was provided by Professor Xiong Dongsheng from the Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College (Tianjin, China). Cells were maintained in Dulbecco's modified Eagle medium (DMEM; cat. no. 1791922; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; cat. no. 25030-081; Gibco; Thermo Fisher Scientific, Inc.) at 37°C. Cells cultured under hypoxic conditions were placed in a hypoxic chamber as previously described (14,15). The air inside the chamber was flushed with a mixture of N₂ (95%) and CO₂ (5%) and when the oxygen concentration decreased to 1%, the chamber was sealed and kept at 37°C.

Peripheral blood lymphocytes (PBLs) were isolated from the blood of healthy donors from the Tianjin Blood Center (Tianjin, China; dataset no. ISCP17021885; tjbc.org.cn) by Ficoll® solution (cat. no. LTS10771; TBD Science, Tianjin, China). The cells were maintained in Roswell Park Memorial Institute-1640 medium (cat. no. 1721503; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 100 U/ml recombinant human IL-2 (cat. no. 202-IL-010; R&D Systems, Inc., Minneapolis, MN, USA) at 37°C every 48 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells or tissue samples using TRIzol® reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA (4 µg) was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was subsequently performed using the SYBR® Premix Ex Taq™ kit (cat. no. RR420L; Takara Biotechnology Co., Ltd., Dalian, China). The primer pairs used are presented in Table II, and β-actin was used as a reference gene for normalization. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 60°C for 34 sec. To analyze the correlation of gene expression in tumor tissues of patients with CRC, the C_q values from patient 1 were used as a baseline to correlate the C_q values of other patients, and the Pearson product-moment correlation coefficient (Pearson's R) was measured. Relative gene expression in CRC cell lines was quantified using the 2^{-ΔΔC_q} method (16). These experiments were repeated in triplicate.

Cell viability assay. Cells were seeded onto 96-well plates at a density of 1×10⁴ cells/well and treated with 0, 12.5, 25, 50, 100 or 200 µM rhein (cat. no. SG8100; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 48 h. Then the purple formazan were dissolved in DMSO (cat. no. D8371-50; Beijing Solarbio Science & Technology Co., Ltd.) and measured at a wavelength of 492 nm. In CRC cell lines, cell viability was measured by MTT reduction assay (cat. no. M2128; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). In PBLs, cell viability was measured by cell counting kit-8 (cat. no. CK04; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) as manufacturer's protocol. Untreated cells were used as a negative control and cell viability was set as 100%.

Establishment of a cell line stably expressing the membrane-bound single chain Fv against CD3 (mCD3scfv). The mCD3scfv sequence was provided by Professor Xiong Dongsheng and amplified by PCR using Pyrobest™ DNA Polymerase (cat. no. R005A; Takara Biotechnology Co., Ltd.) and the following primers: 5'-CGTAGAATTCGCCACCATGGAGACAGACACACTCTG-3' and 5'-CGTAGGATCCCTAACGTGGCTTCTTCTGCC-3'. The thermocycling condition was as follows: Initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 10 sec, 60°C for 60 sec and 72°C for 60 sec. The PCR product was purified and cloned into the lentiviral expression vector pCDH1-CMV-MSC-EF1 α-Puro (cat. no. CD510B-1; System Biosciences, LLC., Palo Alto, CA, USA) using EcoRI and BamHI restriction sites. The lentiviral expression construct was co-transfected with backbone plasmids [5 µg MD2G, 3 µg PAX2 (Invitrogen; Thermo Fisher Scientific, Inc.) and 7 µg pCDH1] in 293T cells using X-tremeGENE™ HP DNA transfection reagent (cat. no. 06366236001; Roche Molecular Diagnostics, Pleasanton, CA, USA) to produce the lentivirus (17). HT29 cells were transduced with the lentivirus for 48 h and selected with 30 µg/ml puromycin (cat. no. P8230; Beijing Solarbio Science & Technology Co., Ltd.) for 2 weeks. The HT29 cell line stably expressing mCD3scfv, was termed HT29-CD3scfv.

Western blot analysis. Total protein was extracted from cells using radioimmunoprecipitation lysis buffer (cat. no. R0010) and the protein concentration was determined by BCA Protein Assay kit (cat. no. PC0020; both Beijing Solarbio Science & Technology Co., Ltd.). The proteins were separated using SDS-PAGE (10% polyacrylamide gel; 40 µg total protein per lane) and transferred onto polyvinylidene difluoride membranes (cat. no. IPVH00010; EMD Millipore, Billerica, MA, USA). Membranes were blocked by 5% skim milk at room temperature for 4 h and then incubated with primary antibodies (1:500) against HIF-1α (cat. no. 100-449), β-tubulin (cat. no. 66240-1; both 1:500; Wuhan Sanying Biotechnology, Inc., Wuhan, China), and HA-tag (cat. no. ab1818; 1:500; Abcam, Cambridge, MA, USA) overnight at 4°C. Following primary incubation, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse (cat. no. SE12) or anti-rabbit (cat. no. SE13) secondary antibodies (both 1:2,000; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature. Protein bands were visualized using the BioVision ECL Western

Table I. Patient information.

Patient no.	Sex	Age, years	Tumor location
1	Female	57	Sigmoid colon
2	Male	83	Ascending colon
3	Male	61	Ascending colon
4	Male	64	Ascending colon
5	Female	50	Descending colon
6	Male	62	Sigmoid colon
7	Female	69	Ascending colon
8	Male	67	Transverse colon
9	Male	58	Descending colon
10	Male	62	Ileocecus
11	Male	70	Descending colon
12	Female	49	Ascending colon

Blotting Substrate kit (cat. no. K820-500; BioVision, Inc., Milpitas, CA, USA).

Immunofluorescence. HT29-CD3scfv or HT29-control cells were fixed with 4% paraformaldehyde for 10 min and blocked with 1% bovine serum albumin (cat. no. A8010; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h. Cells were incubated with mouse anti-HA-tag primary antibody (cat. no. ab18181) for 1 h, followed by incubation with allophycocyanin (APC)-conjugated goat anti-mouse IgG (cat. no. ab130782; both 1:1,000; Abcam) secondary antibody for 1 h. Cells were stained with DAPI (Sigma-Aldrich, Merck KGaA) for 5 min. All the steps were performed at room temperature. Images were captured using a two-photon laser scanning confocal microscope (Olympus Corporation, Tokyo, Japan; FV1200 MPE).

Flow cytometry. HT29-CD3scfv or HT29-control cells were incubated with mouse anti-HA tag primary antibody (cat. no. ab18181; 1:1,000; Abcam) for 30 min at room temperature. Following primary incubation, cells were washed twice using PBS and incubated with APC-conjugated goat anti-mouse IgG (cat. no. ab130782; 1:1,000; Abcam) secondary antibody for 30 min at room temperature. Following one wash by PBS, cells were analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA; FACS LSRII). The data were analyzed by FlowJo software (version 7.6; Tree Star, Inc., Ashland, OR, USA).

Cytotoxicity assays of PBLs. The specific lysis of target cells was measured by lactate dehydrogenase release assay using the CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. HT29 and HT29-CD3scfv cells were seeded onto 96 well plates (1×10^4 cells/well) as target cells. PBLs were added at different effector-to-target (E:T) cell ratios 5:1, 10:1 and 20:1 to 96-well culture plates and co-cultured with target cells for 16 h.

For assessment under hypoxic conditions, HT29-CD3scfv cells were pretreated with or without 50 μ M rhein for 8 h.

Untreated cells in normoxia were used as the negative control. PBLs were subsequently added at different E:T cell ratios and co-cultured with target cells for 16 h, and specific cytotoxicity was detected as described above.

Statistical analysis. The results are reported as the mean \pm standard deviation of at least three independent experiments. All experimental data were analyzed using one-way analysis of variance (ANOVA) with Dunnett's post-test. The strength of a linear association between two variables was calculated with the Pearson product-moment correlation coefficient (Pearson's R coefficient). Pearson's R coefficient and ANOVA were performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HIF-1 α correlates with several immunosuppressive factors in CRC. To determine the effect of HIF-1 α in CRC, the mRNA expression levels of HIF-1 α and six immunosuppressive molecules were determined by RT-qPCR from 12 CRC tumor tissue samples. The correlation between the mRNA expression level of HIF-1 α and the six immunosuppressive molecules was analyzed by Pearson's R coefficient. The mRNA expression levels of PD-L1, VEGF, COX-2, and galectin-1 were positively correlated with that of HIF-1 α in CRC tissues (Fig. 1). However, there was no significant correlation between HIF-1 α and IL-10 or TGF- β 1 in the CRC tissue samples analyzed.

Rhein cytotoxicity. To determine the effect of rhein in CRC, cell viability was examined using the MTT and CCK-8 assays. The MTT assay was performed in CRC cell lines (HT29, HCT116, Colo205, SW620) following treatment with rhein (12.5, 25, 50, 100 and 200 μ M) for 48 h. Rhein (100 and 200 μ M) significantly decreased cell viability in a what appeared to be a dose-dependent manner in all four CRC cell lines ($P < 0.05$; Fig. 2A). The CCK-8 assay was performed in PBLs following treatment with rhein (25 or 50 μ M) for 48 h. Rhein had no affect on PBL cell viability (Fig. 2B).

Rhein downregulates the expression of HIF-1 α and immunosuppressive molecules in hypoxic conditions. To determine the inhibitory effect of rhein in hypoxia, the expression levels of HIF-1 α and six immunosuppressive molecules were examined using western blotting in HT29 cells cultured in 4 conditions: Normoxia, hypoxia, hypoxia + 25 μ M rhein, and hypoxia + 50 μ M rhein for 16 h. The protein expression level of HIF-1 α was increased in hypoxia, compared with normoxia. Additionally, rhein markedly decreased the protein expression level of HIF-1 α under hypoxic conditions in a dose-dependent manner (Fig. 3A). The mRNA levels of six immunosuppressive molecules were detected by RT-qPCR. Similar to HIF-1 α protein expression, the mRNA expression levels of PD-L1, VEGF, COX-2, galectin-1, IL-10 and TGF- β 1 were upregulated in hypoxia, compared with normoxia. Rhein significantly decreased the mRNA expression levels under hypoxic conditions in a dose-dependent manner (Fig. 3B). These results demonstrated that the mechanism underlying the inhibitory effect of rhein may be HIF-1 α -dependent. Although

Table II. Quantitative polymerase chain reaction primer sequences.

Gene	Primer sequence (5'→3')	
	Forward	Reverse
HIF-1 α	TGATTGCATCTCCATCTCCTACC	GACTCAAAGCGACAGATAACACG
PD-L1	TGTACCGCTGCATGATCAG	AGTTCATGTTTCAGAGGTGACTG
VEGF	ACTGAGGAGTCCAACATCAC	GTCTGCATTACATTTGTTG
COX-2	GGTCTGGTGCCTGGTCTGAT	TCCTGTTTAAAGCACATCGCATACT
Galectin-1	CGCTAAGAGCTTCGTGCTGAAC	CACACCTCTGCAACACTTCCAG
IL-10	GCCTTGTCTGAGATGATCCAGTT	TCACATGCGCCTTGATGTCT
TGF- β	GGGAAATTGAGGGCTTTCG	GAACCCGTTGATGTCCACTTG
β -actin	GGACATCCGCAAAGACCTGTA	GCATCCTGTCTGGCAATGC

HIF-1 α , hypoxia-inducible factor 1 subunit α ; PD-L1, programmed cell death 1 ligand 1; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; IL-10, interleukin 10; TGF- β 1, transforming growth factor β -1.

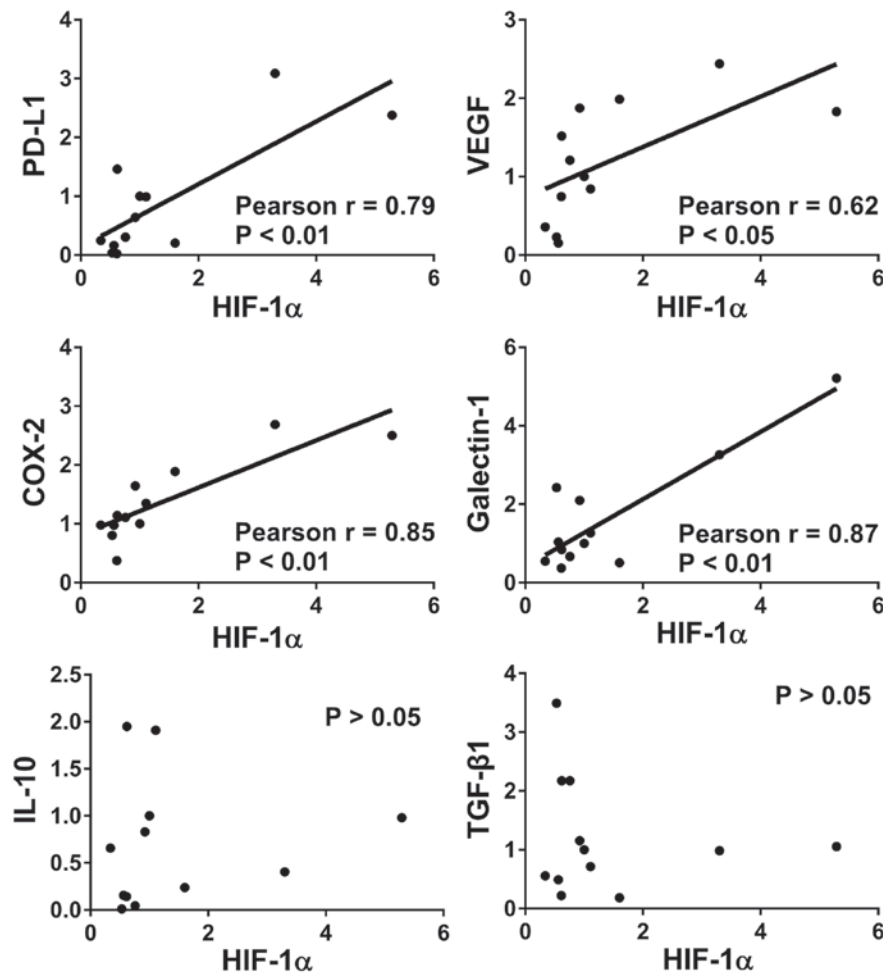


Figure 1. HIF-1 α mRNA expression correlates with immunosuppressive gene expression in colorectal cancer tissue. The expression levels of HIF-1 α and six genes with known immunosuppressive functions were determined by reverse transcription-quantitative polymerase chain reaction from 12 colorectal cancer tissue samples. The Pearson's R coefficient was used to measure the correlation between the mRNA expression level of HIF-1 α and the six immunosuppressive molecules. HIF-1 α , hypoxia-inducible factor 1 subunit α ; PD-L1, programmed cell death 1 ligand 1; COX-2, cyclooxygenase-2; IL-10, interleukin 10; VEGF, vascular endothelial growth factor; TGF- β 1, transforming growth factor β -1.

the mRNA expression levels of IL-10 and TGF- β 1 were also downregulated following treatment with rhein under hypoxic conditions (Fig. 3B), there was no correlation between HIF-1 α

and IL-10 or TGF- β 1 observed (Fig. 1). These results identified a potential HIF-1 α -independent mechanism underlying the inhibitory effect of rhein.

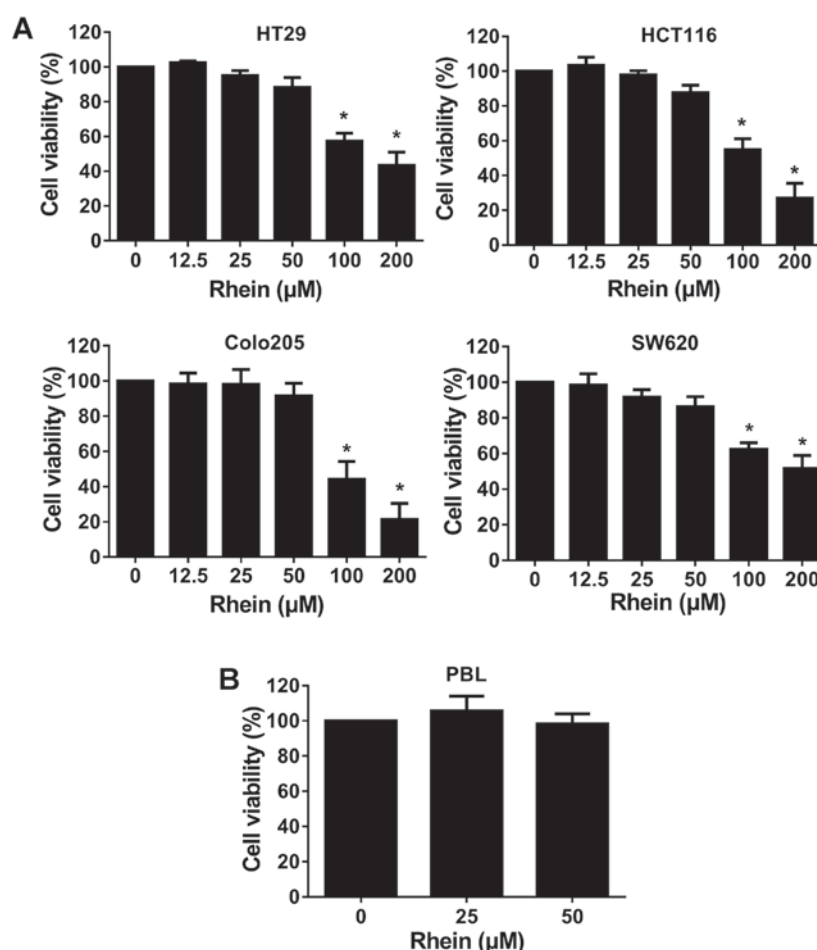


Figure 2. Rhein cytotoxicity in CRC cell lines and PBLs. (A) MTT assay was used to examine cell viability in four CRC cell lines following treatment with various concentrations of rhein for 48 h. * $P < 0.05$ vs. untreated cells. (B) Cell counting kit-8 assay was used to examine cell viability in PBL following treatment with rhein for 48 h. Data are presented as the mean \pm standard deviation and normalized to untreated control. CRC, colorectal cancer, PBL, peripheral blood lymphocyte; HT29, HCT116, Colo205 and SW620, human CRC cell lines.

Cytotoxicity of PBLs toward HT29-CD3scfv cells. To examine the cytotoxicity of PBLs in CRC, a CRC cell model specifically recognized by PBLs was established using a lentivirus containing the membrane-bound anti-CD3scFv expression cassette (Fig. 4A). HT29 cells were transduced with the lentiviral vector or blank control for 48 h and then selected with puromycin for an additional 2 weeks. To identify HT29 cells stably expressing mCD3scfv, western blot analysis, confocal microscopy and flow cytometry were used to detect the N-terminal HA-tag of the fusion protein. The results revealed that mCD3scfv was expressed in HT29-CD3scfv cells and mCD3scfv localized on the cell membrane (Fig. 4B and C). Furthermore, HT29 cells expressing the fusion protein accounted for 96.8% of the total HT29-CD3scfv cells examined (Fig. 4D). Finally, HT29-CD3scfv and HT29-control target cells were co-cultured with PBLs at different E:T cell ratios, respectively for 16 h. CytoTox96® non-radioactive cytotoxicity assays revealed that PBLs were cytolytic toward HT29-CD3scfv cells. Their cytotoxic effect increased as the E:T ratio using PBLs as effector cells and HT29-CD3scfv cells as target cells increased. PBLs were slightly cytolytic toward HT29-control cells. Furthermore, the cytotoxicity of HT29-CD3scfv significantly increase compared with

HT29-control cells at the corresponding E:T ratio ($P < 0.05$; Fig. 4E).

Rhein enhances the cytotoxicity of PBLs in hypoxia. To further examine the inhibitory effect of rhein in hypoxia, the cytotoxicity of PBLs toward CRC cells was examined following treatment with rhein under hypoxic conditions. HT29-CD3scfv cells were cultured under conditions of normoxia, hypoxia and hypoxia + 50 μ M rhein, followed by co-culture with PBLs at different effector-to-target cell ratios (5:1, 10:1 and 20:1). CytoTox96® non-radioactive cytotoxicity assays revealed that PBLs are cytolytic toward HT29-CD3scfv cells, and their cytotoxic effect was inhibited by hypoxia. Treatment with rhein enhanced the cytotoxicity of PBLs in hypoxia, compared with normoxia (Fig. 5).

Discussion

Since the expression of HIF-1 α is inhibited by rhein (13), it may be hypothesized that rhein downregulates immunosuppressive molecules driven by HIF-1 and further enhances the anti-tumor effect of PBLs in hypoxia. The present study investigated the correlation between the mRNA levels of HIF-1 α and the six

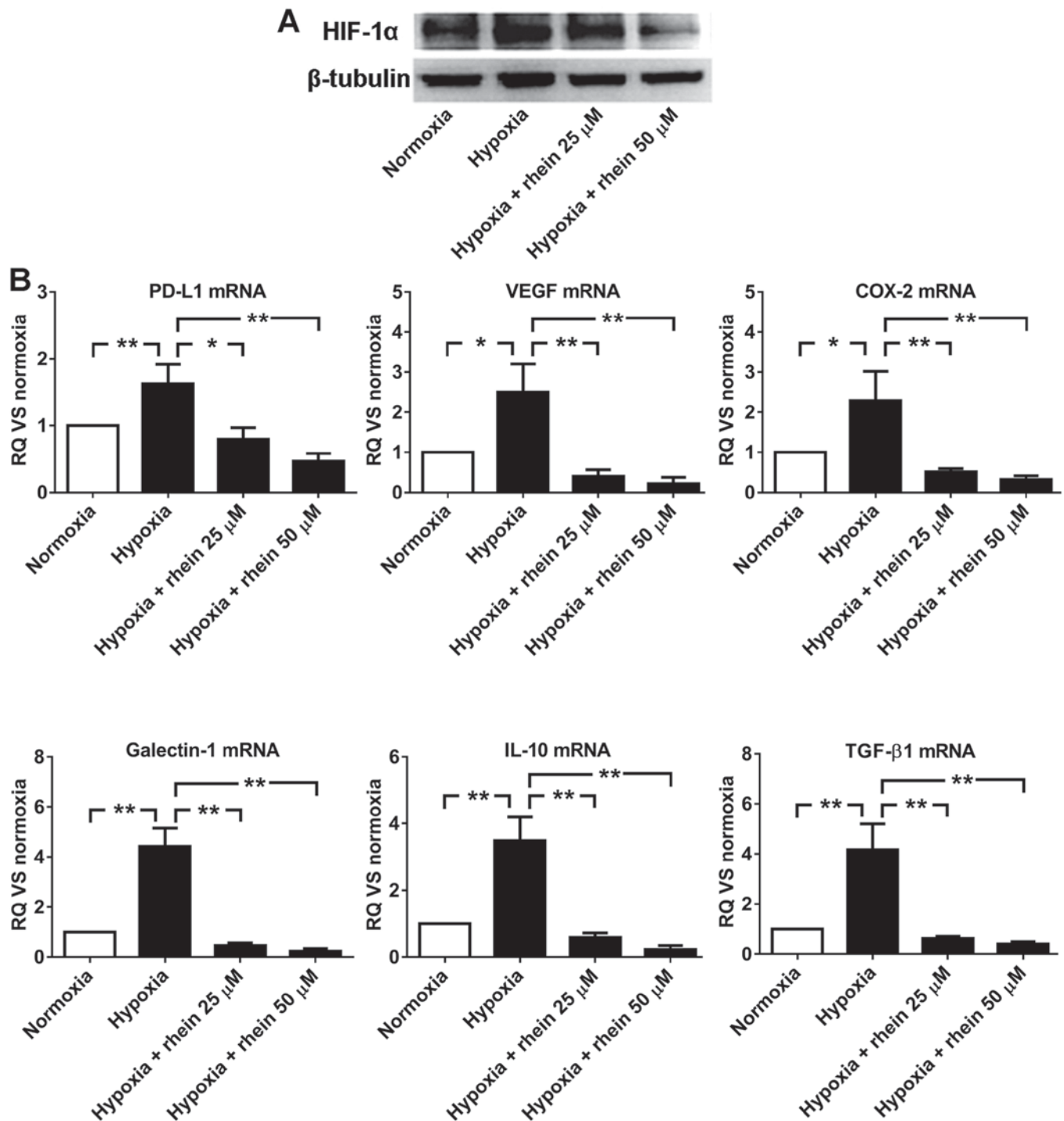


Figure 3. Rhein downregulates the expression of HIF-1α and six immunosuppressive molecules. (A) The protein expression levels of HIF-1α were determined by western blot analysis in HT29 cells cultured under conditions of normoxia, hypoxia, hypoxia + 25 μM rhein, and hypoxia + 50 μM rhein for 16 h. (B) The mRNA expression levels of PD-L1, VEGF, COX-2, galectin-1, IL-10 and TGF-β1 were determined by reverse transcription-quantitative polymerase chain reaction in HT29 cells treated as above. The mRNA expression levels were normalized to that of cells under normoxia. The data are presented as the mean ± standard deviation from three independent experiments. *P < 0.05 and **P < 0.01 as indicated. RQ, relative quantification; CRC, colorectal cancer; HIF-1α hypoxia-inducible factor 1 subunit α; PD-L1, programmed cell death 1 ligand 1; COX-2, cyclooxygenase-2; IL-10, interleukin 10; VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1; HT29, human CRC cell line.

immunosuppressive molecules in tumor tissue samples from patients with CRC. The mRNA expression of four immunosuppressive molecules positively correlated with HIF-1α expression. However IL-10 and TGF-β1 did not correlate with HIF-1α expression. The expression levels of HIF-1α and the six immunosuppressive molecules were upregulated in hypoxia, however the addition

of rhein significantly decreased the mRNA expression levels under hypoxic conditions. These results indicated that the mechanism underlying the inhibitory effect of rhein may be HIF-1α-dependent. However, the mRNA expression levels of IL-10 and TGF-β1 were downregulated following treatment with rhein under hypoxic conditions without the observed correlation

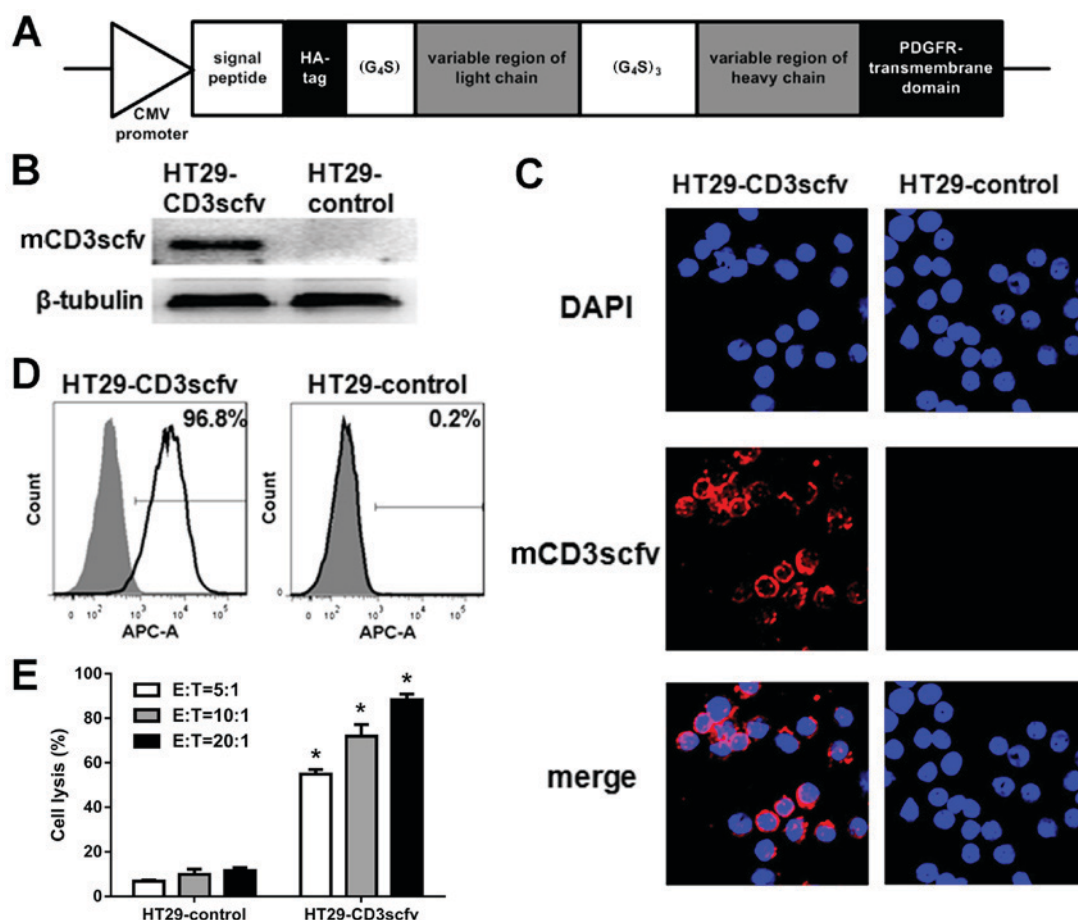


Figure 4. Cytotoxicity of PBLs toward HT29-CD3scfv cells. (A) Schematic representation of the lentiviral vector construct encoding membrane-bound anti-CD3scfv expression cassette. (B) The protein expression levels of CD3scfv in HT29-CD3scfv and HT29-control cells were determined by western blot analysis. (C) The cellular localization of CD3scfv in HT29-CD3scfv and HT29-control cells was detected by confocal microscopy (magnification, x400). (D) The proportion of cells expressing membrane-bound anti-CD3scfv was determined by fluorescence-activated cell sorting analysis. (E) HT29-CD3scfv and HT29-control cells were co-cultured with PBLs at different E:T cell ratios, respectively for 16 h. CytoTox96® Non-Radioactive Cytotoxicity assay was used to determine cell lysis of HT29-CD3scfv and HT29-control cells. The data are presented as the mean ± standard deviation from three independent experiments. *P < 0.05 vs. HT29-control cells at the corresponding E:T ratio. HT29-control, untreated HT29 cells; HT29-CD3scfv, HT29 cells stably expressing mCD3scfv; HT29, human CRC cell line; PBL, peripheral blood lymphocyte; CRC, colorectal cancer; HA, hemagglutinin; E:T, effector-to-target cell ratio.

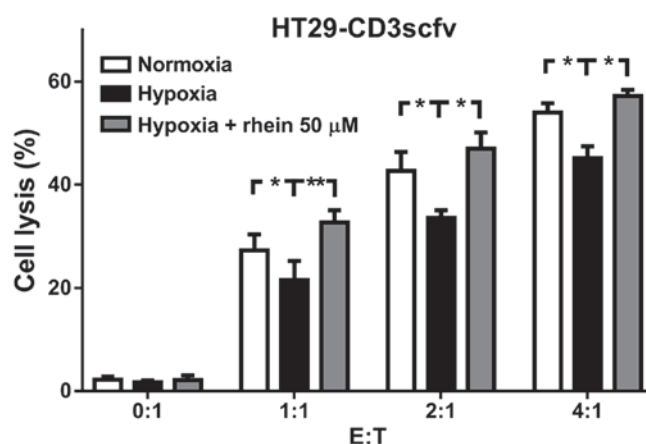


Figure 5. Rhein enhances the cytotoxicity of PBLs in hypoxia. HT29-CD3scfv cells were cultured under conditions of normoxia, hypoxia and hypoxia + 50 μM rhin for 8 h. Cells were subsequently co-cultured with PBLs at different E:T cell ratios for 16 h. HT29-CD3scfv cell lysis was examined by CytoTox96® Non-Radioactive Cytotoxicity assay. The data are presented as the mean ± standard deviation from three independent experiments. *P < 0.05 and **P < 0.01 as indicated. PBLs, peripheral blood lymphocytes; HT29-CD3scfv, HT29 cells stably expressing mCD3scfv; E:T, effector-to-target cell ratio.

with HIF-1α, which indicated a potential HIF-1α-independent mechanism underlying the inhibitory effect of rhin in hypoxia. Furthermore, the current study demonstrated that hypoxic conditions reduced the anti-tumor effect of PBLs. However, the addition of rhin under these conditions enhanced the anti-tumor effect of PBLs in CRC cells.

Although the present study demonstrated that rhin enhanced the cytotoxicity of PBLs on CRC cells in hypoxia, there are several limitations that should be noted. Despite having no effect on the viability of PBLs, other regulatory functions of rhin on effector lymphocytes were not addressed in this study and will need further investigation. Furthermore, the role of HIF-1α in T-cells is controversial and needs further investigation. Some studies suggested that the activity of T-cells in the tumor microenvironment is dependent on the energy supplied by the increased expression of HIF-1 (18,19), however, other studies have demonstrated that the inhibition of HIF-1α may transform T-cells into memory cells, as well as enhancing the effect of cancer vaccines (20-22). The effect of rhin on metabolism and effector T-cell activity is under investigation. Additionally, immunosuppressive molecules and immunosuppressive cells, which include tumor-associated macrophages,

myeloid-derived suppressor cells and regulatory T-cells, are components of the immunosuppressive tumor microenvironment and the immunosuppressive function of these cells is dependent on immunosuppressive molecules and HIF (23-25). Additional *in vivo* studies investigating the effects of rhein with respect to the proportion of immunosuppressive cells in the tumor and infiltration of effector lymphocytes are necessary.

In conclusion, the current study validated the hypothesis that rhein, a down-regulator of HIF-1 α , inhibited the expression of immunosuppressive molecules in CRC cells and enhance the cytotoxicity of effector lymphocytes under hypoxic conditions. It was revealed that HIF-1 α positively correlated with immunosuppressive molecules in CRC tissues, and rhein decreased the mRNA levels of HIF-1 α and immunosuppressive molecules in CRC cell lines, and increased the cytotoxicity of effector lymphocytes to HT29-CD3scfv cells in hypoxia. These results indicate that rhein may have great potential to combine with effector T lymphocytes for the treatment of CRC.

Acknowledgements

The authors would like to thank Professor Xiong Dongsheng from the Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College (Tianjin, China) for providing the 293T cell line and the mCD3scfv sequence.

Funding

National Natural Science Foundation of China (Grant no. 81572318).

Availability of data and materials

Not applicable.

Authors' contributions

XY and FW designed the study. XY, WT and LH performed the experiments. JX and JH analyzed the data. YH and JY collected the human tissue samples.

Ethics approval and consent to participate

The current study was approved by the Tianjin Nankai Hospital ethics commission (approval no. NKYY_YX_IRB_044_01).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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