

BASIC SCIENCE ARTICLE



Propranolol inhibits the angiogenic capacity of hemangioma endothelia via blocking β -adrenoceptor in mast cell

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BACKGROUND: Propranolol, a non-selective blocker of the β -adrenoceptor (AR), is a first-line treatment for infantile hemangioma (IH). Mast cells have been implicated in the pathophysiology of propranolol-treated hemangioma. However, the function of mast cells remains unclear.

METHODS: HMC-1s (Human mast cell line) having been treated with propranolol for 24 h were centrifuged, washed with PBS twice, and maintained in cell culture medium for another 24 h. The supernatants with propranolol which were named as propranolol-treated HMC-1s supernatants were obtained. The expression of cytokines and mediators was examined among HMC-1s dealt with propranolol. HemECs (hemangioma endothelial cells) were co-cultured with propranolol-treated HMC-1s supernatants, and their proliferation and apoptosis were investigated. The autophagic-related protein was examined in HemECs using immunoblot.

RESULTS: In propranolol-treated HMC-1s, the expressions of ADRB1 (β 1-AR) and ADRB2 (β 2-AR) were reduced by 70% and 60%, respectively, and that of cytokines and mediators were reduced. The proliferation was decreased, but apoptosis and autophagy were induced in HemECs treated with propranolol-treated HMC-1s supernatants. However, propranolol can work well in shRNA-ADRB1 or shRNA-ADRB2 transfected HMC-1s.

CONCLUSIONS: Propranolol inhibit the proliferation of HemECs and promote their apoptosis and autophagy through acting on both β 1 and β 2 adrenoceptor in mast cell.

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IMPACT:

- Treated with propranolol, β 1, and β 2 adrenoceptor on human mast cell expression was reduced significantly.
- After hemangioma endothelial cell treated with the supernatants from propranolol-treated human mast cell, its proliferation was decreased, but apoptosis and autophagy were significantly induced.
- Propranolol can work well in shRNA-ADRB1 or shRNA-ADRB2 transfected HMC-1s. Mast cells may have a role in the action of propranolol in infantile hemangioma through both β 1 and β 2 adrenoceptors to inhibit the angiogenic capacity of hemangioma endothelial cells.

INTRODUCTION

Infantile hemangioma (IH) is a benign vascular tumor that commonly occurs in early infancy, with a prevalence of ~5–10% in infants.¹ A significant minority of IH cases in the proliferating phase require medication to prevent complications such as disfigurement, functional impairment, and ulceration.² In 2008, Léauté-Labrèze et al. reported that propranolol, a non-selective β -adrenoceptor (AR) blocker, inhibited the growth of IH. Because of its safety and relatively few side effects, propranolol has replaced corticosteroids as the first-line therapy for problematic IH.³

In recent years, the cellular and molecular mechanisms, by which propranolol exerts its effects, have been widely investigated. Ji et al.

demonstrated that propranolol inhibited the proliferation and induces apoptosis of endothelial cells within hemangiomas, which was mediated by the suppression of vascular endothelial growth factor A (VEGF-A) expression.⁴ IH tissues treated with propranolol were found to be richly infiltrated by mast cells.⁵ Furthermore, β -AR were reported to be highly expressed on mast cells in propranolol-treated IH.⁶ However, the function of mast cells in propranolol-treated IH remains unclear.

Mast cells have been implicated in the pathophysiology of hemangioma. It was found that several mast cell mediators such as VEGF-A, basic fibroblast growth factor (bFGF), histamine, heparin, interleukin-8 (IL-8), and tumor necrosis factor- α (TNF- α) may serve as

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endothelial cell mitogen.⁷ It is also reported that VEGF-A and bFGF are major mediators in mast cell-mediated angiogenesis.⁷

In our previous study, we found that following propranolol treatment, mast cell activation can reduce the proliferation and increase the apoptosis of human umbilical vein blood endothelial cells (HUVEC, Supplementary Data). In this study, we analyzed whether human mast cells treated with propranolol could affect the proliferation, apoptosis, or autophagy of hemangioma endothelial cells (HemECs).

METHODS

HMC-1s culture

HMC-1s were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HMC-1s were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), 0.05 mM beta-mercaptoethanol, 2mM L-glutamine, 0.1 mM non-essential amino acids, 10 ng/mL interleukin-3 (IL-3), and 10 ng/mL human stem cell factor (SCF).

β 1-AR and β 1-AR transduction assays

Lentiviral particles containing β 1-AR (ADRB1) shRNA vectors, β 2-AR (ADRB2) shRNA vectors, or non-silencing control vector DNA (Thermo Scientific-Open Biosystems) were generated by reverse transfection of these constructs, together with Trans-Lentiviral package mix, into HEK293T cells using Arrest-In/Express-In transfection reagent. Approximately 10^5 TU/mL was used to infect HMC-1 cells in the presence of polybrene (10 μ g/mL) and a stable culture was generated by growing these cells in the presence of 1 μ g/mL puromycin, the lowest concentration observed to kill 63% ADRB1- and 55% ADRB2-transduced-HMC-1 cells. All reagents for these transduction assays were purchased from Thermo Scientific. The shRNA sequences were as follows: forward, 5'-CACCG-GAAAGTTTGGGAAGGGATGGCGAACCATCCCTTCCCAAACCTTCC-3' and reverse, 5' AAAAGGAAAGTTTGGGAAGGGATGGTTCGCATCCCTTCCCAAAC TTTCC-3' for ADRB1; forward, 5'-CACCGCATCGTCATGTCTCTCATCGGAAC-GATGAGAGACATGACGATGC-3' and reverse, 5'-AAAAGCATCGTCATGTCTC ATCGTTCGCGATGAGAGACATGACGATGC-3' for ADRB2.

Propranolol treatment

A stock solution of propranolol (Merck, Darmstadt, Germany) was prepared at 100 mM in Dimethyl sulfoxide (DMSO) and stored at 4 °C. For all the treatments, the final concentration of DMSO in the medium was 0.1%. SingleQuot was excluded in the experiments because this agent affected cell survival. Before each treatment, cells were plated on the indicated plates and cultured in standard media as previously mentioned. The HMC-1s supernatants were available. Twenty-four hours later, the medium was replaced with EBM-2 supplemented with FBS and the indicated concentrations of propranolol. Propranolol-treated HMC-1s supernatants were obtained. HMC-1s treated with 0.1% DMSO was set as the control group.

Isolation and culture of HemECs

This study was approved by the Ethics Committee of Children's Hospital of Fudan University. Tissue samples were collected from two subjects suffering from infantile hemangioma (the proliferative phase) treated by surgical resection without any medication. Informed consent was obtained

from the subjects' parents. The clinical diagnosis of vascular neoplasms was confirmed by the Department of Pathology at Children's Hospital of Fudan University through staining for GLUT-1, a marker specific for hemangioma tissue. The hemangioma samples were minced into small pieces using a scalpel and treated with a human tumor cell dissociation kit (Miltenyi Biotec, Cologne, bergesch gladbach, Germany). All cells were filtered through a 40- μ m strainer to obtain a single-cell suspension. HemECs were isolated using human CD31 MicroBead Kit (Miltenyi Biotec, Cologne, bergesch gladbach, Germany).⁸ The HemECs were fluorescently stained with CD31-FITC and analyzed using FACS Cantoll flow cytometer (FACS Cantoll, BD, Franklin Lakes, NJ). These HemECs were plated on gelatin-coated 60-mm plates in Endothelial Basal Medium (EBM-2; Lonza, Walkersville, MD) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY), SingleQuot (Lonza), penicillin (100 units/mL; Gibco), and streptomycin (100 μ g/mL; Gibco, Grand Island, NY). The cells were grown in a humidified atmosphere containing 5% CO₂ at 37 °C.

HemECs proliferation and apoptosis analysis

The HemECs dealt with DMSO-treated HMC-1s supernatants were designated as the control group while that with propranolol-treated HMC-1 supernatants were referred as experimental group. HemECs were seeded at 4×10^3 cells/well in the 96-well plates. Cell proliferation was assessed by CCK-8 proliferation assay (Dojindo, Shanghai, China). After cells were cultured for 1, 2, 3, or 4 d, 10 μ L CCK-8 solution was added to each well of a 96-well plate. After the plate was incubated at 37 °C for another 4 h, optical density (OD) at 450 nm was measured against a solution blank in a microplate reader (MultiSkan FC, ThermoFisher, Foster City). Cell apoptosis was assayed using a commercially available Annexin V-FITC apoptosis detection kit I (BD Biosciences, San Jose, CA) and flow cytometry. Briefly, the cells were trypsinized, harvested, washed, and resuspended with 100 mL 1 \times binding buffer at a final concentration of 1×10^5 cells/mL. Then the cells were stained with 5 μ L Annexin V/FITC followed by 1 μ L PI and incubated in the dark for 15 min at room temperature. The apoptotic cells were analyzed by FACS Cantoll flow cytometer (FACS Cantoll, BD, Franklin Lakes, NJ).

Real-time quantitative polymerase chain reaction analysis

The expression of the cytokines and mediators VEGF-A, bFGF, matrix metalloproteinase (MMP)2, MMP9, and tryptase were evaluated by PCR in HMC-1 cells and transduced-HMC-1 cells. Total RNA was extracted using TriZol reagent (ThermoFisher Scientific, Waltham, MA). Isolated RNA was reverse-transcribed using Reverse Transcriptase M-MLV (Takara, Dalian, China) and then amplified using Takara SYBR Premix Ex Taq (Takara, Dalian, China) according to the manufacturer's instructions. Primers for qPCR assays were from Sangon Biotech Company (Shanghai, China) and their sequences are shown in Table 1. The qPCR assays were performed in triplicate on an ABI 7500 Real-Time PCR instrument (Applied Biosystems, Foster City), according to the manufacturer's protocols. All assays had a single amplification peak on the melting temperature curve with PCR efficiencies >80% and coefficients of variance of less than 15%, in triplicate reactions. Relative expression levels were calculated using the comparative Ct method.

Enzyme-linked immunosorbent assay

The concentrations of VEGF-A, bFGF, MMP2, MMP9, and tryptase in HMC-1 cells and transduced-HMC-1 cells after propranolol therapy were measured

Table 1. Sequences of primers used for qPCR.

| Gene | Forward | Reverse |
|----------------|------------------------|-----------------------|
| ADRB1 | AGGCGCTGAGTCTCTCTCTG | CACCTCCCCATCCCAGTAAA |
| ADRB2 | TGATCGCAGTGGATCGCTAC | GGACACGATGGAAGAGGCAA |
| VEGF-A | AGGGCAGAATCATCACGAAGT | AGGGTCTCGATTGGATGGCA |
| bFGF | GATGTAGAAGATGTGACGCCG | GGTTCACGGATGGGTGTCT |
| MMP2 | GATACCCCTTTGACGGTAAGGA | CCTTCTCCAAAGGTCCATAGC |
| MMP9 | GTACTCGACCTGTACCAGCG | TTCAGGGCGAGGACCATAGA |
| Tryptase | GCCATACTGGATGCACTTC | CGATCTGGGCGGTGTAGAAC |
| β -actin | ACACCCAGCCATGTACGT | ATGGGCACAGTGTGGGTGA |

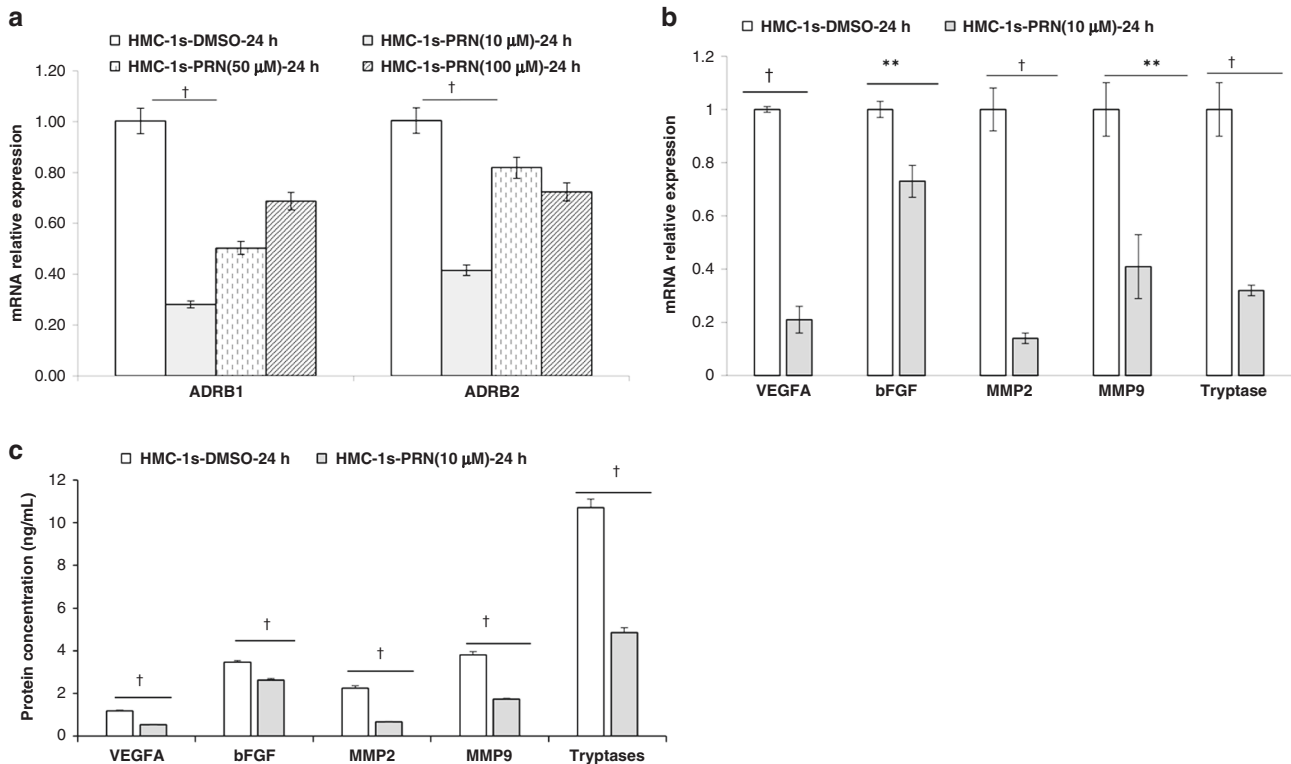


Fig. 1 Propranolol decreased ADRB1, ADRB2, VEGF-A, bFGF, MMP2, MMP9, and tryptase expression in HMC-1s. **a** *ADRB1* and *ADRB2* mRNA expression was significantly decreased and these levels were lowest in HMC-1s treated with 10 μM propranolol. **b, c** Treatment with 10 μM propranolol caused significantly decreased expression of VEGF-A, bFGF, MMP2, MMP9, and TPSAB1 (tryptase) at the mRNA and protein level in HMC-1s. HMC-1s-DMSO-24 h indicated that HMC-1s were treated with 0.1% DMSO for 24 h. HMC-1s-PRN (10 μM)-24 h indicated that HMC-1s were treated with 10 μM propranolol for 24 h. HMC-1s-PRN (50 μM)-24 h indicated that HMC-1s were treated with 50 μM propranolol for 24 h. HMC-1s-PRN (100 μM)-24 h indicated that HMC-1s were treated with 100 μM propranolol for 24 h. All data presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, † $P < 0.001$.

with enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Western blot assay

HemECs treated with propranolol-treated HMC-1 supernatants were collected at 48 h and lysed in M-PER mammalian protein extraction lysis buffer (Thermo Scientific, Hudson, NH) for 30 min. Then total protein was extracted from the collected cells with RIPA lysis buffer (Zhong-Shan JinQiao, Beijing, China). Protein concentrations were evaluated by BCA protein assay kits (CoWin Biotechnology, Beijing, China). Equal amounts of total proteins (40 μg) were subjected to 10% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Boston, MA). Then PVDF membranes were blocked with 5% milk for 1 h and incubated with primary antibodies against ATG5, ATG7, p62, LC-3I, and LC-3II (1:1000; Cell Signaling Technology, Danvers, MA) and GAPDH (1:1000; Cell Signaling Technology) followed by overnight incubation at 4°C. The membranes were further incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) and the protein bands were visualized by chemiluminescence using ECL reagent (Millipore Corp., Billerica, MA). IPP7.0 software (Media Cybernetics, Silver Springs, MD) was used for quantitative analysis, with GAPDH as the internal control. Relative protein expression was calculated from the gray value target band and the gray value internal control.

Statistical analysis

Data are expressed as mean ± standard deviation. An independent sample *t*-test was applied to identify significant differences between the two groups. One-way ANOVA followed by the *S*-*N*-*K* method was used to determine significant differences among multiple groups. All data analyses were performed using SPSS11.0 statistical software and differences were considered significant at $P < 0.05$.

RESULTS

Propranolol decreased the expression of ADRB1, ADRB2, VEGF-A, bFGF, MMP2, MMP9, and tryptase in HMC-1s

The mRNA expression levels of *ADRB1* and *ADRB2* were decreased significantly to the lowest level in HMC-1s treated with 10 μM propranolol (Fig. 1a). When HMC-1s were treated with 10 μM propranolol 24 h, the mRNA and protein expression of VEGF-A, bFGF, MMP2, MMP9, and tryptase were both significantly decreased (Fig. 1b, c).

Propranolol-treated HMC-1s supernatants inhibited the proliferation and induced the apoptosis of HemECs

After incubation with propranolol-treated HMC-1s supernatants for 24 and 48 h, respectively, the proliferations (cell counts) of HemECs were significantly less than those from the control group in HemECs dealt with 0.1% DMSO-treated HMC-1s supernatants ($P < 0.01$) (Fig. 2a). After incubation with propranolol-treated HMC-1s supernatants 48 h, the percentage of Annexin V positive and Annexin V/PI positive HemECs was two times as the control group in which HemECs was dealt with 0.1% DMSO-treated HMC-1 supernatants (34.76% vs. 18.65%; $P < 0.01$) (Fig. 2b–e).

Propranolol-treated HMC-1 supernatants induced autophagy in HemECs

Western blot assay was performed to determine the levels of autophagy-related proteins ATG5, ATG7, P62, and autophagy markers LC-3I and LC-3II of HemECs at 48 h. Compared with the control group, propranolol-treated HMC-1s supernatants increased the expression of LC-3II/I, ATG5, ATG7, and P62 in HemECs (Fig. 3).

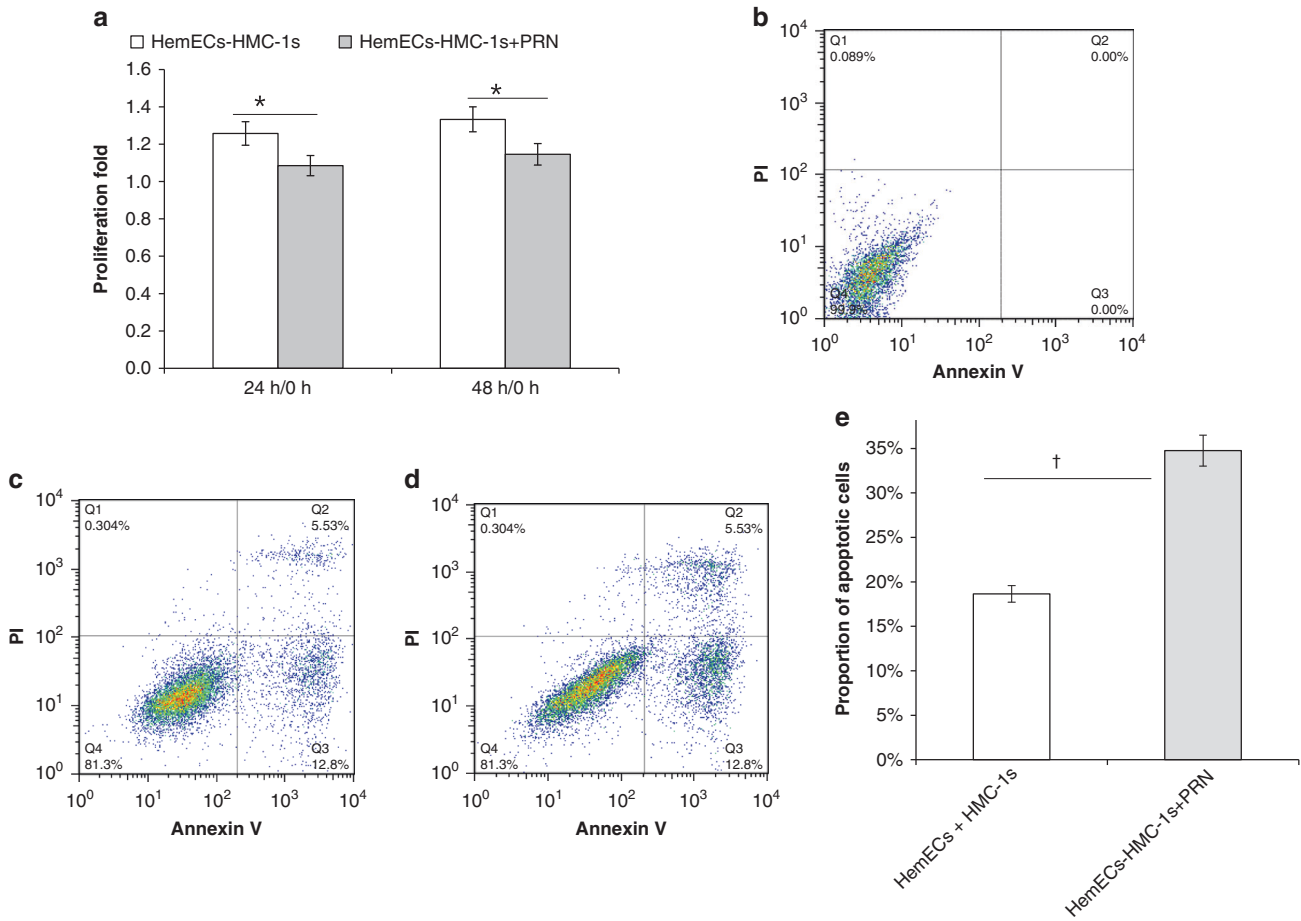


Fig. 2 Propranolol-treated HMC-1s supernatants inhibited proliferation and induced apoptosis in HemECs. HemECs co-cultured with propranolol-treated HMC-1s supernatants showed significantly less proliferation than those from the control group ($P < 0.01$) at 24 and 48 h. HemECs-HMC-1s indicated that HemECs were co-cultured with the 0.1% DMSO-treated HMC-1 supernatants. The apoptosis in HemECs dealt with propranolol-treated HMC-1 supernatants has a one-fold increase in the percentage of Annexin V positive and Annexin V/PI positive HemECs compared with the control group ($P < 0.01$; 34.76% vs. 18.65%). HemECs-HMC-1s-PRN indicated that HemECs were co-cultured with propranolol-treated HMC-1 supernatants. All data presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, † $P < 0.001$.

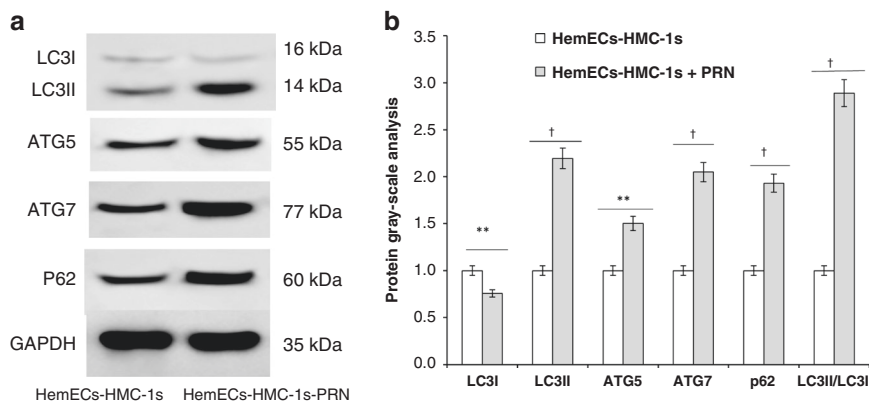


Fig. 3 Propranolol-treated HMC-1s supernatants induced autophagy in HemECs. Compared with the control group, propranolol-treated HMC-1s supernatants increased LC-3II/I, ATG5, ATG7, and P62 protein expression in HemECs. HemECs-HMC-1s indicated that HemECs were co-cultured with the 0.1% DMSO-treated HMC-1 supernatants. HemECs-HMC-1s-PRN indicated that HemECs were co-cultured with propranolol-treated HMC-1 supernatants. All data presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, † $P < 0.001$.

Expression of cytokines and mediators in propranolol-treated HMC-1s transfected with shRNA-ADRB1 and shRNA-ADRB2

In order to explore which subtype β -AR on mast cells was acted upon by propranolol, we use β -AR transduction assay. The mRNA expression of *ADRB1* was decreased by 67% in HMC-1s transfected

with shRNA-ADRB1 while that of *ADRB2* was decreased by 58% in HMC-1s transfected with shRNA-ADRB2 compared with HMC-1s transfected with negative control (NC) at 24 h (Fig. 4a). The mRNA and protein expression of cytokines and mediators VEGF-A, bFGF2, MMP2, MMP9, and TPSAB1 (tryptase) were slightly decreased

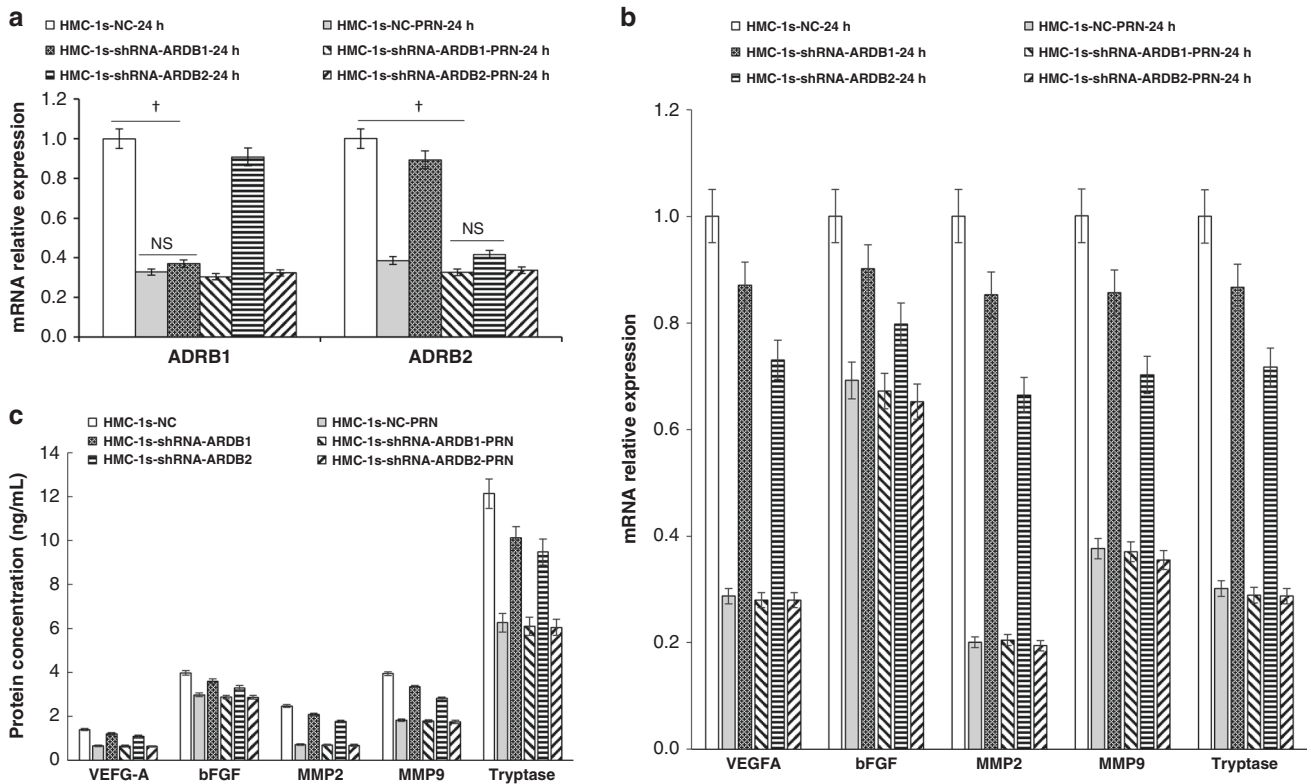


Fig. 4 Expression of cytokines and mediators in shRNA-transfected HMC-1s. **a** mRNA expression of *ADRB1* and *ADRB2* of stably knocked down HMC-1s. **b, c** the mRNA expression and the protein expression of cytokines and mediators VEGF-A, bFGF, MMP2, MMP9, and TPSAB1 (tryptase) of HMC-1s in each group. HMC-1s+NC indicated that HMC-1s were transfected with negative shRNA. HMC-1s+shRNA-ADRB2 indicated that HMC-1s were transfected with shRNA-ADRB2. HMC-1s+NC+PRN-24 h means propranolol was treated in HMC-1s transfected with negative shRNA for 24 h. HMC-1s+shRNA-ADRB1 indicated that HMC-1s was transfected with shRNA-ADRB1. HMC-1s+shRNA-ADRB1+PRN-24 h indicated that propranolol was treated in HMC-1s transfected with shRNA-ADRB1 for 24 h. HMC-1s+shRNA-ADRB2+PRN-24 h indicated that propranolol was treated in HMC-1s transfected with shRNA-ADRB2 for 24 h. All data presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, † $P < 0.001$, ^{NS} $P > 0.05$.

(Fig. 4b, c) in HMC-1s transfected with shRNA-ADRB1 or shRNA-ADRB2 for 48 h. When the shRNA-ADRB1 or shRNA-ADRB2 transfected HMC-1s were treated with propranolol, the expression of cytokines and mediators was decreased sharply.

DISCUSSION

Propranolol, a non-selective adrenergic antagonist, is a first-line treatment for IH.³ The precise mechanism by which propranolol induce accelerated involution of IH remains to be fully elucidated. Potential explanations for the therapeutic effects of propranolol in IH include vasoconstriction, inhibition angiogenesis, induction capillary endothelial cell apoptosis, and increased pericyte contractility.^{9–13} The effects of propranolol on several important cell types in IH, including hemangioma stem cells, hemangioma endothelial cells, hemangioma perivascular cells were reported in recent studies.^{9–13} Mast cell-infiltrating propranolol-treated IH lesions were reported previously.^{6,14,15} In our study, we found that propranolol might affect HemEC proliferation, apoptosis, and autophagy by acting on β -adrenoceptors and modulating the secretion of cytokines and mediator from HMC-1s.

The pathogenesis of IH has not been fully elucidated. It was postulated that IH was formed by the proliferation of immature endothelial cells stimulated by angiogenic factors.^{16–18} Cytokines and growth factors induce the formation of a capillary network by endothelial cells.^{16,17} The most extensively studied cytokines involved in the growth of IH are VEGF-A, bFGF, MMP2, and MMP9.^{18–21} VEGF-A is involved in vascular proliferation and permeability,^{19,21,22} and is produced by various cell types including endothelial cells, macrophages, platelets, and tumor cells. VEGF-A

stimulates the migration of vascular endothelial cells, the proliferation and formation of new vessels, and the inhibition of apoptosis.²² MMPs are secreted by mast cells and tumor cells and are involved in angiogenesis by degrading components of the extracellular matrix, allowing for the formation of new vessels.²³ Increased levels of MMP2 and MMP9 have been observed in tissue, blood, and urine samples from children in the proliferative phase of IH.²³ There is evidence that MMP2 and MMP9 are regulated by β -adrenoceptors.²⁴

Mast cells had been shown to abundantly infiltrate hemangioma tissues.^{25–27} There is evidence that several mast cell mediators, such as VEGF-A, bFGF, histamine, heparin, IL-8, TNF- α , platelet derived growth factor (PDGF), and hepatocyte growth factor (HGF) may serve as endothelial cell mitogens.⁷ VEGF-A and bFGF are potent angiogenic factors in the proliferating phase but not in the latter phases of hemangioma.^{20,28} An in vivo study confirmed that VEGF-A and bFGF are major mediators in mast cell-mediated angiogenesis.^{20,29} In addition, tryptase, MMPs, IL-3, IL-4, IL-8, and other cytokines produced by mast cells might contribute to the angiogenic process through the degradation of the extracellular matrix.^{30,31}

Mast cells expressed high β 1-AR and β 2-AR,⁶ indicating that mast cells be the possible targets of propranolol. However, the function of mast cells in propranolol-treated IH remains unclear. In our study, VEGF-A, bFGF, MMP2, MMP9, and tryptase expression in mast cells treated with propranolol was significantly reduced at the mRNA and protein level. After propranolol-treated HMC-1 supernatants were co-cultured with HemECs for 24 h, it was found that the proliferation of HemECs was decreased, but their apoptosis and autophagy were significantly increased. To our knowledge, our study was the first to explore

the potential mechanism on the act of mast cells in propranolol-treated IH.

Furthermore, we explored which subtype β -AR on mast cells was acted upon by propranolol through the β -AR transduction assay. In this study, the expression of β 1-AR and β 2-AR was decreased by 62% and 56% by *ADRB1* and *ADRB2* knockdown in HMC-1 cells, respectively. The expression levels of VEGF-A, bFGF, MMP2, MMP9, and tryptase in propranolol-treated HMC-1s transfected with shRNA-*ADRB1* or shRNA-*ADRB2* was decreased slightly at the mRNA and protein level much lower than that in propranolol-treated HMC-1s. These findings demonstrated that propranolol can simultaneously act on the β 1 and β 2 adrenoceptors of mast cells.

In the previous study, we found that after propranolol treatment, mast cell can reduce the proliferation and increase the apoptosis of HUVEC (Supplementary Data). However, it was a pity that this study did not include other endothelial cells or other cells of hemangioma tissue.

In conclusion, this study demonstrated that propranolol acted on the mast cell β 1 and β 2 adrenoceptors to inhibit the expression of VEGF-A, bFGF, MMP2, MMP9, and tryptase of mast cells in vitro. Thus, propranolol-treated HMC-1 supernatants can inhibit the proliferation and promote the apoptosis and autophagy of infantile angiomatous endothelial cells. Propranolol may have a role on mast cell in IH, which could provide a new theoretical basis for mast cells as target cells for the treatment of infantile hemangioma.

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AUTHOR CONTRIBUTIONS

Y.Y. and L.W. designed the study. H.Z., Y.Y., and L.D. performed the research and wrote the paper. H.Z. and L.D. analyzed the data. W.S., C.D., and J.L. collected clinical samples and data. W.L. assisted in clinical data collection. L.H. and W.G. assisted in subject enrollment. C.X., G.D., and K.L. offered technical assistance and helped to design the study. L.W. financially supported and revised the paper.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

The study was approved by the Ethics Committee of Children's Hospital of Fudan University. Written informed parental consent was obtained at enrollment.

ADDITIONAL INFORMATION

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