Original Article

Molecular effect of propranolol on cell viability of hemangioma-derived stem cells (HemSCs)

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Abstract: Objectives: Propranolol (PRN) was found by chance very effective in treating hemangioma, yet the therapeutic mechanism remains unclear. This study was aimed to investigate the therapeutic mechanism of propranolol on hemangioma. Materials and Methods: The plasma propranolol concentrations at 2, 6, 10, 24 h were assayed by reversed phase high performance liquid chromatography (RP-HPLC) after oral administration of propranolol (1 mg/kg·d) once a day (qd, n=6) or twice a day (bid, n=6); The hemangioma-derived stem cells (HemSCs) were isolated from Chinese IH patients. The effect of propranolol on proliferation, apoptosis rate of HemSCs was observed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and bi-fluorescence flow cytometry (FCM). With real-time PCR and Western blot, expression of vascular endothelial growth factor (VEGF) was observed after treatment with different concentrations (0, 0.02, 0.2, 2, 20 μM) of PRN at different times. Results: The peak propranolol concentration reached at 2 h in both qd group (207.8±33.9 ng/mL) and bid group (155.2±40.6 ng/mL), the elimination half-life (t1/2) was longer than 6 h. The mean concentration of 10 h was significantly decreased in qd group, while that was elevated in bid group. The proliferation and total apoptosis rate of HemSCs was not obviously affected by PRN in normal concentration; but the expression of VEGF in HemSC was suppressed by PRN. Conclusions: PRN can inhibit HemSC to express VEGF, but cannot inhibit proliferation or induce apoptosis of HemSCs.

Keywords: Hemangioma, stem cell, propranolol, pharmacokinetics, vascular endothelial growth factor (VEGF)

Introduction

Infantile hemangioma (IH) is the most common infancy tumors with nearly 60% localized to the head (40%) and neck (20%) [1, 2]. It is estimated to have an incidence of 1.0~2.6% at birth in Caucasian infants, affecting up to 12% of children by the age of 1 year. Infantile hemangioma presents rapid proliferation in the early neonatal period and have three distinctive clinical and histological phases [3], followed by evolution. Although various treatment options have been described for IH, their clinical aspects are multiple and detailed physiopathology is poorly understood.

Recently a few studies have described that hemangioma-derived stem cells (HemSCs) are multipotential cells isolated from specimens of human proliferating IH, which may contribute to the proliferating and involution of IH. In contrast to endothelial cells (ECs) and endothelial progenitor cells isolated from IH, HemSCs exhibit a mesenchymal morphology and robust proliferation when cultured in vitro. These cells have been used to develop a hemangioma mouse model when injected subcutaneously into nude mice [4, 5], and they also can form human blood vessels with the immunophenotype and dynamics of IH. Thus, HemSCs are the putative progenitors of IH. CD133, a maker of human hematopoietic stem and progenitor cells, was used to isolate stem cells from hemangioma [4].

In the past, the traditional first-line therapy for haemangiomas was systemic corticosteroids. When Léauté-Labrèze et al. [6] occasionally discovered that the propranolol, a β-blocker, had good effects for IH since 2008, corticosteroids (gold standard) [7, 8] was gradually superseded by the use of systemic propranolol. Nowadays,
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the therapeutic options are various and can be personalized based on the hemangioma characteristics. Notably, therapy with propranolol has been a first-option therapy in most of the situations [9-11]. But there is currently not enough known about the mechanism of action of propranolol in IH.

In this study, we investigated the effect of propranolol on proliferation, apoptosis of HemSCs. The aim was to explore the therapeutic mechanism of propranolol on hemangioma.

Materials and methods

Patients

A total of 12 patients from Shanghai Jiao Tong University Affiliated Sixth People's Hospital and Shanghai Children's Hospital Affiliated to Shanghai Jiao Tong University China were involved in the study. Venous blood was collected under a human-subject protocol. The study was approved by the ethics committee and the subjects.

RP-HPLC analysis

12 subjects were randomly divided into 2 groups, qd and bid, each of which comprised of 6 individuals. PRN was orally administered to the patients at a dose of 1 mg/kg/d. Group qd received PRN once a day at halved dose. After the administration, the venous blood was collected at 2, 6, 10 and 24 hrs, and the plasma was isolated. Subsequently, the freed bases of PRN were extracted with 3.5 mL of the extraction solvent (isooamyl-alcohol 1.5 ml: n-heptane 98.5 ml) and were separated using a mixture of a mobile phase consisting of 180 mL methanol, 160 mL water, 70 mL acetonitrile, 2.5 mL acetic acid, and 125 μL triethylamine. 15 μL of sample was injected at a flow rate of 0.5 mL/min. PRN was measured at a wavelength of 291 nm.

MTT assay

The effects of PRN on cell viability were assessed by MTT assay as described previously. Specifically, cells were seeded at a density of 3000 cells per well into 96-well plates. The cells were incubated with PRN of 0, 0.02, 0.2, 2, 20 μM. At the end of treatment, the supernatant was removed, and 20 μL of the tetrazolium compounds, MTT, and 270 mL of fresh IMDM medium were added. After incubation for 4 h at 37°C, 120 μL of DMSO was placed to dissolve the tetrazolium crystals. Finally, the absorbance at 570 nm was recorded using a multiwell plate reader (Tecan, Maenndorf, Switzerland). Each experiment was performed four times. Results were presented as the percentage of growth inhibition with respect to the untreated cells.

Flow cytometry (FCM) analysis

Cells at log phrase were collected at a concentration of 2 × 10^5 cells/ml, and were incubated in 6-well plate for 12 h (2.7 mL for each well). Then 0.3 mL PRN was used to stimulate the cells for 48 h. Cells were sequentially collected, washed with PBS, fixed with 70% ethanol, Cells were to be washed with PBS, and stained with propidium iodide (PI) in dark for 30 min before FCM analysis. Finally, BD FACSCalibur (BD, USA) was used to detect cell cycle. Cells were sampled using sampling software CellQuest 3.0. The proportion of cells in different phases were quantified by ModFitLT 3.0 (25). Each experiment was performed four times.

Quantitative real-time PCR (qPCR) assay

Total RNA was extracted from the cells by the use of the Trizol reagent according to the manufacturer's instructions (Life technologies, USA). Subsequently, 1 μg of RNA was reverse-transcripted into cDNA using a first-strand cDNA using a first-strand cDNA synthesis kit (Takara, China). 1 μL of cDNA was used for quantification by an ABI 7500 real-time machine (ABI, CA, USA) using SYBR Green (Takara, China). The PCR reaction was conducted under the primers: forward: 5'GGGCTGCTGCAATGACGA-3', reverse: 5'CAACGTACACGCTCCAAGACT-3'. The reactions were incubated in a 96-well plate at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 34 s. GAPDH was employed as internal control used for normalization of Ct values and the ∆∆Ct method for relative quantitation of gene expression.

Western blot analysis

Cell lysates were prepared in RIPA buffer (50 mmol/L Tris-HCl buffer, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented
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with protease inhibitor cocktail (Pierce, Rockford, IL). A Bio-Rad protein assay (Bio-Rad) was used to determine protein concentrations. 50 µg of protein was loaded on 12% sodium dodecyl sulfate-polyacrylamide gel and was transferred to PVDF membranes (Whatman, Boston, MA). Membranes were first hybridized with specific primary antibody (SAB3300042, sigma) and then with HRP-conjugated secondary antibodies (Cell Signaling Technology). Protein bands were visualized using ECL Plus reagent (GE Healthcare Amersham, Piscataway, NJ). Beta-actin was detected as internal control.

Statistical analysis

All the results were expressed as means ± SD and data was analyzed with statistical software package SPSS 16.0. The statistical significance of the studies was determined by the parametric unpaired Student’s t test. Differences with P < 0.05 are considered significant and P < 0.01 is extremely significant.

Results

Plasma propranolol concentration in dosed patients

Under oral administration of propranolol (1 mg/kg·d) once a day (qd, n=6) or twice a day (bid, n=6), the plasma propranolol concentrations at 2, 6, 10, 24 h were assayed by reversed phase high performance liquid chromatography (RP-HPLC) (Figure 1A), the RP-HPLC showed that propranolol was detected in the 4.856 minutes. The peak propranolol concentration reached at 2 h in both qd group (207.8 ± 33.9 ng/mL) and bid group (155.2 ± 40.6 ng/mL), and the elimination half-life (t1/2) was longer than 6 h. The mean concentration of 10 h was significantly decreased in qd group, while that was elevated in bid group. After 10 h, propranolol concentration in both qd group and bid group declined rapidly to less than 50 ng/mL until 24 h (Figure 1B).

Effect of propranolol on cell vitality and proliferation

The half-maximal inhibition (IC50) of HemSC, HUVEC, and NHDF was 142, 67.3, 137 μM, respectively. HUVEC was sensitive to propranolol, while HemSC and NHDF showed similar tolerance to propranolol. When dosed with 50 μmol/L propranolol, the activity ratios of HUVEC, HemSC and NHDF to the control decreased to 61 ± 4.2, 81 ± 2.4 and 67.5 ± 1.2%, respectively. While at 100 μmol/L, this ratio decreased to 21.3 ± 4.2, 63.2 ± 5 and
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66.2 ± 0.7%, respectively. When the dosage increased to 200 μmol/L, the activity ratio of HemSC and NHDF significantly decreased to below 20%. However, low concentrations of propranolol (0-10 μmol/L) did not affect the vitality of the three cell lines (Figure 2).

Effect of propranolol on HemSC apoptosis

Dosed with different propranolol concentrations (2, 10, 50, and 100 μmol/L) for 72 h, the apoptosis of HemSC was determined by FCM analysis. Apoptosis rate (total apoptosis rate/early apoptosis rate, %/%) to control, 2, 10, 50, and 100 μmol/L was 4.87/1.96, 6.76/1.51, 5.3/1.31, 6.74/0.95, and 9.68/3.42, respectively (Figure 3). These results indicated that 100 μmol/L could slightly induce cell apoptosis in HemSC.

Expression of VEGF in HemSC treated by propranolol

Previous studies showed that expression of VEGF is more in HemSC than in NHDF and HUVEC [12, 13]. We observed expression of VEGF in HemSC after treatment with different concentrations (0, 0.02, 0.2, 2, 20 μmol/L) propranolol at different times (2 h, 6 h, 12 h, 24 h).

In treated groups, they showed significant down-regulation of VEGF mRNA compared with the untreated group (Figure 4A). Propranolol-mediated inhibition of VEGF mRNA expression was dose-dependent, however, the maximum inhibition achieved at 2 μM of propranolol. When the time of treatment of propranolol in certain group was longer, the inhibition of VEGF mRNA expression was diminished (Figure 4A). We also investigated the VEGF protein production in treated groups compared with untreated group, the tendency was similar to the expression of VEGF mRNA (Figure 4B). These results indicated the inhibitory effect of VEGF mRNA and protein in treated by propranolol.

Discussion

Propranolol has been proved have good effect and acquired satisfactory for treatment of infantile hemangioma (IH). However, the different races present different sensitivity to β-blocking effects of propranolol. As in Chinese individuals, lower-dose propranolol agent for the treatment of IH can acquire satisfied outcome compared with White races. Leaute-Labreze et al. firstly reported the mechanisms of propranolol in treating IH, including the inhibition of proliferation and the promoted apop-
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tosis of hems [14-16], but not reported in hemSC [17-19]. The effect of corticosteroid on hemSCs revealed inhibition of VEGF mRNA expression with the progresses in IHs research. The inhibited vasculogenesis can explain other apoptotic cells previously not understood in the treatment of IH [20-22]. This could explain why corticosteroid had no effect on some patients.

Our results showed that the expression of VEGF was suppressed by propranolol in a dose-dependent pattern. However, the proliferation and apoptosis of HemSCs were not obviously altered after treated with different concentration propranolol. Concerning previous studies, escape of HemSCs from apoptosis might due to induction of anti-apoptotic pathway. Our results further confirmed the present theory. These all might partly provide critical insight into the potential mechanisms of propranolol action on IH.

Figure 4. Expression of VEGF in HemSCs treated by propranolol. Down-regulation of VEGF mRNA in treated group at 0.02, 0.2, 2, 20 μM propranolol concentrations (A). The inhibitory VEGF protein expression in treated group compared with untreated group (B).

Disclosure of conflict of interest

None.

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References


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