cases, IHs grow dramatically and destroy tissue, impair function, or even threaten life. Standard treatment options for IH include corticosteroids or surgical excision or, in life- or sight-threatening cases, treatment with vincristine, interferon or cyclophosphamide. Unfortunately, none of these therapeutic modalities is ideal due to restrictions or potential serious side effects [10-12]. In 2008, Léauté-Labrèze et al. [13] showed that propranolol has an anti-proliferative effect on severe IHs. After this report, a number of studies further demonstrated that β-blockers other than propranolol were effective at halting hemangioma growth with few adverse side effects [14-21]. β-blockers are now the preferred treatment for problematic proliferating IHs.

To date, it is unknown how β-blockers exert its pharmacologic effect on IHs. The β-ARs, a family of G-protein-coupled receptors that are activated by adrenergic catecholamines, can initiate a series of signaling cascades, thereby leading to multiple cell-specific responses. There is evidence suggesting that endogenous catecholamines play a role in basic developmental processes (e.g., embryogenesis and morphogenesis) including the control of cell proliferation, differentiation and migration [22-24]. Recent studies have demonstrated that in endothelial and various cancer cells, a number of β-AR agonists, including epinephrine, norepinephrine and isoprenaline, can induce the proliferation and activation of mitogen-activated protein kinase (MAPK) family members by extracellular signal-related kinase (ERK) [25-28]. ERK and MAPK are serine/threonine kinases that phosphorylate nuclear transcription factors and regulate the expression of multiple genes involved in cell proliferation. VEGF-A participates in this process because a VEGF-A-specific antibody blocks β-AR-mediated cell proliferation and ERK activation [9,25]. Additionally, VEGF-A exerts its pro-proliferative and pro-angiogenic effects, at least partially, by activating the ERK cascade [25,29]. In primary endothelial cells, VEGFR-2 also associates with activated ERK in a Ras-independent manner [30].

The mechanisms of β-AR-stimulated tumor growth have been studied for several years, but the potential role of the β-ARs in IH pathogenesis has not been investigated. Accordingly, this study examined the mechanisms underlying the relationship between the β-adrenergic signaling pathway and the proliferation of HemECs.

Methods

Reagents and antibodies

Endothelial basal medium (EBM-2) and SingleQuot, which contains human epidermal growth factor, vascular endothelial growth factor, human basic fibroblast growth factor, insulin-like growth factor, hydrocortisone, heparin, ascorbic acid, and gentamicin/amphotericin B, were obtained from Lonza (Walkersville, MD). Fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco (Grand Island, NY). The anti-CD31 FITC antibody used in fluorescence-activated cell sorting (FACS) was obtained from BD Pharmingen (San Jose, CA). ICI 118551 (ICI), metoprolol (MET), isoprenaline (ISO), forskolin, collagenase A, bovine serum albumin (BSA), Hoechst 33342, propidium iodide, DNase-free RNase, the ERK/MAPK inhibitor, U0126, the phosphodiesterase inhibitor, 3-isobutyl-methylxanthine (IBMX), and the cAMP antagonist, Rp-cAMP, were purchased from Sigma (St. Louis, MO). The VEGFR-2 inhibitor, PTK787, was obtained from Novartis Pharmaceuticals (Basel, Switzerland). The BrdU cell proliferation assay kit was obtained from Calbiochem (Darmstadt, Germany), and the cAMP assay kit was obtained from Amersham Pharmacia Biotech (Braunschweig, Germany). The primary polyclonal antibodies recognizing the VEGF-A, β_1-AR, β_2-AR, phospho-ERK (Thr202/Tyr204) and ERK were purchased from Santa Cruz Biotechnology (Delaware, CA). The antibodies for cyclin D1, CDK-4, CDK-6, retinoblastoma (Rb), phospho-Rb, phospho-VEGFR-2 (Tyr1175) and VEGFR-2 were purchased from Cell Signaling Technology (Boston, MA). Human umbilical vein endothelial cells (HUVECs) were obtained from Chinese Academy of Sciences (Shanghai, China).

Preparation of hemangioma specimens

This study was approved by the Ethics Committee of the Children's Hospital of Fudan University. Proliferating infantile hemangioma was surgically removed from a 4-month-old female patient who was referred to our department for a rapidly growing mass. Written informed consent was obtained from parents for all tissue obtained for the study. The clinical diagnosis of vascular neoplasm was confirmed by the Department of Pathology at the Children's Hospital of Fudan University based on staining for GLUT-1, a marker specific for hemangioma tissue. The tissues were used immediately in cell isolation and in vitro experiments.

Cell extraction, isolation and culture

HemEC isolation was performed as described previously [7,8]. Briefly, the hemangioma samples were rinsed in PBS, minced, and digested with 0.2% collagenase A at 37°C for 1 h. The tissue was homogenized and filtered through 100 μm cell strainers to dissociate aggregates, and red blood cells were lysed by incubating the samples in NH_4Cl. Next, the samples were filtered through a 40 μm cell strainer to obtain a single-cell suspension. $CD31^+$ HemECs were isolated by FACS using anti-CD31 FITC antibodies and were plated on gelatin-coated 60 mm plates in EBM-2 medium supplemented with 20% heat-inactivated FBS, SingleQuot, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were