Expression and Correlation Analysis of β-adrenoceptors, VEGF, MMP-9 and Caspase-3 in Different Phases of Infantile Hemangioma

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Abstract: Objective: To investigate the expression and correlation analysis of β-adrenoceptors (AR), VEGF, MMP-9 and caspase-3 in the different phases of infantile hemangioma (IH) and to find out the possible mechanism of β-AR antagonist propranolol treatment for IH. Methods: 52 fresh operative IH specimens were collected and were divided into 2 groups: 32 patients of proliferating phase and 20 patients of involuting phase. Ten specimens of normal skin were also collected as the control group. The expression of β-AR, VEGF, MMP-9 and caspase-3 were detected by immunohistochemistry. The integral optical density and positive area rate of β-AR, VEGF, MMP-9 and caspase-3 were examined using a microscopic image analysis system. The correlations among the above markers were also analyzed using Pearson correlation coefficients. Results: β₁-AR and β₂-AR were both strongly expressed in the proliferating and involuting hemangioma tissue. There was a significant difference (P<0.01) for the expression of β₁-AR and β₂-AR between the hemangioma tissue and the normal skin. VEGF and MMP-9 were strongly expressed in the proliferating hemangioma and weakly expressed in the involuting hemangioma, and there was a significant difference (P<0.01) for the expression of VEGF and MMP-9 between the proliferating hemangioma and involuting hemangioma or between the proliferating hemangioma and the normal skin. There was a significantly positive correlation between the expression of β₁-AR and the expression of VEGF and MMP-9 in the proliferating phase of IH. There was a significant difference (P<0.01) for the expression of caspase-3 between the proliferating hemangioma and involuting hemangioma or between the involuting hemangioma and the normal skin. There was a significantly negative correlation between the expression of β₂-AR, VEGF, MMP-9 and caspase-3 have an effect on the pathogenesis of hemangioma. β-AR antagonist propranolol might treat IH by the mechanism of regulating the β-AR/VEGF/MMP-9/caspase-3 pathway, inhibit the proliferation of hemangioma, promote the apoptosis of hemangioma, and eventually bring about the regression of IH.

Key words: Infantile Hemangioma, β-adrenoceptors, VEGF, MMP-9, Caspase-3

Introduction

Infantile hemangiomas (IH) are the most common tumors of early childhood and occur in up to 10% of all infants ¹. They are benign vascular tumours that usually develop shortly after birth and show a unique and characteristic clinical course of proliferation and involution. In severe cases, immediate systemic therapy is indicated. The most common locations for hemangiomas include the head and neck and the superficial parts of limbs. However, the pathogenesis of IH is unclear so far. Nowadays, the treatment methods for IH included oral or local injection of corticosteroids ², radiotherapy³, laser⁴, local injection of sclerosing agent⁵, oral interferon⁶ and surgery. However, the above treatment methods all have some side effects and these methods are not effective for all kinds of hemangiomas⁷. In 2008, Léauté-Labrèze and colleagues reported the serendipitous finding that hemangiomas regress in newborns treated with propranolol, a known nonselective β-adrenoceptors (β-AR) -blocker used in treating infants with cardiac and pulmonary conditions.⁸ This report has been met with great interest and physicians all over the world have started to use propranolol for the treatment of problematic IH. There is evidence from recent publications that the effects of propranolol treatment for IH was very satisfactory and it is now seen as the first-line therapy for severe haemangiomas.⁹ However, the mechanism of this new treatment method and now first-line therapy for severe haemangiomas...
remains unclear. Reports in the literature are pointed out that the expression of VEGF and MMP-9 might be regulated via β-AR.\(^{10,11}\) There were evidence to verify that VEGF and MMP-9 were all involved in the pathological processes of IH.\(^{12,13}\) It is well documented that cell apoptosis played a crucial role in the spontaneous regression of IH.\(^{14}\) It is now generally granted that caspase-3 is the most important terminal shear enzyme in the process of cell apoptosis. It has been reported in the literature that caspase-3 is closely related to the spontaneous regression of IH.\(^{15}\) At the present, there is lack of the study for the expression of β-AR/VEGF/MMP-9/caspase-3 pathway in IH. Combined with the good curative effect of β-adrenergic receptor blocker propranolol for IH, we speculate that β-adrenergic receptor pathway might play a crucial role in the regulation of pathogenesis of hemangioma. We use immunohistochemical methods to detect the β-adrenergic receptor, VEGF, MMP-9, and caspase-3 expression in different phases of infantile hemangioma. And we investigate the correlation analysis between the above cytokines and β-AR. We hope that this study can provide valuable clues and inspiration for the possible mechanism of propranolol treatment for IH.

Materials and Methods

**Materials**

(1) Samples: Fifty two hemangioma paraffin blocks (2010-2012) was collected from Department of Pathology, Shengjing affiliated hospital of China Medical University, including 22 males and 30 females, the youngest patient was 1 month of age and the oldest was 14, the mean age was 2.3 years.

The locations of hemangioma included the face, neck, upper limbs, chest, back and thighs. Archived blocks were resected specimen, which were not received any treatment before surgery and had no any history of endocrine disease or oral corticosteroids. Taking 10 cases of normal skin surrounding hemangioma and normal skin. The main steps of immunohistochemical stain were as follows: dewaxed the paraffin sections in water, and washed in PBS 3 times, 3 minutes every one section; put slice into the freshly prepared 3% hydrogen peroxide solution at the room temperature of 10 min; washed in PBS 3 times, 2 minutes every one section; antigen retrieval according to the requirements of the antibody; dropped 30 μl normal goat serum into each slice to close non-specific tissue antigens; dropped 30 μl first antigen to each slice, and stayed overnight at 4 degrees; every slice was rinsed under PBS 3 times; 30 μl biotinylated secondary antibody was added into each slice, 10 min at room temperature; washed in PBS 3 times; 30 μl horseradish enzyme labeled streptavidin working solution was added into each slice, 10 min at room temperature; washed in PBS 3 times; freshly prepared DAB color solution was added dropwise, controlled the staining time under light microscopy, positive staining was brown; terminated color reaction by water, afterstain 3 min with hematoxylin; dehydrated in alcohol with different gradients, made it transparent by xylene, and closed with neutral gum. Took PBS instead of primary antibody as negative control.

**Determination of results**

Positive cell determination : β-AR, VEGF, MMP-9 and caspase-3 were all located in the cell membrane or cytoplasm. β-AR, VEGF, MMP-9 and caspase-3 will be determined as positive when brown-yellow granules appears in the cell membrane or cytoplasm. There were no brown enlargement granules in the negative control group except that the nucleus was stained for blue. If the backgrounds of paraffin sections were heavier, the positive cells must meet the following requirements: 1. The structures of cells are clear; 2. The location and quantification of positive granules are good; 3. The coloring is obviously higher than the background. The negative alternative control used PBS instead of primary antibodies for synchronous dyeing.

**Methods**

**Hematoxylin and eosin (HE) stain**

The sections were stained with hematoxylin and eosin and examined with the light microscope. The main steps of HE stain were as follows: (1) dewaxed paraffin sections by xylene. (2) stained 5 min in hematoxylin, and then rinsed by running water. (3) differentiated 20s in 1% hydrochloric acid and alcohol, rinsing by running water 1 min intermittently. (4) stained 5 min in Eosin, then rinsed under running water 30s. (5) dehydrated in alcohol with different gradients, made it transparent by xylene, and closed with neutral gum.
Figure 1. Proliferating hemangiomas (1) (HE ×200), Involuting hemangiomas (2) (HE ×200)

Figure 2. β1-AR expression in the proliferating hemangioma (1), involuting hemangioma (2) and normal skin (3). Immunohistochemical staining (SP×400)

Figure 3. β2-AR expression in the proliferating hemangioma (1), involuting hemangioma (2) and normal skin (3). Immunohistochemical staining (SP×400)

Figure 4. VEGF expression in the proliferating hemangioma (1), involuting hemangioma (2) and normal skin (3). Immunohistochemical staining (SP×400)

Figure 5. MMP-9 expression in the proliferating hemangioma (1), involuting hemangioma (2) and normal skin (3). Immunohistochemical staining (SP×400)

Figure 6. Caspase-3 expression in the proliferating hemangioma (1), involuting hemangioma (2) and normal skin (3). Immunohistochemical staining (SP×400)
Immunohistochemical image capture and analysis

The images of β1-AR, β2-AR, VEGF, MMP-9 and caspase-3 expression for immunostaining were captured by SPOT Advanced™ software (Modular Imaging Software for Microscopy, USA). The positive area rate and integrated optical density of β1-AR, β2-AR, VEGF, MMP-9 and caspase-3 expression for immunostaining were quantitated for using microimage analysis system Metamorph/Evolution MP5.0/BX51 (US/JP, UIC/Olympus). Five fields with ×200 power magnification were chosen randomly in every section. And to count the positive area rate and integrated optical density under every field (×200). The mean values of the positive area rate and integrated optical density for every 5 fields (×200) were considered as the measured value of the average positive area rate and the average integrated optical density for every section. Positive area rate = the total area of the positive reaction in every unit area/the total area of the cells in every unit area; Intergrated optical density (IOD) = the area of the measured object × mean optical density.

Statistical analysis

The SPSS 17.0 statistical software was used to store the data and analyze the data. The results were shown by x±s (mean±standard deviations). One-way ANOVA and SNK-q test were used to analyze the average positive area rate and the average integrated optical density of the positive particles in every immunohistochemical reaction. Pearson correlation analysis were used to detect the correlation among the values of the average positive area rate of the positive particles in every immunohistochemical reaction. Statistics difference was considered to be significant when P-values were less than 0.05.

Results

HE stain

In the proliferating phase of IH, there were a large number of vascular endothelial cells. Some of the vascular endothelial cells proliferated into cord-like shape. The vascular endothelial cell nuclei were hypertrophic. Some endothelial cells were rounded
into vascular cavity and the vascular cavity was small and the number of the vascular cavity was less. (Fig1-2) In the involuting phase of IH, there were significantly declining number of vascular endothelial cells. The vascular endothelial cell nuclei were flat and the vascular vessel lumens were larger and the number of vessel lumens was increasing. The connective tissue and the fat tissue among the vascular vessels increased markedly. (Fig1-2)

**Immunohistochemical staining**

(1) β1-AR expression: there were strong expression of β1-AR and β2-AR both in the proliferating phase and involuting phase of IH (Fig2-1,2, Fig3-1,3). The expression of β1-AR and β2-AR were mainly localized in the cytoplasm of vascular endothelial cells and they were weak expression in normal skin tissue. (Fig2-3, Fig3-3) There was significant difference for the expression of β1-AR and β2-AR between the hemangioma tissue and the normal skin tissue (P<0.01).

(2) VEGF expression: there were a large number of brown particles deposited in the vascular endothelial cell cytoplasm in the proliferating hemangioma. (Fig4-1) There were few brown particles deposited in the vascular endothelial cell cytoplasm in the involuting hemangioma and the color was slightly stained. (Fig4-2) There were no brown particles in vascular endothelial cell cytoplasm of normal skin tissue. (Fig4-3) There was statistically significant difference between the proliferating hemangioma and the involuting hemangioma (P<0.01) or between the proliferating hemangioma and the normal skin tissue (P=0.01).

(3) MMP-9 expression: there were strong expression of brown particles in the cytoplasm of vascular endothelial cell in the proliferating hemangioma. (Fig5-1) There were few brown particles deposited in the vascular endothelial cell cytoplasm in the involuting hemangioma. (Fig5-2) There were significantly weak expression in normal skin tissue. (Fig5-3) There was statistically significant difference between the proliferating hemangioma and the involuting hemangioma (P<0.01) or between the proliferating hemangioma and the normal skin tissue (P<0.01).

(4) Caspase-3 expression: small amount of brown particles could be seen in the cytoplasm of vascular endothelial cells in the proliferating hemangioma. (Fig6-1) There were a large number of brown particles in the vascular endothelial cell cytoplasm of involuting hemangioma. (Fig6-2) There were a few brown particles in vascular endothelial cell cytoplasm of normal skin tissue. (Fig6-3) There was statistically significant difference between the involuting hemangioma and the proliferating hemangioma (P<0.01) or between the involuting hemangioma and the normal skin tissue (P<0.01).

(5) The average positive area rate and average optical density of positive particles of β1-AR, β2-AR, VEGF, MMP-9 and caspase-3 are shown in Table 1, 2.

The correlation coefficient values of the average positive area rates for the above 5 indicators (β1-AR, β2-AR, VEGF, MMP-9 and caspase-3) by means of Pearson correlation analysis are shown in Table 3. According to P=0.05 level, it can be considered that the average positive area rates of β-AR expression had significantly positive linear relationship with those of VEGF and MMP-9 expression in the the proliferating phase of infantile hemangioma (P<0.01). And the average positive area rates of caspase-3 expression had significantly negative linear relationship with those of β-AR, VEGF and MMP-9 expression in the the proliferating phase of infantile hemangioma (P<0.01).

**Discussion**
IH comprise the majority of vascular anomalies and are considered the predominant vascular tumor type. Hemangiomas affect nearly 4% to 10% of infants and have been found to have unique, but natural phases of proliferation and involution.\(^{17}\) If present in inconspicuous sites, hemangiomas are frequently left untreated and allowed to follow their natural involuting course. However, problematic hemangiomas occur when they ulcerate, have massive growth, cause disfigurement, or impact normal function or cosmetic development.\(^{11}\) Common locations for problematic hemangiomas include the face, ear, orbit and airway. These hemangiomas subsequently require early and aggressive treatment for ideal functional and cosmetic outcomes. However, current treatment options have limited therapeutic benefit with their own side-effect profile and risks.\(^{19}\) In 2008, Léauté-Labrèze and colleagues reported the serendipitous finding that hemangiomas regress in newborns treated with propranolol, a known nonselective beta-blocker used in treating infants with cardiac and pulmonary conditions.\(^9\) Physicians all over the world have started to use propranolol for the treatment of problematic IH. There is evidence from recent publications that the effect of propranolol treatment for IH was very satisfactory.\(^{20}\) However, the exact mechanism of oral propranolol treatment for IH is still not clear.

Propranolol is a nonselective beta-adrenergic antagonist, which competitively inhibits \(\beta_1\) and \(\beta_2\)-AR with the same affinity. The subtypes of \(\beta\)-AR are all located on the cell membrane and belong to the G protein-coupled receptors. \(\beta_1\) and \(\beta_2\)-AR were activated by adrenaline and other hormones and then coupled with the stimulated G protein (Gs) on the cell membrane and made the latter change the conformation and generate the activity. Then this can activate adenylylase (AC) and increase the generation of cyclic adenosine monophosphate (cAMP) and thereby activate protein kinase A (PKA). On the one hand, \(\beta\)-AR can be coupled with the stimulated G protein (Gs) and make cAMP increasing; on the other hand, \(\beta\)-AR can also be coupled with the inhibitory G protein (Gi) and make cAMP declining. In addition, propranolol is a nonselective \(\beta_1\) and \(\beta_2\)-AR antagonist and this can basically rule out the role of \(\beta_3\)-AR in the above biological process. In our study, we found that \(\beta_1\)-AR and \(\beta_2\)-AR were strongly expressed in the proliferating phase and involuting hemangioma tissue. And \(\beta_1\)-AR and \(\beta_2\)-AR were located in the cytoplasm of vascular endothelial cell. There were weakly expressed in the normal skin for \(\beta_1\)-AR and \(\beta_2\)-AR. And there was significant difference (P<0.01) for the expression of \(\beta_1\)-AR and \(\beta_2\)-AR between the hemangioma tissue (including proliferating phase and involuting phase) and the normal skin. According to the above results, there is plenty of reason to speculate that \(\beta_1\)-AR and \(\beta_2\)-AR and their related pathway played a crucial role in the development of IH. Therefore, it is necessary for the researchers to conduct further study for the regulation mechanism of \(\beta\)-adrenergic signaling pathway in the pathogenesis of IH.

VEGF is one of the most potent angiogenic factor involved in hemangioma growth, which increases vascular permeability, stimulates endothelial cell proliferation and prevents endothelial cell apoptosis.\(^{21,22}\) There is evidence from recent publications that the expression of VEGF is not only controlled by the oxygen partial pressure in the tissue (via HIF-1\(\alpha\)), but also by \(\beta\)-adrenergic stimulation: as demonstrated in different in vitro and in vivo models catecholamines such as adrenaline and noradrenaline can induce the expression of VEGF.\(^{11}\) Conversely, beta blockers like propranolol might lead to a reduced expression of VEGF and thus to an inhibition of angiogenesis.\(^{23}\) MMPs are a family of soluble and membrane-anchored proteinases involved in degradation and transformation of extracellular matrix proteins. They play a key role in different physiological and pathophysiological processes, e.g. cell proliferation, migration and adhesion, embryogenesis, wound healing and also in angiogenic processes relevant for tumour growth and metastasis.\(^{24}\) Elevated concentrations of MMP-9 were found in tissue samples and in the blood of infants in the proliferative phase of hemangiomas.\(^{13}\) MMP-9 is important for the migration of endothelial cells and tubulogenesis.\(^{25}\) It has been shown that inhibition of MMP-9 impedes angiogenesis of human microvascular endothelial cells.\(^{26}\) There is evidence from recent publications that \(\beta\)-adrenoceptor agonists such as adrenaline or nor-adrenaline increase the expression of MMP-2 and MMP-9 and this illustrates that the expression of MMP-9 and MMP-2 might be regulated via \(\beta\)-adrenoceptors.\(^{10,11}\) Reduced expression of MMP-9 by propranolol leads to an inhibition of tubulogenesis of endothelial cells and thus also provides a conclusive mechanism for the antiangiogenetic effect of propranolol.\(^{26}\) In our study, we found that VEGF and MMP-9 were strongly expressed in the proliferating hemangioma tissue and weakly expressed in the involuting hemangioma tissue and normal skin tissue. And there was significant difference (P<0.01) for the expression of VEGF and MMP-9 between the proliferating hemangioma and involuting hemangioma. There was also significant difference (P<0.01) for the expression of VEGF and MMP-9 between the proliferating hemangioma and the normal skin. By means of Pearson correlation analysis, we found that there was significantly positive correlation between the expression of \(\beta\)-AR and the expression of VEGF and MMP-9 in the proliferating phase of IH. According to our experimental results along with the above related references, we can speculate that the stimulation and activation of \(\beta\)-adrenergic signaling pathway might promote the expression of VEGF and MMP-9 in IH and then stimulate the massive proliferation of vascular endothelial cells and thus result in the rapid growth of hemangioma. This might explain the curative effect of propranolol treatment for IH possibly by means of impeding the \(\beta\)-adrenergic signaling pathway and then inhibiting the expression of VEGF and MMP-9 in IH.
Weili Yuan et al.: β-adrenergic Receptor Pathway in Infantile Hemangioma

Recently, a number of studies have shown that spontaneous regression of hemangioma is the result of cell apoptosis. The apoptosis of endothelial cell in hemangioma begins at around 1 year old and goes to peak at 2 years old. Degradation of hemangioma endothelial cells are 3-5 times of proliferating period, and at least 1/3 of apoptosis cells of hemangioma is the vascular endothelial cells. The terminal part of cell apoptosis is the activation of caspase cascade. Caspase cascade is the central part of cell apoptosis and it is also the point of convergence of multiple apoptotic pathways (Bcl-2/Bax and Fas/FasL). Caspase-3 is a caspase protein that interacts with caspase 8 and caspase 9. It is encoded by the CASP3 gene. The caspase-3 protein is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. Caspase-3 protein cleaves and activates caspases 6 and 7; and the protein itself is processed and activated by caspas 8, 9, and 10. Caspase 3, 6, 7 are cracking cytoskeleton and functional proteins, make the cells disintegrated, known as the effector caspase. Mitochondrial pathway of apoptosis depends on the caspase 9, and the death receptor pathway is mainly mediated by the caspase 8. And there was evidence from recent publications that propranolol induces apoptosis in PC-2 cells (pancreatic cancer cell line) via the β2-AR principally and β-AR antagonists therapy affected caspase-3 and caspase-9 expression. In our study, we found that caspase-3 were strongly expressed in the involuting hemangioma tissue and weakly expressed in the proliferating hemangioma tissue and normal skin. And there was significant difference (P<0.01) for the expression of caspase-3 between the proliferating hemangioma and involuting hemangioma. There was also significant difference (P<0.01) for the expression of caspase-3 between the involuting hemangioma and the normal skin. By means of Pearson correlation analysis, we found that there was significantly negative correlation between the expression of β-AR, VEGF and MMP-9 and the expression of caspase-3 in the proliferating phase of IH (P<0.01). This study might explain the curative effect of propranolol treatment for infantile hemangioma possibly by means of blocking the cell surface β-AR, activating the caspase apoptotic signaling pathway and then inhibiting the expression of VEGF and MMP-9 in IH. In conclusion, based on our experimental results and the related references, we speculate that the possible mechanism of causing regression of hemangioma by propranolol was as follows: (1) on the one hand, propranolol blocked the cell surface β1-AR and β2-AR, promoted β2-AR coupled with inhibitory G protein (Gi) and activated caspase 3 and then promoted hemangioma endothelial cell apoptosis by means of the two cell apoptosis pathways of Bcl-2/Bax and Fas/FasL; (2) on the other hand, propranolol might act synergistically with repressed β1-AR coupled with the stimulated G protein (Gs), activated the ERK/MAPK pathway by means of G protein/adenylcyclase (AC)/cAMP/ PKA signal pathway, inhibit the production of VEGF, resulted in the declining of the vascular capacity in hemangioma and eventually brought about the regression in IH.

Reference


