Original Article
The adipogenesis in infantile hemangioma and the expression of adipogenic-related genes

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Abstract: Infantile hemangioma, a common benign tumor of infancy, grows quickly in six months to one year after birth, then slowly involutes into fibrofatty tissue childhood. In this study, we observed the adipogenesis in hemangioma and investigated the expression of adipogenic differentiation-related genes. 33 fresh resected hemangioma samples were collected, including 18 proliferating cases (less than one year old), 9 involuting cases (from one to five years old), and 6 involuted cases (more than five years old). The pathological evolution of hemangioma was observed by H-E staining. The expression of Perilipin A was showed by immunohistochemistry staining. The expression and location of PPAR-γ (a key transcription factor in adipogenesis) was displayed by Immunofluorescence staining, with the co-staining of α-SMA and CD31. The expression of adipose differentiation-related genes including PPAR-γ2, LPL, CEBPA, and Perilipin A was detected by Quantitative real time PCR. The results of H-E and Immunohistochemical staining showed the increase of adipose cells as hemangioma developed from the proliferative phase to involuting phase and later to involuted phase. Immunofluorescence staining showed that PPAR-γ wa expressed in the perivascular cells in hemangioma. Quantitative PCR analysis showed a significant increase of PPAR-γ2, LPL, CEBPA and Perilipin A genes' expression in the involuting and involuted heangioma. In conclusion, the PPAR-γ(+) perivascular cells (specific mesenchymal stem cells or pericytes) contribute to the adipogenesis in hemangioma. The siginificantly increased expression of adipogenic differentiation-related genes in the involuting and involuted phase suggested that they played a role in the adipogenesis in hemangioma.

Keywords: Infantile hemangioma, involution, adipogenesis, PPAR-γ

Introduction

Infantile hemangioma (IH), a common benign tumor in children, grows quickly in the six months to one year after birth, then regresses slowly in childhood, and is replaced by fibrofatty tissue finnaly [1]. The accumulation of fibrofatty tissue in IH indicates the adipogenesis in the involution. Studies have showed that mesenchymal stem cells (MSCs) contributed to the adipogenesis in IH [2]. PPAR-γ signal pathway plays important role in MSCs' adipogenic differentiation into adipocytes [3]. In this study, we observed the adipogenesis in IH by H-E staing and immunohistochemistry (IHC) staining of perilipin A, displayed the location of PPAR-γ by Immunofluorescence (IF) staining, and detected the expression of adipogenic-related genes including PPAR-γ, LPL, CEBPA, and Perilipin A gene by quantitative real time PCR (QPCR). The

results may widen our understanding of this unique phenomenon of IH and inspire new ideas for its treatment.

Materials and methods

Samples

Thirty three fresh removed hemangioma samples were got from Children's Hospital and Jinling Hospital in Nanjing, China, under a human subject's protocol approved by the Committee on Clinical Investigation. Informed consent was provided according to the Declaration of Helsinki. The samples included 18 cases in the proliferating phase, 9 cases in the involuting phase, and 6 cases in the involuted phase. Each sample was cut into two parts. One part was fixed by 10% formaldehyde solution and embeded by paraffin for H-E stain-

Table 1. The primers of PPAR-γ2, LPL, CEBPA, Perilipin A and GAPDH genes

Gene	Forward primer	Reverse primer	Size of products (bp)
PPAR-γ2	AGAAAGCGATTCCTTCACTGAT	AGAATGGCATCTCTGTGTCAAC	80
LPL	AATGTACCTGAAGACTCGTTCT	GTTCTCCAATATCTACCTCTGTG	224
CEBPA	CCAGAAAGCTAGGTCGTGGGT	TGGACTGATCGTGCTTCGTGT	174
Perilipin A	TGTGCAATGCCTATGAGAAGG	AGGGCGGGGATCTTTTCCT	154
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA	121

ing, IHC staning and IF staining. Another part was frozened for QPCR.

Routine H-E stainig was performed to observe the pathological structure of IH.

Immunohistochemical (IHC) staining

Paraffin-embedded samples were cut into 5 μm sections, immersed in 0.1 mol/L citrate, and incubated in an microwave oven for 15 min for antigen retrieval. MaxVisionTM IHC kit (KIT-5001, Maixin Biotech., China) and DAB detection kit (DAB-0031, Maixin Biotech., China) were used according to the manufacture's introduction. In briefly, The sections were blocked for 30 minutes in 5% serum and incubated with the first antibodies goat anti-human perilipin A (Ab61682, Abcam), followed by the second antibodies. In the negative control, PBS was used to replace the first antibody. The brown-yellow deposit in cytoplasm was the sign of positive staining.

Immunofluorescence (IF) staining

The cutting of sections and antigen retrieval was done as described above. The sections was incubated with the first antibody rabbit anti human PPAR-γ antibody (BS1587, Bioworld Technology) with the co-staining of rat anti human α-SMA antibody (1A4, Maixin Biotech., China) and CD31 antiboby (JC/70A, Maixin Biotech., China) for 24 hours, followed by the second antibodies Alexa Fluor® 555 donkey anti-mouse IgG antibody (A-31570, Invitrogen) and Alexa Fluor® 488 donkey anti-rabbit IgG antibody (A-21206, Invitrogen), counterstained with DAPI (D-21490, Invitrogen). Fluorescent staining was observed by a fluorescence microscope (BX51, OLYMPUS). The images were taken with a digital microscope camera (ProgRes MFcool, JENOPTIK) and analyzed by an image system (IMSTAR KARYO, IMSTAR).

Quantitative real time PCR (QPCR)

Total RNA was extracted from unfrozen hemangioma sample by Trizol total RNA isolation kit (3101-100, Shanghai Pufei Biotech, China). Purity of the RNA samples was assessed by determining the optical density at 260:280 nm. And reverse transcription was done to get cDNA with M-MLV RT kit (M1705, Promega). Then QPCR was performed with SYBR Master Mixture (DRR041B, TAKARA) and other reagents on a real time PCR machine (LightCycler480, Roche). The primers of PPAR-γ2, LPL, CEBPA, Perilipin A, and internal control GAPDH genes were listed in **Table 1**. The two steps PCR reactions were performed with the following temperature profiles: 95°C for 30 seconds; then 95°C for 5 seconds and 60°C for 30 seconds, 40 cycles; and 95°C for 15 seconds, 60°C for 30 seconds, 95°C 15 seconds. Size of the PCR products was examined by running 5 μl products on 3% agarose gel. In the analysis of data, the relative expression of genes was showed as $2^{-\Delta\Delta Ct}$ ($\Delta Ct = Ct_{tageted\ gene} - Ct_{internal\ gene}$; $-\Delta\Delta Ct = \Delta Ct_{contral\ group} - \Delta Ct_{experimental\ group}$).

Statistics

Statistics was performed with Microsoft® Office Excel 2007. Data were expressed as mean ± SD and analyzed by Student's two-tailed test where appropriate. Differences were considered significant at $P<0.05$.

Results

Clinical evolution of IH

IH may occurr everywhere in the body, with high incidence in the head and neck. IH in the proliferating phase appears bright red or pink with rough surface. It grows quickly and may swell like a "strawberry". About one years old, IH tends to regress spontenously. The regression begin at the center of the lesion, and extend to

Case 1   IH in the upper abdomen

| Proliferating phase | Involuting phase | Involuted phase |

Case 2   IH in the left forearm

| Proliferating phase | Involuting phase | Involuted phase |

Figure 1. Clinical evolution of two cases of IH in the upper abdomen and left forearm.
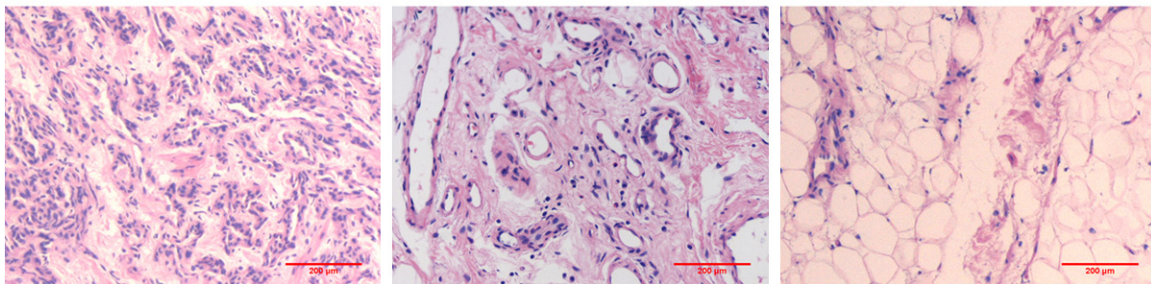
| Proliferating phase | Involuting phase | Involuted phase |

Figure 2. Pathological evolution of IH. In the proliferating IH, there were numerous clusters composed of capillaries. In the involuting IH, the number of capillaries decreased and the adipose tissue increased. In the involuted IH, the tumor tunned into fibrofatty tissue with a few of microvessels. Scale bar: 200 μm.

the margin. The involution of IH lasts for a long time, even more than five years. IH may involutes without any sequela, or with telangiectasis and local hypertrophy. **Figure 1** showed two cases treated in our clinic.

H-E staining

In the proliferating phase, there were numerous capillary clusters in IH, arounded by a little of adipose tissue. In the involuting phase, the number of capillary decreased and the adipose tissue increased. In the involuted phase, most of IHs turned into fibrofatty tissue with a few of microvessels (**Figure 2**).

IHC staining

Perilipin A positive adipocytes increased with IH's involution. In the involuting phase, there were some dilated capillaries in the clusters, and perilipin A expression at the margin of clus-
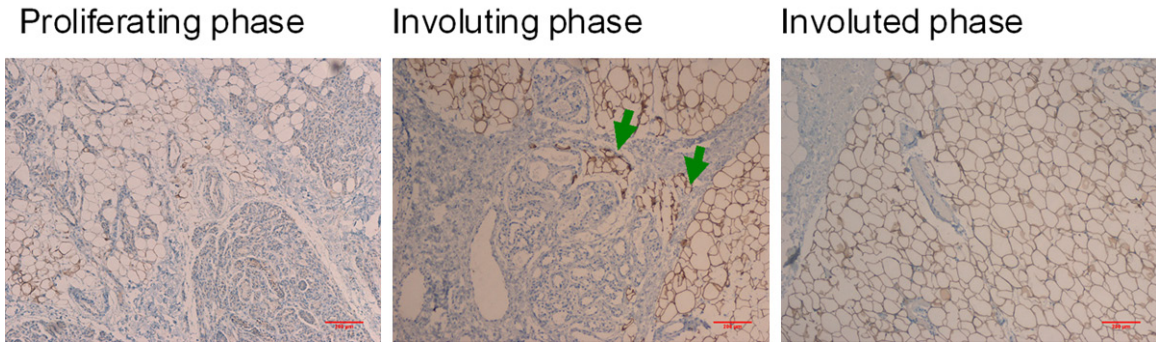
Figure 3. The expression of perilipin A in IH. Perilipin A expression increased with IH's involution, indicating the accumulation of adipose tissue. In the involuting phase, there were the dilated capillaries in the clusters, and perilipin A expression at the margin of clusters (blue arrow), which indicated the adipogenesis here. Scale bar: 200 μm.
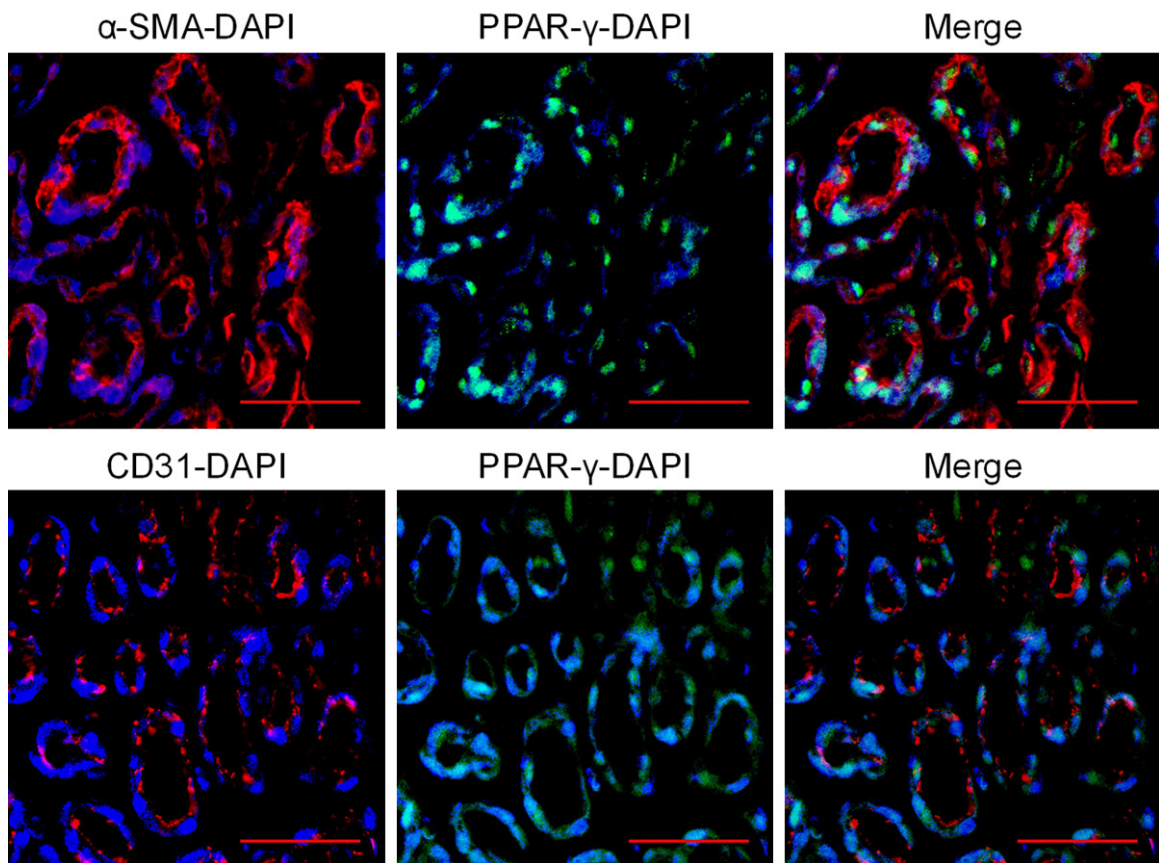

Figure 4. The expression of PPAR-γ in the proliferating IH. In the merged image of α-SMA (red) and PPAR-γ (green), the expression of PPAR-γ was located in the perivascular cells. And in the merged image of CD31 (red) and PPAR-γ (green), the expression alos was showed in a few of endothelial cells. Scale bar: 50 μm.

ters, which indicated the adipogenesis here (**Figure 3**).

IF staining

In the merged image of α-SMA (red) and PPAR-γ (green), the expression of PPAR-γ was located in the nuclei of the perivascular cells. And in the merged image of CD31 (red) and PPAR-γ (green), the expression alos was showed in a few of the nuclei of endothelial cells (**Figure 4**). The results suggested that PPAR-γ in IH was mainly expressed in the perivascular cells, and also in a few of endothelial cells.
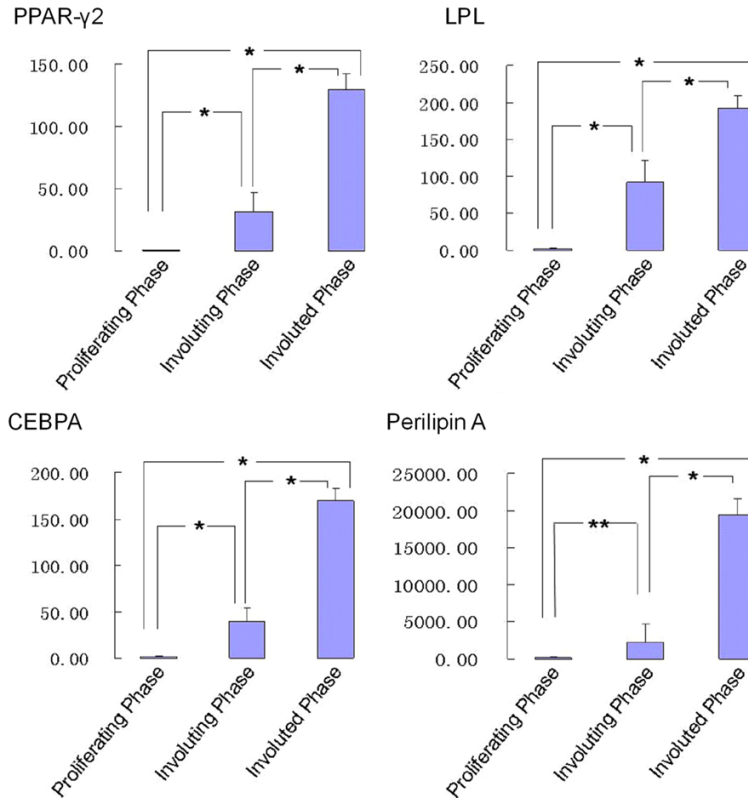
PPAR-γ2

LPL

CEBPA

Perilipin A

Figure 5. The expression of adipogenic-related genes in IH. A of PPAR-γ2, LPL, CEBPA genes' expression increased continously and significantly in IH's involution. Perilipin A gene's expression increased in the involuting phase, and increased very significantly in the involuted phase. *P<0.01, ** P<0.05.

QPCR

A significant and continous increase of PPAR-γ2, LPL, CEBPA genes' expresssion was showed in IH's involuting and involuted phase. Perilipin A gene's expression increased in the involuting phase, and increased very significantly in the involuted phase (**Figure 5**). The enhanced expression of adipogenic-related genes was consistent with the accumulation of adipose tissue in IH's involution.

Discussion

Although it's so common in children, the pathogenesis of hemangioma isn't totally clear till now. The mutation of some genes, abnormal differentiation of stem cells/progenitor cells, changes of hormones, hypoxia, and many other factors have been reported to contribute to the occurrence of hemangioma [4]. In the early hemangioma, active vasculogenesis and angiogenesis lead to the rapid growth of tumor. Unbalanced VEGFR1/VEGFR2 signals, altered

ratios of pro- and anti-angiogenic VEGF-A variants, and proangiogenic properties of pericytes are invovled [5-7]. The involution of IH is still a mystery. Decrease of growth factors [8] and downregulation of anti-apoptotic genes [9] were observed in the involution, but we don't know the real causes behind these phenomena. The accumulation of fibrofatty tissue in IH's involution indicates the adipogenesis during this period. Adipogenesis is one of the impetus of IH's involution, and lead to its final outcome.

Studies have showed that MSCs contributed to the adipogenesis in IH [2]. MSCs in IH reside in the perivascular region [10]. In fact, Hem-MSCs display the features of pericytes, such as the expression of α-SMA and PDGFR-β [11]. Studies have reported the multi-differentiation potential of the pericytes from many tissues or organs [12]. Therefore, we think the perivascular MSCs in IH are pericyts in fact, which have the proagiogenic properties [7] and take part in the vasculogenesis and angiogenesis together with endothelial cells, but also maitain the multi-differentiation potentials, including adipogenic potential. In IH's involution, the apoptosis of endothelial cells leads to the collapse of capillaries and the shrinkness of tumor [4, 13]. Simutaneously, perivascular MSCs (also pericytes) differentiate into adipocytes so hemangioma ivolutes into fibrofatty tissue finnally.

PPAR-γ is an very important transcription factor controlling the adipogenic differentiation of MSCs. Hem-MSCs expressed PPAR-γ2 gene [2, 10]. In this study, co-staining of PPAR-γ/α-SMA and PPAR-γ/CD31 showed that PPAR-γ was mainly expressed in the α-SMA(+) perivascular cells, also in a few of CD31(+) endothelial cells. The results was the further evidences that perivacular MSCs (also pericytes) contributed to the adipogenesis in IH, which may be controlled by PPAR-γ pathway. QPCR showed the signifi-

cant increase of PPAR-γ2, LPL, CEBPA, and Perilipin A genes' expression from the proliferating, involuting, to involuted phase. These genes play their roles in the serial steps of MSCs' adipogenic differentiation [14, 15]. The enhanced expression of above genes was consistent with the accumulation of adipose tissue in IH's involution.

In recent years, the role of PPAR-γ in angiogenesis attracted the attention [16]. PPAR-γ agonists, TZDs, can suppress the angiogenesis by inhibiting the chemotaxis of endothelial cells and promoting their apoptosis through Erk5 activation [17]. PPAR-γ ligands can induce the growth inhibition and apoptosis through p63 and p73 in tumor cells [18]. In this study and our previous study, PPAR-γ expression was observed in the endothelial cells in IH [10]. PPAR-γ pathway may also play a role in the apoptosis of ECs in IH's involution.

Our results indicated the possible role of PPAR-γ pathway in MSCs' adipogenic differentiation and ECs' apoptosis, both of which lead to IH's involution. So, can we treat IH via PPAR-γ pathway? Can PPAR-γ agonists, TZDs, be used to treat IH? Rationally, TZDs may induce ECs' apoptosis and the sbusequent collapse of capillaries, promote MSCs' adipogenic differentiation, and then accelerate IH's involution. TZDs may become the ideal drugs for IH.

In summary, this study observed the adipogenesis in IH, located the expression of PPAR-γ, and detect the expression of adipogenic-related genes. The results may be the priliminary evidences for the targeted therapy of IH via PPAR-γ pathway. In our future study, rosiglitazon, one of TZDs, will be used to investigate the potential of PPAR-γ pathway in the treatment of IH.

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Disclosure of conflict of interest

None.

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