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Propranolol Promotes Accelerated and Dysregulated Adipogenesis in Hemangioma Stem Cells

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Abstract

Background—Infantile hemangiomas are the most common tumor of infancy, yet there are no FDA-approved therapeutics to date. Recently, the non-selective beta-adrenergic-blocker propranolol has been shown to be a safe and effective means of treating infantile hemangiomas, though its mechanism has yet to be elucidated. We have previously demonstrated that propranolol induces early and incomplete adipogenesis in stem cells derived from hemangiomas. We hypothesize that propranolol promotes dysregulated adipogenesis via the improper regulation of adipogenic genes.

Methods—Hemangioma stem cells isolated from resected infantile hemangioma specimens were treated with adipogenic medium for 1 or 4 days in either propranolol or vehicle. Cell death was measured by the incorporation of annexin V and propidium iodide by flow cytometry. Adipogenesis was assessed by visualizing lipid droplet formation by Oil Red O staining. Pro-adipogenic genes C/EBP β , C/EBP δ , PPAR δ , PPAR γ , RXR α , and RXR γ were analyzed by quantitative reverse transcription and polymerase chain reaction.

Results—Hemangioma stem cells treated with propranolol increased lipid droplet formation compared to vehicle-treated cells indicating increased adipogenesis. Cell death as measured by FACS analysis indicated that the propranolol-treated cells died due to necrosis and not apoptosis. During adipogenesis, transcript levels of PPAR δ , PPAR γ , C/EBP β , and C/EBP δ were significantly increased ($p < 0.01$) in propranolol-treated cells relative to control cells. In contrast, RXR α and RXR γ levels were significantly decreased ($p < 0.05$), and C/EBP α , a gene required for terminal adipocyte differentiation, was strongly suppressed by propranolol when compared to vehicle-treated cells ($p < 0.01$).

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Conclusions—In hemangioma stem cells, propranolol accelerated dysregulated adipogenic differentiation characterized by improper adipogenic gene expression. Consistent with accelerated adipogenesis, propranolol significantly increased the expression of the pro-adipogenic genes, PPAR γ , C/EBP β and C/EBP γ compared to control. However, propranolol treatment also led to improper induction of PPAR δ and suppression of C/EBP α , RXR α and RXR γ . Taken together this data indicates that propranolol promoted dysregulated adipogenesis and inhibited the hemangioma stem cells from becoming functional adipocytes, ultimately resulting in cell death. Understanding this mechanism behind propranolol's effectiveness will be a vital factor in producing more effective therapies in the future.

Introduction

Infantile hemangiomas (IHs) are the most common benign tumors of infancy, affecting approximately 5% of children.¹ These vascular tumors present 2-3 weeks after birth and proliferate for approximately one year. The clinical appearance of IH can be highly variable and range from a small, superficial lesion to a large, deep tumor.² Beginning at 10-12 months and lasting up to 10 years, most IHs involute and resolve without intervention. The lesions that result from atrophy of IHs can form permanent residual changes including scarring and fibro-fatty residuum.³ The current hypothesis for cellular origin of IH is the hemangioma stem cell (HemSC). HemSCs have a high proliferation rate, can differentiate into multiple cell types, and can form functional, GLUT1+ hemangioma endothelial cell-lined blood vessels in matrigel-implanted animal models.⁴

While IHs are benign, these tumors can become problematic for infants, causing visual or airway impairment, disfigurement, or ulceration.² The use of propranolol has been added to the treatment armamentarium of long-standing conventional therapies including corticosteroids and surgery for high-risk IHs.^{5,6} Since its serendipitous discovery in 2008 as an effective treatment for severe IH,⁷ propranolol has been become used worldwide and for various types of compromising hemangiomas, even replacing corticosteroids as a first-line therapy in many cases.^{8,9,10,11} Despite the widespread use of this effective therapy, its mechanism of action in IH is not yet understood.

Studies have shown that propranolol increases vasoconstriction while also decreasing proliferation rates of hemangioma endothelial cells (HemEC) in vitro.^{4,10,12,13,14,15} Recently, we have shown that propranolol also affects HemSCs, causing accelerated adipogenesis. In the absence of adipogenic induction, propranolol increased proadipogenic gene expression in HemSCs as early as 24 hours after starting treatment.⁴ Our results showed that propranolol had a pro-adipogenic effect in HemSCs, in addition to its “anti-angiogenic” effects in IH.¹⁶ In this study, we further demonstrate that during adipogenic induction, propranolol promotes dysregulated adipogenesis, by improper regulation of proadipogenic genes, followed by eventual cell death. This may in part explain propranolol's influence in initiating early tumor involution, as well as its overall effectiveness as a therapeutic for IH.

Methods

Cell Isolation

Institutional review board approval for collection of resected human hemangiomas was obtained from Columbia University College of Physicians and Surgeons (IRB #AAAA9976). Written informed consent was obtained for all tissue obtained. Tissues were used immediately for cell isolation for *in vitro* experiments. HemSCs were isolated from clinically resected specimens using the Miltenyi Biotec (Auburn, CA) magnetic cell sorting system selecting for CD133+ cells.^{4,17} HemSC identity was confirmed by successful differentiation into adipocytes, endothelial cells, and osteocytes *in vitro* and recapitulation of IH histology *in vivo* in a nude mouse model.¹⁷

Tissue Culture and Treatment with Propranolol

HemSCs were maintained on fibronectin-coated, 10-cm plates in EGM-2 and 20% fetal bovine serum. Medium was changed every other day, and cells at passage number 10 to 20 were used for all experiments.

Propranolol hydrochloride (Sigma, St. Louis, MO) was reconstituted at 100 μ M in pH 3.0 dH₂O (hereafter referred to as “vehicle.”) For experimentation, fresh propranolol was added to culture media during media changes at indicated concentrations.

RNA Isolation and cDNA generation

Total RNA was isolated with the RNeasy Mini Prep Kit (Qiagen, Germantown, Md.). cDNA was synthesized by reverse transcription of 2 μ g of total RNA, performed using the Superscript II Reverse Transcriptase Kit (Invitrogen, Grand Island, NY).

Quantitative Real-Time Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction was performed in triplicate utilizing the CFX Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and Asolute Blue QPCR SYBR Green Mix (Applied Biosystems, Foster City, CA). The results were normalized to β -actin expression levels and samples were analyzed in triplicates. Gene expression levels for C/EBP α , C/EBP β , and C/EBP δ , PPAR δ , PPAR γ , RXR α , and RXR γ were determined (Table 1).

Adipogenic Differentiation

Adipogenic differentiation was performed as previously described.⁴ Briefly, 10⁴ HemSC were seeded on fibronectin-coated, eight-well Nunc Lab-Tek II chamber slides (Thermo Fisher Scientific, Waltham, MA) in EGM-2/20%FBS. The following day cells were washed once with phosphate-buffered saline and then cultured in adipogenic media [Dulbecco's Modified Eagle Medium low glucose (Invitrogen), 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen), 5 μ g/ml of insulin (Sigma), 1 μ M dexamethasone (Sigma), 0.5 mM isobutylmethylxanthine (Sigma), and 60 μ M indomethacin (Sigma)] for 4 days in the presence of 50 or 100 μ M propranolol or vehicle. Adipogenic differentiation was assessed by Oil Red O staining for lipid droplets after 4 days of treatment in adipogenic media.

Annexin V Measurement by Fluorescence-Activated Cell Sorting

HemSCs were grown to 75% confluence in EGM-2/FBS 20%. Cells were then exposed to vehicle, 10, or 100 μ M propranolol for 24 hours. Levels of annexin V and propidium iodide (PI) expression by hemangioma stem cells were measured using the Annexin V-FITC Apoptosis Detection Kit (BioVision, Milpitas, Calif.) according to the manufacturer's instructions. Differentiation between apoptosis and necrosis was made by comparing levels of annexin V and PI, respectively. The FACSCalibur flow cytometer (BD Biosciences, San Jose, Calif.) was used for antibody detection.

Results

Propranolol Induced Cell Death in Hemangioma Stem Cells during Adipogenesis

HemSCs were grown in adipogenic medium with either propranolol (50 or 100 μ M) or vehicle for 4 days (Fig. 1). Differences in cell density were observed after 24 hours in the propranolol treated cells in a dose dependent manner. Vehicle-treated HemSCs were 90-95% confluent, while HemSCs in 100 μ M propranolol were 40-50% confluent and had begun to detach. By day 4, vehicle-treated cells were still 90-95% confluent. In contrast, HemSCs treated with 50 μ M propranolol were less confluent (80-85%) and beginning to detach, while cells treated with 100 μ M propranolol were less than 10% confluent, and displayed an altered cell morphology.

Adipogenesis was also determined by Oil Red O staining. HemSCs treated with 50 μ M propranolol had an increase in lipid droplet formation relative to vehicle-treated cells (Fig. 2), consistent with increased adipogenic differentiation. Thus, we found that propranolol increased the rate of adipocyte differentiation and promoted HemSC detachment characteristic of increased cell death.

Cell Death in Propranolol-treated Hemangioma Stem Cells was not Caused by Apoptosis

Next we wanted to determine the mechanism of cell death after 2 days in adipogenic media. Cell death was assessed by the Annexin V Apoptosis Detection Kit and propidium iodide uptake. Consistent with the increased HemSC cell detachment, propidium iodide uptake was increased in propranolol-treated HemSCs in a dose dependent manner (Fig. 3). However, levels of annexin V remained unchanged for each treatment type. Taken together, this data suggests that propranolol induced cell death by an apoptosis-independent mechanism.

Propranolol Induced Dysregulated Adipogenesis in HemSCs

We hypothesize that the cell death observed in response to propranolol may be due to defective or incomplete adipocyte differentiation. To assess this, we evaluated the expression of adipogenic genes at day 1 and day 4 post-treatment with 50 μ M propranolol relative to day 0 prior to adipogenic induction. The gene expression levels were determined for the CCAAT/enhancer-binding proteins C/EBP α , C/EBP β , and C/EBP δ (transcription factors commonly up-regulated during adipogenesis), peroxisome proliferator-activated receptors PPAR δ , PPAR γ (receptors well established for modulating adipocyte differentiation), and retinoid X receptors RXR α , and RXR γ (nuclear receptors known to heterodimerize with PPAR proteins).

We found three distinct gene expression patterns (Fig. 4). At days 1 and 4, transcript levels of *C/EBP β* , *C/EBP δ* , and *PPAR γ* , were significantly increased above vehicle-treated cells (3.4, 3.3, and 2.8-fold, respectively, at day 1), consistent with the observation of early fat vacuole formation ($p < 0.01$). In vehicle treated cells, *PPAR δ* levels decreased at days 1 and 4 relative to day 0. In contrast, *PPAR δ* was significantly upregulated in the propranolol treated HemSCs at days 1 and 4 relative to day 0. Propranolol-treated HemSCs showed a significant decrease in *RXR α* , and *RXR γ* transcript levels when compared to vehicle (1.6, and 3.2-fold, respectively, by day 1; $p < 0.05$). Finally, *C/EBP α* , a gene required for terminal differentiation, had an attenuated induction in propranolol-treated cells (48-fold at day 4) when compared to vehicle (840-fold increase at day 4) ($p < 0.005$) (Fig. 4).

Discussion

Since 2008, propranolol has been known to be an effective empirical treatment for problematic IHs. There are multiple studies on the anti-angiogenic effects of propranolol on endothelial cells (including HemECs).^{16,18} We have shown that propranolol also has other effects, including changing HemSCs proliferation and differentiation into adipocytes.⁴ Here, we further demonstrate that the propranolol induced dysregulated adipogenesis associated with increased cell death and improper induction of pro-adipogenic genes. As fibrofatty tissue is typically found in involuting or involuted infantile hemangioma, our results suggest that propranolol acts on both the endothelium and the fatty residuum associate with IHs.

While accelerated adipogenesis was observed as early as 24 hours after propranolol treatment, treated HemSCs also showed decreased cellular confluence over the time course of the experiment, indicating a departure from the typical mitotic clonal expansion (MCE) normally found in early adipogenesis of committed preadipocytes.^{19,20} Our results suggest that there was increased cell death in propranolol-treated HemSCs, and this was confirmed with increased propidium iodide expression. Our analysis of annexin V expression showed that levels remained unchanged during treatment, suggesting that mechanisms of cell death other than apoptosis may be involved. Léauté-Labrèze et al. postulated that propranolol-induced IH cell death may be due to apoptosis of endothelial cells.⁷ Our study concentrated on propranolol's effects in HemSCs, and our findings suggest that propranolol induces necrosis versus apoptosis in these cells. Future experiments on HemECs will help clarify the role of apoptosis in propranolol-induced IH involution. Furthermore, we will study other possible triggers of cell death that are distinct from apoptosis, such as anoikis (a mechanism of cell death induced by detachment) or paraptosis (a caspase-independent mechanism characteristic of extensive cytoplasmic vacuolization).²¹

The dysregulation in adipogenesis seen in propranolol-treated HemSCs was confirmed by the changes in gene expression profile during adipogenesis. We have previously shown that propranolol increased transcripts in multiple pro-adipogenic genes in basal media even in the absence of adipogenic stimulation. The only exception was *C/EBP α* , which was not changed.⁴ This study shows the adipogenic gene expression was perturbed during early adipogenesis. Preadipogenic genes such as *C/EBP β* , and *C/EBP δ* are known to be increased early in the differentiation process,^{22,23,24} and these were all upregulated in propranolol-treated HemSCs as compared to vehicle treated HemSCs. Elevated expression levels of

PPAR γ were seen at the 24-hour mark, a gene that is usually expressed later in adipogenesis during terminal differentiation.^{20,25} While PPAR γ was increased in the propranolol treatment group at both time points, the difference was more dramatic at day 1, supporting the early acceleration seen in propranolol-treated cells. This could explain the increase in lipid formation observed in the 50 μ M propranolol treated HemSCs.

Other genes required for differentiation, including RXR α , RXR γ , were found to be downregulated in propranolol treated HemSCs, and may play a vital role in the cell death of HemSCs under propranolol treatment. Under normal circumstances, RXRs are important factors in the epigenetic control of adipogenesis, as PPARs regulate transcription by forming heterodimers with RXR.^{20,22} As treatment with propranolol appears to significantly down-regulate the expression of RXR factors, it can be inferred that differentiation is at least partially hindered through the limitation of PPAR-RXR heterodimer formation. Thus, the increase in PPAR levels may not result in increased signaling as RXRs are decreased.

Perhaps the most drastic departure from normal physiological transcription levels during treatment is that of C/EBP α , an integral factor that oversees terminal adipogenesis alongside PPAR γ .^{23,24} Under physiological conditions, C/EBP α is induced by C/EBP β and C/EBP δ , and its expression is associated with terminal adipocyte differentiation. While propranolol treatment increased expression of C/EBP β and C/EBP δ , they failed to induce C/EBP α expression. In knockdown models, white adipose tissue fails to develop in C/EBP α $-/-$ mice.^{26,27} The loss of C/EBP α induction therefore may explain the failure in adipocyte differentiation and subsequent cell death observed in propranolol-treated HemSCs.

Understanding the observed departure in gene expression profile of normal adipocyte development may help understand the mechanism by which propranolol acts on infantile hemangiomas. Our studies have shown upregulation of genes that cause accelerated early adipogenesis. In addition, we have shown that propranolol downregulated genes that are necessary for completion of differentiation. However, further study is needed in order to find exactly how these departures take place in order to find a more effective and targeted therapy for future use with infantile hemangioma.

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Figure 1. Propranolol induced HemSC cell death

HemSC cultured in adipogenic media in the presence of vehicle, 50 μ M propranolol, and 100 μ M propranolol for 1 day and 4 days after the addition of adipogenic medium. (Scale bar = 10 μ m).

Figure 2. Propranolol accelerated HemSC adipocyte differentiation

Oil Red O staining of HemSC in adipogenic media in the presence of vehicle vehicle, or 50 μ M or 100 μ M propranolol for 4 days showing lipid droplets in red. [Magnification: 20x (left), scale bar = 10 μ m; 40x (right), scale bar = 5 μ m].

Figure 3. Analysis of Propranolol induced cell death of HemSC

Propidium iodide (PI) and annexin V incorporation were analyzed by FACS analysis of HemSCs grown in adipogenic media for 24 hours. HemSCs (vehicle only) without annexin V or PI added was used as the control (gray). HemSCs treated with vehicle only are shown (thin black line). HemSCs treated with propranolol (10 or 100 μ M) are shown (thick black line).

Figure 4. Adipogenic gene expression analysis of propranolol treated HemSCs

Treatment of HemSC with 50 μ M propranolol (grey) resulted in significantly deregulated gene expression in HemSC when compared to vehicle (black) after 1 and 4 days of treatment. Quantitative RT-PCR was performed to determine the expression of C/EBP α , C/EBP β , C/EBP δ , PPAR δ , PPAR γ , RXR α , and RXR γ . B-actin qRT-PCR performed to normalize between samples. Values are normalized to Day 0 values in growth media prior to addition of adipogenic differentiation media. *p < 0.05; **p < 0.01.

Table 1

Primers

Gene	Primer (5' to 3')
<i>PPARδ</i>	
Forward	CTT GCC CTC CCT TTC TCT CT
Reverse	AGG TGT GCA AAA GCA GAG GT
<i>PPARγ</i>	
Forward	GCT GGC CTC CTT GAT GAA TA
Reverse	TTG GGC TCC ATA AAG TCA CC
<i>C/EBPα</i>	
Forward	TGG ACA AGA ACA GCA ACG AG
Reverse	TTG TCA CTG GTC AGC TCC AG
<i>C/EBPβ</i>	
Forward	TTT CGA AGT TGA TGC AAT CG
Reverse	CAA CAA GCC CGT AGG AAC AT
<i>C/EBPδ</i>	
Forward	ACT CAG CAA CGA CCC ATA CC
Reverse	CGC TCC TAT GTC CCA AGA AA
<i>RXRα</i>	
Forward	TGG ACG CCT TCT CCA TAG TC
Reverse	GAC ACT TTC TTC CCC ACC AA
<i>RXRγ</i>	
Forward	TGT GGT CAA CAG TGT CAG CA
Reverse	GTC TCC ACA GAT GGC ACA GA