Infantile Hemangioma—Mechanism(s) of Drug Action on a Vascular Tumor

Shoshana Greenberger¹ and Joyce Bischoff²

¹The Department of Dermatology and Sheba Cancer Research Center, Sheba Medical Center, Ramat-Gan 52621, Israel

²Vascular Biology Program and Department of Surgery, Children's Hospital Boston and Harvard Medical School, Boston, Massachusetts 02115

Correspondence: joyce.bischoff@childrens.harvard.edu

Infantile hemangioma (IH), a benign vascular tumor, is the most common tumor of infancy, with an incidence of 5%–10% at the end of the first year. The tumor displays a distinctive life cycle consisting of a proliferating phase, occurring in the first months of life, followed by an involuting phase. Thus, IH represents a unique model of postnatal vasculogenesis, angiogenesis, and vessel regression. Traditionally, corticosteroids were the drug of choice when treatment of IH was indicated. In recent years, beta-blockers, most specifically propranolol, have serendipitously been shown to be an effective pharmacological treatment. This article will focus on the mechanism of action of these two drugs, the old and the new treatments, in slowing the growth and accelerating involution of IH.

Infantile hemangioma (IH), a benign vascular tumor, is the most common tumor of infancy, with an incidence of 5%–10% at the end of the first year. There is increased risk in premature neonates under the weight of 1500 g (Amir et al. 1986), in females and in Caucasians (Haggstrom et al. 2007). The tumor displays a distinctive life cycle that can be separated, both clinically and histologically, into three phases (Enjolras and Mulliken 1993; Frieden et al. 2005). The proliferating phase starts within a few weeks from birth and ends within the first year of life, with the most growth occurring during the first 4–6 months of life (Fig. 1) (Chang et al. 2008). In the proliferating phase the tumor is composed of densely packed cells that express endothelial and pericytic markers,

with features of immature vessels such as plump endothelium and high nuclei/cytoplasmic ratio. Multipotent progenitor-like cells reside outside of the vessels (Khan et al. 2008; Boscolo and Bischoff 2009). The involuting phase typically begins at 1 yr of age. Clinically, the infantile hemangioma shrinks centrifugally from the center of the lesion, changes color to less red, and softens. The vascular channels appear more mature, with flattened endothelial cells, an organized perivascular layer, and basement membrane. There is a prominent apoptosis, some occurring in endothelial cells (Iwata et al. 1996; Razon et al. 1998; Dosanjh et al. 2000), and an increase in the number of mast cells. Finally, at the involuted phase, tumor growth has stopped and the tumor has

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Figure 1. Proliferating, involuting, and involuted phases of infantile hemangioma. Hematoxylin and eosin stain (H&E) stained sections from each phase. (A) Proliferating phase is highly cellular with immature vessels, (B) involuting phase contains well-formed blood vessels with endothelial and perivascular layers, and (C) involuted phase contains adipocytes, fibrous deposits, and few remaining vessels. Scale bar, 100 μ m.

regressed. The regression is either complete or, more often, leaves scar tissue, telangiectasia, or redundant or anetodermic skin (Jackson 1998). When viewed in histological sections, fat, fibroblasts, and connective tissue replace the vascular tissue at this stage, with few large feeding and draining vessels evident.

IH is a benign tumor, and in most patients no specific treatment is required. However, in 10% of cases IH is problematic or even endangering to the child, owing to its location or owing to excessive growth. In these circumstances that carry the risk of irreversible disfigurement, airway obstruction, or decreased vision, treatment is indicated. Corticosteroids have been the first-line treatment for IH for more than 40 years (Zarem and Edgerton 1967; Cohen and Wang 1972). In recent years, beta-blockers, most specifically propranolol, have serendipitously been shown to be an effective pharmacological treatment of proliferating IH. Other, less common medications are interferon-2 α and vincristine (Maguiness and Frieden 2010). This article will focus on the mechanism of action of corticosteroids and propranolol, the old and the new treatments, in slowing the growth and accelerating involution of IH. For each drug, a general mechanism of action will be reviewed, then its specific mechanism against IH, and finally, broader mechanisms in antiangiogenic therapy.

CORTICOSTEROIDS

General Mechanism of Action

Glucocorticoids, natural or synthetic, belong to a class of steroid hormones that bind to the glucocorticoid receptor (GR). Unbound GR resides within the cytoplasm in an inactive state, as an oligomeric complex with regulatory proteins such as the HSP90 (heat shock protein-90 KD) (Dalman et al. 1989), HSP70 (heat shock protein-70 KD), and the p59 immunophilin. On diffusion of the glucocorticoid ligand into the cytoplasm and its binding to high-affinity GR, the receptor is activated, and a nuclear localization signal is unmasked (Pelaia et al. 2003). The GR then translocates to the nucleus

where it binds to a glucocorticoid response element (GRE) within the controlling region for glucocorticoid responsive target genes (Adcock et al. 2006). Once bound to DNA, GR can elicit either transactivation or transrepression of gene expression. Transactivation occurs through the recruitment of complexes containing basal transcription factors, coactivators, chromatin modifiers, and RNA polymerase II, which together induce histone modifications and chromatin remodeling that lead to increased production of messenger RNAs (mRNAs) (Adcock et al. 2006). As for transrepression, GR can bind to a GRE that overlaps the DNA-binding site for a transcription factor or the start site of transcription, thus preventing gene expression. Importantly for IH, GR can repress AP-1/NF- κ B-mediated gene expression. Furthermore, it can interact with corepressors such as NCoR (nuclear receptor corepressor receptor) or dephosphorylate RNA polymerase II (Schaaf and Cidlowski 2002; Schoneveld et al. 2004). Finally, GR can induce rapid nongenomic effects through a membrane-associated receptor (Norman et al. 2004) or by modulating the activity of kinases such as ERK, Akt, or phosphatidylinositol 3-kinase and protein kinase Akt.

Mechanism of Action in Infantile IH

When studying the effect of a drug on a tumoral process, one has to consider the activity on the different cellular compartments as well as on the inter-relationships between the cells. Thus, a brief description of the major cell types in IH is provided. Endothelial cells constitute about 20% of the cell population within the tumor (Yu et al. 2004). These hemangioma-derived endothelial cells have been shown to be clonal, have immature morphology, and display increased growth and migratory properties compared to normal dermal endothelial cells (Dosanjh et al. 2000; Boye et al. 2001; Yu et al. 2001; Dadras et al. 2004). Pericytes, or mural cells, are arranged around the endothelial cell layer in a proliferating IH tissue section (Li et al. 2003; Boscolo and Bischoff 2009). Several cell types have been identified in the tumor's interstitium. Among them are mast cells, which

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increase in number during involution (Qu et al. 1995; Tan et al. 2004), and myeloid cells (CD45⁺/CD14⁺), which are present during the proliferative phase. Hemangioma progenitor cells or hemangioma-derived stem cells (HemSCs) have been identified and isolated by us based on the expression of the human stem/progenitor cell marker CD133 (Shmelkov et al. 2005). These cells have self-renewal capacity and display multipotential activity, being able to differentiate in vivo into endothelial cells and adipocytes (Khan et al. 2008). When injected subcutaneously into nude mice, HemSC form human blood vessels that express GLUT-1, a specific IH marker. Hence—the HemSC are vasculogenic—able to form blood vessels de novo. Furthermore, 2 mo after injection the vessels are replaced by fat tissue, indicating the HemSCs can recapitulate key features of the life cycle of IH.

Evidence from In Vitro and In Vivo Studies of IH

Blocking of the Vasculogenic Potential of HemSC

To study the effect of corticosteroids on vasculogenic processes that occur when HemSC are implanted in vivo, we used our animal model. HemSC were injected subcutaneously into nude mice, and dexamethasone (a synthetic glucocorticoid) was injected intraperitoneally thereafter for 7 d. Dexamethasone inhibited the formation of blood vessels in a dose-dependent manner (Greenberger et al. 2010b). Furthermore, by pretreating the HemSC and endothelial cells with dexamethasone, each separately in vitro and before implantation, we were able to show that HemSCs, and not the endothelial cells, are the target of corticosteroids. This indicated that steroids might work by inhibiting the vasculogenesis that occurs in IH.

Down-Regulation of Vascular Endothelial Growth Factor A

Vascular endothelial growth factor A (VEGF-A), a master regulator of angiogenesis and vasculogenesis, has been shown by us and by others

to be present at higher levels in IH tumors in the proliferating phase compared to the involuting phase (Takahashi et al. 1994; Chang et al. 1999; Kleinman et al. 2007; Greenberger et al. 2010a,b). It has also been shown that corticosteroids suppress the expression of VEGF-A in a variety of cell types (Bandi and Kompella 2001; Kompella et al. 2003; Kim et al. 2008). We found that the corticosteroids dexamethasone, prednisone, prednisolone, methylprednisolone, and hydrocortisone were each able to dramatically down-regulate VEGF-A secretion by HemSC. The suppression was reversed by RU-486, a synthetic steroid hormone antagonist, indicating a GR-dependent mechanism (Greenberger et al. 2010b). These findings are consistent with reports that show decreased levels of VEGF-A in the serum of IH patients following systemic steroid therapy (Zhang et al. 2005). Silencing the expression of VEGF-A in HemSC by short hairpin RNA (shRNA) was sufficient to block blood vessel formation in vivo (Greenberger et al. 2010b). The effect of the steroids was not mediated by the induction of apoptosis as dexamethasone did not lead to reduced viability of HemSC or endothelial cells, even at concentrations 1000 times higher than those that led to VEGF suppression.

Modification of Proangiogenic Profile

The direct target genes of corticosteroids in vivo are still largely unknown and might be tissue or even cell specific (Phuc Le et al. 2005; van Batenburg et al. 2010). Examining HemSC and endothelial cells in vitro revealed that several corticosteroid-modified genes with relevance for angiogenesis, in addition to VEGF-A, were expressed (Greenberger et al. 2010b). Monocyte chemotactic protein-1 (MCP-1), which has been reported to be overexpressed in proliferating versus involuting infantile hemangioma (Isik et al. 1996), was suppressed by dexamethasone. Interleukin-6, which was detected in in vitro hemangioma models (Hasan et al. 2003), was also suppressed, as were matrix metalloproteinase-1 (MMP-1) and urokinase-type plasminogen activator receptor (uPAR). These target genes, MCP-1,

uPAR, and IL-6, were found to be differentially expressed in proliferating versus involuting IH tissue by quantitative real-time polymerase chain reaction (PCR) measurements (Greenberger et al. 2010b). In sum, corticosteroids seem to affect key molecules in the angiogenesis process when analyzed in HemSC and hemangioma-derived endothelial cells cultured in vitro.

Suppression of Nuclear Factor κ -Light-Chain-Enhancer of Activated B Cells Activity

Corticosteroids are known to be regulators of nuclear factor κ -light-chain enhancer of activated B cells (NF- κ B) activity, through several distinct mechanisms (Tao et al. 2001), for example, (1) physical interaction between NF- κ B subunits and GR (Caldenhoven et al. 1995), (2) increased transcription of the NF- κ B inhibitor I κ B α (Auphan et al. 1995), and (3) competition for interactions with coactivators (Kamei et al. 1996; Lee et al. 1998). In HemSC, corticosteroids suppress NF- κ B activity. This suppression in turn leads to down-regulation of VEGF-A, as well as other proangiogenic cytokines MCP-1, uPAR, MMP-1, and IL-6 (Greenberger et al. 2010a). As VEGF-A is critical for hemangiogenesis, this finding suggests that the effect of corticosteroids is to interfere with NF- κ B regulation. In addition, suppression of NF- κ B may also have direct antiendothelial effects, as NF- κ B has been shown to contribute to embryonic carcinoma (EC) survival in vitro, and NF- κ B blockade in vivo sensitizes ECs to stress-induced apoptosis (Kisseleva et al. 2006).

Antagonizing Estrogen's Effects

Corticosteroids have been shown to antagonize estrogen responses (Bever et al. 1956; Rhen et al. 2003), including the prosurvival effects of estrogen (Zhou et al. 1989) and its stimulatory effects on insulin growth factor-1 (IGF-1) (Sahlin 1995). This inhibition might occur through corticosteroid induction of the expression and activity of estrogen sulfotransferase, an enzyme that deactivates estrogens (Gong et al. 2008). In

infants with proliferating IH, serum levels of estrogen (estradiol 17 β) have been shown to be increased compared to normal infants. (Sasaki et al. 1984; Liu et al. 1999). Furthermore, estrogen receptor (ER) expression and binding activity were reported to be higher in patients with proliferating IH (Sasaki et al. 1984; Kleinman et al. 2007) and to decrease following corticosteroid treatment (Sasaki et al. 1984). As estrogen has been shown to be essential for neoangiogenesis (Das et al. 2009) and vasculogenesis (Masuda et al. 2007) in the reproductive system, it might then be the case that corticosteroids exert an additional effect on IH—that of antagonizing the activity of estrogen.

PROPRANOLOL

Propranolol as a pharmacological treatment of IH was first reported in 2008, in two children who showed rapid regression of disease when treated with propranolol for cardiopulmonary indications (Leaute-Labreze et al. 2008). Since then, propranolol treatment for IH has become popular worldwide and positive results with its use are reported by many groups (Cheng et al. 2010; Mazereeuw-Hautier et al. 2010; Rosbe et al. 2010). Propranolol is a noncardioselective β -adrenergic receptor blocker used traditionally for other indications such as hypertension, angina pectoris, myocardial infarction, migraines, anxiety disorders, and tremor. The mechanism of action of propranolol on IH remains elusive. To date there are no studies on the effects of propranolol using IH animal models or cultured IH-derived cells. Therefore, we will summarize here the general mechanism of action of propranolol and suggest possible mechanisms based on data derived from studies performed using angiogenesis assays and endothelial cells.

General Mechanism of Action

When catecholamines, adrenaline and noradrenaline, interact with the β -adrenergic receptors β 1 or β 2, an agonist-promoted binding of the receptor to the heterotrimeric guanosine

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triphosphate-binding protein Gs occurs, causing dissociation of $G\alpha$ -GTP and $G\beta\gamma$ subunits. This in turn leads to activation of adenylate cyclase and production of cyclic adenosine monophosphate (cAMP). Downstream effectors of cAMP include cAMP-dependent protein kinase (PKA) as well as cAMP-gated ion channels (Benovic 2002). Propranolol is an orthosteric antagonist of both β 1- and β 2-adrenergic receptors. In addition, it has been shown to function as central serotonin 5-HT receptor antagonist, inhibitor of noradrenaline reuptake and indirect agonist of α -adrenergic receptors (Young and Glennon 2009). The drug exists as a pair of optical isomers: *S*(-)-propranolol and *R*(+)-propranolol. The enantiomers bind with relatively large differences in affinity to the β -adrenoceptors (Young and Glennon 2009). Most commercial preparations contain a mix of the two isomers.

Hemodynamic Effects

Following the administration of propranolol, a rapid change in the tumor is typically noticed, including decreased redness and softening (Sans et al. 2009; Rosbe et al. 2010). This raises the question of whether propranolol exerts its effect via vasoconstriction of the high-flow blood vessels feeding the IH tumor. Propranolol has been shown to decrease blood flow to many tissues following single administration (Nies et al. 1973; McSorley and Warren 1978; Vandenburg et al. 1981). Particularly in the skin, adrenaline-induced vasoconstriction has been shown to be increased by oral propranolol (Doshi et al. 1984). However, as prolonged treatment probably does not affect vascular resistance in subcutaneous tissues (Jensen et al. 1983), it is reasonable to assume that there are additional mechanisms that account for the prolonged effects of propranolol on the course of IH.

VEGF-A

VEGF-A is a critical factor for the growth of IH. Thus, suppression of VEGF-A by propranolol is a plausible mechanism for its activity. Noradrenaline has been shown to enhance VEGF-A

production by several cell types, both normal and cancerous (Fredriksson et al. 2000; Lutgendorf et al. 2003; Park et al. 2011). Furthermore, in an ovarian carcinoma animal model, catecholamines up-regulated tumoral VEGF and increased angiogenesis (Thaker et al. 2006). These effects of the catecholamines are mediated by the β 1 and β 2 adrenergic receptors and are blocked by propranolol (Fredriksson et al. 2000; Park et al. 2011). In addition to VEGF, noradrenaline up-regulates HIF-1 α protein (Park et al. 2011). The signal transduction pathway leading to HIF-1 α and VEGF-A up-regulation is mediated through cAMP and PKA (Fredriksson 2000). In some cell lines, Src tyrosine kinase, a downstream effector of PKA, is involved, however, not through activation of the mitogen-activated protein (MAP) kinases Erk1/2 (Fredriksson et al. 2000). Importantly, propranolol does not affect baseline level of VEGF-A production by the cells, but rather opposes catecholamine stimulation (Fredriksson et al. 2000; Thaker et al. 2006). Thus, if VEGF suppression is a proposed mechanism of action, one must assume increased noradrenaline stimulation or increased sensitivity to noradrenaline in the tumor. Studies testing these assumptions in vitro and in vivo on IH models are needed to begin to gain some insight into how propranolol exerts its effects on IH. In our laboratory, we examined the effect of propranolol on VEGF-A production in hemangioma-derived stem cells and endothelial cells, but found no evidence that propranolol affected VEGF-A under basal cell culture conditions (S Greenberger et al., unpubl.).

Down-Regulation of Other Proangiogenic Cytokines

Angiogenesis is a process in which a coordinated set of events leads to the formation of new blood vessels by sprouting from pre-existing vessels. Among those events is the proteolysis of components of the extracellular matrix allowing the endothelial cells to migrate and sprout (Aznavorian et al. 1993; Bergers et al. 2000). Accordingly, matrix metalloproteinases (MMPs), secreted both by tumor cells

and by tumor-associated macrophages (TAMs) have been shown to be critical for angiogenesis (Bergers et al. 2000; Fang et al. 2000; Huang et al. 2002). In a vascular tumor model, using endothelioma cells, a synthetic inhibitor of MMPs inhibited in vivo tumor growth (Tarabozletti et al. 1995). Noradrenaline increases the expression of MMP-2 and MMP-9 by tumor cells and by macrophages, and these effects could be inhibited by the beta-blocker, propranolol (Lutgendorf et al. 2003; Guo et al. 2009). Propranolol has also been reported to block the up-regulation of MMP-7 in a gastric tumor (Shi et al. 2010). In brain vascular endothelial cells, propranolol blocked the induction of MMP-9 by PMA (a phorbol ester that causes an extremely wide range of effects in cells (Blumberg 1981; Annabi et al. 2009). Thus, it is possible that propranolol's effect is mediated, at least in part, by MMPs regulation.

In addition to MMP down-regulation, propranolol might inhibit hemangiogenesis by the control of another proangiogenic cytokine, IL-6. Recent reports have shown IL-6 is up-regulated by catecholamines, both in the transcriptional and in the translational levels, and that the up-regulation is blocked by propranolol. In-depth analysis of this signaling pathway also suggests a cAMP and Src kinase-dependent mechanism (Nilsson et al. 2007). It is of interest that both corticosteroids and propranolol suppress VEGF-A, MMPs, and IL-6. This overlap in activity may provide a clue to the roles of these proteins in the pathogenesis of IH.

Effects on Differentiation

IH is a tumor in which new blood vessels form, presumably de novo, from immature progenitor (stem) cells. Thus, it is possible that propranolol prevents the hemangioma stem cells from differentiating into endothelial cells or pericytes (Frieden and Drolet 2009). Another possibility is that propranolol hastens the differentiation of the progenitor cells into adipocytes, which would in effect convert the tumor stage from “proliferating” to “involuting.” Although support for this hypothesis has not yet been

provided by experimentation with in vitro or in vivo models of IH, effects have been reported on mesenchymal stem cells. For example, noradrenaline has been shown to push mesenchymal progenitors within white adipose tissue toward a brown adipocytic phenotype (Vegiopoulos et al. 2010). Also, an effect of noradrenaline on mesenchymal stem cell adipogenesis has been shown in vitro (Li et al. 2010).

CONCLUDING REMARKS

In recent years, our understanding of the pathological vasculogenic process leading to IH proliferation has expanded with the identification of hemangioma stem cells and the corticosteroid-sensitive targets. The emergence of propranolol as a new therapeutic option that is both safe and effective provides a crucial alternative therapy for disfiguring, and at times, life-endangering hemangiomas. A major challenge for the future years will be to elucidate the mechanism of action of propranolol on proliferating IH. Insights gained from such studies will enable the development of target-specific drugs, not only for IH but also for malignant vascular tumors and tumor angiogenesis.

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