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The potential effect of human chorionic gonadotropin (hCG) on vasoproliferative disorders of the immature retina

Tammy Z Movsas^{a,b} and Arivalagan Muthusamy^a

^aZietchick Research Institute, Plymouth, MI

^bDept of Pediatrics & Human Development, College of Human Medicine, Michigan State University, East Lansing,

Abstract

Objective: Human chorionic gonadotropin (hCG) is known to be a powerful VEGF-regulating hormone. It stimulates vascularization of the gravid uterus by upregulating VEGF expression. In the body, hCG activates the same receptor as luteinizing hormone (LH). Like hCG, LH is also strongly pro-angiogenic. Recently, it has been shown that LH/hCG receptors are present in the retina and that both LH and hCG are found in the eye. In fact, the human eye can synthesize its own hCG. We have previously shown that LH and VEGF are significantly correlated in mammalian eyes, potentially implicating LH/hCG receptor activation in intraocular VEGF regulation. Given that elevated VEGF is associated with progression of two vasoproliferative pediatric retinal disorders, retinopathy of prematurity (ROP) and retinoblastoma, our objective was to determine whether hCG may potentially affect VEGF production and pathologic retinal vascularization in vasoproliferative disorders affecting the immature retina.

Methods: (a) Use of oxygen-induced retinopathy (OIR) mouse model (standard model for ROP) and (b) use of Y79 retinoblastoma cells (a human cell line derived from immature retinal cells)

Results: (a) In OIR model, number of pre-retinal nuclei (representing pathologic retinal neovascularization) significantly increases by 57% ($p < 0.05$) in hCG-treated mice. (b) In Y79 cells, VEGF production significantly increases by 37% ($p < 0.05$) in hCG-treated cells.

Conclusions: These findings suggest that hCG is potentially able to influence retinal vascularization and VEGF production and thus, the hCG receptor may potentially represent a therapeutic target for vasoproliferative retinal disorders affecting the young eye.

Keywords

chorionic gonadotropin; hCG; retinoblastoma; retinopathy of prematurity; ROP; Y79; retina; luteinizing hormone; VEGF; vascular endothelial growth factor

Corresponding Author (and requests for reprints): Tammy Z Movsas, MD, MPH Zietchick Research Institute, 46701 Commerce Center Dr, Plymouth, MI, 48170 Cell Phone: 1-248-445-2661; tmovsas@gmail.com.

Potential Conflicts of Interest:

Dr. TZ Movsas is the Director and Principal Investigator at Zietchick Research Institute (ZRI), a private (for-profit) research institute, and has pending patent applications for the use of both gonadotropins and gonadotropin antagonists for the treatment of ocular disorders. Some of the data contained within this manuscript had been utilized in these patent pending applications. Dr. A Muthusamy is a salaried employee (no equity) at ZRI.

INTRODUCTION:

Vascular endothelial growth factor (VEGF) is a potent mediator of retinal angiogenesis during ocular maturation and in the years beyond.[1] Tight regulation of intraocular VEGF is critical for both eye development and maintenance of ocular health. Dysregulation of VEGF can lead to neovascularization, the formation of pathologic vasculature. Highly elevated VEGF expression is strongly associated with the vasoproliferative progression of at least two vision-threatening disorders affecting the young eye, retinopathy of prematurity and retinoblastoma.[2] [3]

Retinopathy of prematurity (ROP), a leading cause of pediatric blindness in the U.S. and abroad, affects premature infants of all races, ethnicities and socioeconomic groups.[4] In advanced stages of ROP, high levels of intraocular VEGF levels stimulate the formation of abnormal retinal blood vessels in a process known as retinal neovascularization.[5] Though the induction of increased VEGF expression in ROP has been mainly attributed to retinal ischemia, other factors contributing to dysregulation of VEGF expression in this disorder have not been completely identified [5]

Retinoblastoma, the most common intraocular malignancy of childhood, is derived from retinoblasts, immature retinal cells of neuroepithelial origin.[6, 7] The vast expression of VEGF seen in retinoblastoma allows the tumor to induce new vessels as it outgrows its existing blood supply.[7] The growth rate of retinoblastomas is more dependent on the tumor's ability to induce neovascularization than on the inherent growth rate of the malignant cells. [8] Thus, identifying factors, other than low oxygen tension and tissue ischemia, that influence retinoblastoma's VEGF regulation is of importance in slowing the tumor's rapid growth rate.[9]

Human chorionic gonadotropin (hCG) and luteinizing hormone (LH), closely related to one another, are powerful pro-angiogenic hormones, responsible for VEGF regulation in several tissue types.[10, 11]. Of key relevance to this study, both of these gonadotropic hormones are present in the eye. Though hCG is generally regarded as a pregnancy-related hormone, the human retina (both healthy and neoplastic retinal tissue) has been shown to synthesize its own hCG (unrelated to pregnancy).[12–15] In addition, we recently showed that LH was detectable in all vitreous fluid samples collected from human adult patients undergoing vitrectomies for a variety of indications.[16] Since LH is produced by the male and female anterior pituitary, it most likely enters the eye from the systemic circulation.

In the body, both LH and hCG bind to the same G-coupled receptor. LH/hCG receptors, here on in referred to as LHRs, have been identified in the retina.[12] We recently examined the naturally-occurring levels of LH and VEGF in vitreous fluid extracted from healthy mammalian eyes. We found a strong and significant LH-VEGF correlation in bovine and porcine eyes, potentially implicating LHR signaling in intraocular VEGF regulation.[17]

In the present investigation, we aimed to examine the potential effect of hCG on two vasoproliferative disorders affecting the immature retina, ROP and retinoblastoma. This study complements our recent findings that LHR signaling participates in VEGF regulation and retinal vascularization during normal eye development.[18] In this current study, we

utilized oxygen-induced retinopathy (OIR) mouse model (i.e. a standard model for the study of ROP)[19] to demonstrate that hCG can increase retinal neovascularization in the neonatal mouse. Second, we utilized the Y79 retinoblastoma cell line to demonstrate that hCG can increase retinal VEGF secretion in a human pediatric tumor line derived from immature photoreceptor cells.

MATERIALS & METHODS:

Oxygen-Induced Retinopathy (OIR) Investigation:

Iris Pharma (LaGaude, France), a contract research organization (CRO) specialized in ophthalmic studies, has >20 years of experience in OIR investigations; thus, we chose this CRO to perform this OIR investigation. This animal investigation was approved by the Iris Pharma's Animal Review Board. All animals were treated according to the European convention and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The minimum number of animals were used to attain significant results and all possible steps were taken to avoid animals' suffering at each stage of the experiment.

OIR Experimental Procedure:

Three litters, each consisting of 5 C57BL/6J neonatal mouse pups, were received from the breeder Elevage Janvier (FR-53940 Le Genest-St-Isle-France) and included in this study. All animals were housed under identical environmental conditions and routinely exposed to a 12 hour light/12 hour darkness controlled cycle. The mouse pups were divided into 3 groups, with no significant differences in mean starting weights between the groups; all groups were subjected to the standardized OIR protocol.[20] In brief, retinopathy was induced in Day-7 postnatal animals (P7) by a 5-day exposure to 75% oxygen atmosphere from P7 to P12. After this, animals were transferred back to normoxia for the duration of the experiment. Depending upon group assignment, animals were treated by once daily intraperitoneal (IP) administrations of either 1320 IU/kg hCG (Sigma Aldrich) or 1600 IU/kg of hCG (Sigma-Aldrich) or vehicle control for 10 consecutive days (from P5 to P14). Treatments were performed at approximately the same time each morning. Animals were euthanized 72 hours $\pm$ 2 hours after last dosing on P17. Both eyeballs were removed from each animal (totaling 10 eyes per group) for histological processing.

Histology:

Eyeballs were fixed in 4% paraformaldehyde/phosphate buffered saline solution and embedded in paraffin. *The microtome used by Iris Pharma for this study has $\pm$ 1 μ m thickness variability; thus, the blocks were cut into serial sagittal section of 6 $\pm$ 1 μ m (i.e. 5–7 μ m) along a superior to inferior plane, through the optic nerve in accordance with the well-established OIR protocol [20]. An average section thickness of 6 μ m is utilized in the OIR protocol because it matches the average diameter of a mammalian nucleus. The range of 5–7 μ m in section thickness may have some effect on the number of pre-retinal nuclei counted per section. However, the consistent use of the same microtome at the same setting for all sections should eliminate any potential significant difference in mean number of pre-*

retinal nuclei between groups caused by the microtome's inherent $\pm 1 \mu\text{m}$ variation in tissue sectioning.

In the central half of the globe, two sagittal 5–7 μm sections at 30 and 90 micrometers apart of the optic nerve were done; a total of 4 sections were selected for staining and evaluation. Sections with the optic nerve were excluded. They were stained with Periodic Acid Schiff (PAS), hematoxylin and picroindigocarmin. Using light microscopy, preretinal nuclei (nuclei of vessels extending into the vitreal side of the internal limiting membrane) were counted by a masked investigator, exactly as per described in the OIR protocol described by Smith, et al. [20] Average nucleus count per eye was calculated from the results of the 4 sections. After that, the average nucleus count per animal was calculated by averaging together the nucleus counts from each eye.

hCG- VEGF Y79 Human Retinal Cell Line Investigation:

This Y79 cell line investigation was performed at the laboratory of the Zietchick Research Institute, located within the Michigan Life Science and Innovation Center (MLSIC) in Plymouth, MI. For reliability, reproducibility and robustness, the experiment described below was repeated twice

Y79 Cell Culture and hCG treatments:

Y79 human retinoblastoma cell line (#HTB-18) was procured from ATCC (Manassas, VA) and cultured per ATCC recommendations. In brief, the Y79 cells were cultured in RPMI media (GIBCO #A1091) containing high glucose, L-glutamine and HEPES in the presence of 20% fetal bovine serum with 1% penicillin and streptomycin and incubated at 37°C under 5% CO₂ under a tissue culture hood. hCG treatments were prepared as follows: 1500 units of highly purified human hCG from pregnant urine (MBS173051; MyBioSource, San Diego, CA) was resuspended in 0.1% BSA in sterile PBS to treat Y79 retinoblastoma cells at 3 different concentrations of hCG (1 mIU/ml, 10 mIU/ml, 100 mIU/ml). These hCG doses were chosen based upon prior published hCG dosages that resulted in Y79 cell proliferation. [13]

Y79 retinoblastoma cells (50,000 cells/ well) were seeded into a laminin coated (2 $\mu\text{g}/\text{ml}$) 48- well plates. The cells were initially cultured in complete media with 10% FBS and then cultured overnight with stepdown media containing 1% FBS, 1% penicillin/ Streptomycin with 250 μM cAMP. For each condition, multiple replicates were used (n=8). The cell culture media was replaced after 24 hours of treatment with either fresh non-treated media (controls) or with fresh hCG-treated at the indicated concentrations. Cell culture supernatant was collected by centrifugation of the cells at 1000 rpm for 5 min after 24 and 48 hours of treatment and stored at -80°C immediately.

Determination of VEGF protein levels:

To determine the VEGF protein levels upon the hCG treated retinoblastoma cells, cell culture supernatants were analyzed for VEGF by Human ELISA Kit (R & D Systems: DVE00) as per the manufacturer's instructions. Mean VEGF level in each treatment group

of individual experiment was normalized to their control group (vehicle treated), and the values were averaged between the experiments.

Statistical Analyses:

The non-parametric, Mann-Whitney U test was utilized for all statistical analyses using Prism 4.0 (GraphPad Software, San Diego, CA,). For OIR study, the Mann-Whitney U test was performed on the pre-retinal nucleus counts; each treated group was compared to that of the control group. For the Y79 analysis, the Mann-Whitney U test was performed to assess the relative difference in VEGF levels between the control group (hCG=0) and each of the aforementioned conditions; p-values <0.05 were considered as statistically significant. Corrections for multiple comparisons were not performed because the experiments were focused on planned comparisons which had been designed into the experiment and were not decided upon after inspecting the data.

RESULTS:

Effect of hCG on Oxygen-Induced Retinopathy (OIR) :

The mean pre-nuclei counts for vehicle-treated control, 1320 hCG IU/kg and 1600 hCG IU/kg groups were 36.1 [SD 7.5], 56.9 [SD 14.0] and 53.0 [SD 6.6] respectively. 1320 hCG IU/kg and 1600 hCG IU/kg significantly increased pre-retinal nucleus count by 57.6% [U (5,5) =2, p<0.05] and by 46.8% [U (5,5)=1, p<0.05] respectively. Thus, IP-administered hCG significantly increased retinal neovascularization in the neonatal mouse model of OIR.

Effect of hCG on Y79 human retinoblastoma VEGF production:

After 48 hours of hCG exposure, there was a significant increase in relative VEGF levels (compared to control) at 100 mIU/ml of hCG [U(16,16)=73, p<0.05]. (**Fig 1**). In addition, after 48 hours of hCG exposure, there was a visible, increasing trend in VEGF levels at 1 mIU/ml [U(16,16) =114.5] and 10 mIU/ml [U(16,14)=87.5]. (**Fig 1**).

DISCUSSION:

As aforementioned, LH and hCG activate the same LHR in the body to upregulate VEGF expression and promote blood vessel formation in a variety of tissue types. [10, 11]. LHRs are found in a multitude of organs including the eye. [12] To the best of our knowledge, our team has been the first to show that LHR signaling potentially impacts intraocular VEGF regulation. In prior work, we demonstrated a strong and significant correlation between LH and VEGF in healthy adult bovine and porcine eyes.[17] We also previously showed that human proliferative diabetic retinopathy is significantly associated with elevated vitreous LH levels (compared to non-diabetic levels).[16]]. Taken together, these observations suggest that increased LH/hCG signaling potentially impacts intraocular VEGF production and retinal vascularization in mature retinas under both physiologic and pathophysiologic circumstances. Recently, we have also shown that LHR signaling impacts physiologic VEGF levels and retinal vascularization in developing mouse eyes.[18]

In this current study, we examined the potential effect of hCG on the pediatric retina under pathologic conditions. By utilizing a neonatal rodent model of proliferative retinopathy [the OIR model], we showed that IP-injection of hCG significantly increases retinal neovascularization. Also, in this current study, we utilized a retinoblastoma cell line derived from immature photoreceptor cells and showed that hCG significantly increases retinal VEGF secretion. Taken together, these observations suggest that hCG likely exacerbates VEGF levels and pathologic neovascularization in vasoproliferative retinal disorders in the young eye.

For this study, we used the OIR model to examine how increased hCG affects neonatal eyes with co-existing retinal pathology. The OIR neonatal rodent model is known to yield reproducible findings of retinal neovascularization via the well established protocol of counting of pre-retinal nuclei as described by Smith et al.[20] Neonatal mouse eyes, not exposed to the OIR model, exhibit, on average, less than two pre-retinal nuclei; [20] the OIR protocol is known to reliably increase the presence of pre-retinal nuclei in neonatal mouse eyes and is widely accepted as a suitable animal model for the study of ROP and other proliferative retinopathies.[19]

For practical considerations, we dosed the neonatal mice with daily hCG (or vehicle control) via IP-injection; systemic hCG has been previously shown to penetrate the blood-retinal barrier to enter the posterior segment of the eye.[21] We based the IP- hCG dosing for our OIR study on dosing utilized in prior hCG-mouse brain studies by ourselves and others.[22, 23] In a prior study, we utilized the Rice-Vannucci model of neonatal cerebral hypoxia-ischemia to show that IP- injection of hCG (1500 IU/kg) results in a significant decrease in hippocampal and striatal tissue loss follow brain injury. Given that the brain and retina share similar embryological origins, anatomical origins and physiological properties, we reasoned that an IP-hCG dose that had been shown to exert a neurological effect would probably exert a retinal effect as well. Of note, 1500 IU/kg dose of hCG closely approximates the hCG levels seen in human amniotic fluid at term.[24] Based upon prior study results, we expect approximately 1% of the IP-dosing to enter the eye.[25] Because of the unknown therapeutic window of hCG dosing, we closely bracketed the treatment doses in this study on either side of the 1500 IU/kg dosage.[22] With the doses of IP -hCG utilized in this current study, we found approximately 50% increase in preretinal neovascular tufts in the neonatal mouse eyes, representing pathologic neovascularization.

An entire litter was selected as either a vehicle-treated control group or as a treatment group. This was done because a mother mouse may reject mouse pups who smell differently than others. It is unlikely that our results were significantly impacted by this grouping method. Importantly, the results from one of our prior studies have also shown that LHR signaling impacts retinal vascularization in the neonatal eye [18]; in our recent study of neonatal LHR knockout (LHRKO) mice, the elimination of LHR signaling significantly reduced retinal vascularization.[18] Thus, two separate neonatal studies (the LHRKO study and this current study) together show that less LHR signaling decreases retinal vascularization whereas more LHR signaling increases it.

Though our groups contained unsexed mouse pups, the role of gonadotropins in VEGF regulation in the eye is not gender related. In healthy bovine, porcine and non-diabetic human eyes, neither LH nor VEGF levels are significantly impacted by gender. [16, 17] Furthermore, in our recent study involving LHR knockout (LHRKO)s and wildtype (WT) siblings, we did not find any significant gender-related difference in VEGF in LHRKO/WT mouse eyes.[18] Thus, a difference in gender ratios between groups is unlikely to have mediated our reported results.

The Y79 cell line utilized in this study is a human retinoblastoma cell line with cone photoreceptors properties.[26] Cone photoreceptors are the main cells that have been shown to express the hCG receptor not only in retinoblastoma but also in normal, human retina.[26] Y79 cells not only express both hCG and its receptor[13] but also produce VEGF (our outcome measurement).[27] This Y79 in vitro study nicely complements the in vivo study by demonstrating that hCG directly increases VEGF in a human retinal cell line; the in vivo study alone (when not combined with this study) does not allow us to determine whether the mouse eyes are directly affected by increased retinal LHR signaling or indirectly by downstream effects resulting from increased gonadal organ stimulation.

Given our findings that hCG-treated Y79 cells have increased VEGF production, we would expect hCG-treated Y79 cells to also exhibit increased proliferation. A prior hCG-Y79 study observed exactly that; when treated with increasing concentrations of hCG (0.001 to 100 IU/ml), Y79 cells have been shown to have increased proliferation (via wst1 assay) and when treated with hCG together with anti-LH/hCG-receptor, the proliferation induced by hCG is completely abolished. [13] Thus, proliferation of Y79 by hCG treatment is indeed secondary to LH/hCG-receptor activation.

In conclusion, our findings suggest that hCG may influence retinal vascularization and VEGF production in pediatric retinal disorders such as ROP and retinoblastoma. Given that the retina synthesizes its own hCG (under both physiologic and pathologic circumstances) [12–15], future studies should evaluate the therapeutic potential of LH/hCG receptor as a target for vasoproliferative retinal disorders affecting the young eye.

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Figure 1: hCG increases VEGF levels in Y79 human retinoblastoma cells:

After 48 hours of hCG exposure, there was a 37% significant increase ($p < 0.05$) in relative VEGF levels in retinal Y79 cells exposed to media enriched with 100 mIU/ml of hCG compared to controls (hCG=0). In addition, there was an increasing trend in VEGF production seen in retinal Y79 cells enriched in media enriched with 1 mIU/ml and 10 mIU/ml of hCG. Before normalizing and combining the 2 control groups, the mean VEGF level of (a) 1st control group, (N=8) was 200.2 (SD: 83.0) and (b) 2nd control group (N=8) was 99.9 (SD 65.9).