The Effect of Increased VEGF Levels on Limb Bud Angiogenesis in a Developing Chick Embryo

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Summary

Vascular endothelial growth factor (VEGF) is a necessary regulator of normal angiogenesis as it serves as a potent mitogen for vascular endothelial cells derived from arteries and veins.[1] In this study, the effect of augmented levels of VEGF on limb bud angiogenesis in a developing chick embryo will be demonstrated. Alkaline phosphatase staining was used to show the presence of endothelial tissue originating from angiogenesis. We report that increased levels of VEGF resulted in an increase in dark blue alkaline phosphatase staining, suggesting that there is more enzymatic activity where endothelial cells are present. These findings support the documented role of VEGF in promoting angiogenesis.

Introduction

The establishment of a vascular supply is essential for organ development and differentiation during embryogenesis, as well as for reproductive functions and wound healing.[1] During development, the vascular system is dependent upon the formation of new blood vessels from pre-existing ones, a phenomenon known as angiogenesis. Abnormal angiogenesis has been implicated in various disorders such as rheumatoid arthritis, psoriasis, proliferative retinopathies, tumors, and age-related muscular degeneration.[2]

The foremost mitogen involved in the promotion of neovasculature is vascular endothelial growth factor (VEGF). VEGF is a heparin-binding, glycoprotein[1] that directs multiple angiogenic developmental systems such as retinal development,[3] carcinoma metastasis,[4,5] and embryonic limb bud formation[6] by binding to specific tyrosine-kinase receptors.[7] VEGF comes in one of four isoforms of varying amino acid lengths (121, 165, 189, 206).[2] VEGF typically binds to one of two receptors, VEGFR-1 or VEGFR-2, respectively known as flt1 and KDR in humans. These VEGF receptors are exclusively found on endothelial tissue in high density.[7]

In retinal development, VEGF is released by astrocytes migrating away from pre-existing vasculature.[7] Hypoxic conditions increase as the astrocytes migrate further from the blood vessels, triggering their release of VEGF to make a chemotaxic gradient between the astrocytes and proximal endothelial tissue.[7] VEGF binding to this endothelial tissue promotes angiogenesis in the developing retina. In the neovascular disease, retinopathy of prematurity, VEGF is inhibited by hyperoxia, causing blindness in the developing organism.[3] However, hypoxia is just one of many possible stimuli demonstrated to trigger expression of VEGF. Other factors that induce VEGF production are epidermal growth factor (EGF), TGF-β, and keratinocyte growth factor (KGF).[1]

VEGF is thought to function on a number of levels in promoting angiogenesis. Foremost, VEGF expression is thought to function in angiogenesis by inducing vascular permeability,[8] which would facilitate the release of fibronectin – the substrate for endothelial growth[9] - into the extracellular matrix. Additionally, excess concentrations of VEGF present in cancerous tumors are shown to increase permeability,[8] as well as promoting proliferation and chemotaxis of endothelial cells.[10] Still, the role of VEGF and similar vascularizing molecular mechanisms in other developmental processes are only vaguely understood.

The limb bud vascular system is known to develop from endothelial precursor cells of the paraxial mesoderm, which later move into the limb bud.[11] Additionally, this vascular system shows regression during the condensation of mesenchyme tissue in the limb bud during chondrogenesis and eventual skeletogenesis.[6] These processes imply the need for molecular mechanisms behind the vascularization of the limb bud. One such avian specific VEGF-related mitogen, Retinoic Acid-induced Growth Factor (RIGF), has been cloned and shown to aid in patterning limb bud vascularization.[12] Still, revealing VEGF's functioning in angiogenesis is of particular relevance to understanding human limb bud development, where a factor like RIGF remains undiscovered.

To explore how VEGF patterns angiogenesis, we used the chick model for embryonic limb bud development. Acrylic VEGF soaked beads were placed directly underneath the vitelline envelope and proximal to the regions of the developing fore-and hindlimb buds. Based on the aforementioned angiogenic abilities of VEGF, we hypothesize that VEGF will trigger augmented angiogenesis in the chick embryo's developing limb bud.

Results

Staining of alkaline phosphatase enzyme activity using a BCIP/NBT chromogen protocol should produce a dark blue granular precipitate.[15] Overall, we found a visible difference in alkaline phosphatase staining between the sham and experimental surgery chicks.

The cross sections of the sham surgery chicks, with the exception of Figure 1B, only show a purple staining of the epithelial layer. Gray crystallization exterior to the tissue sample was produced by residual paraffin wax adhering to the sample.

A greater amount of alkaline phosphatase activity was visible among chick embryos implanted with the VEGF soaked beads, most notably in the region of tissue between the purple epithelium and gray mesenchyme.

Discussion

The alkaline phosphatase staining was successful because the experimental limb cross-sections revealed dark blue coloration, (Figures 3 & 4) compared to the sham limb cross sections that did not (Figures 1 & 2). This indicates that the VEGF beads caused an increase in enzymatic activity. The lack of dark staining in the interior of the cross-sections hints the beginning of vascular regression. Vascular regression happens when mesenchymal cells condense and chondrogenesis occurs.[6] Chondrogenesis is the formation of cartilage, a non-vascular tissue. Therefore, we conclude that the whitish-blue coloration in all of the figures indicates the presence of cartilage.

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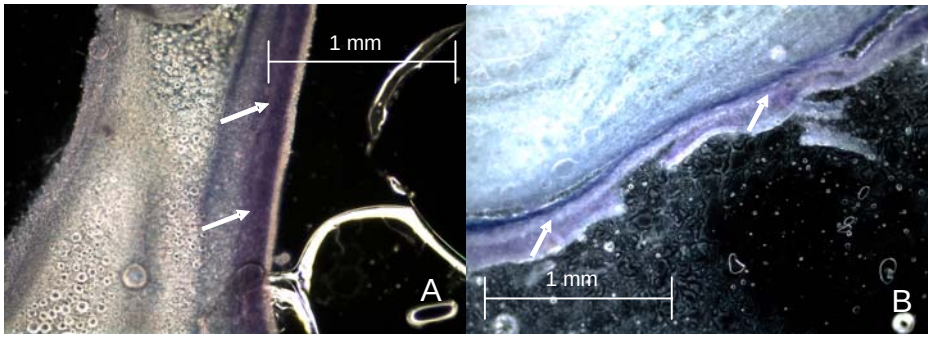

Figure 1. Cross sections of hindlimb sham surgery chick. (A) Wing section with purple staining of epithelium. (B) Leg section with purple staining of epithelium and blue staining of alkaline phosphatase activity.

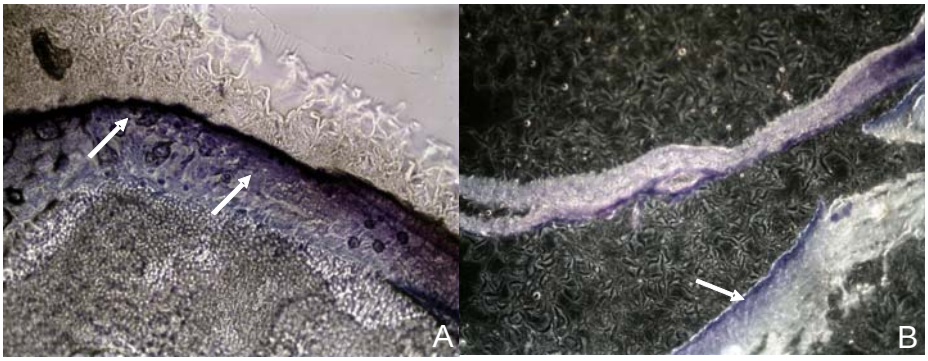

Figure 2. Cross Sections of forelimb sham surgery chick. (A and B) Wing Sections with purple staining of epithelium.

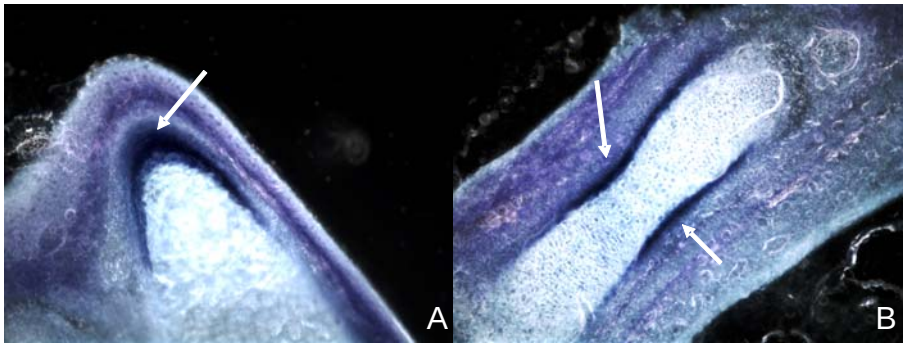

Figure 3. Cross sections of hindlimb experimental surgery chick. (A) Wing section shows dark blue alkaline phosphatase activity between epithelial layer stained purple and mesenchymal tissue stained whitish-blue. (B) Leg section shows dark blue alkaline phosphatase activity between epithelial layer stained purple and mesenchymal tissue stained whitish-blue

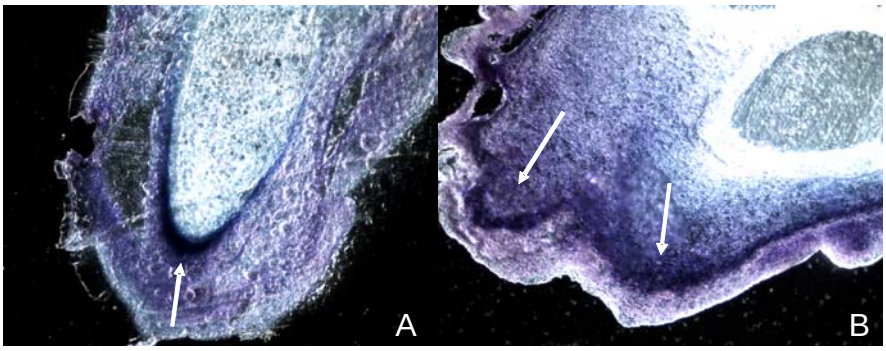

Figure 4. Cross sections of forelimb experimental surgery chick. (A) Wing section shows dark blue alkaline phosphatase activity between the epithelial layer stained purple and the mesenchymal tissue stained whitish-blue. (B) Leg section shows epithelium stained purple.

Due to the lack of knowledge in the field of VEGF staining, we encountered a few problems during our experiment. First and foremost, the two hour BCIP/NBT staining rendered all the chick embryos pitch black. Even after the PBT, glycerol, and Histoclear washes, the embryos remained black. A way of circumventing this problem would have been to stain the embryos for only 30 to 45 minutes. Also, the fact that we did not know the exact concentration of the stain may have caused the embryos to turn so black. The protocol we used explained the composition of the stain in mass whereas the solution we actually used had the composition in millimoles. Since we did know the molecular mass of the stain, we could not know whether or not we had the correct concentration of the BCIP/NBT stain. Furthermore, the VEGF beads that were implanted were not strictly VEGF, but also included other unknown mitogens. This may have resulted in the staining of other factors besides VEGF. Another issue that we faced involved the cutting of cross-sections. Since we placed all the limbs with the anterior facing the closed side of the plastic mold, we were only able to view one perspective of the staining. However, had we placed the limbs within the mold at a different angle, we may have yielded different views of the staining. For example, we could have placed the posterior side of the limbs facing the closed side of the plastic mold. As a result, we could have had a better view of the heavily stained subclavian artery, which supplies the developing limb bud with blood.[12] Another area of the methods that we could have ameliorated was the implantation of the beads. Ideally, we wanted to insert only one bead, but instead, on average we inserted 10-15 beads. As the distance of VEGF diffusion was unknown, the addition of numerous beads may explain the unexpected staining of the limbs distal to VEGF bead implantation. Lastly, the embryos could have been stained at an earlier or later point in development, in order to determine the time required before VEGF's angiogenic effects came into view.

The scientific field of VEGF and VEGF-related topics is open to a number of new areas of research due to its importance in angiogenesis and the development of the vascular system. In order to understand the biological functions of molecules such as VEGF, it is necessary to examine the mechanisms involved in their signal transduction. Presently, areas of the VEGF pathway remain unclear to researchers. For instance, the mechanisms of signal transduction of the VEGF receptors as well as the importance of Flt-1 receptor have not yet been identified.[1] The use of VEGF as a form of therapy in treating ischemic tissues needs to be further examined. One study has shown that the addition of VEGF into a rabbit model with an ischemic hindlimb produced angiogenesis in the ischemic region.[16] These findings must be further analyzed in order to help treat people who have atherosclerosis or a history of blood clotting.

Methods

Thirty chick embryos were used in this experiment. Sixty hours post-fertilization, ten embryos underwent sham surgeries, where heparin-acrylic beads were implanted proximal the developing hind- or forelimb bud. The other twenty embryos underwent experimental surgery where VEGF soaked heparin-acrylic beads were implanted proximal to the developing hind- or forelimb bud. The sham and experimental surgery groups were divided in half such that the chicks either had the beads implanted near their forelimb (n = 5 sham; n = 10 experimental) or their hindlimb (n = 5 sham; n = 10 experimental). For the bead implantation protocol, each egg was sterilized with 70% ethanol and approximately 2 ml of albumin was removed from the blunt of the egg with an 18 gauge needle. The egg was then opened with either a scalpel or scissors. Using a pair of fine forceps, the beads were placed either near the wing or the leg. Around 10-15 beads were placed in each egg. The egg was then closed by taping glass cover slips over the window. All the embryos were allowed to develop for seven days in a 37° Celsius incubator.

Of the 30 chicks, only 8 survived: 2 sham (1 leg and 1 wing) and 6 experimental (3 leg and 3 wing). The embryos were removed from the eggs and placed into glass vials. The embryos were then washed with PBS and paraformaldehyde (PFA) overnight. The next day they were rinsed with PBT (PBS, 0.1% Tween detergent). The embryos were then washed with methanol. First there was a 50% methanl/50% PBT wash for 15 minutes. Next, the embryos were washed in 100% methanol, twice for 15 minutes, and then stored over night at -20° Celsius. The following day, the embryos were washed with PBS and NTMT (100mM Tris pH 9.5, 100mM NaCl, 50 mM $MgCl_2$, 0.1% Tween) buffer three consecutive times for 15 minutes each. Once washed, alkaline phosphatase activity in the embryos was stained with BCIP (5-Bromo-4-chloro-8-indolilphosphate) and NBT (4-Nitroblue-tetrazolium) in the NTMT buffer for two hours. To clear the embryos, a series of washes were performed. First, they were rinsed with PBT three times for five minutes each. Then they were rinsed with 70% glycerol/30% PBT overnight. Following the 70% glycerol, they were rinsed with 100% glycerol overnight again to completely clear the BCIP/NBT stain.[13] Lastly, they were rinsed with 50% Histoclear/50% PBT and then 100 % Histoclear as a secondary measure for clearing the stain from the embryo.

Following this procedure, the embryos were ready for cross-sectioning. In order to dehydrate the embryos, they were placed in 70% ethanol and then 100% ethanol for 20 minutes per wash. Once dehydrated, the wings and legs were removed from the embryos. They were then placed in a plastic mold (anterior of the limb facing the closed end of the mold) with melted paraffin wax. The molds were cooled to room temperature and placed in -20° Celsius. With the use of a microtome, 15 micrometer thick cross-sections were cut. A hot bath of electrolyzed water was used to displace the wax from the tissue slice.[14] Afterwards, the cross-sections were placed on a slide to be observed under a microscope. A Leica microscope camera was used to take pictures of the slides. The microscope was set at 40x magnification.

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References

1. Ferrara, Napoleone. (1999). Molecular and biological properties of vascular endothelial growth factor. *Journal of Molecular Medicine, 77*: 527-543.

2. Ferrara, Napoleone, and Davis-Smyth. (1997). The Biology of Vascular Endothelial Growth Factor. *Endocrine Reviews, 18*(1): 4-25.

3. Alon, Tamar, Hemo, Itzhak, Itin, Ahuva, Peter, Jacob, Stone, Jonathan, & Keshet, Eli. (1995). Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels, and has implications for retinopathy or prematurity. *Nature Medicine*, *1*(10): 1024-1028.

4. Takahashi, Yukata, Kitadai, Yasuhiko, Bucana, Corazon D., Cleary, Karen R., & Ellis, Lee M. (1995). Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Research, 55*: 3964-3968.

5. Warren, Robert S., Yuan, Hui, Matli, Mary R., Gillett, Nancy A., & Ferrara, Napoleone. (1995) Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis

in a mouse model of experimental liver metastasis. *Journal of Clinical Investigation, 95*: 1789-1797.

6. Yin, Melinda, & Pacifici, Maurizio. (2001). Vascular regression is required for mesenchymal condensation and chondrogenesis in the developing limb. *Developmental Dynamics, 222*: 522-533.

7. Neufield, Gera, Cohen, Tzafra, Gengrinovitch, Stela, & Poltorak, Zoya. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *The FASEB Journal, 13*: 9-22.

8. Senger, Donald R., Galli, Stephen J., Dvorak, Ann M., Perruzzi, Carole A., Harvey, V.S., & Dvorak, Harold F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science, 219*: 983-985.

9. Wilting, Jorg, & Christ, Bodo. (1996) Embryonic angiogenensis: a review. *Naturwissenchaften, 83*: 153-164.

10. Waltenberger, Johannes, Claesson-Welsh, Lena, Siegbahn, Agneta, Shibuya, Masabumi, & Heldin, Carl-Henrik. (1994). Different signal transduction properties of KDR and flt1, two receptors for vasculat endothelial growth factor. *The Journal of Biological Chemistry, 269*(43): 26988-26995.

11. Pardanaud, L., Altmann, C., Kitos, P, Dieterlen-Lievre, F., & Buck, C.A. (1987). Vasculogenesis in the early quail blastodisc as studied with monoclonal antibody recognizing endothelial cells. *Development, 100*: 339-349.

12. Tamura, Koji, Amano, Takanori, Satoh, Taeko, Saito, Daisuke, Yonei-Tamura, Sayuri, & Yajima, Hiroshi. (2003). Expression of *rigf*, a member of avian VEGF family, correlates with vascular patterning in the developing chick limb bud. *Mechanisms of Development, 120:* 199-209.

13. Fujita, Misato. (2007). In *Medaka Book*, Alkaline Phosphatase Staining. Tokyo Institute of Technology. Retrieved April 29, 2008, from http://www.shigen.nig.ac.jp/medaka/medakabook/index.php?4.5.1%20Alkaline%20phosphatase%20staining.

14. Sun, Xin. (2007). Paraffin sectioning procedure. *Genetics at UW-Madison*. Retrieved April 29, 2008, from http://sun.genetics.wisc.edu/Paraffin%20Sectioning%20Procedure.htm.

15. De Jong, A.S.H., Van Kessel-Van Vark, M., and Raap, A.K. (1985). Sensitivity of various visualization methods for peroxidase and alkaline phosphatase activity in immunoenzyme histochemistry. *Histochemical Journal, 17*: 1119-1130.

16. Witzenbichler, Bernhard, Asahura, Takayuki, Murohara, Silver, Marcy, Spyridopoulos, Ioakim, Magner, Meredith, Principe, Nicole, Kearney, Marianne, Hu, Jing-Shan, & Isner, Jeffrey M. (1998). Vascular endothelial growth factor-c (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *American Journal of Pathology, 153*(2): 381-394.