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Vascular Endothelial Growth Factor (VEGF) Isoforms may Regulate Sex-Specific Vascular Development, Cord Formation and Follicle Progression in Developing Gonads

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VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) ISOFORMS MAY REGULATE SEX-SPECIFIC VASCULAR DEVELOPMENT, CORD FORMATION AND FOLLICLE PROGRESSION IN DEVELOPING GONADS

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Summary

The ratio of VEGF angiogenic to anti-angiogenic isoforms appears to determine different biological functions in reproduction. Reduced amounts of angiogenic VEGF isoforms inhibit testis sex-specific vasculature and normal cord formation in organ cultures while reduction of inhibitory isoforms increased vasculature and perturbed cords. In the female, using peri-natal ovarian cultures, inhibition of angiogenic VEGFisoforms reduced vascular development and inhibited follicle progression while conversely reductions in inhibitory isoforms or increases in angiogenic isoforms enhanced follicle development. Thus, regulation of the *Vegfa* gene to produce angiogenic or anti-angiogenic isoforms may be a mechanism to alter sex-specific vascular development, formation of seminiferous cords, and/or follicle progression within mammalian gonads.

Introduction

Infertility affects 40-70 million couples; of those approximately 50% of the infertility problems are attributed to male-related factors which include: low sperm count, abnormal spermatogenesis, and reduced androgen production [1, 2]. Formation of testicular cords, and sex-specific vasculature, are the two morphological hallmarks that distinguish a testis from an ovary. Female-related infertility factors include: ovulatory disorders, anovulation due to polycystic ovarian disease and ovarian hyperstimulation syndrome, or premature ovarian failure. Some or all of these female infertility problems may be caused by improper prenatal development of the fetal gonad, reduction of number of primordial follicles or disruption of progression of folliculogenesis. Neovascularization of the ovary and continued formation of follicle vasculature are critical events in normal reproductive function.

The VEGF family is composed of five ligands: VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D and Placenta Growth Factor. VEGF (VEGF-A) is the best characterized and most potent VEGF molecule. VEGF works through both Fms-like tyrosine kinase 1 (FLT1) and Kinase

domain region receptor (KDR), to elicit its effects on endothelial cell migration, differentiation, proliferation and survival and apoptosis. VEGF is transcribed from a single gene that has 8 exons and is alternatively spliced into different isoforms each containing a different number of amino acids. The most common angiogenic isoforms are VEGF205, 188, 164, 144, and 120 [3].

In 2002, an additional isoform, VEGF164b, was identified which contained part of the 3' UTR that is now determined to be exon 8b. Furthermore, recent studies have demonstrated that the human VEGF165b isoform is anti-angiogenic in function and inhibits signal transduction through KDR [4, 5]. Thus, this isoform is inhibitory to the actions of VEGF. Therefore, it appears that for every angiogenic isoform there is a sister inhibitory isoform that is formed when exon 8a is replaced with exon 8b. These inhibitory (anti-angiogenic) isoforms serves to modulate the functions of the angiogenic VEGF isoforms.

Materials and Methods

Rat Testis Organ cultures: E13 testes with attached mesonephros were placed on Millicell CM filters (Millipore, Bedford MA) in drops of medium floating on the surface of 0.4 ml of CMRL 1066 media (Gibco BRL, Gaithersburg, MD) at conditions reported [6, 7]. One organ from each animal was designated as a vehicle control, while its pair was subjected to a VEGF receptor signal transduction inhibitor, VEGFR-TKI (8 μM), or a VEGF antagonist, Je-11 (10 μg/ml) [8]. *Whole-mount IHC and Confocal Microscopy:* After culture, the organs were fixed in 4% paraformaldehyde. Samples were washed, blocked and whole-mount IHC was conduted as reported [8]. *Vascular Density Quantification was conducted as reported* using the staining index in Scion Image [8]. *Ovarian Organ Cultures:* Ovaries were dissected from postnatal day 3 and 4 (P3/4) rats (day of birth was considered to be P0). One ovary from each animal was designated as a control, while its pair was subjected to Treatment with 8 μM VEGFR-TKI; Calbiochem, La Jolla, CA or KDR signal transduction inhibitor, V1, (30 μM; Calbiochem, La Jolla, CA), VEGFA164 (R & D Systems Inc., Minneapolis, MN) or VEGF165b antibody (5ng/ml or 50ng/ml) (Abcam, Cambridge, MA). All of these treatments were added daily to the culture medium of the treated wells.

Results

Treatment of testis organ cultures with tyrosine kinase inhibitors to the VEGF receptor signal transduction pathway (VEGFR-TKI) or to VEGF antagonists (Je11) disrupted both sex-specific vascular development and seminiferous cord formation. Vascular density was reduced by 90 and 46%, respectively ($P < 0.01$). Conversely, treatment

with VEGF angiogenic isoforms: VEGF164, VEGF120 or an antibody to the exon 8b which binds inhibitory isoforms increased vascular density 50-100% over controls and resulted in swollen and perturbed testis cord formation. Thus angiogenic VEGF isoforms are important in establishing the sex-specific vascular development and too much inhibitory isoforms may alter the ability for this vasculature and subsequent testis cord formation to occur.

In the female, treatment of perinatal rat ovaries signal transduction inhibitors (VEGFR-TKI), antagonists to KDR (V1) arrested follicle development to later secondary follicle stages ($P < 0.05$). In contrast, treatment with angiogenic VEGF isoforms or an antibody to inhibitory isoforms increased vascular development and accelerated follicle progression to later secondary follicle stages ($P < 0.05$). Thus, we propose that amount of angiogenic to inhibitory VEGF isoforms modulates follicle progression and may determine whether an ovarian follicles continues to progress or undergoes atresia.

Conclusion

Approximately two million couples seek treatment for infertility every year and less than half find successful treatments [9, 10]. Infertility problems in at least half of these couples are a result of male-related factors that are created by testicular dysgenesis. Many of the problems associated with testicular dysgenesis are proposed to involve a disruption in embryonic differentiation of cells within the indifferent gonad resulting in altered testicular development. Elucidating the factors involved in sex-specific vascular development will allow for a better understanding of how transcription factors coordinate regulation of growth factors to result in a testis-specific vascular system. Furthermore, delineating the interaction of VEGF angiogenic and inhibitory isoforms in ovarian follicle arrest and or progression may also be an interesting piece in the puzzle of disorders that result in female infertility.

References

1. Skakkebaek, N.E., *Testicular dysgenesis syndrome: new epidemiological evidence.* Int J Androl, 2004. **27**(4): p. 189-91.
2. Skakkebaek, N.E., et al., *Association between testicular dysgenesis syndrome (TDS) and testicular neoplasia: evidence from 20 adult patients with signs of maldevelopment of the testis.* Apmis, 2003. **111**(1): p. 1-9; discussion 9-11.
3. Veikkola, T. and K. Alitalo, *VEGF's receptors and angiogenesis.* Semin Cancer Biology, 1999. **9**: p. 211-220.

4. Bates, D., et al., *$VEGF_{165}b$, an Inhibitory Splic Variant of Vascular Endothelial Growth Factor, Is Down-Regulated in Renal Cell Carcinoma.* Cancer Research, 2002. **62**: p. 4123-4131.
5. Woolard, J., et al., *$VEGF_{165}b$, an Inhibitory Vascular Endothelial Growth Factor Splice Variant: Mechanism of Action, In vivo Effect on Angiogenesis and Endogenous Protein Expression.* Cancer Research, 2004. **64**: p. 7822-7835.
6. Cupp, A., G. Kim, and M. Skinner, *Expression and Action of Transforming Growth Factor Beta (TGFβ1, TGFβ2, and TGFβ3) during Embyonic Rat Testis Development.* Biology of Reproduction, 1999. **60**: p. 1304-1313.
7. Cupp, A.S., et al., *Effect of transient embryonic in vivo exposure to the endocrine disruptor methoxychlor on embryonic and postnatal testis development.* J Androl, 2003. **24**(5): p. 736-45.
8. Bott, R.C., et al., *Vascular endothelial growth factor and kinase domain region receptor are involved in both seminiferous cord formation and vascular development during testis morphogenesis in the rat.* Biol Reprod, 2006. **75**(1): p. 56-67.
9. Carlsen, E., et al., *Longitudinal changes in semen parameters in young Danish men from the Copenhagen area.* Hum Reprod, 2005. **20**(4): p. 942-9.
10. Shaban, S., *Male Infertility Overview.* http://www.ivf.com/shaban.html., 2000.

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