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14. ABSTRACT
VEGF signaling is critical to neovascularization of tumors and metastatic progression. In fact, alterations in VEGF levels or in receptor phosphorylation results in suppression of vascular expansion and concomitant reduction of tumor growth and metastasis. In this proposal we explore the ability of matrix metalloproteases to modulate VEGF signaling in tumors. We found that excess of MMPs results in the release of VEGF from matrix binding sites, with increased permeability and aberrant neovascular growth. Genetic inactivation of MMP9 in the context of tumors did not affect VEGF processing, as other MMPs compensated functionally for the lack of the protease. We thus, generated a mutant VEGF protein unable to be cleaved. In this case, permeability was suppressed, but growth of vessels continued from matrix-bound VEGF.

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Angiogenesis, tumor angiogenesis, VEGF signaling

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Introduction

The contributions of matrix metalloproteases (MMPs) to extracellular matrix remodeling, tumor expansion and metastatic events has been highlighted in multiple studies. However, the direct effect of MMPs in direct regulation of growth factors is not as well understood. The goal of this fellowship was to further understand the relationship between MMPs and vascular endothelial cell factor (VEGF) signaling in the context of angiogenic growth in prostate tumors. During the process of this fellowship, we have been able to clarify the contributions of MMPs to the to release VEGF from extracellular stores. Specifically, we found that VEGF can be cleaved intramolecularly by several MMPs. MMP3 in particular is able to release fragments capable to activate the receptor leading to increased permeability and modulation of tumor growth.

The guiding hypothesis behind this proposal is that suppression of VEGF availability through modulation of MMP levels should be able to restrain its access to endothelial cells and thus restrain tumor growth and metastasis. While pursuing this hypothesis, and as outlined in the proposal, we identified the specific cleavage sites and generated proteins that were able to mimic both the cleaved form, as well as a form unable to be cleaved. A surprise came when we found that recombinant proteins from both the soluble and MMP3-resistant VEGF forms were equally able to phosphorylate VEGFR2 *in vitro* (discussed in report 2). Therefore, if bound VEGF is actually able to signal effectively, then suppressing its release might have no consequence to tumor growth and angiogenic abilities. It turned out that the outcome was more interesting than anticipated. While bound VEGF is actually able to signal, the relative abundance of bound versus soluble VEGF regulates vascular branching, permeability status of tumors, and metastatic spreading. Some of these aspects have been communicated in a publication (Lee et al., 2005) and others are still under investigation.

During the last year of this fellowship, we have concluded task 3 which includes the xenografts assays using tumor cells transfected with the different forms of VEGF and the cross with MMP9 null mice. Interestingly, we found that in the absence of MMP9, PTEN null tumors upregulate MMP3 and MMP19, both capable of cleaving VEGF, as shown in our publication. Thus, these compensatory mechanisms make it impossible to test our hypothesis. Additional experiments were then performed to overcome these difficulties and rather than targeting the MMPs themselves, we decided to focus on the substrate: VEGF. Our goal was to mutate VEGF to render it unable to be cleaved by MMPs and plasmin, while preserving its activity and ability to bind VEGF receptors. Here we communicate our strides towards that goal that expanded beyond the tasks originally presented. In addition, and as stated in the last report, we also generated an inducible VE-cadherin Cre recombinase model for studies on tumor endothelium. This was a very profitable task that resulted in one first author publication (Monvoisin et al., 2006). The mouse has been already used in 5 published studies and has been sent to 57 laboratories with the USA and abroad.

Body

As stated in previous reports tasks 1 and 2 were completed in years 1 and 2.

During the last year of the fellowship, we have completed experiments associated with Task 3 and expanded on these experiments to also explore intracrine activation of VEGF in prostate tumors .

Task 3 – To ascertain the contribution of the MMP3-VEGF axis towards prostate cancer progression and establishment of metastatic disease.
As stated in the previous report, we had partially completed this aim by generating stable human cell lines that express wild-type VEGF 165, a mutant form that resemble the fragment of VEGF after cleavage by MMP3 (named VEGF 113) and a mutant form of VEGF unable to be cleaved by this protease (delta 109-118). Cell lines were further characterized and tested in xenograft assays using nude mice. As reported (Lee et al., 2005), the tumors from VEGF113 showed the smallest size, in contrast, tumors from uncleavable VEGF that resulted in significant tumor expansion. These experiments were expanded with reproducible results.

Subsequently, we moved on to PTEN transgenic mice that spontaneously develop prostate cancer. The mice were crossed with MMP9 null mice with the goal to directly test our hypothesis that MMP9 regulates VEGF bioavailability. Unfortunately, after execution of the aim and analysis of the results we found that VEGF was cleaved in MMP9 null tumors similarly to controls. Further investigation on the cause of such outcome let us to find that indeed MMP3 and MMP19 became increased and compensated for the absence of MMP9. We confirmed that indeed both MMP3 and MMP19 were able to cleaved VEGF (Lee et al., 2005). While the outcome was not predicted based on the xenograft studies, we found that most of the MMPs were produced by inflammatory cells that were not as predominant in the immunocompromised mouse model.

Albeit disappointed, we decided to switch our strategy to target VEGF instead of the proteases. The idea was to make the growth factor unable to be cleaved by MMPs. This entailed designing either deletions or mutations that would render VEGF uncleavable, while preserving its activity. An initial delelion was done in our published study (Lee et al., 2005), however its included 11aa, further, more settle mutations were needed, as that deletion expanded the splicing region if made in the VEGF gene and our long term goal was to generate a mutant mouse. The MMP cleavage site was mapped to aa113 and aa114 (Figure 1) and splicing of Exon 5-6 takes place exactly in residue 114.

In our advantage was the fact that unlike most enzymes, MMPs tend to recognize and target conformational structures rather than specific sites. Considering structural information, we generated a total of nine mutants. The mutants were made in the cDNA, transfected into 293T cells, protein was purified and tested for its ability to be cleaved by MMPs (data not shown). From all those mutants, only one (5aa deletion between R109 and E113) was resistant to MMP cleavage (Figure 2).

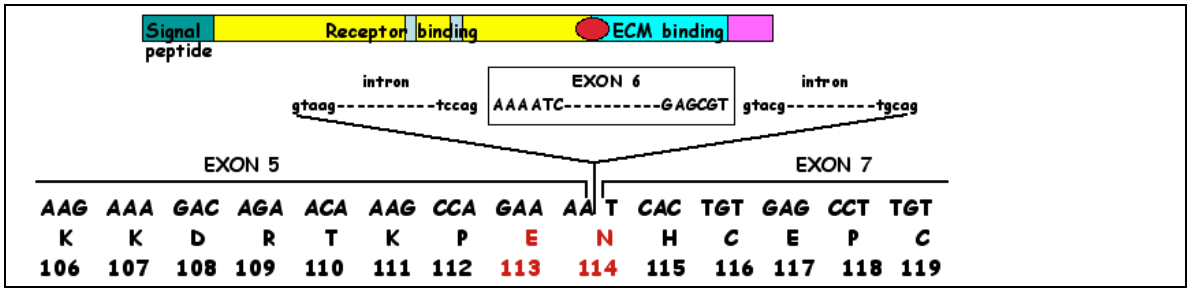

Fig 1. MMP cleavage sites. (top) Schematic diagram of the functional domains present in VEGF protein. Cleavage region is shown by filled red circle. (bottom) Partial structure of mVEGF gene and localization of MMP cleavage site. MMPs cleave $mVEGF_{164}$ at amino acid residue 113 (between glutamic acid and asparagine).

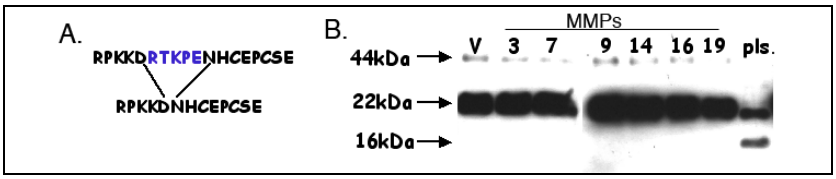

Fig 2. Mutant VEGF is resistant to MMP cleavage. (A) Schematic structure of mutant VEGF. (B) Processing of mutant VEGF forms by MMPs. Mutant $VEGF_{164}$ was incubated with the indicated MMPs and plasmin at molar ratio of 4:1 (VEGF:proteinases). VEGF cleavage was visualized by SDS-PAGE followed by immunoblotting with N-terminal VEGF specific antibody. Lanes: V: VEGF; MMPs -3, -7, -9, -14, -16, -19, Pls, plasmin. On the left are indications of molecular weight: 44kD – VEGF dimer; 22kD – VEGF monomer; 16kDa cleaved product.

Because of its location, the mutant has been termed VEGF-DRE. The next important evaluation was that of activity. We tested the ability of VEGF-DRE to promote VEGFR2 phosphorylation in comparison to the wild-type form of VEGF. Figure 3 shows that indeed VEGF-DRE can phosphorylate VEGFR2 at levels indistinguishable from the wild-type protein.

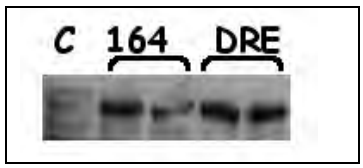

Fig. 3- VEGFR2 phosphorylation by mutant VEGF. VEGFR2 phosphorylation was assessed on porcine aortic endothelial cells. Cells were exposed to either vehicle (c); VEGF164 (164) in duplicate; or VEGF mutant (DRE) in duplicate for 5 min. Cell lysates were resolved by SDS-PAGE, transferred and probed with antibodies against P-VEGFR2. Both 164 and DRE were able to phosphorylate VEGFR2. No phosphorylation was detected in vehicle-treated cells.

We then proceeded to generate the genomic construct. Arms for the generation of the genomic construct were provided by Andras Nagy. His laboratory generated the knock-out construct (Carmeliet et al., 1996), and has subsequently made three additional transgenics that target of this same locus. A scheme of the construct is shown in Figure 4.

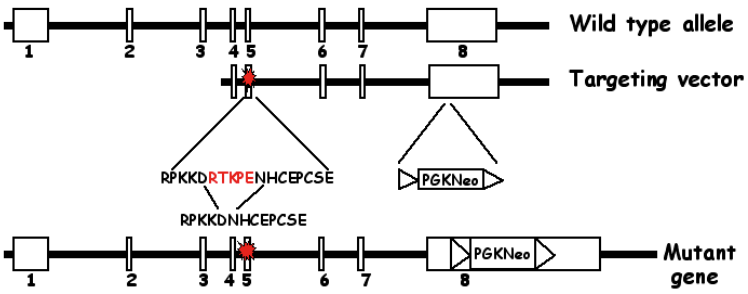

Fig 4. MMP-resistant VEGF knock-in strategy. mVEGF gene structure of wild type allele (top), targeting vector (middle) and the predicted structure of the targeted VEGF allele. The white box represents exon and black line intron. Numbers below correspond to exon numbers.

We are currently screening ES cell lines to identify clones with the targeted vector. It is our prediction that this reagent will be extremely valuable to understand the interplay between cancer and angiogenesis.

In summary, through the execution of this fellowship, we were able to gain a more detailed understanding of how MMPs can regulate the biology of VEGF in tumor growth. Some of the information obtained from this proposal has now been used to further understand resistance to VEGF

therapy in cancer progression. We are extremely thankful to the Department of Defense for the opportunity to perform these experiments and gain additional training experience in prostate cancer biology. Thanks to this training, Dr. Monvoisin is currently faculty at the Institut de Physiologie et Biologie Cellulaires, Université de Poitiers, France.

Key Research Accomplishments

Task 1 – To determine the MMP3 and MMP9 cleavage sites in VEGF and the sequence of the released VEGF peptides –

Status: Concluded, findings were described in report #1.

Task 2 – To determine the relevance of released peptides to VEGF receptor signal transduction

Status: Concluded, findings were described in reports #1 and 2.

Task 3 – To ascertain the contribution of the MMP3-VEGF axis towards prostate cancer progression and establishment of metastatic disease.

Status: Concluded, findings were described in reports #2 and 3 (present report).

- We have been able to generate five cell lines that express different types of VEGF (wild-type, cleaved, and MMP-resistant)
- These cell lines were injected in the flank of nude mice and evaluated in xenograft assays. A cohort of five mice per cell line showed that the MMP-resistant form induces the growth of thin and frequently branched capillaries. In contrast, cleaved VEGF results in few and enlarged tumor vessels.
- In addition, we generated double transgenics PTEN / MMP9 null and explored the effect of the different VEGF isoforms in tumor growth
- Developed and tested a cohort of mutations to identify mutations that will prevent cleavage without blocking growth factor activity.
- Developed a targeted vector for the generation of a knock in mouse to be use as a platform for the investigation of MMP-cleavage and VEGF function.

All tasks were concluded and expanded.

Reportable Outcomes

Research

Publications:

- Monvoisin, A., Alva, J.A., Hofmann, J.J., Zovein, A.C., Lane, T.F., and Iruela-Arispe, M.L. 2006. VE-cadherin-CreER(T2) transgenic mice: A model for inducible recombination in the endothelium. Dev. Dyn. 235:3413-3422.

- Lee, S., Jilani, S.M. Nikolova, G.V., Monvoisin, A., and Iruela-Arispe, M.L. 2005. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J. Cell Biol. 169(4):681-691.

Products

Cell Lines, Tissues or Serum Repositories Developed

- Generated stable human cell lines expressing wild-type VEGF165 ; a mutant VEGF that resembles the fragment of VEGF generated by MMP-3 cleavage (VEGF113); or a mutant VEGF that cannot be cleaved by MMP-3 (delta 109-118).

Animal Models

- Generated a transgenic mice expressing Cre in mature endothelial cells and endothelial progenitor cells; this transgenic mouse line will allow us to induce mutations specifically in the endothelium.
- Generated dual transgenic mice PTEN / MMP9 null that spontaneously develop prostate cancer.
- We are in the process of generating a knock-in model for evaluating the relevance of MMP9 cleavage to tumor progression and expansion.

Conclusions

The project was completed and findings were communicated in two manuscripts. We were able to show that a cohort of MMPs participate in the release of VEGF fragments from matrix-bound sites. We were able to show that these fragments are indeed active. Nonetheless, the ability of VEGF to interact with matrix enables the formation of gradients that are not conveyed by soluble VEGF. Therefore it is the balance and relative abundance of bound over soluble VEGF that modulates the total outcome of capillary growth. Relative levels of MMP can upset this balance in a manner that facilitates tumor expansion. Our data argues for a direct contribution of tumor microenvironment in the modulation of angiogenesis.

References

Monvoisin, A., Alva, J.A., Hofmann, J.J., Zovein, A.C., Lane, T.F., and Iruela-Arispe, M.L. 2006. VE-cadherin-CreER(T2) transgenic mice: A model for inducible recombination in the endothelium. Dev. Dyn. 235:3413-3422.

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U.S. Army Medical Research and Materiel Command Animal Use Report

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AAALAC* Accreditation Status (circle one): (Full) Provisional Not Accredited
Initial date of Accreditation (MM/YYYY) 05/1976 Date of Most Recent Site Visit (MM/YYYY) 03/2008

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Cats					
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NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS *(X)* d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
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(1) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(2) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
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(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
none	none		
(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*	(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*		
none	none		

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

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I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)*	b. TITLE	c. SIGNATURE	d. DATE SIGNED