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TITLE: Modulation of Anaplastic Lymphoma Kinase Upon Tumor-Stroma Interaction and Its Implications for Tumor Growth and Metastasis in Breast Cancer

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Induction of neovascularization is needed for a growing tumor as well as for its metastasis. Angiogenic and growth promoting factors like Pleiotrophin (PTN) act on endothelial and epithelial cells and on fibroblasts. We identified the receptor for PTN as anaplastic lymphoma kinase (ALK). In individual tissues the presence of ALK is elevated in tumor stroma (endothelium) while adjacent normal tissue lacked ALK. In cultured endothelial cells or human fibroblasts ALK is upregulated in response to supernatants from human breast cancer cells. Our hypothesis is that ALK from stromal cells, upregulated in response to factors from tumor cells, constitutes a marker and a potential therapeutic target in breast cancer. We will investigate the specificity of ALK modulation in tumor stroma versus normal endothelium in response to growth factors and breast cancer cell lines supernatants. Also, we will determine the functional effects of the differences in ALK signaling and uncover the differences in drug sensitivity in cells that have an increased ALK level versus untreated. We expect a lowering of the effective dose thus lowering the side effects of these drugs. The completion of the study will translate in establishing ALK as a new target for the breast metastatic cancer therapy.
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Introduction

Growth factors that are secreted by the tumor cells are eliciting host responses crucial for survival, proliferation and metastasis (1). A critical event in this respect is the induction of neovascularization needed for a growing tumor as well as for its metastasis. In breast cancer microvessel density is an independent prognostic indicator and there is a direct correlation between blood vessel density in primary tumors and their metastasis. Solid tumors cannot develop in absence of new blood vessels; therefore, the malignant cells would co-opt endothelial cells and fibroblasts to sustain their growth. The latter cells are sensitive to angiogenic and growth promoting factors like Pleiotrophin (PTN) which supports the transition from the avascular to the vascular stage. We identified the receptor for PTN as anaplastic lymphoma kinase (ALK), a formerly orphan tyrosine kinase receptor (2). Preliminary studies showed that in individual tissues the presence of ALK is elevated in tumor stroma (endothelium) while adjacent normal tissue lacked detectable ALK expression. Moreover, experiments performed in cultured endothelial cells (HUVEC) or human fibroblasts (WI-38) showed that ALK is upregulated in response to basic FGF (b-FGF) and supernatants from human breast cancer cells.

Our hypothesis is that ALK from stromal cells, upregulated in response to factors from tumor cells, constitutes a marker and a potential therapeutic target in breast cancer.

Proposal body:

In the approved statement of work the following tasks were outlined:

Task 1 Investigate the specificity of ALK up regulation in tumor stroma versus normal endothelium (months 1-6)
Task 2 Substantiate the ALK up regulation in stroma in response to growth factors and breast cancer cell lines supernatants (months 1-6)
Task 3 Demonstrate the effects of ALK up regulation at the signal transduction level. (months 2-8)
Task 4 Investigate the functional effects of the differences in ALK signaling (months 7-11)
Task 5 Detect the differences in drug sensitivity in endothelial cells that have an increased ALK level versus untreated cells. (months 10-11)

Task 6 Analyze data and prepare report for DOD (month 12)

Due to complexity of our tasks and unforeseen circumstances (medical leave of one of the participants in the project) we have requested and were granted a one year no-cost extension from DOD-USAMRMC.

Task 1
Investigate the specificity of ALK up regulation in tumor stroma versus normal endothelium (months 1-6)

We completed task 1 in the months 1-5. We performed a detailed analysis of the presence of PTN in breast cancer specimens and the expression of ALK in the epithelium and Stroma of breast cancer tissue arrays. Moreover, we correlated these two markers with the long term survival of the patients from which the tissues were prevailed (Figure 1). Our results indicate that there is a significant difference between the 10 year survival for patients expressing high levels of PTN versus the negative cases (~ 50 % in the Kaplan-Meier survival curve). 35% of the patients that did not express PTN survived for 20 years while only 10% of the patients with high levels of PTN survived that amount of time. A similar conclusion may be drawn for ALK expression: for high expressers the 10-year survival was ~30% while 60% of the patients in which ALK was not detected survived 10 years or longer. At 20 years, 30% of the negative for ALK cases were living while no patients who expressed high levels of ALK survived more than 160 months.

Task 2 Substantiate the ALK up regulation in stroma in response to growth factors and breast cancer cell lines supernatants (months 1-6)

a) We had great difficulties in obtaining and establishing human breast reduction primary fibroblasts. While postponing this subtask we decided to focus our resources on cancer cell lines in tissue culture.

b) We collected conditioned media from the supernatants of the breast cancer cell lines MDA MB 231, Hs578 T, MCF-7, as well as other cancer lines and we used them to stimulate endothelial cells and fibroblast.
c) Using RT PCR and Western blotting we measured the ALK levels (mRNA and protein) in HUVEC cells in response to bFGF, IGF, PDGF, EGF, Herregulin or conditioned media (obtained as above) treatment as presented in figure 2. The experiments on HMVEC, WI-38 and another normal human lung fibroblastic cell line were not conclusive yet, but we are working on optimizing the conditions for these cells. We will expend the range of growth factors and the cells stimulated by them.

d) Regarding the identification of the growth factors responsible for ALK regulation by using blocking antibodies we performed preliminary experiments which will allow us to choose the best antibodies to use as well as a reliable and robust assay.

**Task 3** Demonstrate the effects of ALK up regulation at the signal transduction level.

a) Monitor the activation of ALK by tyrosine phosphorylation of the receptor and by activation of downstream signaling molecules like Akt and MAPK in cellular lysates obtained as in Task 2.

We are in the stage of setting up a high throughput assay for detection of ALK, Akt and MAPK phosphorylation based on DELFIA assays. This will enable us to handle a substantial number of sample and thus to asses a multitude of conditions.

b) Perform dose response studies with small molecules that inhibit ALK, AKT, and MAPK phosphorylation to detect the extent to which the ALK signaling is enhanced/modified.

We started a very promising collaboration with Dr. Alan Kozikowsky from University of Chicago and we already have some lead compounds that act as inhibitors of ALK. (Figure 3)

**Task 4** Investigate the functional effects of the differences in ALK signaling (months 7-11)

We are currently pursuing the subtasks a)-d). For Subtask c) we were able to demonstrate the change in the migration rate of Huvec cells in response to PTN after pretreatment with growth factors and cancer cell conditioned media (Figure 4) and the blocking of the effect with anti ALK mouse monoclonal antibody 8B10.

a) Test the proliferative effects of PTN on pretreated versus not treated endothelial cells (HUVEC and HMVEC) or fibroblasts (WI-38) by WIST system. (months7-8)
b) Test the anti apoptotic effects of PTN on pretreated versus not treated endothelial cells (HUVEC and HMVEC) or fibroblasts (WI-38) by Annexin V-FITC system. (months 7-8)
c) Test the potential differences in PTN induced cell migration of fibroblasts and endothelial cells by Boyden chamber assays using standard protocols from the Developmental Therapeutics Program-NCI/NIH (months 8-9)
d) Uncover potential differences in PTN induced tube formation of endothelial cells, using standard protocols from the Developmental Therapeutics Program-NCI/NIH. (months 9-10)
We are currently pursuing the subtasks a)-d). For Subtask c) we were able to demonstrate the change in the migration rate of Huvec cells in response to PTN after pretreatment with growth factors and cancer cell conditioned media. (Figure 4) and the blocking of the effect with anti ALK mouse monoclonal antibody 8B10.

**Task 5** Detect the differences in drug sensitivity in endothelial cells that have an increased ALK level versus untreated cells. (months 10-11)
   a) Perform dose-response growth assay (DTP-NIH) using the fumagillin derivative TNP-40, paclitaxel (Taxol) and doxorubicine on the HUVEC and HMVEC cells pretreated as in Task 2. (months 10-11)
This task will be actively pursued an the incoming months.

**Task 6** Analyze data and prepare report for DOD (month 12)

Key research accomplishments
- Establishing a correlation between the expression of PTN and ALK in breast cancer stroma and long term survival.
- Underscore the role of tumor secreted growth factors in upregulation of ALK

Reportable outcomes:
- Upregulation of ALK in the tumor stroma
- Uncovering of specific small molecule inhibitors for ALK
Conclusion

We investigated the ALK upregulation in tumor stroma versus normal endothelium in response to growth factors and breast cancer cell lines supernatants. Also, we targeting our work towards determination of the functional effects of the differences in ALK signaling and uncover the differences in drug sensitivity in stromal cells that have an increased ALK level versus untreated. The study will establish ALK as a new target for the breast cancer therapy and especially metastatic cancer.

References:
Figure 1: Kaplan Meier survival curves for breast cancer patients.

NEG, LOW, HIGH: Curves of patients with the indicated expression level, ALL: all tumors regardless of expression. In brackets: number of samples per group. P-values: Results of log rank test for trend Kaplan Meier analysis.
Modulation of ALK in HUVEC

Figure 2. Upregulation of ALK in HUVEC in response to growth factors and conditioned media from various cell lines. a) The levels of ALK mRNA, as determined by RT-PCR, upon treatment with bFGF 10 ng/ml, IGF-I, PDGF, EGF 100ng/ml, and conditioned media from SW-13, MCF-7, MDA-MB-231, Hs578T, U 87 MG cell lines. EBM-2 is the basal medium and EGM-2 is the growth medium for HUVEC (Clonetics, Walkersville, MD) b) ALK modulation at the protein level after treatment of HUVEC with bFGF 10 ng/ml, IGF-I 140 ng/ml, PDGF 140 ng/ml, EGF 100ng/ml, and conditioned media from SW-13, SW-13 transfected with PTN (SW-13/PTN), MCF-7, MDA-MB-231, Hs578T, U 87 MG, PC3 and DU 145 cell lines.
Figure 3
Dose response curves for ALK inhibition in cultured cells

Log curve 1

- ING-9
- ING 10
- ING 12
- ING 13
- ING 14
- Ing 15

Inhibition (% of control)

[Inhibitor] nM

0 0.1 1 10 100 1000 10000 100000
Figure 4

Boyden chamber assay: collagen IV coated Φ=8 μm membrane; X axis pretreatment for 16 hrs and then starved for 4 hrs; legend = lower chamber medium