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TITLE: A Molecular Connection Between Breast Cancer Proliferation and Metastasis Mediated by Akt Kinase

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A Molecular Connection Between Breast Cancer Proliferation and Metastasis Mediated by Akt Kinase

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Death from cancer is most frequently caused by metastases. While research of the past 20 years has identified genes whose malfunction causes cancer to grow, it has not been clear why these defects also induce the ability to metastasize. We have defined molecules that form a connection between signals that make cancer cells grow and signals that cause them to metastasize. In breast cancer cells, a molecule called Akt kinase bridges these two functions. Here we study the exact mechanism by which Akt kinase connects mechanisms of growth with mechanisms of cancer spread. The identification of a single defined defect as responsible for growth and metastasis enhances our molecular insights into cancer and it defines candidate targets for therapeutic intervention.
Table of Contents

Cover.................................................................1
SF 298.................................................................2
Note.................................................................4
Introduction.........................................................4
Body.................................................................5
Key Research Accomplishments.........................9
Reportable Outcomes..........................................10
Conclusions.......................................................11
References.........................................................12

Appendices

Revised S.O.W..................................................13
Research to be Completed................................15
At the turn of the year 2003/2004, this laboratory moved from the New England Medical Center, Boston, to the University of Cincinnati. The transfer of the grant has not yet been completed, mostly because of delays in relinquishing it from the New England Medical Center. Therefore, this report essentially covers the first half of the second year of funding. As part of the request for transfer, the Statement of Work was revised. A copy of this revised S.O.W. is attached at the end of this document.

Introduction

The defining characteristics of benign and malignant tumors are excessive growth and immortalization. In contrast, only malignant tumors express gene products that mediate invasiveness. Uncontrolled proliferation is a consequence of gain-of-function mutations of proto-oncogenes or loss-of-function mutations of tumor suppressor genes. Metastatic dissemination is a consequence of aberrant expression or splicing of stress response genes (Weber/Ashkar 2000). The consistent topology of metastasis formation by specific cancers, such as the high frequency of colony formation in bone and brain by malignant breast tumors, implies that metastasis gene expression is an inevitable consequence of gain of function by specific oncogenes. This raises the question: What molecular mechanisms connect the signal transduction pathways associated with dysregulated growth to the expression of metastasis genes in malignant, but not in benign tumors?

Receptor ligation by the epidermal growth factor can induce osteopontin gene expression (Malyankar et al. 1997; Atkins et al. 1997) through signal transduction that proceeds via protein kinase C and tyrosine kinases (Chackalaparampil et al. 1996). This implies that gain-of-function mutations in the EGF receptor pathway in breast cancer, causing dysregulated growth, may also mediate the over-expression of osteopontin, leading to dissemination. We find osteopontin to be constitutively expressed in malignant, but not in benign transformed breast cells. Here, we map the cause for this to constitutive activation of Akt kinase, an enzyme that is part of the EGF signaling pathway.
Body

Specific Aim 1: Molecular characterization of the role of AKT in proliferation and induction of metastasis genes

Task 1: Transfection of Akt, dnAkt, constitutively active Akt, or vector control into breast epithelial cells. Cloning and characterization of the transfectants. Deliverable: Stably transfected cell lines. 3 months.

We stably transduced murine breast epithelial cells with various Akt kinase constructs. Benign cells were transfected with vector, wild-type Akt, or constitutively active Akt, while malignant cells were transfected with vector, wild-type Akt, or a dominant negative Akt mutant. The expression levels of the transfected genes were measured on the RNA and protein levels. Expectedly, osteopontin was constitutively expressed in Akt kinase-transduced cells and the constitutive osteopontin gene expression in malignant cells was suppressed by the dominant negative Akt kinase mutant according to RNase protection assay, Northern blotting, and Western blotting. The levels of osteopontin induced by wild-type Akt and constitutively active Akt are comparable, likely reflecting the substantial overexpression. At these amounts, the baseline activity of wild-type Akt kinase is sufficient to transduce a signal. It may be important to note that the reduction of osteopontin gene expression by dominant negative Akt kinase is partial. Only moderate levels of over-expression can be achieved for dominant negative Akt kinase because this mutant also slows down cell divisions, consistent with the hypothesis that the constitutive activation of Akt kinase in MT2994 cells is causative for the transformation of these cells (Zhang et al. 2003).

Task 2: Analysis of the changes in osteopontin expression, expression of cyclins, and changes in cell cycle profile after transfection of Akt kinase, constitutively active Akt kinase, or dominant negative Akt kinase. Deliverable: Definition of the role of Akt in proliferation and osteopontin expression on the genetic level. 6 month.

In order to differentiate between osteopontin-dependent and osteopontin-independent consequences of Akt kinase overexpression we generated doubly transduced lines. Benign cells that stably expressed constitutively active Akt kinase were infected with a retrovirus containing osteopontin antisense. Similarly, we generated malignant
dominant negative Akt/osteopontin cells. All relevant singly and doubly transfected controls were included. As before, the expression levels of the modulated genes were measured by Western blotting. The elevated osteopontin expression in transfectants of constitutively active Akt kinase could be reversed by co-transfection of antisense osteopontin, and the suppression of osteopontin by dominant negative Akt was reversed by co-transfection with the osteopontin gene. We tested the growth rates of doubly transduced cells by plating 5000 cells per well in 24-well plates followed by daily cell counts in quintuplicates. Remarkably, within only one day, transfection of constitutively active Akt kinase into benign cells significantly induced the growth rate, while transfection of dominant negative Akt kinase into malignant cells significantly reduced it. The differences remained significant throughout the four days of measurement. In contrast, the stable transfection of osteopontin into cells overexpressing dominant negative Akt kinase or of osteopontin antisense into cells overexpressing constitutively active Akt kinase had no effect on the growth rates. We also analyzed cell motility by in vitro wounding and transwell chemokinesis. Benign cells transfected with constitutively active Akt displayed enhanced migration. Co-transfected osteopontin antisense completely reversed this effect (Zhang et al. 2003). These results are consistent with osteopontin-independent roles of Akt as a growth promoting gene product and osteopontin-dependent roles of Akt as a migration promoting gene product.

**Specific Aim 2: Identification of downstream targets of Akt in osteopontin induction and cell cycle progression.**

**Task 4:** Cloning of the osteopontin promoter into a reporter construct and investigation into reporter activity in various transfectants of Akt, constitutively active Akt, or dominant negative Akt. Electrophoretic mobility shift assays of nuclear extracts from the transfectants with oligonucleotides derived from the osteopontin promoter. Deliverable: Identification of Akt-induced transcription factors that mediate osteopontin gene expression. 8 months.

Previous studies have shown that the induction of osteopontin in renal epithelial cells by TGF-β or EGF is caused by increased transcription (Malyankar et al. 1997). Akt kinase activity has previously been associated with activation of the AP-1 transcription...
factor and Ets-family transcription factors, both of which are known to bind to the osteopontin promoter. We set out to further map the molecular connections between Akt kinase activity and osteopontin transcription in breast epithelial cells. For this purpose, we took advantage of the breast epithelial cell lines constitutively expressing various Akt constructs that we had generated. We used benign cells stably expressing wild-type Akt kinase, constitutively active Akt kinase, or vector in luciferase reporter assays under the control of full length or truncated osteopontin promoter sequences. Expectedly, Akt kinase activity can induce the transactivation of the osteopontin promoter (Zhang et al. 2003). The Akt responsive promoter domain was mapped to a region between base -600 and base -777. Consistent with a previous report (Guo et al. 1995), a far distal promoter element (between bases -777 and -882) appeared to contain a repressor.

Specific Aim 3: Analysis of metastatic potential dependent on Akt and osteopontin

Task 5: Study of colony formation of transfected cells (vector, Akt, constitutively active Akt, dominant negative Akt) in soft agar in the presence or absence of anti-OPN antibody or after co-transfection with osteopontin antisense. Deliverable: Data on dependence of growth in soft agar on Akt and osteopontin. 3 months.

Because osteopontin is necessary for soft agar colony formation and Akt kinase induces osteopontin expression we asked whether the constitutive over-expression of Akt kinase in benign cells was sufficient to convey the ability of anchorage independent growth. Expectedly, Akt transfected breast epithelial cells displayed clone formation in soft agar, whereas vector transfected cells did not. The repeated addition of an anti-osteopontin antibody to the cell culture reduced the numbers and sizes of clones formed. In contrast, a control immunoglobulin had no effect (Zhang et al. 2003). We also analyzed the doubly transduced cells for colony formation in soft agar. Co-expression of osteopontin antisense in constitutively active Akt expressing cells lead to a partial reduction of colony formation, while cells transduced with vector constructs did not form clones in soft agar. In a complementary approach, the expression of dominant negative Akt in malignant cells caused a partial reduction in the numbers and sizes of clones formed. Co-expression of osteopontin virtually completely reversed this inhibition (Zhang et al. 2003).
Task 6: In vivo analysis of dissemination of cells transfected with Akt, constitutively active Akt, dominant negative Akt, vector control after orthotopic injection. Deliverable: Data on dependence of metastasis formation on Akt and osteopontin. 9 months. This task will use a maximum of 100 mice.

We tested in vivo tumorigenesis and dissemination by the doubly transduced benign cells. Expectedly, the vector/vector and vector/antisense osteopontin transduced cells generated small hyperplastic lesions. In contrast, caAkt expressing cells generated larger tumors. Only the cells containing Akt plus vector, but not the cells expressing Akt plus antisense osteopontin, formed distal lesions (Zhang et al. 2003).
Key Research Accomplishments

* We have identified the constitutive activation of Akt kinase as a molecular defect in malignant breast cancer.

* Our laboratory and others have shown that osteopontin gene expression is essential for generating a malignant phenotype in breast tumors.

* We have found osteopontin gene expression to be a downstream target of Akt activity.

* We can differentiate two signal transduction cascades downstream of Akt that lead to either growth dysregulation or invasiveness.
Weber, Georg F.

Reportable Outcomes


Conclusions

Like other aspects of cancer, metastasis is caused by the dysregulation of specific genes.

Metastasis genes are stress response genes, which suggests that malignancy constitutes mimicry of leukocyte homing.

In cancer, metastasis genes are aberrantly expressed or spliced.

The dysregulation of metastasis genes in cancer occurs secondary to oncogene activation.
References


Revised Statement of Work

Specific Aim 1: Molecular characterization of the role of AKT in proliferation and induction of metastasis genes

Task 1: Transfection of Akt, dnAkt, constitutively active Akt, or vector control into breast epithelial cells. Cloning and characterization of the transfectants. Deliverable: Stably transfected cell lines. 3 months. Completed, ref. Zhang et al. 2003

Task 2: Analysis of the changes in osteopontin expression, expression of cyclins, and changes in cell cycle profile after transfection of Akt kinase, constitutively active Akt kinase, or dominant negative Akt kinase. Deliverable: Definition of the role of Akt in proliferation and osteopontin expression on the genetic level. 6 month.

Specific Aim 2: Identification of downstream targets of Akt in osteopontin induction and cell cycle progression.

Task 3: Analysis of changes in proliferative and anti-apoptotic signaling molecules that act downstream of Akt. Deliverable: Data on changes in p27kip, Bad, caspase-9, and FKHR after transfection with Akt or its variants in the presence or absence of EGF. 7 months.

Task 4: Cloning of the osteopontin promoter into a reporter construct and investigation into reporter activity in various transfectants of Akt, constitutively active Akt, or dominant negative Akt. Electrophoretic mobility shift assays of nuclear extracts from the transfectants with oligonucleotides derived from the osteopontin promoter. Deliverable: Identification of Akt-induced transcription factors that mediate osteopontin gene expression. Only in part completed 5 months.

Specific Aim 3: Analysis of metastatic potential dependent on Akt and osteopontin

Task 5: Study of colony formation of transfected cells (vector, Akt, constitutively active Akt, dominant negative Akt) in soft agar in the presence or absence of anti-OPN antibody or after co-transfection with osteopontin antisense. Deliverable: Data on dependence of growth in soft agar on Akt and osteopontin. 3 months.
Task 6: In vivo analysis of dissemination of cells transfected with Akt, constitutively active Akt, dominant negative Akt, vector control after orthotopic injection. Deliverable: Data on dependence of metastasis formation on Akt and osteopontin. 9 months. This task will use a maximum of 100 mice.

Completed, ref. Zhang et al. 2003
Description of Research to be Completed

The remaining 1.5 years of the funding period will focus on the remaining tasks:
Specific Aim 1, Task 2: Analysis of the changes in osteopontin expression, expression of cyclins, and changes in cell cycle profile after transfection of Akt kinase, constitutively active Akt kinase, or dominant negative Akt kinase.
Specific Aim 2, Task 3: Analysis of changes in proliferative and anti-apoptotic signaling molecules that act downstream of Akt. Deliverable: Data on changes in p27kip, Bad, caspase-9, and FKHR after transfection with Akt or its variants in the presence or absence of EGF.
Specific Aim 2, Task 4: Cloning of the osteopontin promoter into a reporter construct and investigation into reporter activity in various transfectants of Akt, constitutively active Akt, or dominant negative Akt. Electrophoretic mobility shift assays of nuclear extracts from the transfectants with oligonucleotides derived from the osteopontin promoter.