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PRINCIPAL INVESTIGATOR: Renee M. Marshall, Ph.D.

CONTRACTING ORGANIZATION: Temple University
Philadelphia, PA 19140

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Renee M. Marshall, Ph.D.

E-Mail: rmarshall@wistar.org

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14. ABSTRACT:
Positive transcription elongation b (P-TEFb) is a general transcription elongation factor and is composed of a catalytic subunit, CDK9, and a regulatory subunit, a T-type cyclin. The complex phosphorylates the C-terminal domain of RNA polymerase II as well as negative elongation factors to allow for the transcriptional elongation of paused transcripts. We have investigated the regulation and role of cyclin T1 in breast cancer cells. While cyclin T1 expression is regulated by multiple signaling pathways in T cells, it is constitutively expressed in breast cancer cells. Also, cyclin T1 associated kinase activity is not regulated in PMA treated MCF-7 and T47D cells. Flavopiridol (FVP), a drug being evaluated in clinical trials as an anti-cancer agent, and a potent inhibitor of HIV transcription, is believed to act, at least in part, by inhibiting CDK9. We have compared the effects of FVP with those effects induced by direct inhibition of CDK9 by a dominant negative (dnCDK9) in breast cancer cells and found that both treatments result in p53-independent apoptosis of breast cancer cells.

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Introduction

Androgen receptor (AR) is a nuclear receptor that belongs to the steroid receptor family. Two androgens, testosterone and the testosterone metabolite, dihydroxytestosterone (DHT), bind with different affinities to the receptor and activate AR. When activated AR, a transcription factor, will bind to the androgen response element (ARE) in its target genes to facilitate transactivation of those genes.

A number of findings suggest that the AR may play a role in the development of breast cancer: (1) 80% of breast cancer patients have AR+ tumors, and these tumors retain their growth potential even when the estrogen receptor and the progesterone receptor are lost, as exhibited by MDA-MB-453 cells (Cailleau *et al*., 1978; Hall *et al*., 1992; Brinkley *et al*., 1980; Bae *et al*., 1993; Hall *et al*., 1994), (2) a greater proportion of breast cancer patients with AR+ tumors respond to treatment and survive than those with AR- tumors (Bryan *et al*., 1984), (3) there is evidence of male breast cancer patients having mutations in the AR gene (Wooster *et al*., 1992), and (4) a CAG-repeat in AR is known to be inversely related to AR transcription, and women with one allele coding for a long CAG-repeat and a mutation in BRCA1 seem to have a higher incidence of diagnosis at an earlier age for breast cancer (Rebbeck *et al*., 1999).

Positive transcription elongation factor b (P-TEFb) is composed of a catalytic subunit, CDK9, and a regulatory subunit, T-type cyclin or cyclin K. CDK9 is a serine-threonine proline directed kinase. P-TEFb is a transcription elongation factor that phosphorylates the C-terminal domain of RNA polymerase II, as well as negative elongation factors, to allow for productive elongation of transcripts (figure 1) (Garriga and Graña, 2004).

It was demonstrated that the transactivation of a reporter gene under the control of an androgen response promoter was increase following treatment with PMA, a PKC activator, in CHO cells (De Ruiter, *et* al., 1995) and T47D cells (Darne *et al*., 1998), demonstrating AR response in a ligand-independent pathway. Our lab has demonstrated that PMA stimulation of human PBLs resulted in an increase in cyclin T1, a regulatory subunit of P-TEFb, at the protein and mRNA level (Garriga, *et al*., 1998b; Marshall, *et al*., 2005). Also it has been suggested that P-TEFb interacts with AR in prostate cells *in vivo* (Lee *et a*l., 2001). Therefore, it is possible that P-TEFb activity is necessary for productive elongation of androgen response genes in breast cancer cells.

Given these results, I had proposed the following aims in my original proposal to determine the role of P-TEFb in AR-mediated transactivation in breast cancer cells:

(1) *To determine the effects of PKC activation and androgen-mediated AR stimulation on P-TEFb in breast cancer cells.*

(2) *To dissect the components of the signal transduction pathway leading to P-TEFb activation in breast cancer cells.*

(3) *To determine whether AR is a substrate for P-TEFb.*

(4) *To establish whether the interaction between P-TEFb and AR is upregulated by ligand-dependent and/or ligand-independent mechanisms.*

In my annual review submitted July 2004, I reported that PMA stimulation of MCF-7 and T47D breast cancer cells did not result in upregulation of cyclin T1 protein levels or P-TEFb activity. Given that, to date, the only physiological cellular system in which cyclin T1 is upregulated is during T-cell activation, this result is not that surprising. However, a growing body of evidence shows that P-TEFb interacts with many transcription factors and that activation of these transcription factors sequesters P-TEFb for productive elongation of specific genes. One of these transcription factors is the proto-oncogene c-myc (Majello, *et al*., 1999., Eberhardy, *et al*., 2001., Eberhardy, *et al*., 2002., Kanazawa, *et al*., 2003). Therefore, although we have demonstrated that cyclin T1 protein levels and P-TEFb activity are not regulated by PMA stimulation in breast cancer cells, this does not preclude the complex's function from contributing to breast cancer cell growth.

Therefore, I revised my proposal to focus on aims 3 and 4 using chemical inhibitors selectively for CDK9 as well as a dominant negative CDK9 mutant to determine the effects of inhibiting P-TEFb function on the transactivation of AR response genes and other cellular genes in breast cancer cells. I also proposed to study the signaling pathways responsible for maintaining basal levels of cyclin T1 in breast cancer cells. We had also proposed to P-TEFb-dependent genes in MCF7 breast cancer cells.

BODY

Cyclin T1 and P-TEFb activity are not regulated following PMA treatment of MCF-7 breast cancer cells: PMA stimulation of PBLs did not result in upregulation of cyclin T1 protein or mRNA. Neither did treatment result in upregulation of P-TEFb activity. However, given that P-TEFb is recruited to promoters by transcription factors, such as the oncogene, myc, it is necessary to determine if P-TEFb target genes are upregulated in breast cancer cells.

Effects of a CDK9 selective inhibitor in MCF-7 breast cancer cells: MCF-7 breast cancer cells were treated with a CDK9 inhibitor, flavopiridol (25-300nM). Flavopiridol treatment prevented the phosphorylation of RNA polymerase II on serine 2 and serine 5, which have been suggested to be phosphorylated by P-TEFb and TFIIH, respectively. Interestingly, we observed an upregulation of p53 protein levels by 6 hours with 150nM flavopiridol and PARP cleavage, which demonstrates that the cells are apoptotic. We thought that the upregulation of p53 protein levels may be the result of a downregulation of its regulator, mdm2, which targets p53 for degradation. However, the increase in p53 precedes the downregulation of mdm2.

Effects of a dominant negative CDK9 mutant in MCF-7 breast cancer cells: Our results with flavopiridol suggest that P-TEFb function may be essential in MCF-7 breast cancer cells and that inhibition of this complex results in p53-independent apoptosis. However, it is not clear whether inhibition of other targets contributes to the effects of flavopiridol. Thus, we used a dominant negative CDK9 mutant to determine whether inhibition of CDK9 is responsible for the observed results. Flavopiridol is a potent CDK9 inhibitor. It has been shown to inhibit other CDKs, such as CDK4 and CDK7, at significantly higher concentrations than those required for inhibition of CDK9. *In vitro*, at the concentrations we used in our experiments ($\leq$300nM) CDK9 is inhibited, but the functions of other CDKs may also be could be inhibited. Therefore, to confirm our results were due to inhibiton of CDK9 we used a dominant negative CDK9 mutant (dnCDK9). MCF-7 breast cancer cells infected with a recombinant adenovirus expressing dnCDK9 exhibited upregulation of p53 and PARP cleavage comparable to cells treated with flavopiridol. These results demonstrate that specific inhibition of P-TEFb function results in p53-independent apoptosis in MCF-7 breast cancer cells.

Gene targets of P-TEFb in breast cancer cells: We had planned to determine which genes require P-TEFb activity for expression by using microarray technology. Samples were submitted for microarray analysis to compare gene expression in MCF-7 cells treated and untreated with flavopiridol as well as those expressing dnCDK9. We expected that these results would help us to identify gene targets of P-TEFb, including whether AR response genes require P-TEFb function. Unfortunately, these results were not conclusive and are currently being repeated in our laboratory.

Using T-cells as a model for determining signal transduction pathways required for maintaining cyclin T1 basal levels in MCF-7 breast cancer cells: T-cell activation is the only known physiological system in which regulation of cyclin T1 protein and mRNA levels have been observed. Because P-TEFb activity is inducible in T-cells, they are the best-suited model to identify targets of this important transcription factor as well as to dissect the molecular events necessary for expression and activation of P-TEFb. Our results strongly suggest that p53-independent apoptosis can be induced by inhibition of P-TEFb function in MCF-7 breast cancer cells. Interestingly, Actinomycin D, a chemotherapeutic drug, and other cellular stresses can upregulate P-TEFb activity. Thus, a detailed understanding of the mechanisms responsible for assembling active P-TEFb in breast cancer cells is essential. We have determined that the JNK and calcineurin pathways are required for cyclin T1 upregulation at the mRNA and protein levels. Also, we have shown that PMA-mediated upregulation of cyclin T1 is at the level of protein stability and requires calcium-independent PKCs (PKC theta and PKC delta), but not the ERK pathway.

Key Research Accomplishments

1. We have demonstrated that cyclin T1 protein levels and activity are not upregulated during stimulation of MCF-7 breast cancer cells with PMA.

2. We have demonstrated that inhibition of P-TEFb function, through the use of a CDK9 inhibitor and a dominant negative CDK9 mutant induces p53-independent apoptosis.

3. We have demonstrated that JNK and calcineurin are required for PHA-mediated upregulation of cyclin T1 mRNA and protein levels during T-cell activation. We have also demonstrated that the calcium-independent PKCs, but not the ERKs, are required for PMA-mediated upregulation of cyclin T1 protein levels during T-cell activation.

4. We determined that PHA-mediated upregulation of cyclin T1 is at the level of RNA stability, whereas, PMA-mediated upregulation of cyclin T1 is at the level of protein stability.

Reportable Outcomes

As previously reported, I collaborated on a study on CDK9 stability. These data demonstrated that CDK9 expression is not regulated during the cell-cycle in a SKP2-dependent manner. This work was published in Molecular and Cellular Biology in August 2003. Citation: Garriga J, Bhattacharya S, Calbo J, **Marshall RM**, Truongcao M, Haines DS, Grana X. (2003). CDK9 Is Constitutively Expressed throughout the Cell Cycle, and Its Steady-State Expression Is Independent of SKP2. Mol Cell Biol. 2003 Aug;23(15):5165-73.

The signaling data using the T-cell model was accepted for publication in the Journal of Immunology in November 2005. Citation: **Marshall RM**, Salerno D, Garriga J, Grana X.Cyclin T1 expression is regulated by multiple signaling pathways and mechanisms during activation of human peripheral blood lymphocytes. J Immunol. 2005 Nov 15;175(10):6402-11.

This work also resulted in an invited review published in FEBS Letters in September 2006. Citation: **Marshall RM**, Grana X. Mechanisms controlling CDK9 activity. Front Biosci. 2006 Sep 1;11:2598-613. Review.

Finally, this support also resulted in me receiving my Ph.D. in Molecular Biology and Genetics from the Fels Institute for Cancer Research and Molecular Biology at Temple University in August 2005. I am currently at the Wistar Institute as a post-doctoral fellow in the laboratory of Dr. Anthony Capobianco where I am studying the role of Notch in tumorigenesis, including assisting in the development of a breast cancer mouse model.

Conclusions

We have demonstrated that PMA stimulation of MCF-7 breast cancer cells does not result in upregulation of cyclin T1 expression or P-TEFb. However, our results strongly suggest that P-TEFb is required for MCF-7 breast cancer cell survival. Inhibition of P-TEFb activity via a chemical inhibitor, flavopiridol, or a dominant negative CDK9 leads to p53-independent apoptosis. Using T-cells as a model, given that this is the only physiological cellular system where cyclin T1 has been shown to be regulated, we determined pathways critical for cyclin T1 upegulation. Our data show that PHA stimulation results activation of the calcineurin and JNK pathways, both of which required for upregulation of cyclin T1 at the level of mRNA stability. We also demonstrate that PMA treatment results in upregulation of cyclin T1 at the level of protein stability. Our laboratory is currently determining whether inhibition of the pathways we demonstrated are necessary for upregulation of cyclin T1 during T-cell activation are required to maintain basal levels of cyclin T1 in MCF-7 cells. Our laboratory is also continuing to pursue the mechanism by which p53-independent apoptosis occurs after inhibition of P-TEFb activity by examining known pro-apoptotic genes as well as other potential targets. Given that flavopiridol is currently being used in clinical trials as a cancer therapy it is very important to understanding these effects in MCF-7 breast cancer cells. Importantly, of laboratory is still trying to determine what cellular genes require P-TEFb activity in order to maintain MCF-7 breast cancer cell survival.

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