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TITLE: BTG2 Antiproliferative Gene and Prostate Cancer

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Levels of the BTG2 tumor suppressor protein diminish during the transition of normal prostate epithelial cells into prostate cancer cells and restoration of BTG2 expression in prostate cancer cells significantly reduces cell proliferation and tumorigenicity. Our working hypothesis being tested is that the tumor suppressive activity of BTG2 is diminished as an early event in prostate carcinogenesis due to increased proteasomal degradation, leading to compromised cell cycle regulation, increased cell invasion and cancer progression. To date we have shown that BTG2 protein expression is lost as a very early event in prostate cancer and that prostate cancer tissue and cells degrade BTG2 at a greater rate than non-cancer tissue and cells. We have also shown that BTG2 has a predominantly nuclear localization consistent with its antiproliferative function, but that BTG2 is transiently sequestered in the nucleolus at 4 hours following growth stimulation of quiescent cells indicating that BTG2 might additionally influence some aspect of ribosome biosynthesis. In the present reporting period we have shown that steady state levels of BTG2 during the cell cycle are influenced by changes in BTG2 ubiquitination (consistent with proteasomal degradation) and not by changes in the level of the deubiquitinating enzyme USP4.
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INTRODUCTION: The protein product of the antiproliferative B-cell translocation gene-2 (BTG2) exerts its effect at the G1 and G2/M checkpoints via Rb- and p53-dependent and independent mechanisms. BTG2 appears to function in the prevention of malignant transformation of prostate epithelial cells.\textsuperscript{1,2} Forced expression of BTG2 in PC-3 prostate cancer cells (which do not normally express detectable levels of BTG2) is associated with significantly reduced rates of both cell proliferation and tumorigenicity.\textsuperscript{1,2} Furthermore, BTG2\textsuperscript{-/-} cells continue to proliferate in the presence of DNA damaging agents\textsuperscript{3} predisposing cells to the accrual of genetic damage. Steady state levels of BTG2 protein are regulated post-translationally and increased rates of proteasomal degradation may account for loss of BTG2 protein expression during prostate carcinogenesis.\textsuperscript{2} In addition, we have shown that ectopic expression of BTG2 in BTG2 null PC-3 prostate cancer cells causes reduced expression of cyclin D1, cyclin B, and thymosin \( \beta 4 \) indicative of a role in reducing cell migration as well as blocking cell cycle progression. Consistent with this finding, forced expression of BTG2 in PC-3 cells was associated with reduced local tumor growth and absence of metastases following orthotopic injection in the prostates of nude mice. These findings have provided the basis for formulating our working hypothesis that the tumor suppressive activity of BTG2 is lost as an early event in prostate carcinogenesis due to increased proteosomal degradation, leading to compromised cell cycle regulation, increased cell invasion and cancer progression. We proposed 3 tasks to test this hypothesis: Task 1: Define BTG2 expression during the morphological transition of normal prostate epithelial cells into prostate cancer precursors (PIA and HGPIN); Task 2: Compare the mechanism of BTG2 protein degradation in non-cancerous and cancerous prostate tissue and epithelial cells and Task 3: Determine the effects of BTG2 on prostate cell attachment and invasion.

BODY: Research accomplishments associated with the approved Statement of Work are described below. Only new research accomplishments since the previous report are described.

Task 1: Define BTG2 expression during the morphological transition of PIA (proliferative inflammatory atrophy) into HGPIN (high-grade prostatic intraepithelial neoplasia) (months 1-5).
This has been completed.

Task 2: Compare the mechanism of BTG2 protein degradation in non-cancerous and cancerous prostate tissue and epithelial cells (months 5-26).
This task has now been completed.

a. Histologically characterize and isolate adjacent regions of non-cancerous and cancerous prostate tissue (months 5-9).
This has been completed.

b. Determine the rates of BTG2 protein degradation in extracts of non-cancerous prostate tissue specimens and prostate cancer and determine the contribution of the ubiquitin-proteasome pathway to the degradation process (months 5-9).
This has been completed.
c. Establish and maintain primary cultures of prostate epithelial cells and the LNCaP and PC-3 cell lines for study (months 10-26).
This has been completed.

d. Determine the rates of BTG2 protein degradation in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines and determine the contribution of the ubiquitin-proteasome pathway to the degradation process (months 10-14).
This has been completed.

e. Determine the rates of BTG2 protein degradation during the cell-cycle in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines (months 14-18).
This has been completed.

f. Determine if there are differential rates of BTG2 protein synthesis in addition to differential rates of protein degradation (months 18-23).
This has been completed.

g. Determine the extent of ubiquitination of BTG2 in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines (months 23-25).
This has been completed. We modified the originally proposed in vitro ubiquitination assay due to technical difficulties we experienced. Recombinant $^{35}$S-labeled BTG2 protein was synthesized in E. coli by incubating the bacteria in sulfate-free medium containing $^{35}$S-sulfate. The labeling phase was chased with cold sulfate to ensure full-length BTG2 protein products. The $^{35}$S-labeled BTG2 product was purified from bacterial lysates by ion-exchange and size exclusion chromatography. Aliquots of $^{35}$S-labeled BTG2 were incubated with proteasome inhibitors and extracts of prostate epithelial cells prepared at quiescence and at various time periods into the growth cycle. Reactions were quenched by boiling in SDS-PAGE sample buffer and the products were examined by SDS-PAGE and fluorography (Figure 1). We determined that the extent of ubiquitination of BTG2 was cell-cycle dependent in all cell-lines examined. The results for primary cultures of non-cancerous human prostate epithelial cells are shown in Figure 1. BTG2 ubiquitination was undetectable by this assay in quiescent cells and in cells that had been stimulated to enter the growth cycle for 1 hour.

Ubiquitination of BTG2 was seen to gradually increase in cells at 2 hours through 24 hours into the growth cycle and is generally consistent in explaining the levels of BTG2 protein seen during the cell cycle. However, our findings from the previous reporting period also indicate that BTG2 is sequestered into the nucleolus at 4 hr following entry into the cell cycle and that the protein accumulates transiently in this organelle (possibly due to reduced degradation in the nucleolus). These findings indicate that the regulation of steady state levels of BTG2 protein during the cell cycle is more complex that we originally anticipated and suggests an additional function for BTG2 in the nucleolus.
Figure 1. Extent of BTG2 protein ubiquitination during the cell cycle of primary cultures of non-cancerous human prostate epithelial cells. [35S]-labeled BTG2 protein was incubated with extracts of primary cultures of non-cancerous human prostate epithelial cells that were quiescent (0 hr) or had been stimulated to enter the growth cycle by addition of EGF (1 hr to 24 hr). The products were then analyzed by SDS-PAGE and fluorography. The BTG2 and ubiquinated BTG2 (Ub-BTG2) regions of the same gel are shown (exposure times for the Ub-BTG2 region were longer).

h. Determine the levels of de-ubiquitinating enzyme Unp-1 in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines (months 24-26).
This has been completed. During the tenure of this award, antibodies to the human ubiquitin specific protease USP4 became commercially available from Bethyl Laboratories (Montgomery, TX). USP4 is the human analog of murine Unp-1.

Figure 2. Levels of the ubiquitin specific protease USP4 during the cell cycle of primary cultures of non-cancerous human prostate epithelial cells. Extracts of primary cultures of non-cancerous human prostate epithelial cells that were quiescent (0 hr) or had been stimulated to enter the growth cycle by addition of EGF (1 hr to 24 hr) were analyzed by SDS-PAGE and immunoblotting using antibodies to USP4 and p42/p44 MAPK (as loading control).

Task 3: Determine the effects of BTG2 on prostate cell attachment and invasion (months 26-36).
Due to technical difficulties these studies took much longer than planned. The PC-3/Retro-Off BTG2 cells that express BTG2 under the control of an inducible tetracycline promoter were prepared before the original proposal was submitted. These cells were frozen down for use in
task 3 of the proposal, however the cells did not maintain adequate levels of inducible expression upon thawing. These cells therefore had to be rederived in order to initiate Task 3. Because of this delay we requested a no-cost extension of this award until 12/15/2007 in order to complete these studies. We are pleased to inform that these studies are now ~75% complete and we plan to report the results in our final report.

a. Maintain cultures of PC-3/Retro-Off BTG2 cells that express BTG2 under the control of an inducible tetracycline promoter (months 26-36).
   This is in progress

b. Determine whether BTG2 expression in PC-3/Retro-Off BTG2 cells increases cell attachment to different extracellular matrices (months 26-31).
   This is almost complete

c. Determine whether BTG2 expression in PC-3/Retro-Off BTG2 cells decreases tumor cell invasion through the extracellular matrix (months 31-36).
   This is in progress

KEY RESEARCH ACCOMPLISHMENTS:

- Shown that BTG2 protein is increasingly ubiquitinated after prostate epithelial cells enter the cell cycle.
- Shown that BTG2 protein levels during the cell cycle are likely regulated by ubiquitination [and by nucleolar sequestration (previous annual report)] and not by alterations in the level of the ubiquitin specific protease USP4.
- In other studies beyond the scope of the present award we have shown that in breast cancer cells BTG2 protein levels are also regulated by methylation of the BTG2 promoter. The possibility that promoter methylation may also account for lower levels of BTG2 expression in prostate cancer cells will be investigated in future studies.

REPORTABLE OUTCOMES:

- We are in the process of revising a manuscript to include the new data from this annual report that will be submitted this year.
- We have generated methodologies and reagents from our studies on cell cycle synchronized cancerous and non-cancerous prostate epithelial cells that are amenable to further study in other cancers.

CONCLUSIONS: The research completed to date shows that the BTG2 tumor suppressor is lost as an early event in prostate carcinogenesis. Our research completed to date also supports our working hypothesis that the reason for BTG2 protein loss in prostate cancer is due to increased proteasomal degradation of the protein. We have shown that there is a general increase in ubiquitination of BTG2 during the cell cycle, but there are no corresponding changes in the expression of the ubiquitin specific protease USP4. Thus increased targeting for degradation appears to be a major method of regulation, however transient sequestration of BTG2 in the nucleolus at 4 hours into the cell cycle, appears to be an additional regulatory mechanism.
discovered during this award. While the general mechanisms for BTG2 degradation are similar in non-cancerous and cancerous prostate cells, they result in lower levels of BTG2 protein in cancer cells.

Loss of BTG2 expression has been shown to occur as an early event in carcinogenesis in several solid tumors including prostate,² breast,⁶,⁷, kidney,⁸ and most recently in medulloblastoma.⁹ Thus loss of BTG2 expression may represent an important general mechanism in carcinogenesis. The goal of our final no-cost extension award period is to complete studies aimed at determining the effects of BTG2 on prostate cell attachment and invasion.

REFERENCES: