Award Number: DAMD17-03-1-0322

TITLE: Investigating the Role of Celecoxib as a Chemopreventive and Chemotherapeutic Agent for Breast Cancer

PRINCIPAL INVESTIGATOR: Randy J. Levitt

CONTRACTING ORGANIZATION: Jewish General Hospital Montreal, Quebec H3T 1E2

REPORT DATE: May 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
13. ABSTRACT (Maximum 200 Words)

Although I obtained some interesting data from the experiments outlined in Year 1 of my original statement of work, my statement of work for Year 2 was revised and accepted on 12/10/04. The rationale for my proposed change was based on the recent announcement by Merck and Co. that they have pulled their COX-2 inhibitor rofecoxib off of the market due to unreasonable risks for heart attack and stroke. For my experiments, I had been working with celecoxib, a sister compound of rofecoxib, and several experts in the field fear as if long-term celecoxib administration may also pose a risk to the health of patients. Due to this unexpected announcement, I felt as if the work outlined in my Year 2 statement of work may not be clinically relevant. For Year 2 of my proposal, instead of extending my results with celecoxib, I focused my research on the role of another insulin-like growth factor binding, namely IGFBP-2, in breast cancer. We have obtained from the laboratory of Dr. Martin Gleave (University of British Columbia) the MDA-MB-231 breast cancer cell line transfected with a lentiviral vector expressing IGFBP-2 (231/BP-2), as well as a mock transfected MDA-MB-231 cell line (231/mock). We have determined that the 231/mock cells do not express any IGFBP-2 protein, whereas the 231/BP-2 cells express large amounts. Interestingly, we have observed that the 231/BP-2 cell line proliferates significantly faster than the 231/mock cells. Therefore, my results obtained in Year 2 focused on determining the mechanism of the growth promoting effect of IGFBP-2 in breast cancer cells.
# Table of Contents

Cover .................................................................................................................. 1  
SF 298 .................................................................................................................. 2  
Table of Contents ............................................................................................... 3  
Introduction ......................................................................................................... 4  
Body ...................................................................................................................... 5  
Key Research Accomplishments ......................................................................... 7  
Reportable Outcomes .......................................................................................... 8  
Conclusions .......................................................................................................... 9  
References ............................................................................................................ 10  
Appendices .......................................................................................................... 11
Introduction

Although I obtained some interesting data from the experiments outlined in Year 1 of my original statement of work, my statement of work for Year 2 was revised and accepted on 12/10/04. The rationale for my proposed change was based on the recent announcement by Merck and Co. that they have pulled their COX-2 inhibitor rofecoxib (Vioxx) off of the market due to unreasonable risks for heart attack and stroke (1,2). For my experiments, I had been working with celecoxib (Celebrex), a sister compound of rofecoxib, and several experts in the field fear as if long-term celecoxib administration may also pose a risk to the health of patients (1,2).

Due to this unexpected announcement, I felt as if the work outlined in my Year 2 statement of work may not be clinically relevant. The basis for my original proposal was our observation that celecoxib induces insulin-like growth factor binding protein-3 (IGFBP-3) in breast epithelial cells. IGFBP-3 is a known inhibitor of breast cancer proliferation, so we wished to examine the relevance of this finding. The work which I performed in Year 1 has examined this issue in detail, and I summarized those results in my first Annual Report (submitted on June 1, 2004). For Year 2 of my proposal, instead of extending my results with celecoxib, I focused my research on the role of another insulin-like growth factor binding, namely IGFBP-2, in breast cancer.

Insulin-like growth factor binding protein-2 (IGFBP-2) is the second most abundant IGFBP in the circulation and is found in a variety of human fluids and tissues, including some breast cancer cells (3). Classically, IGFBP-2 was considered to be a growth inhibitor, as it had a well-defined role in sequestering the mitogens IGF-I and IGF-II, therefore preventing binding and subsequent activation of mitogenic and anti-apoptotic pathways downstream of the IGF-I receptor (IGF-IR) (3). However, increasing evidence indicates that under certain conditions, IGFBP-2 can act as a growth stimulator, and both IGF-dependent and independent mechanisms have been proposed (for a review, see ref. (4)).

We have obtained from the laboratory of Dr. Martin Gleave (University of British Columbia, Vancouver, B.C., Canada) the MDA-MB-231 breast cancer cell line transfected with a lentiviral vector expressing IGFBP-2 (231/BP-2), as well as a mock transfected MDA-MB-231 cell line (231/mock). We have determined that the 231/mock cells express very low levels of IGFBP-2 protein, whereas the 231/BP-2 cells express large amounts. Interestingly, we have observed that the 231/BP-2 cell line proliferates significantly faster than the 231/mock cells. Therefore, my results obtained in Year 2 focused on determining the mechanism of the growth promoting effect of IGFBP-2 in breast cancer cells.
The 231/BP-2 cell line is a breast cancer cell line which was transfected with a lentiral vector expressing IGFBP-2. We determined that the 231/mock cells express very low levels of IGFBP-2 protein, whereas the 231/BP-2 cells express large amounts (figure 1b). Interestingly, the 231/BP-2 cell line proliferates significantly faster than the 231/mock cells, as determined by a MTT assay (figure 1a). Classically, IGFBP-2 was considered to be a growth inhibitor, as it had a well-defined role in sequestering the mitogens IGF-I and IGF-II, therefore preventing binding and subsequent activation of mitogenic and anti-apoptotic pathways downstream of the IGF-IR (3). However, increasing evidence indicates that under certain conditions, IGFBP-2 can act as a growth stimulator, and both IGF-dependent and -independent mechanisms have been proposed. Hypothesized mechanisms for IGF-dependent growth stimulatory effects include IGFBP-2 acting as a chaperone, presenting the IGFs to the IGF-IR, or IGFBP-2 acting by binding to and increasing the half-life of the IGFs. Several proposed mechanisms have been suggested for the IGF-independent growth effects of IGFBP-2, and for a review see ref. (4).

We observed the growth stimulatory effect of IGFBP-2 in 5% fetal bovine serum (FBS), which intrinsically contains IGFs, therefore we wished to determine if IGFBP-2 acts as an IGF-dependent growth stimulator in our system. As seen in figure 1b, transfection of IGFBP-2 did not increase levels of activated (phosphorylated) Akt or ERK-1/2, two downstream effectors of IGF-IR signalling. These results suggest that the growth stimulatory actions of IGFBP-2 in our system are IGF-independent. Consistent with this data, transfection of IGFBP-2 or addition of recombinant human IGFBP-2 (rhBP-2) to 231 cells did not increase IGF-IR expression (figure 2). Furthermore, addition of 50 ng/ml recombinant human IGF-I to either 231/mock or 231/BP-2 cells did not enhance their growth (data not shown), indicating that neither cell line is IGF-I responsive. Task 1d in my revised statement of work would have determined the effect of blocking IGF signalling at different levels of the pathway in both 231/mock and 231/BP-2 cells. However, since previous data suggested that the growth promoting effect of IGFBP-2 is IGF-independent, I did not perform this task.

After having established that the growth promoting effects of IGFBP-2 are IGF-independent, we then wished to determine if IGFBP-2, a secreted protein, acts as a growth promoter intracellularly or extra-cellularly. As seen in figure 3a, very high levels of IGFBP-2 are detected in serum-free conditioned media from 231/BP-2 cells, whereas very low levels are detected in 231/mock cells. Using ELISA, we quantified the amount of IGFBP-2 secreted by 231/BP-2 cells after 48 hours at ~250 ng/ml (figure 3b). Addition of 250-500 ng/ml rhBP-2 to 231/mock cells did not stimulate the growth of 231/mock cells in both serum-free and 5% FBS conditions (figure 3c), suggesting that IGFBP-2 acts as a growth promoter intracellularly. To further test this hypothesis, serum-free conditioned media from 231/mock and 231/BP-2 cells was collected at 48 and 72 hours, and was then added to 231/mock cells in culture. As seen in figure 3d, there was no difference in the ability of 231/mock and 231/BP-2 conditioned media to stimulate growth of 231/mock cells, once again providing evidence that IGFBP-2 acts as
a growth promoter intracellularly. A role for intracellular IGFBP-2 in various biological processes has been suggested in the literature (4,5) and recently, an intracellular binding partner of IGFBP-2 named IIP45 has been isolated (6), but its physiological role requires further clarification. The possibility exists that IIP45 is involved in the growth stimulatory role of IGFBP-2 in our system.

In order to confirm that intracellular IGFBP-2 is acting as a growth promoter in 231/BP-2 cells, we wished to downregulate IGFBP-2 expression with siRNA. We formed a collaboration with the siRNA company Atugen (Berlin, Germany), and they sent us various siRNAs which targeted IGFBP-2. We determined the most potent siRNA in terms of downregulating IGFBP-2 expression (data not shown), and we used this siRNA for all subsequent experiments. A siRNA which did not effect IGFBP-2 expression whatsoever was used as a negative control. As seen in figure 4a, treatment of 231/BP-2 cells with IGFBP-2 siRNA resulted in a significant decrease in IGFBP-2 expression as early as 24 hours. This decreased expression was observed up to 72 hours post-treatment. As seen in figure 4b, although the siRNA resulted in a significant decrease in IGFBP-2 expression, no decrease in IGF-IR expression or P-ERK expression was observed. These results are consistent with our data from figures 1 and 2, where we showed that increasing expression of IGFBP-2 in 231 cells did not increase IGF-IR expression or downstream signalling, suggesting an IGF-independent growth stimulatory role for IGFBP-2.

Although decreasing IGFBP-2 expression in 231/BP-2 cells with siRNA did not inhibit IGF-IR signalling, it significantly decreased growth as seen in figure 5a. To determine if the growth inhibitory effect of the siRNA was specific to 231 cells, we treated MCF-7 cells, a natural IGFBP-2-expressing breast cancer cell line, with the IGFBP-2 siRNA. As seen in figure 4b, treatment with the siRNA abolished IGFBP-2 expression in MCF-7 cells, and this correlated with a ~35% decrease in cell viability (figure 4a). The IGFBP-2 siRNA had no effect on the proliferation of the 184htert cell line, an immortal breast epithelial cell line that was created by transfecting a retrovirus expressing the human telomerase reverse transcriptase gene into normal breast epithelial cells (7). However, we could not detect any IGFBP-2 expression in this cell line, therefore it serves as a good control to show that the growth inhibitory effect of the IGFBP-2 siRNA is not due to non-specific toxicity. We attempted to extend our studies with the siRNA to another natural IGFBP-2 expressing breast cancer cell line, MDA-MB-468, however we observed that the lipid transfection reagent used was toxic to this cell line (data not shown).
Key Research Accomplishments

1) Determined that transfection of IGFBP-2 into the MDA-MB-231 breast cancer cell line results in a significant increase in proliferation.

2) Determined that this increase in proliferation is due to an IGF-independent effect of IGFBP-2.

3) Determined that this increase in proliferation is due to IGFBP-2 acting intracellularly.

4) Determined that downregulating IGFBP-2 expression with siRNA decreases the growth of 231/BP-2 cells, as well as the growth of the natural IGFBP-2 expressing breast cancer cell line MCF-7.
Reportable Outcomes

Manuscripts


Abstracts and poster presentations


Employment opportunities received based on experience/training supported by this award

1) Postdoctoral Fellow, laboratory of Dr. Martin Gleave, The Prostate Centre, University of British Columbia, Vancouver, B.C., Canada.
Conclusions

In this report, we show that transfection of IGFBP-2 in the MDA-MB-231 cell line (which expresses very low levels of IGFBP-2) results in a significant increase in proliferation. We determined that the mechanism of IGFBP-2 induced proliferation is IGF-independent, as overexpression of IGFBP-2 did not increase levels of IGF-IR or two downstream effectors of IGF-IR signalling: P-Akt and P-ERK. Furthermore, we determined that IGFBP-2 induced proliferation is due to intracellular IGFBP-2, as rhBP-2 did not effect the proliferation of 231/mock cells, nor did 231/BP-2 conditioned media. Finally, we show that downregulating IGFBP-2 expression with siRNA significantly inhibits the proliferation of 231/BP-2 cells, as well as the proliferation of the natural IGFBP-2 expressing breast cancer cell line MCF-7. In aggregate, these results provide evidence that IGFBP-2 functions as a growth stimulator for breast cancer cells, and therefore therapies that target IGFBP-2 expression, such as siRNA or antisense oligonucleotides, may have therapeutic value for breast cancer.
References


Appendix I:

Figures
Figure 1: Transfection of IGFBP-2 into MDA-MB-231 breast cancer cells results in increased proliferation. (A) 231/mock and 231/BP-2 cells were plated in DMEM/5% FBS. After 72 hrs, a MTT assay was performed to quantify cell viability (*P<0.05). (B) 231/mock and 231/BP-2 cells were plated in DMEM/5%FBS. After 48 hrs, whole cell lysates were collected, separated by SDS-PAGE, and western blots were performed.
Figure 2: IGFBP-2 does not effect IGF-IR expression in MDA-MB-231 cells. (A) 231/mock and 231/BP-2 cells were plated in DMEM/5%FBS. After 48 hrs, cells were collected by trysinization, stained with a FITC-conjugated IGF-IR antibody, and analyzed by flow cytometry. (B) 231/mock and 23/BP-2 cells were plated in DMEM/5%FBS. The following day, the cells were washed with PBS and switched to serum-free DMEM with or without human recombinant IGFBP-2 (rhBP-2). Cell lysates were collected after 48 hrs, separated by SDS-PAGE, and western blots were performed.
Figure 3: Extracellular IGFBP-2 does not stimulate the growth of 231/mock cells. 231/mock and 231/BP-2 were plated in DMEM/5%FBS. The following day, the cells were washed with PBS and switched to serum-free DMEM. Conditioned media was collected after 48 hrs and analyzed by western blotting (A) or ELISA (B). (C) 231/mock cells were treated with or without rhBP-2 in the indicated media for 72 hrs and then cell viability was assayed by MTT. (D) 231/mock cells were treated with serum-free 48 or 72 hr conditioned media from either 231/mock or 231/BP-2 cells for 72 hrs, and then cell viability was assayed by MTT.
Figure 4: Downregulation of IGFBP-2 by siRNA in 231/BP-2 cells does not inhibit IGF-IR expression or signalling. 231/BP-2 cells were treated with 30 nM control siRNA or 30 nM IGFBP-2 siRNA (Atugen, Berlin, Germany) with profectin-50 lipid transfection reagent (Atugen) in DMEM/8% FBS. At the indicated time points, whole cell lysates were collected, separated by SDS-PAGE, and western blots were performed.
Figure 5: Downregulation of IGFBP-2 by siRNA in 231/BP-2 and MCF-7 cells inhibits proliferation. Cells were treated with 30 nM control siRNA or 30 nM IGFBP-2 siRNA (Atugen, Berlin, Germany) with profectin-50 lipid transfection reagent (Atugen) in DMEM/8% FBS. (A) After 96 hrs, cell viability was assayed by MTT (*P<0.05). (B) After 72 hrs, whole cell lysates were collected, separated by SDS-PAGE, and western blots were performed.