REGULATION OF COLONOCYTE APOPTOSIS BY BCL-2:

INFLUENCE OF CHEMOTHERAPEUTIC DIETARY AGENTS

A Senior Scholars Thesis

by

HARMONY FAITH TURK

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2008

Major: Nutrition

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Approved by:

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Regulation of Colonocyte Apoptosis by Bcl-2:
Influence of Chemotherapeutic Dietary Agents (April 2008)

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This study intends to elucidate a mechanism of cancer prevention by certain nutrients. The health benefits of n-3 polyunsaturated fatty acids, e.g., docosahexaenoic acid (DHA), and fiber are well documented. It has been previously documented that DHA and butyrate, a fiber by-product, work synergistically to prevent colorectal cancer by increasing cell death (apoptosis). The resulting increase in apoptosis could be caused in part by down-regulation of the anti-apoptotic protein bcl-2. This study aimed to determine the correlation of bcl-2 and the apoptotic effects of butyrate and DHA. Immortalized mouse colonocytes were pre-treated with DHA, transfected with human bcl-2 cDNA to overexpress full length bcl-2, and then co-treated butyrate. Apoptosis was measured using an enzyme-linked immunosorbent assay (ELISA), and western immunoblot was used to quantify bcl-2 levels. Apoptosis was significantly different ($P<0.05$) in DHA and butyrate treated cells depending on whether or not cultures were transfected with bcl-2. The DHA and butyrate treated cells transfected with bcl-2 contained significantly fewer apoptotic cells compared to untransfected DHA and

butyrate treated cells. These data suggest that DHA and butyrate induce apoptosis in part by decreasing bcl-2 expression.

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I would like to thank my research advisor, Dr. Robert S. Chapkin, for guiding me through this process and assisting me in every way that he could. I would also like to thank Dr. Satya Kolar for teaching me numerous techniques and always helping me when I needed it. I would like to thank Dr. Yang-Yi Fan for her assistance and being tough on me when I needed it. I would also like to thank everyone else in the lab, especially Dr. Laurie Davidson and Evelyn Callaway, for pointing me in the right direction and always offering support and advice.

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CHAPTER I

INTRODUCTION

Colon cancer

Colon cancer is one of the leading causes of death from cancer in almost all developed countries.[1] Colon cancer is thought to be affected by numerous environmental aspects, and it is estimated that up to 70% of colorectal cancers can be prevented by moderate changes in lifestyle and eating habits.[2] Since the colon is a key aspect of the digestive tract, nutrients that are consumed can regulate homeostatic mechanisms within the colon. Therefore, it is likely that colon cancer is heavily influenced by dietary constituents. Overall, certain nutrients play an important role in regulating carcinogenesis.

Apoptosis

Carcinogenesis is a complex process that perturbs the homeostasis of intestinal epithelial cell proliferation, differentiation, and programmed cell death.[3] An important aspect of preventing carcinogenesis is prohibiting the perpetuation of DNA damaged cells through processes of programmed cell death. One particular type of programmed cell death is apoptosis. Apoptosis is utilized to remove cells that are in excess, old, or damaged

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This thesis follows the style of *Cancer*.

beyond repair, and it is critical that proliferation and apoptosis are balanced in order to maintain the steady-state number of cells in the colon.[4] Apoptosis results from a series of reactions that signal for the cell to die. When apoptosis is impeded, damaged cells are no longer removed. Apoptosis is crucial in preventing carcinogenesis because it eliminates unwanted and damaged cells that could potentially become tumorigenic.[5] Several different factors exist that have an effect on the overall regulation of apoptosis.

Chemotherapeutic nutrients

Dietary fatty acids and fiber are two highly investigated regulators of colon carcinogenesis. Epidemiological studies have shown that there is an inverse relationship between high intake of fish and colorectal cancer.[6] Substantial evidence demonstrates that n-3 polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are chemopreventative agents in fish oil that protect against colon tumorigenesis. The combination of n-3 PUFA with certain fiber sources is potentially more chemoprotective than n-3 PUFA alone. Our lab has shown that feeding rats a diet rich in n-3 PUFA (fish oil) with pectin as a fiber source results in more than twice the apoptotic index in the colon compared with diets high in n-6 (corn oil) and n-9 (olive oil) dietary lipids.[7] It has recently been shown that the bioactive compound in pectin is butyrate, a four carbon fatty acid fermentation product of pectin.[8] Furthermore, a significant increase in colonocyte apoptosis was observed when rats were fed a diet containing fish oil and butyrate pellets for targeted release in the colon.[8] Subsequently, our lab determined *in vitro* that DHA and butyrate work synergistically to enhance

apoptosis by up to 43% in colonocyte cultures compared with butyrate alone, indicating that DHA is a bioactive component of fish oil.[9] Furthermore, our lab has shown that DHA and butyrate synergize via a Ca^{2+}-mediated intrinsic mitochondrial pathway to induce apoptosis.[9] Overall, n-3 PUFA and butyrate are very important chemoprotective nutrients.

Bcl-2

Bcl-2 is a 26-kDa protein which has been found to be closely related to apoptosis. It has been shown to be mainly localized in the mitochondrial membrane, nuclear membrane, and the endoplasmic reticulum.[10] Bcl-2 is expressed in cells that are rapidly dividing and differentiating, and it has also been shown that bcl-2 has the ability to inhibit apoptosis by conserving the integrity of the mitochondrial matrix so that pro-apoptotic molecules cannot escape from the mitochondria.[11] Bcl-2 is one of the most important regulators of both spontaneous and damage-induced apoptosis.[12] By inhibiting programmed cell death, bcl-2 plays a critical role in the process of tumorigenesis. High levels of bcl-2 are capable of keeping cells alive after tumorigenic mutations have been incurred, and overexpression of bcl-2 has been linked to many types of human cancer, including colorectal cancer.[11] Bcl-2 levels have been shown to be affected by diet. Our lab has shown that bcl-2 levels in rats fed fish oil containing diets with pectin as a fiber source are lower compared to rats fed a corn oil based diet.[13] In the same experiment, bcl-2 levels were shown to be negatively correlated to apoptosis. These results suggest that the downregulation of bcl-2 could be responsible for the increase in apoptosis.

Purpose

The purpose of this project was to determine if the ability of DHA and butyrate to induce apoptosis is in the result of a decrease in colonic bcl-2 levels. The goal was to measure the level of apoptosis in a mouse colonocyte line (YAMC) after treating cells with bioactive nutrients and overexpressing bcl-2. Changes in apoptosis resulting from a treatment of DHA (active component in fish oil) and butyrate (active component in pectin) were compared with other treatments. Overall, we determined the change in apoptosis that results from a diet high in n-3 PUFA and butyrate is due in part to decreased levels of bcl-2.

CHAPTER II

MATERIALS AND METHODS

Materials

RPMI 1640 and Hanks' balanced salt solution (HBSS) were purchased from Mediatech (Herndon, VA). Dulbeco's phosphate buffered saline (PBS), glutamax, and interferon-γ were purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Insulin, transferrin, selenium without linoleic acid were purchased from Collaborative Biomedical Products (Bedford, MA). Fatty acids were purchased from NuChek (Elysian, MN). Butyrate was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA). NaN_3 was received from Sigma-Aldrich Co. (St. Louis, MO). Rabbit Bcl-2 polyclonal antibody was purchased from Assay Designs (Ann Arbor, MI). Alexa 568 goat anti-rabbit IgG and ProLong Antifade Kit were purchased from Molecular Probe (Eugene, OR). Nucleofector device II and nucleofector kit L were obtained from Amaxa (Gaithersburg, MD). Lab-Tek II 2-well glass chamber slides were purchased from Nalge Nunc, Inc. (Rochester, NY). Cell detection ELISA kit and fatty acid free bovine serum albumin (BSA) was from Roche Applied Science (Indianapolis, IN). ABI PRISM® 3100 Genetic Analyzer was purchased from Applied Biosystems (Foster City, CA).

Plasmid sequencing

Three human bcl-2 constructs were received from Dr. Maria Hoyer-Hansen, Danish Cancer Institute.[14] The plasmids had been cloned into the episomal vector pCEP4 from Invitrogen. One plasmid encoded full-length human bcl-2. Another plasmid contained a 35 amino acid sequence of the endoplasmic reticulum (ER)-specific isoform of rat hepatic cytochrome b5 which replaced the 21 amino acid carboxy-terminus of bcl-2 to target bcl-2 predominantly to the ER.[15] The last construct also contained a 27 amino acid insert from the protein Act-A from *Listeria monocytogenes* to target bcl-2 to the endoplasmic reticulum. [15] All three constructs were sequenced using an ABI PRISM® 3100 Genetic Analyzer. Only the full-length bcl-2 was used in this experiment.

Cell culture

YAMC cells, immortalized mouse colonocytes, were originally obtained from R. H. Whitehead, Ludwig Cancer Institute (Melbourne, Australia). Cells were cultured under permissive conditions, 33 ^{o}C and five percent atmospheric CO_2 with interferon-γ, as previously described.[16, 17] Bovine serum albumin (BSA) complexed fatty acid was added to cultures 24 hours after cell plating as previously described.[17] Select cultures were treated with BSA-complexed DHA (22:6, n-3) (50 μM) for 72 hours. Protocol for cell culture techniques can be found in Appendix A.

Transfection

Forty-eight hours after seeding (24 hours after DHA treatment), cells underwent transfection using Amaxa Nucleofector Device II and Amaxa Nucleofector Kit L. The cells were transfected with full length human bcl-2 plasmid. YAMC cells were transfected with 0 or 2 μg of bcl-2 DNA for the purpose of overexpressing bcl-2. Program A-020 was used on the nucleofector device for the transfection. After transfection, cells were seeded onto 2-chambered cover glass slides at a density of 2.0 x 10^5 for further analysis. For apoptosis assays, cells were seeded into 6-well plates at a density of 1.2 x 10^5. Select cultures were initially treated with BSA-complexed DHA for 48 hours. Cells that were treated with DHA were co-treated with butyrate (5 mM) in RPMI media for the final 24 hours of fatty acid pre-treatment. Protocol for transfection techniques can be found in Appendix B.

Fluorescent imaging

Overexpression of bcl-2 was measured by immunohistochemistry. Cells were plated onto 2-chambered cover glass slides. After 48 hours, cells were fixed with 4% paraformaldehyde for twenty minutes and incubated at 4^oC overnight in blocking solution (1%BSA/0.1%NaN_3 in PBS). Next, cells were incubated with rabbit anti-bcl-2 [20 μg/μL] for one hour. Subsequently, cells were incubated with Alexa 568 goat anti-rabbit IgG [10 μg/μL] as a secondary antibody for one hour. Fluorescence was viewed with TE 300 Nikon Eclipse fluorescence microscope. Cells were excited at 595-605 nm and fluorescence emission was collected at 620 nm. Images were taken with an inverted

with a digital Cool SNAPEX Monochrome camera from Photometrics with 12 bits @ 20 mghz, a DEI-750D 3-chip Optronics color camera. Protocol for immunohistochemistry can be found in Appendix C.

Apoptosis assay

Apoptosis was measured using cellular fragmentation enzyme linked immuno-sorbent assay (ELISA) (Roche) as previously described.[17] Apoptosis in floating and adherent cells was measured 24 hours after adding the butyrate and BSA-complexed DHA co-treatment. Apoptotic index was calculated by dividing 100 times the absorbance calculated by the ELISA by the number of adherent cells. Techniques for ELISA can be found in Appendix D.

CHAPTER III

RESULTS

Sequencing

The full-length bcl-2 was sequenced. Basic Local Alignment Search Tool (BLAST) was used to calculate homology. The full-length bcl-2 had 99% homology with the B-cell lymphoma protein 2 (bcl-2) alpha isoform from *Homo sapiens*. The sequence is listed in **Figure 1**. The sequences for the other two constructs are located in Appendix E.

A

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ATGGCGCACGCTGGGAGAACAGGGTACGATAACCGGGAGATAGTGATGAAGTACATCCAT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ATGGCGCACGCTGGGAGAACAGGGTACGATAACCGGGAGATAGTGATGAAGTACATCCAT

TATAAGCTGTCGCAGAGGGGCTACGAGTGGGATGCGGGAGATGTGGGCGCCGCGCCCCCG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TATAAGCTGTCGCAGAGGGGCTACGAGTGGGATGCGGGAGATGTGGGCGCCGCGCCCCCG

GGGGCCGCCCCCGCACCGGGCATCTTCTCCTCCCAGCCCGGGCACACGCCCCATCCAGCC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGGGCCGCCCCCGCACCGGGCATCTTCTCCTCCCAGCCCGGGCACACGCCCCATCCAGCC

GCATCCCGGGACCCGGTCGCCAGGACCTCGCCACTGCAGACCCCGGCTGCCCCCGGCGCC
|||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
GCATCCCGGGACCCGGTCGCCAGGACCTCGCCGCTGCAGACCCCGGCTGCCCCCGGCGCC

GCCGCGGGGCCTGCGCTCAGCCCGGTGCCACCTGTGGTCCACCTGACCCTCCGCCAGGCC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GCCGCGGGGCCTGCGCTCAGCCCGGTGCCACCTGTGGTCCACCTGACCCTCCGCCAGGCC
```

FIGURE 1. Sequence of full-length human bcl-2. The aligned sequence (A) shows that the plasmid matched 705 of 720 nucloetides; the top line is the plasmid and the bottom line is the given bcl-2 alpha isoform from BLAST. The derived amino acid sequence (B) shows that two out of 239 amino acids did not correlate to the amino acid sequence given by BLAST. Differences are highlighted in red.

```
GGCGACGACTTCTCCCGCCGCTACCGCCGCGACTTCGCCGAGATGTCCAGCCAGCTGCAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGCGACGACTTCTCCCGCCGCTACCGCCGCGACTTCGCCGAGATGTCCAGCCAGCTGCAC

CTGACGCCCTTCACCGCGCGGGGACGCTTTGCCACGGTGGTGGAGGAGCTCTTCAGGGAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CTGACGCCCTTCACCGCGCGGGGACGCTTTGCCACGGTGGTGGAGGAGCTCTTCAGGGAC

GGGGTGAACTGGGGGAGGATTGTGGCCTTCTTTGAGTTCGGTGGGGTCATATGTGTGGAG
|||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
GGGGTGAACTGGGGGAGGATTGTGGCCTTCTTTGAGTTCGGTGGGGTCATGTGTGTGGAG

AGCGTCAACCGGGAGATGTCGCCCCTGGTGGACAACATCGCCCTGTGGATGACTGAGTAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AGCGTCAACCGGGAGATGTCGCCCCTGGTGGACAACATCGCCCTGTGGATGACTGAGTAC

CTGAACCGGCACCTGCACACCTGGATCCAGGATAACGGAGGCTGGGATGCCTTTGTGGAG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CTGAACCGGCACCTGCACACCTGGATCCAGGATAACGGAGGCTGGGATGCCTTTGTGGAA

CTCTACGGCCCCAGCATGCGGCCTCTGTTTGATTTCTCCTGGCTGTCTCTTAAGACTCTG
|| |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
CTGTACGGCCCCAGCATGCGGCCTCTGTTTGATTTCTCCTGGCTGTCTCTGAAGACTCTG

CTCTCGCTAG-CACTAGTGGGAGCATGCATCACCCTGGGTGCCTATCTGTCCCACAAGTGA
|||  | | | | || |||||||| |||||||||||||||||||||||||  ||||||||||
CTCA-GTTTGGCCCTGGTGGGAGCTTGCATCACCCTGGGTGCCTATCTGGGCCACAAGTGA
```

B

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MAHAGRTGYDNREIVMKYIHYKLSQRGYEWDAGDVGAAPPGAAPAPGIFSSQPG
HTPHPAASRDPVARTSPLQTPAAPGAAAGPALSPVPPVVHLTLRQAGDDFSRRYR
RDFAEMSSQLHLTPFTARGRFATVVEELFRDGVNWGRIVAFFEFGGVICVESVNR
EMSPLVDNIALWMTEYLNRHLHTWIQDNGGWDAFVELYGPSMRPLFDFSWLSL
KTLLSLALVGACITLGAYLSHK
```

FIGURE 1, Continued.

Fluorescence microscopy

Fluorescence microscopy was used to examine transfection efficiency. An antibody for bcl-2 was used to detect bcl-2 expression 24 hours following transfection. As shown by **Figure 2**, the cells that were transfected with bcl-2 exhibited much more fluorescence.

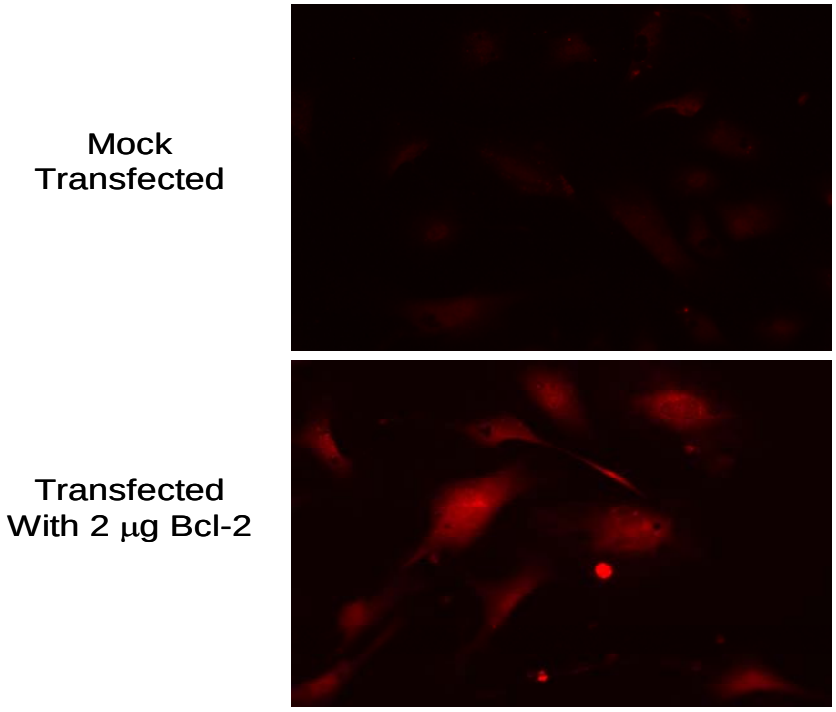

FIGURE 2. Bcl-2 expression as shown by fluorescence microscopy. YAMC cells were transfected with either an empty vector or a vector containing bcl-2. YAMC cells were subsequently cultured for 48 h. Cells were then fixed and treated with rabbit anti-bcl-2 for 1 h followed by Alexa 568 conjugated goat anti-rabbit IgG for 1 h. Bcl-2 levels were elevated 48 h following transfection (lower panel).

These data indicate that bcl-2 was overexpressed in the transfected cells. Approximately 75% (76% $\pm$ 0.075; n=3) of the transfected cells showed significantly enhanced bcl-2 levels.

Apoptosis

Intranucleosomal degradation of genomic DNA, a biomarker for apoptosis, was detected by an ELISA assay. As shown in **Figure 3**, the apoptotic index was lowest in the mock

transfected control(C) and bcl-2 transfected control (CT) cells, i.e., no DHA or butyrate added.

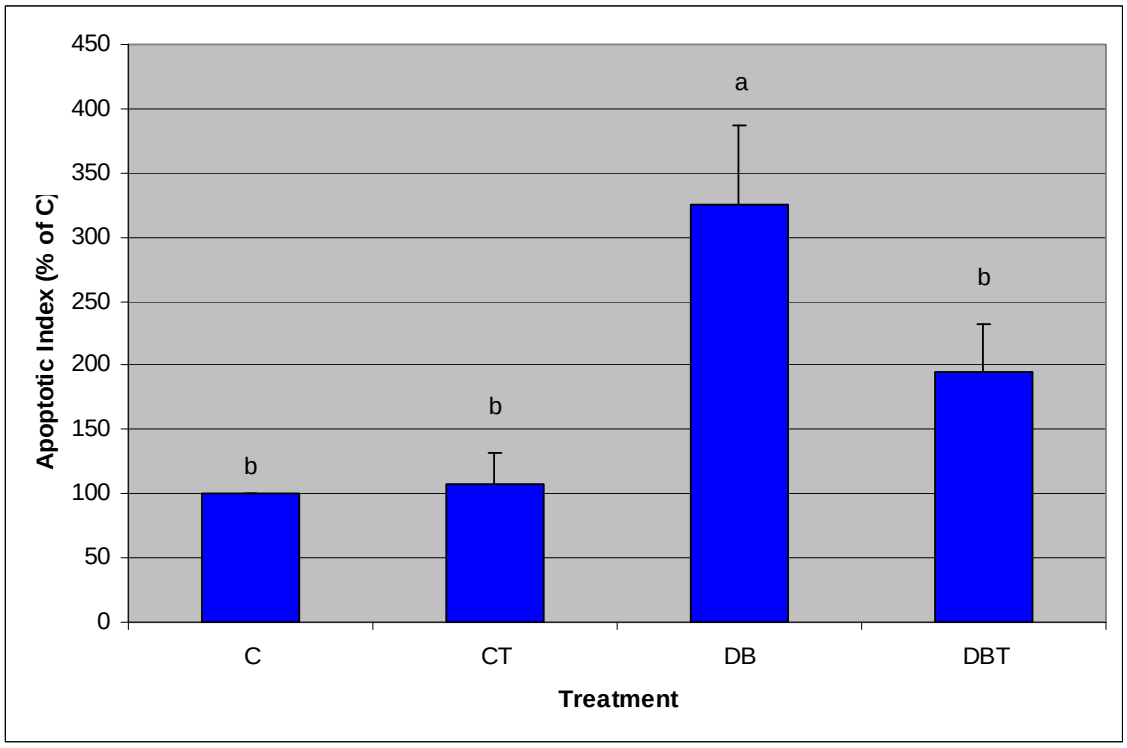

FIGURE 3. Apoptotic index of treatments as measured by ELISA assay. C, mock transfected control; CT, bcl-2 transfected control; DB, mock transfected and treated with DHA and butyrate; DBT, bcl-2 transfected and treated with DHA and butyrate. n=8-12 separate wells for each group from 2 separate experiments. Statistical significance was measured using Fisher's Protected Least Significant Difference test (LSD). $P<0.05$.

Mock transfected cells incubated in the presence of DHA and butyrate (DB) exhibited the highest apoptotic index. Apoptotic index for control (C) was set at 100%, and all other groups were compared to C. The apoptotic index for mock transfected cultures treated with DHA and butyrate increased approximately three-fold relative to control (DB vs. C). Bcl-2 transfected cells treated with DHA and butyrate (DBT) exhibited

significantly decreased (~ 50%) apoptotic index, compared to DB. In comparison, DBT had almost two-fold higher levels of apoptosis compared to both C and CT.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

DHA and butyrate are well known chemopreventative agents that promote apoptosis. Bcl-2 is a protein that is capable of inhibiting apoptosis. When colonocytes are cultured in the presence of DHA and butyrate, bcl-2 levels are reduced and apoptosis is increased. We showed in this study for the first time that the ectopic expression of bcl-2 can partially block the induction of apoptosis in colonocytes treated with DHA and butyrate. **Figure 4** illustrates how overexpressing bcl-2 can block the effects of DHA and butyrate. Even though overexpression of bcl-2 in DHA and butyrate treated cells significantly decreased apoptosis compared to mock transfected DHA and butyrate cells, overexpression of bcl-2 alone did not bring the levels of apoptosis back to control. This suggests that there may be multiple apoptotic pathways induced by DHA and butyrate. By determining the mechanisms employed by these chemotherapeutic nutrients, we hope to learn how to optimally use these nutrients to reduce the abundance of cancers, e.g., colorectal cancer.

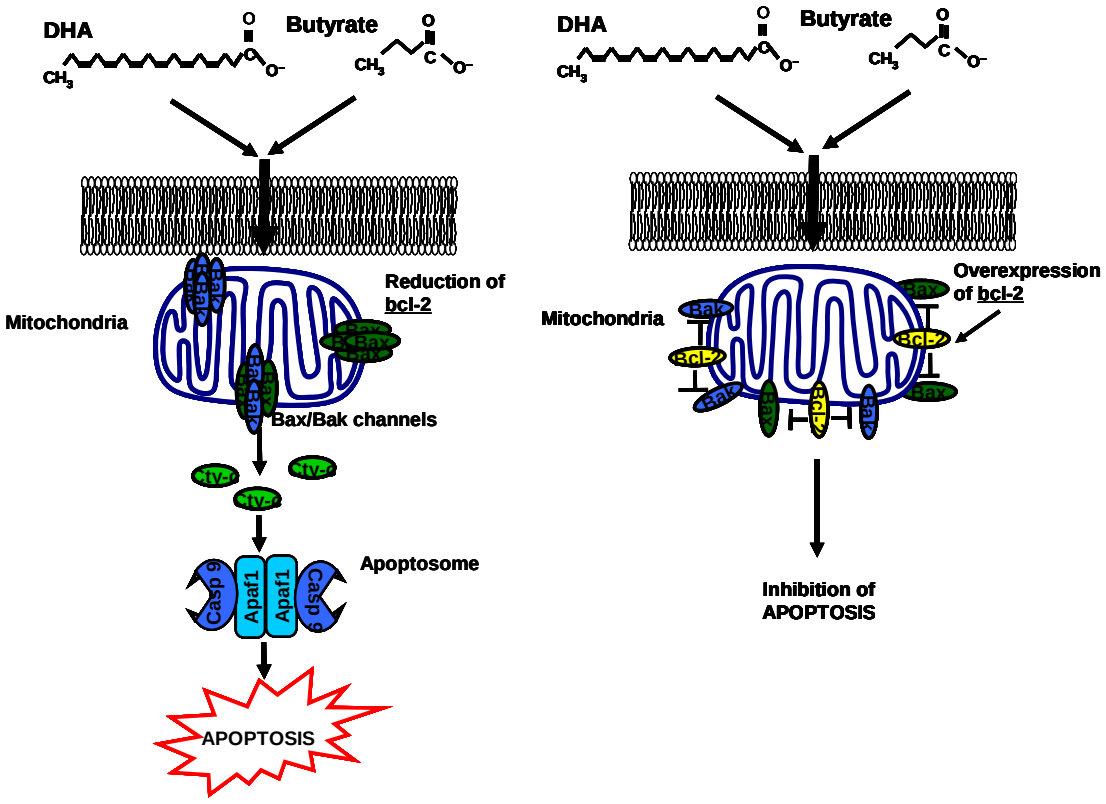

FIGURE 4. Putative mechanism by which overexpression of bcl-2 modulates DHA and butyrate-induced apoptosis in colonocytes. The left panel shows how DHA and butyrate treatment suppresses bcl-2 levels and therefore promotes apoptosis. The right panel illustrates how forced overexpression of bcl-2 partially blocks cell death.

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APPENDIX A

PROTOCOL: CELL LINE SEEDING AND FEEDING

Purpose: To seed cells for apoptosis assay

Materials:

- Complete RPMI 1640 media
- γ-IFN (Gibco BRL#13284-021)
- HBSS
- Trypsin
- 2 T-175 flask
- 50 mL conical tube
- Plastic pipettes
- Glass pipettes

Procedure:

1. Sterilize the hood.
2. Warm complete RPMI 1640 media to room temperature.
3. Aliquot 20 mL of media into a 50 mL conical tube in which to spin down the cells.
4. Make up 50 mL of complete media with 5 μL γ-IFN in two 50 mL conical tubes.
5. Aspirate media from flask. Wash with 15 mL HBSS. Aspirate HBSS.
6. Add 10 mL trypsin and incubate at 37^{o}C for 3 minutes.
7. Add media into the flask to stop the trypsinization. Put trypsinized cells into the conical tube to spin down cells.
8. Centrifuge the cells in the tube at 200 x g (1096 rpm) in the tabletop centrifuge for 5 min at 6 Acc./Dcc.
9. Open the two T-175 flasks under the hood using sterile techniques
10. Add approximately 22 mL of complete media (with γ-IFN) from the 50 mL conical tube into each flask.
11. After completion of the spin, aspirate the supernatant to remove the media from the cells (be careful not to disturb the pellet at the bottom).
12. Resuspend the pellet in 5 mL of complete media from the 50 mL conical tube.
13. Count the cells and seed approximately 8.0 x 10^5 cells into two flasks.
    Need approximately 1.5 x 10^6 cells for each transfection (2 transfections per flask so need 3.0 x 10^6 cells per flask on day 2).
    3.0 x 10^6/2 = 1.5 x 10^6/2 = 7.5 x 10^5

13. Add the resuspended cells to the T-175 flask and rotate it gently to distribute the cells evenly.
14. Label the flask—specify the cell type, passage number, initials, and date.
15. Incubate the flask at 33^{o}C under 5% CO_2 atmospheric pressure.

APPENDIX B

PROTOCOL: TRANSFECT YAMC CELLS WITH BCL-2 CONTAINING PLASMIDS

Purpose: To transfect cells with bcl-2 in order to overexpress bcl-2.

Materials:

Amaxa Nucleofector Device II

Amaxa Nucleofector Kit L (cat# VCA-1005)

100 μL Nucleofector solution L (per reaction-total of 1mL)

2-Lab-Tek II 2-well glass chamber slide (Nalge, cat# 154461)

Glass pipettes

Complete Culture Medium (RPMI 1640 (cat #15-040-CV) with FBS, ITS, and Glutamax-1)

γ-IFN (Gibco BRL#13284-021)

PBS (Gibco, cat#14190)

Trypsin (cat # 25300054)

10 μL and 200 μL pipettes and tips

0.6 μL Eppendorf tubes (one per reaction)

15 mL conical tubes

1-2 x 10^6 YAMC cells (per reaction)

Bcl-2 DNA (2 μg (4.52 μL) wild type Bcl-2 per reaction with DNA)

4 6-well plates

Procedure:

1. From the Nucleofector kit L, mix the Supplement to Nucleofector solution L at the ratio of 2:9 (v/v)-(100 µL supplement and 450 µL of solution L). Mix gently. (It is stable for 3 months at 4°C.) This is now the Nucleofector solution **(SN).**
2. Pre-warm **SN** to room temp. Pre-warm 25 mL complete culture medium (with γ-IFN) (12.5 mL in 2-15 mL conicals) and 25 mL of FA-BSA-Media (12.5 mL in 2-15 mL conicals) at 33°C.
   12.5 mL (50 µM) / 2.5 mM = .25 mL = 250 µL FA in 12.5 mL media
3. Aspirate old media from the flasks and wash cells with PBS (about 20 mL for a T-175).
4. Harvest cells with trypsin (about 10 mL). Incubate at 37°C for about 5 minutes then stop the reaction by adding 20 mL of complete RPMI medium to the flask then putting the entire mixture into a 50 mL conical tube.
5. Take an aliquot of cell suspension for cell counting and count to determine the concentration (will need approximately 3.0 x 10^6 cells per tube). Some of the cells can be thrown out at this point if the concentration is too high.
6. Centrifuge at **90 x g** for 10 min at room temperature.
7. Select program "**A-020"** from the Nucleofector device II.
8. Remove pre-warmed media from the incubator.
9. Use the special long pipette (provided in the kit) to suck up 500 µL (to the line above the ball area of the pipette) of pre-warm complete media or FA-BSA-Complete Media (depending on which treatment) from one of the 15 mL conical tubes. Keep it in a sterile place.
10. After centrifugation, resuspend the cell pellet with **SN** at the final concentration of 1.6 x 10^6/100 µL **SN**. (Avoid storing the cells in SN for more than 15 min, as this will reduce cell viability and transfection efficiency).
11. Mix 100 µL of cell suspension with either 4.52 µL 1xTE (0 µg DNA) or 4.52 µL of wild type Bcl-2 (2 µg) DNA in a sterile 0.6 mL eppy-tube.

12. Transfer 100 µL of the cell/DNA mixture to an Amaxa certified cuvette (provided in the kit). Make sure that the sample covers the bottom of the cuvette and avoid air bubbles. Close the cuvette with the blue cap.
14. Insert the cuvette into the cuvette holder on the Nucleofector device and press the "**X**" button to start the program.
15. Remove the cuvette from the device immediately after it is finished and add the 500 µL medium from the long pipette into the cuvette. Then immediately transfer the entire sample to pre-warm media (or FA treatment) in the conical tube from which the 500 µL was taken to dilute to the appropriate concentration (approximately 120,000 cells per mL).
    1.5×10^6 cells / 12.5 mL = 1.2×10^5 cells/mL
16. Transfer 1 mL of cells into each well on the 6-well plate.**Be sure to add the cells drop wisely and distribute evenly.**
17. Also, transfer 1 mL from each control (no FA treatment) into a well on a 2-well glass chamber slide for fluorescent microscopy.
17. Incubate at 33^{o}C for approximately 24 hours.

APPENDIX C

PROTOCOL: BCL-2 WT IMAGING IN YAMC CELLS

Purpose: To image Bcl-2 WT that has been overexpressed in YAMC cells

Materials:

Rabbit anti Bcl-2 (Stressgen, cat# AAS-070E) Ref. Hoyer-Hansen M., et. al. 2007, Mol Cell, Vol 25, No. 2, p.193-205.

Alexa 568 goat anti-rabbit IgG(H+L), [2 mg/mL] (Molecular Probe, cat# A-11036)

ProLong Antifade Kit (Molecular Probe, cat# P-7481)

Coverglass (No. 1, 22 x 50 mm, Corning)

Fresh 4% PFA in PBS (dilute from 20% PFA, EMS#15713-S)

Lab-Tek II 2-well glass chamber slide (Nalge, cat# 154461)

Procedure: (n=2)

Approximately 24 hours after transfection:

1. Wash cells (on the chamber slide) with PBS 3x
2. Immediately fix in Fresh 4% paraformaldehyde in PBS for **20 min** at RT.
3. Rinse samples with PBS 2x
4. Incubate cells with 10 mM glycine in PBS for **10 min** at RT (to quench the aldehyde groups)
5. Wash coverglass with PBS 2x
6. Permeabilize cells in PBS/0.2% Triton X-100 for **5 min** at RT
7. Wash with PBS 3x.
8. Incubate cells in Blocking solution (1%BSA/0.1%NaN_3 in PBS) at 4°C **overnight**
9. Wash coverglass with PBS 3 x.

(Dilute AB in Blocking solution to make <u>20 µg/mL</u>)

(For 1 well):

10. (Bcl-2) 1°AB mix:

    ***<u>20 µg/mL:</u>* 4** µL of α–Bcl-2 [stock 1 mg/mL] (1:20 dil) + **196** µL buffer

    Incubate all cells with 200 µL AB/well, incubate at room temp for **1 hr <u>(Protect from light)</u>**

11. After **1 h** incubation, wash chamber slide with PBS 3 x, and the last wash with blocking solution.

    2° AB: **1** µL of Alexa 568 goat anti-rabbit IgG [stock 2 mg/mL] (1:200 dil) + **199** µL buffer

12. Incubate cells with 2° AB Alexa 568 goat anti-rabbit IgG [10µg/mL] 200 µL AB/well at RT for **1 hr** at a humidity chamber.
13. Prepare the ProLong medium and labeling slides during the 1 hr incubation
14. Mix 1 mL of ProLong mounting medium (Component B) to one brown vial of Prolong antifade reagent (Component A). (Mix well by pipetting or vortexing, remove bubbles that have formed during the mixing procedure by sonication)
15. Use the slide separator to remove the chamber. Put the slides on the metal slide holder for the following wash.
16. Wash chamber slide with PBS 3 x.
17. Incubate chamber slide in 70% ethanol 1 x.
18. Incubate chamber slide in 95% ethanol 1 x.
19. Incubate chamber slide in 100% ethanol 1 x.
20. Incubate chamber slide in fresh xylene 1 x.
21. Apply a small amount of antifade reagent/mounting medium mixture to the chamber slide, cover the slide while it is still wet. (Use glass rod to put a line of medium on the side of the coverglass, then cover the slide at 45° angle to avoid bubbles) use razor blasé to help coverslipping.
22. Place the coverglass/slide on a flat surface in the dark to dry **overnight** at RT.

---

23. Once dry, seal the coverglass to the slide with fingernail polish, to prevent shrinkage of the mounting medium and subsequent sample distortion.
24. After sealing, store the coverglass/slide upright in a covered slide box at -20°C. Desiccant may be added to the box to ensure that the slide remains dry.
25. Examine sample under Fluorescence microscopy.

APPENDIX D

PROTOCOL: ELISA APOPTOSIS ASSAY

Purpose: To determine the amount of apoptotic cells

Materials:

ELISA kit: Cell death detection ELISA Plus from Roche- (BM# 1774425)

Reconstitute working solutions *(refer to manual, pg 7)*

Bottle 1: anti-histone-biotin: each bottle, reconstitute in 450 μL double distilled H_2O for 10 min and mix well *(store at 2-8°C up to 2 months)*

Bottle 2: anti-DNA-POD- reconstitute with 450 μL double distilled H_2O for 10 min and mix well *(store at 2-8°C upto 2 months)*

Bottle 3: Positive control - reconstitute with 450 μL double distilled H_2O for 10 min and mix well *(store at 2-8°C upto 2 months)*

Bottle 7: ABTS Tablets- dissolve 1 tablet in 5 ml of substrate buffer (vial 6). (100 μL / sample). This is light sensitive and hence store container in a foil wrap *(store for 1 month protected form light)*

Complete RPMI media (without γ- INF)

Label 3 sets of 2 ml or 1.7 ml eppys (depending on the volume in the plate): floaters collection (step 1), supernatant collection (step 11) and counting.

Procedure:

1. Under sterile conditions in the hood: swirl the 35 mm dish and collect the media and floating cells into the 1st set of 2 ml eppy-tubes.

2. Add 1ml of sterile PBS into the plates and put them in the 33°C incubator for counting later.

3. Centrifuge the first set of eppy-tubes in the Eppendorf centrifuge at **5,000 rpm** for 5 min at RT.

4. Remove supernatant using 1ml pipette manually without disturbing the pellet *(pellet is imaginary most of the time)***.**

5. Resuspend pellet in 1ml media without γ-IFN.

6. Finger flick the tubes to mix.

7. Centrifuge again at 5,000 rpm for 5 min at RT to wash and remove the excess media.

8. Remove as much of the supernatant as possible (without disturbing the pellet) and discard it.

9. Resuspend cell pellet with **125 µL** lysis buffer (bottle 5 of Cell death detection ELISA kit)

10. Mix thoroughly and **incubate** sample for **30 min** at **4°C (cell culture fridge)**

11. During incubation, prepare the immunoreagent (manual: pg 9)

    For 10 tests- 40 µL of reconstituent from bottle 1 + 40 µL of reconstituent from bottle 2 + 720 µL of incubation buffer from bottle 4. Mix well.

12. After incubation, centrifuge lysate at max speed (**13,600 x g**, eppendorf centrifuge) for 10 min, at **4°C.** *(Pellets out the intact cells and nuclei etc leaving the cytosolic fraction in the supernatant for the assay)*

13. Transfer **100 µL** of the supernatant carefully into another eppy-tube without shaking the pellet. Keep tubes on **ice**.

Continue in the main lab

14. Transfer **20µL** of all samples into MP. Pipette into the **middle** of the MP well.

15. Use 20µL of
    - Well (A –1) – Background (Bottle 4 of kit)
    - Well (B-1) – Blank (ABTS solution - Tablet dissolved in substrate-reconstituted tablet)
    - Well (C-1) – Positive control (Bottle 3 of kit)

16. Add to each well **80µL** of the immunoreagent using multiple channel pipette/ regular (**no immuno-reagent into the blank** (B-1)

17. Remove all bubbles from the wells in the MP with a pipette tip.

18. Cover the MP with an adhesive cover and foil. Incubate on a MP shaker under gently shaking (300 rpm) for **2hr** at 15-25°C.

19. Empty the solution from the MP thoroughly by **tapping** (tape both sides of the modules to secure them).

20. Rinse each well **3x with 250µL** of incubation buffer (bottle 4).

21. Empty the solution carefully by tapping.

22. In the dark, pipette to each well **100µL ABTS** solution (light sensitive).

23. Incubate on a plate shaker at 250 rpm **in the cold room** until the color development is sufficient for a photometric analysis (approx 2-5min). Check every minute.

24. Measure at **405nm** against ABTS solution as a blank (reference wavelength approx 490nm)

Counting:

26. Remove the plates from the incubator
27. Swirl the plates with the PBS and aspirate the PBS from the plates
28. Add 0.5 ml Trypsin and put them in the incubator for 3 minutes @ 33^0C.
29. Add 1ml media into each of the plates with the trypsin.
30. Swirl the plate and collect all the cell/ trypsin and media into the eppy-tubes.
31. Count the cells using hemocytometer.
32. Calculate the cell density and total number of adherent cells.
33. Use this count to calculate the apoptotic index.
    a. Apoptotic Index = Optical Density/ # adherent cells
34. Plot and analyze data using super-ANOVA

APPENDIX E

SEQUENCE OF UNUSED BCL-2 CONSTRUCTS

The first 654 nucleotides (218 amino acids) are the same in all three constructs. They all have the same differences in those first nucleotides from the sequence given by BLAST. Below is the remaining nucleotide and amino acid sequence for the organelle specific plasmids.

ER-targeted

Nucleotide sequence:

```
ATC ACT ACC GTT GAA TCG AAC TCT AGT TGG TGG ACT ACT GGG TTA TCC CTG CGT
CTC TGC TCT GGT TTA AGC CCT GAT GTA CCG TCT GTA CAT GCT GAA GAT AAT
```

Amino acid sequence:

```
ITTVESNSSWWTTGLSLRLCSGLSPDVPSVHAEDN
```

Mitochondrial-targeted

Nucleotide sequence:

```
CTG ATT CTC GCC ATG CTG GCA ATT GGG GTG TTC TCC CTC GGC GCC TTC ATT AGA
TCA TTC AGC TCC GGA AGA ACA ATT GAT
```

Amino acid sequence:

```
LILAMLAIGVFSLGAFIRSFSSGRTID
```

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