THE ADENOMATOUS POLYPOSIS COLI TUMOR SUPPRESSOR GENE SUPPRESSES DEOXYCHOLIC ACID INDUCTION OF THE CHEMOTACTIC CYTOKINE CXCL8 IN HUMAN COLORECTAL CANCER

by
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A Dissertation Submitted to the Faculty of the

GRADUATE INTERDISCIPLINARY PROGRAM IN CANCER BIOLOGY

In partial fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2007

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

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ACKNOWLEDGEMENTS

I would like to acknowledge those people who have helped me with my dissertation. First, I would like to thank both Dr. Gerner and Dr. Lance who were simultaneously my sharpest critics and greatest allies. They challenged me to think critically of my own research and encouraged me to exceed my own expectations. I would also like to thank the members of my committee: Dr. Bowden, Dr. Martinez, and Dr. Nelson. Their insightful questions helped to direct and focus my research.

This work would not have been possible without the help by the members of the lab. I would especially like to thank Upal Basu Roy for technical advice on procedures but also philosophical discussions about science and rationale. Similarly, I would like to acknowledge the assistance and advice of Dr. Natalia Ignatenko, David Stringer, Dipti Metha, Rebecca Henkhaus and Karen Kachel for their insights into scientific procedures.

I would like to thank my parents and brothers for their support. In addition, I would especially like to thank my wife, Claire, who has been a pillar of strength in our family. Not only has she completed her Master's of Public Health, but has been a wonderful role model to our children as she balanced work, relationships and school. Our two young daughters Annika and Emma have grown up having attended graduate school classes with either Claire or myself, having visited the lab or sat in on a Final Defense. I hope to afford them as many opportunities as have been afforded to me.

DEDICATION

This dissertation is dedicated to the memory of my grandfathers; Chester Edward Abbey and William Smith Rial II. Their battles with cancer are a constant reminder of the importance of scientific research and its application towards a greater understanding of the disease. May we treat the patients with this knowledge and with compassion.

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ABSTRACT

Elevated deoxycholic acid (DCA) and mutations in the Adenomatous Polyposis Coli (*APC*) tumor suppressor gene have been associated with increased risk of colorectal cancer (CRC). Chronic inflammation has also been associated with increased risk of CRC. It is unclear if DCA mediates inflammation in the normal or transformed colonic mucosa. The status of *APC* was manipulated in human CRC cell lines to study the role of DCA mediated inflammation. The chemotactic cytokine, CXCL8, was used as a marker of inflammation. Addition of DCA to the HT29-parental cell line with mutant-*APC* increased the steady state mRNA and protein levels of CXCL8. Conversely, addition of DCA to the HT29-APC cell line with wild type-*APC* was protective for increased steady state RNA and protein levels of CXCL8. DCA activated transcription factors which had binding regions in the CXCL8 5'-promoter. To elucidate the mechanism of induction, the 5'-promoter of CXCL8 was investigated. DCA increased promoter-reporter activity of the CXCL8 gene in HT29-parental cell line but wild type-*APC* blocked this effect. Chromatin immunoprecipitation (ChIP) revealed that DCA activated transcription factors, AP-1 and NF-κB were bound to the 5'-promoter of CXCL8. The transcription factor, β-catenin, was also bound to the 5'-promoter of CXCL8. Phenotypic effects were measured. Increased CXCL8 lead to matrix metalloproteinase-2 (MMP-2) production and increased invasion by HT29-parental cells on laminin coated filters. The DCA-mediated invasion was blocked by antibody directed against CXCL8 and wild type-*APC*. Therefore DCA-mediated inflammation occurs in transformed colonic epithelium and increases the invasive phenotype of CRC cells by CXCL8.

CHAPTER 1:

INTRODUCTION AND BACKGROUND

Cancer Statistics

Cancer is the leading cause of mortality in the world [11]. Cancer is also the leading cause of death in Americans under the age of 85 [12]. In 2007, colorectal cancer (CRC) contributed to an estimated 55,170 deaths in men and women in the United States [13]. Risk for CRC are derived from factors in the environment and from genetic factors [14].

Environmental Risk Factors for Colorectal Cancer

CRC incidence has been associated with highly processed and high fat foods of the Western diet [15]. As shown in Fig. 1.1.A., bile acid metabolism follows a circular pathway known as enterohepatic recycling. Primary bile acid is made by the liver, from cholesterol, secreted into the duodenum, emulsifies fats from the diet and is absorbed from the ileum [16]. From the ileum, bile acids are returned to the liver [16]. Enterohepatic recirculation is approximately 95% efficient with some bile acids lost to the large intestine [8]. In the colon, enteric bacteria convert the primary bile acids; cholic acid (CA) and chenodeoxycholic acid to the secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (CA).

The major difference between primary and secondary bile acid is the conversion of a hydroxyl group (-OH) to a hydrogen moiety (-H), as shown in Fig. 1.1.B. Ursodeoxycholic acid (UDCA) is a tertiary bile acid found in small amounts in humans that has been evaluated for its chemopreventative properties [17, 18]. As shown in Fig. 1.1.B. the structure of UDCA is similar to the primary bile acid, CA.

The tumorigenic potential of bile acid was established by Cook et al in 1940 [19, 20]. Elevated bile acid concentration, acting as a carcinogen, and risk of cancer has been substantiated by epidemiological [15], physiological [21, 22] and laboratory models [23]. Further evidence in clinical [24] and animal [25] models associated incidence of CRC with the secondary bile acid DCA. Elevated DCA is a has been shown to increase proliferation, dysplasia, invasion and metastatic cancer [26]. As shown in Fig. 1.1.C., the bile acid, DCA is recognized to alter intracellular signaling pathways [27] through the activation of activator protein-1 (AP-1) [28], CAAT enhancer binding protein (C/EBP) [29] as well as nuclear factor-kappa B (NF-κB) [30].

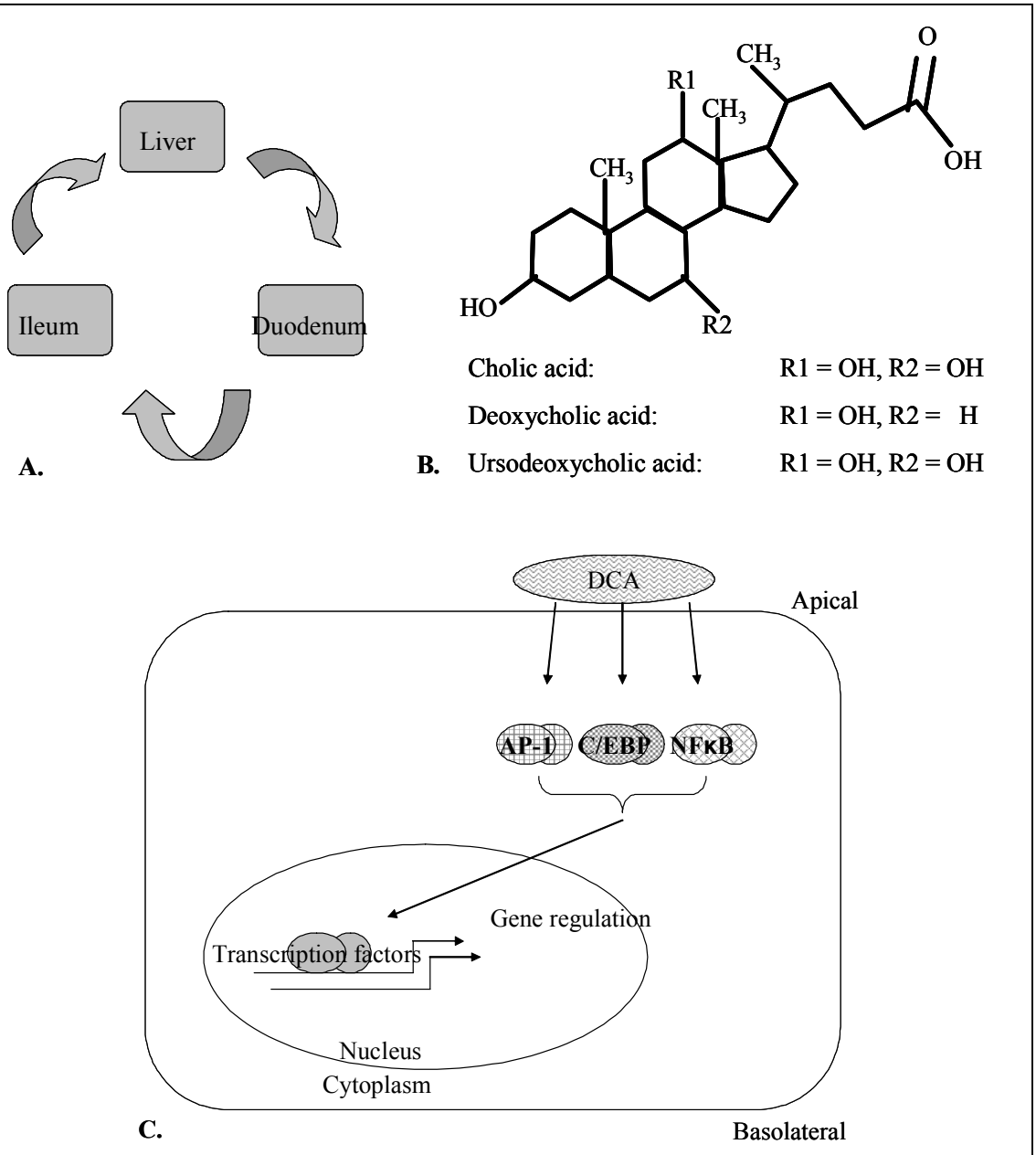


Figure 1.1. Bile acid physiology, structure and deoxycholic acid signaling. (A) Enterohepatic circulation is the process whereby bile acid is secreted by the liver into the duodenum, solubilizes fats and is reabsorbed by the ileum and returned to the liver by the portal blood. (B) Bile acid structure for the primary, secondary and tertiary bile acids. *Adapted from [3, 6, 8].* (C) The secondary bile acid, DCA has been shown to activate transcription factors. The activation of transcription factors regulates gene expression. Abbreviations: AP-1, activator potein-1; C/EBP, CAAT enhancer binding protein; NF-κB, nuclear factor kappa B.

Genetic Risk Factors for Colorectal Cancer

In addition to luminal risk factors for CRC, genetic risk factors also exist. CRC is a multistep disease and takes many years to develop [31]. The accumulation of mutations in tumor suppressors and/or proto-oncogenes parallels the alterations from normal to dysplastic then carcinoma in situ and finally metastatic disease [32]. Adenomatous Polyposis Coli (*APC*), is a tumor suppressor gene [31] relevant to CRC is shown schematically in Fig. 1.2.A. Patients with Familial Adenomatous Polyposis (FAP) harbor a germline mutation in *APC* in the mutation cluster region (MCR) which has a regulatory region of β-catenin [9, 33]. Over 80% of sporadic CRC harbor somatic mutations in the *APC* gene or mutations of its binding partner β-catenin [9].

Both APC and β-catenin are integral members of the Wnt signaling family. Shown in Fig. 1.2.B., is a representation of Wnt signaling in normal development. Upon binding of the Wnt ligand to the Wnt receptor, activation of the frizzled protein inactivates glycogen-synthase kinase-3β (GSK-3β). Inactivation of GSK-3β allows for the accumulation of β-catenin in the cytoplasm and translocation into the nucleus. Along with its binding partners T-cell factor (Tcf)/lymphoid enhancing factor (Lef), β-catenin can regulate gene expression.

As shown in Fig. 1.2.C., the normal adult tissue does not produce the Wnt ligand. The absence of the Wnt ligand allows for GSK-3β to remain active, marking β-catenin for proteosomal degradation. β-catenin does not accumulate in the cytoplasm or the nucleus, and does not alter gene expression. In cancer cells as shown in Fig. 1.2.D, mutant *APC* can lead to an inability to regulate β-catenin and thereby activate the Wnt signaling pathway. β-catenin is not degraded in the proteosome and can accumulate in the cytoplasm and lead to nuclear translocation. Along with its binding partners Tcf/Lef, β-catenin can regulate gene expression. *APC* mutations are initiating events in sporadic CRC [34] in genetic models [32] and are found in clinical samples [35]. *APC* mutations have led to activation of survival genes [36] as a result.

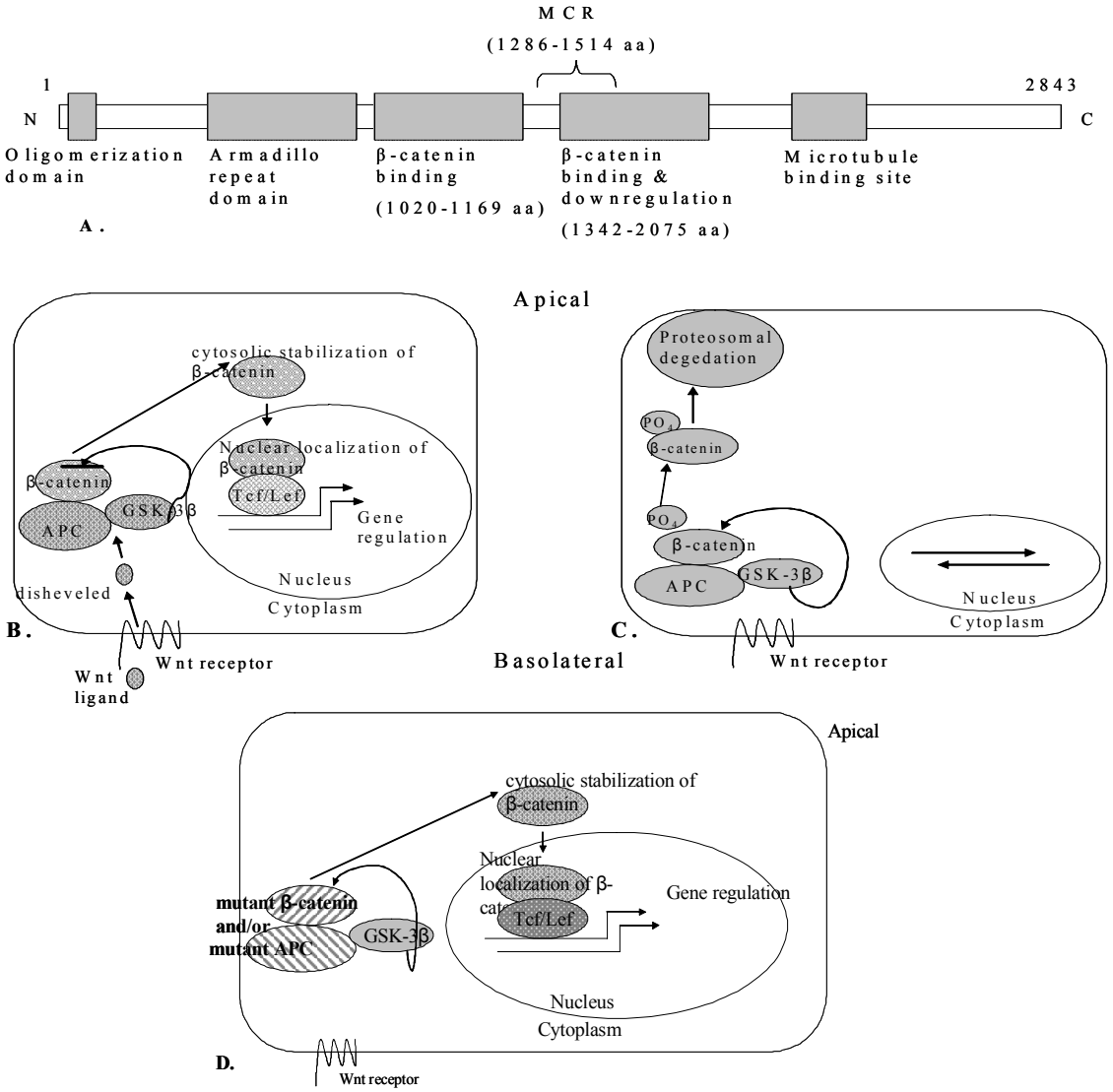


Figure 1.2. Adenomatous Polyposis Coli tumor suppressor gene and role in Wnt signaling. (A) *APC* gene structure and the regulatory regions. Most mutations in *APC* occur in the mutator cluster region (MCR) leading to truncated proteins. (B) Wnt signaling in normal developmental tissue involves the Wnt ligand activating the receptor to inactivate GSK-3β. β-catenin accumulates in the cytoplasm and leads to the nuclear translocation of β-catenin. With its binding partners T-cell factor (Tcf)/lymphoid enhancing factor (Lef), β-catenin can regulate gene expression. (C) Wnt signaling in normal adult tissue, the absence of the Wnt ligand allows for GSK-3β to remain active, marking β-catenin for proteosomal degradation. β-catenin does not accumulate in the cytoplasm or the nucleus, and does not alter gene expression. (D) Mutant APC can lead to Wnt signaling. β-catenin is not degraded in the proteosome and can accumulate in the cytoplasm lead to nuclear translocation. With its binding partners Tcf/Lef, β-catenin can regulate gene expression. *Adapted from [7, 9].*

Inflammation and Cancer

A causal interaction between chronic inflammation and cancer was hypothesized in 1863 by Virchow and may contribute to the tumor microenvironment [37]. Inappropriate inflammation may cause diseases such as, cardiovascular, inflammatory and autoimmune, neurodegenerative and cancer [38]. The milieu of chronic inflammation may be conducive for the development of cancers. The activation of NF-κB by inflammation may be an intermediary in the carcinogenic process [39]. Activation of gene targets by NF-κB can lead to a microenvironment filled with specialized leukocytes, cytokines and chemokines that facilitate cancer growth, invasion and metastasis [40]. Mouse models in which the NF-κB axis was disrupted, decreased tumor incidence [41, 42]. While some of these inflammatory molecules may exert an anti-tumor activity, in the context of genomic instability, mutations and selection pressures upon these molecules they may direct tumor growth [43].

Inflammatory Bowel Disease

Excessive recruitment of inflammatory molecules that leads to injury of the tissue has underscored the development of inflammatory bowel disease [44]. Patients with either inflammatory bowel disease, often have elevated cytokine levels [45]. The elevation in cytokine levels may contribute to the pathophysiology of the disease, notably IL-1-β elevation in ulcerative colitis and abnormal gut motility [46]. The levels of pro-inflammatory molecules may be related to diet, with low fat elemental diets leading to significant decreases in the systemic, endoscopic and histological inflammatory markers

[47]. Conversely, animal models that were fed high fat diets, showed increased inflammatory markers by gene expression and histology that were consistent with inflammatory bowel disease [48]. Patients with ulcerative colitis and Crohn's disease have an increased risk of developing colon cancer [49].

Metabolic Syndrome

In 2005 over 50% of all U.S. adults are overweight, or obese [13]. Metabolic Syndrome is described with factors of excess caloric intake (notably highly processed foods), obesity and decreased physical activity [50]. Obesity itself is a risk factor for developing cancers of the colon, breast, endometrium, kidney, and esophagus [51]. The risk for colorectal cancer is increased with smoking, reduced fiber intake and inactivity [52]. In addition, the risk of adenomas is increased among people who gain, rather than maintain, weight over time [53]. Activation of NF-κB also occurs through Metabolic Syndrome which involves a number of proinflammatory cytokines [54]. The clustering of these risk factors may provide a more plausible connection among obesity, chronic inflammation and cancer [55].

Inflammatory Molecules - Chemotactic Cytokines

Markers of inflammation include chemotactic cytokines, also known as chemokines [2]. Chemokines normally act to recruit leukocytes [43], aid in blood vessel remodeling [56], but also disrupt the basement membranes [57] and aid in metastasis [58]. Patients with CRC demonstrate elevated chemokines [59]. The expression level of many chemokines

is regulated by NF-κB [60] and substantiates their role in carcinogenesis [39]. Knock-out models of the NF-κB axis are associated with decreased tumor burden in mice [41, 61] while modulating NF-κB activation has been targeted for therapy [62]. The expression of many inflammatory molecules is governed by NF-κB and provides evidence for a strong link between chronic inflammation and cancer [60, 63]. A super-family of inflammatory molecules, known as chemotactic cytokines is found in normal and disease states. The chemotactic cytokines, or chemokines, are classified into four highly conserved groups based upon conserved cysteine residues found in the N-terminus portion of the molecule; C, CC, CXC, CXXXC [56]. The chemokines are small proteins (8-10 kDA) that bind to seven-transmembrane G-protein coupled receptors [58]. As shown in Table 1.1. is a summary of the 'CXC' family of chemotactic cytokines. CXCL8 was previously reported as interleukin-8 (IL-8). Currently chemokines are being evaluated as targeted therapies in a variety of cancers [64-66].

Class	New name	Old name	Abbreviaiton
CXC	CXCL1	Growth-related oncogene a	GROa
	CXCL2	Growth-related oncogene ß	GROß
	CXCL3	Growth-related oncogene γ	GROγ
	CXCL4	Platelet factor-4	PF-4
	CXCL5	Epithelial cell-derived neuthrophil-activating factor 78	ENA-78
	CXCL6	Granulocyte chemoattractant protein-2	GCP-2
	CXCL7	Neutrophil-activating protein-2	NAP-2
	CXCL8	Interleukin-8	IL-8
	CXCL9	Monokine induced by γ-interferon	Mig
	CXCL10	γ-Interferon -inducible protein-10	IP10
	CXCL11	Interferon-inducible T-cell a-chemoattractant	ITAC
	CXCL12	Stromal cell-derived factor-1	SDF-1
	CXCL13	B-cell-activating chemokine 1	BCA
	CXCL14	Breast and kidney chemokine	BRAK

Table 1.1. CXC Chemotactic Cytokines. The chemotactic cytokine super-family is classified according to the conserved, N-terminus, cysteine residues. The CXC family have an intervening amino acid between the cysteine residues. *Adapted from [2]*

Inflammatory Molecules - CXCL8

DCA has been shown to activate the proinflammatory cytokine CXCL8 (previously named IL8) in the HT29-parental cell line through the activation of NF-κB [67]. In addition, CXCL8 has been identified as a β-catenin-Tcf-4 target in hepatoma [10] and a Wnt/β-Catenin target in endothelial [7] cell lines, but not in colorectal cancer cell lines.

In vitro data has implicated CXCL8 in angiogenesis [68], as an autocrine growth factor [69], migration [70], and epithelial-mesenchymal-transition (EMT) [71] in cell culture models of the colon cancer. CXCL8 has been associated with the clinical progression of CRC. CXLC8 increased angiogenesis in normal tissue [72] and the hyperplastic colonic mucosa adjacent to colon cancer [73] as well as increased the risk for metastatic lesions [74].

Epithelial-Mesenchymal Transition (EMT)

Epithelial-mesenchymal transition (EMT) is an essential component of embryonic development, tissue remodeling, wound repair and is implicated in cancer progression [75]. Features of EMT are consistent with a loss of polarity and loss of adhesive constraints as well as morphology that is appropriate for migration [76]. These features are consistent with increased malignancy and therefore EMT provides a mechanism by which carcinoma cells may acquire a more aggressive phenotype [77]. Notably, nuclear localization of NF-κB is recognized as a feature of EMT [4].

CXLC8 has been identified as an integral part of EMT in colon carcinoma cells [71]. Increased CXCL8 has also been shown to up-regulate the expression and activity of matrix metalloproteinase-2 (MMP-2) [65, 78, 79]. Degradation of the extracellular matrix by matrix metalloproteinase has been the proposed mechanism by which MMP-2 exerts its effects [4, 80].

Statement of the Problem

Colorectal cancer has both environmental and genetic influences. These influences may lead to an inappropriate inflammatory cascade. Further, features of the inflammatory reaction contribute to the carcinogenic process. The purpose of this study is to determine the role of DCA in the carcinogenic sequence. It is unclear whether DCA acts on the normal colonic mucosa, or the genetically altered epithelium to affect inflammation. Therefore this research asks whether the bacterial derived, luminal risk factor, DCA can induce an inflammatory reaction in the normal or transformed colonic epithelium. DCA and Wnt signaling have been shown to increase CXCL8, but there is no evidence for wild-type *APC* to suppress CXCL8. Furthermore the aim of the study is to determine the role of the chemotactic cytokine, CXCL8, in CRC.

Hypothesis: Functional APC tumor suppressor gene suppresses DCA-mediated induction of the chemokine CXCL8 in human colorectal cancer.

Specific Aims:
1. Determine the mechanism of CXCL8 regulation
2. Determine the effects of CXCL8 in colorectal carcinogenesis
3. Determine if CXCL8 expression in human CRC is correlated with nuclear localization of β-catenin

CHAPTER 2:

MATERIALS AND METHODS

MATERIALS

Cholic acid, deoxycholic acid, zinc chloride, crystal violet, McCoy's 5a medium and citric acid were purchased from Sigma (St. Louis, MO). Ursodeoxycholic acid was purchased from Calbiochem (San Diego, CA). Dulbecco's Modified Eagle Medium (DMEM), hygromycin B, penicillin, streptomycin and HEPES buffer solution were purchased from Invitrogen Corporation (Carlsbad, CA). Recombinant human CXCL8 was purchased from R&D Systems (Cat # 208-IL) (Minneapolis, MN). Rabbit antihuman CXCL8 was purchased from Affinity BioReagents (cat #OMA1-03351) (Golden, CO). APC antibody (cat # OP44) was purchased from Calbiochem (San Diego, CA). β-catenin (cat #9562), c-Fos (cat #4384) and NF-κB p65 (cat #3034) antibodies were purchased from Cell Signaling (Danvers, MA). β-actin antibody was purchased from Sigma (cat #A 4700). NF-κB p50 antibody (cat #sc-8414) and β-catenin (cat #sc-1496) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MMP-2 antibody (cat # MS-8-4-P1ABX) was purchased from Lab Vision Corporation (Fremont, CA). Normal rabbit IgG (cat #sc-2027), horseradish peroxidase-conjugated goat anti-rabbit IgG (cat #sc-2004) and horseradish peroxidase-conjugated goat anti-mouse (cat #sc-2005) were purchased from Santa Cruz Biotechnology. Chromatin immunoprecipitation kits were purchased from Active Motif (Carlsbad, CA). Dual luciferase reporter (DLR) assay and Reverse Transcription kits were purchased from Promega (Madison, WI). RNA purification kits were purchased from Qiagen (Valencia,

CA). Plasmid DNA purification kits, NucleoBond, were purchased from BD Biosciences (Palo Alto, CA). PCR primers were purchased from Invitrogen Corporation. The 8.0 μM inserts were purchased from Falcon (Franklin Lakes, NJ). Nuclear and cytoplasmic extraction kit was purchased from Pierce (Rockford, IL).

METHODS

Cell Cultures. The HCA7 carcinoma cell line was a generous gift from Mark Nelson. The HCA7 cells were maintained in DMEM with 10% FBS, 1% P/S with glutamine, and 5 mL of 100 mM sodium pyruvate. The colon adenocarcinoma cell lines HCT116 and HT29 cell lines were purchased from American Type Culture Collection (ATCC) (Rockville, MD). Both the HCT116 and HT29 cell lines were maintained in McCoy's 5a media supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 μg/mL streptomycin. The HT29-APC and HT29-β-Gal were maintained in McCoy's 5a media with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, and 0.6 mg/mL hygromycin B for selection [81]. All cell cultures were kept in a humidified incubator at 37˚C with 5% CO_2.

The HCA7 cell line has mutant *APC* and wild type *β-catenin*. The HCT116 cell line has wild type *APC* and mutant *β-catenin* The HT29-parental cell line has mutant *APC* and wild type *β-catenin*. The HT29-APC cell line has an inducible wild-type *APC* gene under control of a metallothionine promoter. Wild-type *APC* was induced by exposure to 300 μM $ZnCl_2$ [82]. The HT29-β-Gal cell line served as a transfection control, with the

β-Galalctosidase gene inserted in place of wild type *APC*. Both the HT29-APC and HT29-β-Gal cell lines were pre-treated for 3 hours with 300μM $ZnCl_2$ and 1% FBS for optimal induction of wild type *APC* without significant Zn-induced toxicity [82]. A summary of the cell lines and conditions is found in Table 2.1.

Cell line	Characteristics	Media conditions
HCA7 *APC*^{mut}, β-catenin^{WT}	Colorectal carcinoma cell line	DMEM, 10% FBS, 1% P/S with glutamine, and 5mL of 100mM sodium pyruvate
HCT116 *APC*^{WT}, β-catenin^{mut}	Colorectal carcinoma cell line	McCoy's 5A, 10% FBS, 1% P/S
HT29-parental *APC*^{mut}, β-catenin^{WT}	Colorectal carcinoma cell line	McCoy's 5A, 10% FBS, 1% P/S
HT29-APC *APC*$^{WT\text{-}inducible}$, β-catenin^{WT}	Colorectal carcinoma cell line with a stably transfected, full length APC gene, driven by a zinc inducible metallothionien promoter.	McCoy's 5A, 10% FBS, 1% P/S with glutamine, and 600 μg/mL Hygromycin B
HT29-βGal *APC*^{mut}, β-catenin^{WT}	Colorectal carcinoma cell line with a stably transfected, β-galactosidase gene, that serves as a transfection control	McCoy's 5A, 10% FBS, 1% P/S with glutamine, and 600 μg/mL Hygromycin B

Table 2.1. Human CRC Cell Lines Multiple cell line models of colorectal cancer were used in the experiments. Mutational status of members of the Wnt signaling pathway, *APC* and β-catenin are listed with the cell lines. *Adapted from [5, 6]*

Cells were plated at a density of 2.5 x 10^6/10 cm dish with media that contain 10% FBS. On day 2, HCA7, HCT116 and HT29-parental cells were serum starved overnight, the HT29-APC and HT29-β-Gal cells were kept in media that contained 1% FBS overnight. On day 3, cells were treated with the appropriate compound. Cells were harvested at the indicated time for RNA or protein analysis.

Cell Cultures Treatments. All inhibitory or stimulatory agents were solubilized at 100X concentration stocks and then diluted in the medium. Compounds were sterilized by passing through a 0.2 μM sized pore at the end of a syringe, before they were used.

RNA Isolation. Cells were trypsinized, pelleted and total RNA was isolated by the Qiagen (Valencia, CA) RNeasy protocol. Briefly, the pellets were disrupted and lysed with a buffer containing guanidine thiocyanate and homogenized by passing the lysate 5 times through a 21 ½ gauge needle fitted to an RNase-free syringe. The column was spun and washed to remove the contaminants before eluting the sample.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR). The samples were quantified, reverse transcribed using Promega (Madison, WI) Reverse Transcription System (A3500) according to instructions by the manufacturer. Samples were incubated at room temperature for 10 min, heated to 42˚C for 15 min, 95˚C for 5 min and then incubated on ice for 5 min. The product served as a template (at 100 ng/μL) for the subsequent PCR reactions that utilized puRe Taq Ready-To-Go PCR Beads (Amersham

Biosciences, Piscataway, NJ). The template and primers (5 pmol/uL) were denatured at 94˚C for 5 min, then cycled at 94˚C for 1 min, 60˚C for 1 min and 72˚C for 2 min for 35 cycles. The CXCL8 primers were designed using Vector NTI, Suite 8 (Bethesda, MD), that yielded a 246 bp product: Sense -ATG ACT TCC AAG CTG GCC and Antisense -CAG ACA GAG CTC TCT TCC. GAPDH was used as a loading control with the 450 bp product: Sense-ACC ACA GTC CAT GCC ATC AC and Antisense TCC ACC ACC CTG TG CTG TA [83]. Products were visualized on agarose gels stained with ethidium bromide. A summary of the primer sequences and conditions is found in Table 2.2.

			Sequence	denature	anneal	polymerase	cycles
CXCL8	246 bp	For.	ATGACTTCCAAGCTGGCC	94°C	60°C	72°C	35
		Rev.	CAGACAGAGCTCTCTTCC				
GAPDH	450 bp	For.	ACCACAGTCCATGCCATCAC	94°C	60°C	72°C	35
		Rev.	TCCACCACCCTGTGACTGTA				
CXCL8 ChIP	313 bp	For.	CACCAAATTGTGGAGCTTCA	94°C	50.5°C	72°C	35
		Rev.	GGTGGTTTCTTCCTGGCTCT				
GAPDH	166 bp	For.	TACTAGCGGTTTTACGGGCG	94°C	59°C	72°C	35
		Rev.	TCGAACAGGAGGAGCAGAGAG CGA				

Table 2.2. Primer Sequences For PCR. Steady state CXCL8 mRNA was determined using primers designed to amplify across three of the four exons. CXCL8 ChIP primers were designed to amplify within the 5' promoter region that included binding sites for β-catenin, AP-1, C/EBP and NF-κB. [3] [7] [10]

Nuclear and Cytoplasmic Extraction. Cells were plated at a density of 2.5 x 10^6 cells per 10 cm dish in normal media. Cells were serum starved overnight, and then treated with vehicle or DCA for 2 hours. The packed cell volume was determined to be between 20-40 mg. The manufacturer's instructions were followed.

RIPA Lysis and TCA Precipitation. Cells were lysed on ice in radio immunoprecipitation assay (RIPA) buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 μg/mL phenylmethylsulfonyl fluoride, 50 mM sodium orthovanadate, and 10 μg/mL apoprotinin) and centrifuged for 10 minutes at 14,000 rpm at 4˚C. In this study 50 μg of total cellular protein extract was loaded per lane of a 15% polyacrylimide gel and electrophoresed on a sodium dodecyl sulfate polyacrylimide gel electrophretic (SDS-PAGE) apparatus. Alternatively, proteins were precipitated from the media by trichloroacetic acid (TCA) precipitation. A stock solution was made with 500 grams TCA in 350 mL deionized water. Proteins in the medium were precipitated with 1 volume TCA stock to 4 volumes sample. Samples were incubated at 4˚C for 10 minutes, and then centrifuged at 14,000 rpm at 4˚C for 15 minutes. The supernatant was aspirated and the pellet was washed with 200 μL cold acetone. Samples were centrifuged at 14,000 rpm at 4˚C for 15 minutes and the supernatant was aspirated. Cracking buffer was added and the samples were boiled for 10 minutes and then placed on ice for 10 minutes.

Western Analysis of Protein Expression. Samples were loaded onto a polyacrylimide gel and electrophoresed on an SDS-PAGE apparatus. The proteins were transferred electrophoretically to Hybond-C (Amersham Biosciences, Arlington Heights, IL) nitrocellulose membrane overnight. The blots were blocked in Blotto A (5% w/v non-fat dry milk, 0.05% Tween-20, and Tris-buffered saline (TBS) consisting of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 1 hr at room temperature. The primary antibodies were incubated in Blotto A with the blots for 2 hrs at room temperature. The CXCL8 antibody was used at a final concentration of 0.2 μg/mL and β-actin antibody at 1:1000 dilution. The blots were washed in TBS/0.05% Tween-20 (TBST). Secondary antibodies were incubated at room temperature for 1 hour and then washed with TBST. Detection was achieved with enhanced chemiluminescence (ECL) detection reagent (Amersham Biosciences, Arlington Heights, IL). Blots were stripped and reprobed with β-actin antibody as a loading control. All western blots shown are a representative of three or more experiments, unless otherwise noted.

The protocol for the APC western blot was provided by Calbiochem (San Diego, CA) and was carried out with slight modifications. Briefly, the cells were grown in 10 cm plates and lysed directly in cracking buffer (5% glycerol, 50 mM Tris (pH 6.8), 2 mM EDTA, 2% SDS, 144 mM 2-mercaptoethanol and 0.001% bromophenol blue). The lysate was sonicated to reduce viscosity, and 100 μL of extract was loaded per lane and run on a 5% polyacrylimide gel on SDS-PAGE apparatus. The protein was then transferred to Hybond-C nitrocellulose membrane overnight. The blots were blocked in

Blotto A for 1 hr at room temperature. The primary antibody was used at a dilution of 1:75 in Blotto A and incubated for 2 hours at room temperature. The blots were washed in TBST, three times for 10 minutes each. The primary antibody was then detected with a goat anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase for 1 hour at room temperature. Blots were washed with TBST, as described above and detection was achieved with ECL detection reagent. A summary table of the antibodies and conditions is compiled in Table 2.3.

Western

Antibody	Supplier	Cat #	Dilution	Diluant (TBST)	Temp	Time
CXCL8	ABR	OMA1-03351	0.2 µg/mL	5% NFDM	RT	2 hrs
β-catenin	Cell Signaling	9562	1:1000	5% BSA	4 ℃	O/N
AP-1, c-Fos	Cell Signaling	4384	1:1000	5% BSA	4 ℃	O/N
NF-κB, p65	Cell Signaling	3034	1:1000	5% BSA	4 ℃	O/N
NF-κB, p50	Santa Cruz	sc-8414	1:300	5% NFDM	RT	2 hrs
MMP-2	Lab Vision	MS-804-P1ABX	1:1000	5% NFDM	RT	2 hrs
APC	Calbiochem	OP44	1:100	5% NFDM	RT	2 hrs
β-actin	Sigma	A4700	1:2000	5% NFDM	RT	2 hrs

ChIP

Antibody	Supplier	Cat #	Quantity
β-catenin	Cell Signaling	9562	10 µL
AP-1, c-Fos	Cell Signaling	4384	10 µL
NF-κB, p65	Cell Signaling	3034	10 µL
NF-κB, p50	Santa Cruz	sc-8414	10 µL

Table 2.3. Antibodies used for experiments. The antibodies, dilutions and incubation conditions are listed for Western experiments as well as ChIP experiments.

Plasmids. The parent plasmid pXP2m was utilized in the construction of the promoter-reporter assays, as designed and constructed by Dr. Lazennec [3]. The promoter-mutants utilized the sequence from -1481 bp upstream from the start of transcription through to +44 bp beyond AUG transcriptional start site and sub-cloned into the BamH1 sites. As shown in Fig. 2.1., that backbone served as the template for promoter-reporters. The wild-type (WT) promoter-reporter contained the full promoter region (-1481, +44), without mutations. The other promoter-reporters: AP-1^{mutant}, C/EBP ^{mutant}, NF-κB ^{mutant} contained mutated sequences for the binding of the different transcription factors.

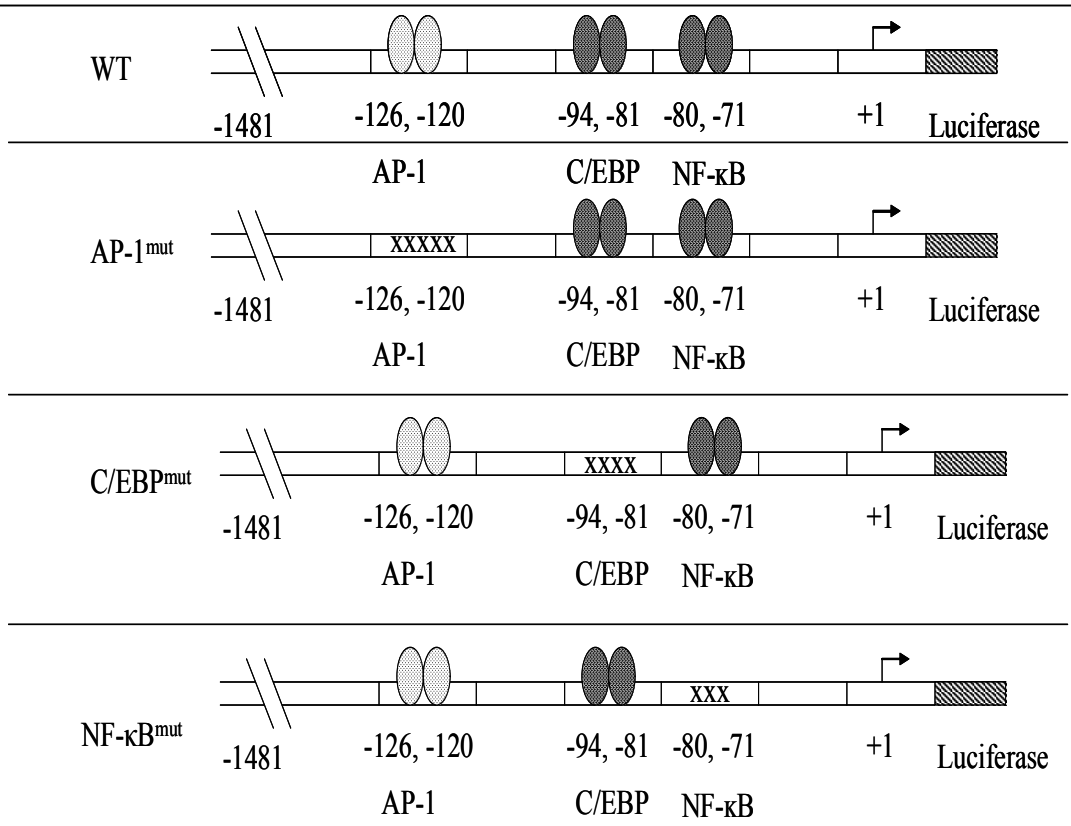


Figure 2.1. CXCL8 Promoter-Reporter Constructs. CXCL8 promoter-reported constructs that have mutations of AP-1, C/EBP or NF-κB were transfected into CRC cell lines. *Adapted from [3]*

Transient Transfection of Luciferase Reporter Constructs. For this study, 4.5 x 10^5 HT29-parental, -APC, or –β-Gal cells were plated per well of a 6-well dish and transfected with 2.5 μg plasmid DNA/well using Lipofectamine 2000 (cat #11668-027) according to manufacturer's instructions. For the HCT116 cells, 3.0 x 10^5 cells were plated per well and 1.0 μg plasmid DNA/well was transfected using Lipofectamine 2000 The HT29-parental, HT29-APC and HT29-β-Gal cells were transfected with a plasmid that contained the full length CXCL8 promoter luciferase reporter (-1481, +44) [3]. A renilla luciferase reporter was used as a transfection control. After transfection, cells were incubated for 24 hours. HT29-parental cells were exposed to 300 μM deoxycholic acid or control. The HT29-APC and HT29-β-Gal cells were treated with 300 μM $ZnCl_2$ or control for 3 hours and then were exposed to 300 μM deoxycholic acid or control. All cells were incubated overnight at 37˚C with 5% CO_2 and lysed the next day. The samples were analyzed using the dual luciferase reporter method. The ratio of firefly to renilla luciferase was reported.

Chromatin Immunoprecipitation (ChIP). Cells were plated at a density of 2.0 x 10^7 per 15cm plate in normal media. Cells were serum starved overnight, and then treated with DCA, or control for 8 hours. The manufacturer's protocol was followed with 3 plates per treatment group. DNA was sheared for at 37 ˚C for 10 minutes with enzyme. The chromatin was pre-cleared on day 2 of the protocol for 1.5 hours. To each 150 μL aliquot of pre-cleared chromatin, 10 μL (2 μg) of antibody was added. The tubes were incubated overnight, on a rotator, at 4˚C. On day 3, tubes were incubated for 1.5 hours at

4˚C with beads. Subsequent washes and elution were done according to instructions. RNA was removed with RNAse treatment and cross-links were reversed in a 65˚C water bath overnight. Each of the aliquots was then treated with Proteinase K for 2 hours. The purified DNA was eluted as per the manufacturer's instructions. Amplification of the DNA was achieved with 10 pmol of each primer. Chromatin Immunoprecipitation primers were designed with Vector NTI and yielded a 313 bp product: Sense - CAC CAA ATT GTG GAG CTT CA and Antisense – GGT GGT TTC TTC CTG GCT CT. For CXCL8, DNA was denatured at 94˚C x 5 min, then: 94˚C x 1 min, 50.5˚C x 1 min, and 72˚C x 2 min for 35 cycles followed by a 72˚C x 10 min extension. For GAPDH, DNA was denatured at 94˚C x 3 min, then: 94˚C x 20 sec, 59˚C x 30 sec, and 72˚C x 30 sec for 35 cycles followed by a 72˚C x 10 min extension. Products were visualized on a 2% agarose gel, stained with ethidium bromide.

Invasion Assays. In a 24-well plate, 500 μL of serum free media was aliquoted into each well. Cell culture inserts were pre-treated with 1μg of laminin-1 on each side and allowed to dry. Inserts were then placed into the cell plate. The HT29-parental cells were grown to 80% confluence and then harvested by trypsinization. The HT29-APC and –β-Gal cells were pretreated with 300 μM Zn for three hours and then trypsinized. The suspension was spun in a centrifuge at 1800 rpm at 4˚C for 5 min. The supernatant was removed and the cell pellet was resuspended in serum free media, at a concentration of 1.0×10^6 cells/mL. After cells were resuspended, they were dosed with the appropriate compound. Into the upper portion of the insert, 200 μL of cell suspension was

plated. The HT29-parental cell line was allowed to migrate for 48 hours. The HT29 APC/β-Gal cell lines were allowed to migrate for 24 hours, based on apoptosis data [82]. The inserts were removed, inverted and washed 3 times with HEPES buffer. Cells were removed from the inside of the insert with cotton swabs. Cells that had invaded through the insert were stained with 100 μL crystal violet solution (0.5% crystal violet, 20% methanol, 80% water). The crystal violet solution was washed with deionized water after 1 minute. The inserts were dried overnight. Then the membrane was cut out of the insert and placed into a 96-well plate. The crystal violet was dissolved with 200 μL 0.1 M citric acid. The dye was completely dissolved and then the solution was transferred to a new well. The optical density was read at 562 nm on BIO-TEK Instruments EL800 universal microplate reader.

Statistical Analysis. P-values were generated by either Student's t-test or Analysis of Variance (ANOVA), dependent upon the qualification of the data. Contingency tables were also constructed to determine the association of variables. Fisher's Exact values were reported. A p-value of 0.05 or less considered significant.

Immunohistochemisty (IHC). The Human Subjects Protection Program, at the University of Arizona reviewed an approved the Immunohistochemistry protocol. Informed consent from each participant was obtained at the time of the procedure. Patients undergoing treatment for colorectal cancer had a colonic resection. Tissue samples were obtained from the cancer, adjacent tissue and tissue 10 cm from the anal

verge that was considered to be normal. Samples were fixed in 10% neutral buffered formalin for 24 hours, processed and paraffin embedded. Immunohisochemistry (IHC) was performed by the Tissue Acquisition and Cellular/Molecular Analysis Shared Service (TACMASS) Core facility at the Arizona Cancer Center. IHC was performed on three micron sections of the tissue cut from the formalin fixed, paraffin embedded blocks (FFPE) and placed on glass slides. IHC was performed using the Affinity BioReagents CXCL8 antibody (cat #OMA1-03351) diluted to 1:100. IHC was also performed using the Santa Cruz β-catenin antibody (cat # sc-1496) diluted to 1:100. The slides with the samples were stained on a Discovery XT Automated Immunostainer from Ventana Medical Systems, Inc. (VMSI) (Tucson, AZ). Deparffinization, cell conditioning (antigen retrieval), primary antibody staining, detection with biotinylated-streptavidin-HRP and DAB, and hematoxylin counterstaining were performed on this instrument using VMSI validated reagents. Following staining on the instrument, slides were dehydrated through graded alcohols to xylene and cover slipped using Pro-Texx mounting medium. Images were captured using an Olympus BX50 and Spot camera (Model 2.3.0.). Images were standardized for light intensity. No automated analysis of the data was performed. The slides were read by a pathologist who was blinded to the study.

CHAPTER 3

Nuclear Factor κB ACTIVATION BY DCA

INTRODUCTION:

The causal effects of chronic inflammation upon carcinogenesis were suspected long before many of the molecular mechanisms had been identified [84]. Nuclear factor κ-B (NF-κB) is a transcription factor that is activated through various signals and leads to the production of molecules associated with inflammation. NF-κB can be activated by bacteria, viruses and inflammatory molecules and may lead to the cells evading apoptotic mechanisms as well as inducing growth signals and angiogenic factors [85]. NF-κB plays a central role in disease and significantly in carcinogenesis [61, 86]. Mouse models of colon carcinogenesis have confirmed the role of inflammation, via NF-κB [41]. As such, the NF-κB pathway is considered a target for anti-cancer therapeutics [62, 87].

CRC incidence has been associated with highly processed and high fat foods of the Western diet [15]. Bile acid, specifically DCA can induce NF-κB nuclear translocation, in esophageal and colon models [67, 88]. Early evidence of DCA's carcinogenic potential by Cook et al in 1940 [19, 20] has been substantiated by epidemiological [15], physiological [21, 22] and laboratory models [23]. Further evidence in clinical [24] and animal [25] models associated increased risk of CRC with elevated bile acid. DCA is a secondary bile acid that is a bacterial metabolite and has been associated with hyperproliferation, dysplasia, invasion and metastatic cancer [26] and stimulates migration of CRC cell lines [23, 89, 90]. DCA alters intracellular signaling pathways

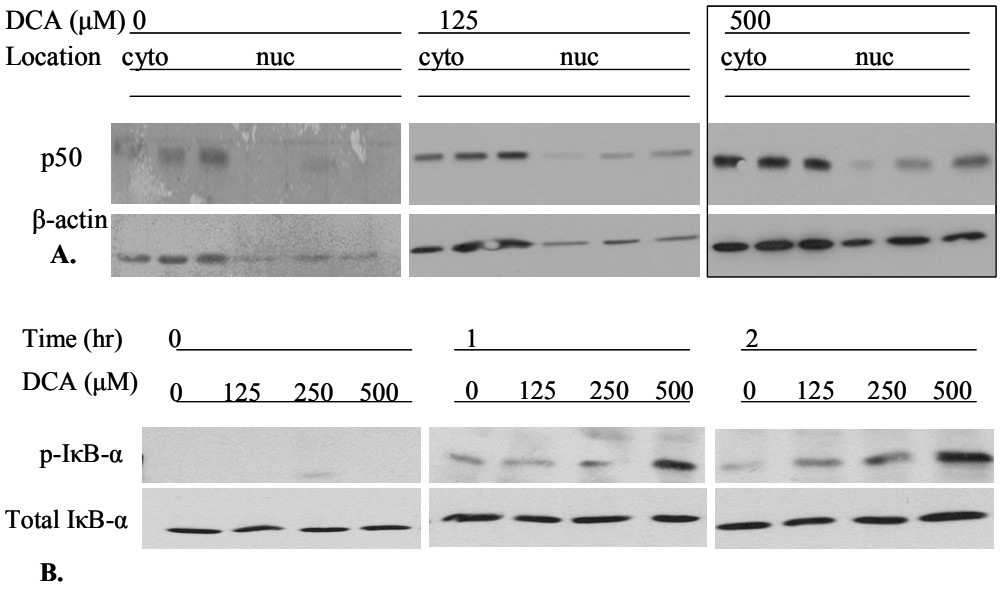
[27, 91] through the expression of activator protein-1 (AP-1) [28] as well as C/EBP [29] and NF-κB [30].

The purpose of the study was to determine the role of DCA-mediated inflammation. Colorectal carcinogenesis is a complex series of steps. Throughout carcinogenesis, NF-κB has been identified to play a role through its nuclear localization and activation of survival genes. This study investigated how DCA activated NF-κB, and what were its effects. Further, the aim of the study was to determine whether inflammation was sensitive to primary, secondary or tertiary bile acids.

RESULTS:

DCA increases NF-κB nuclear localization in CRC cell lines. The effects of DCA on NF-κB were assessed in the HCA7 cell line. As shown in Fig. 3.1.A., treatment of the HCA7 cell line with 125 μM DCA as well as 500 μM DCA increased the nuclear translocation of the NF-κB p50 subunit within 2 hours. This is consistent with previous reports that DCA could affect the translocation, and activation of NF-κB [67].

DCA increases IκB-α phosphorylation (Ser 32/36) in CRC cell lines. To determine the mechanism of NF-κB activation, the status of the negative regulator IκB was investigated. As shown in Fig. 3.1.B., the data revealed that the phosphorylation occurred at serine residues 32 and 36, when using an antibody that was raised against the serine phosphorylated IκB-α subunit.

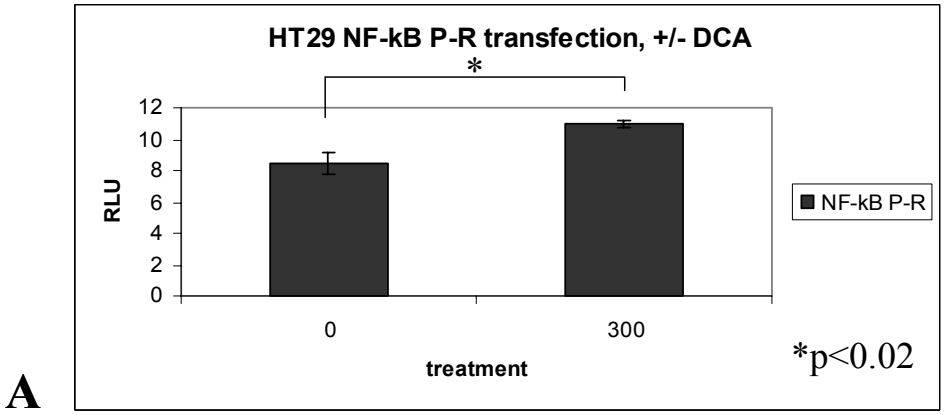


Western analysis

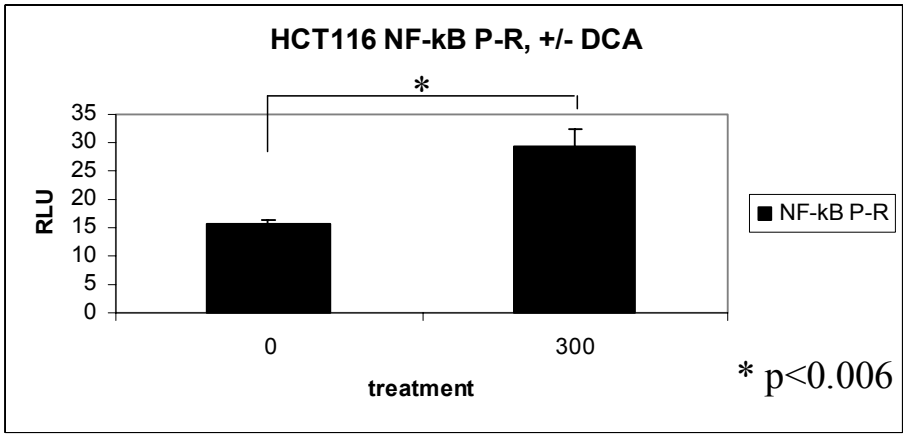
Figure 3.1. DCA increases NF-κB activation by inactivating its repressor. HCA7 cells were plated, serum starved and dosed with various concentrations of DCA. Lysates were collected, separated by SDS-PAGE and probed with the appropriate antibody. (A) The NF-κB p50 subunit increased nuclear localization with increased DCA and time. (B) The ratio of the phosphorylated to non-phosphorylated protein, increased with DCA exposure over time

DCA increases NF-κB promoter-reporter activity in CRC cell lines. To validate the role of DCA to activate NF-κB, promoter-reporter studies were done. As shown in Fig. 3.2., the addition of 300 μM DCA in both HT29-parental (3.2.A.) and HCT116 (3.2.B.) cell lines activated the NF-κB promoter-reporter.

DCA-mediated induction of inflammatory molecules. A group of inflammatory molecules have NF-κB binding sites in the promoter sequences of the genes. The inflammatory genes; NOS-2, IL-1β, TNF-α and CXCL8 were investigated for changes in their expression profiles in response to DCA. As shown in Fig. 3.3., the pro-inflammatory molecule, NOS-2, IL-1β, TNF-α did not appear to change their expression levels. The larger pro-IL1-β was consistent over time, without significant production of the active IL1-β. Similarly, the production of TNF-α and *NOS-2* did not appear to have a significant change over time. By contrast to the previous negative data, DCA-mediated CXCL8 was significant. This was not expected as the pro-inflammatory molecules; TNF-α, IL-1-α, IL-6 and CXCL8 are all NF-κB-dependent cytokines [92, 93].

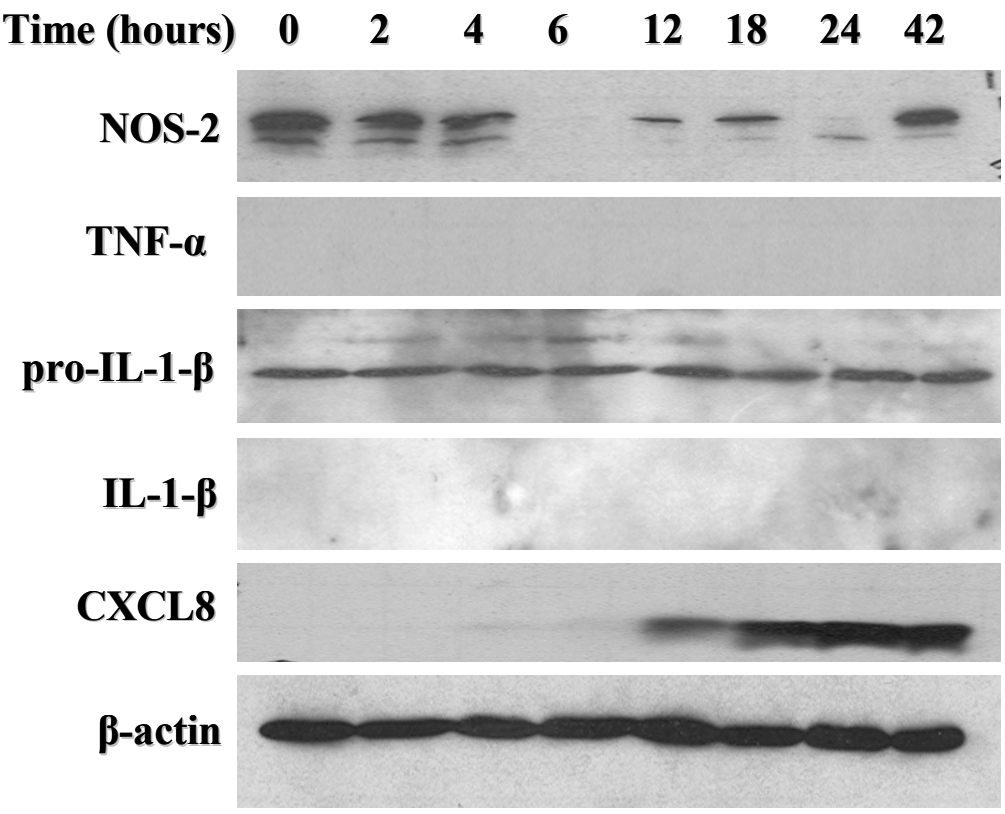


A



B

Figure 3.2. DCA activation of NF-κB promoter-reporter assay. Cells were plated in 6-well plates, transfected , treated with DCA for twenty four hours and then lysed. (A) The HT29 cell line had an induction of the NF-κB promoter-reporter system by DCA. (B) The HCT116 cell line had an induction of the NF-κB promoter-reporter system by DCA.



Western analysis

Figure 3.3. DCA-mediated inflammatory molecules with NF-κB binding sites. The HCA7 cell line was plated, serum starved, exposed with DCA for twenty four hours and then total cell lysate was run on an SDS-PAGE. Antibodies for the inflammatory molecules were used to probe the blots.

DCA, not CA or UDCA, increases CXCL8 protein in HT29-parental cell lines. To determine if the DCA-mediated CXCL8 production could be generalized to all bile acids, the HT29-parental cell line was plated in the presence of three different bile acids. The first bile acid was cholic acid (CA), the primary bile acid from which DCA is derived. The second bile acid was DCA, the bacterial metabolite that is a luminal risk factor for CRC. The third bile was the chemopreventative, tertiary bile acid, ursodeoxycholic acid (UDCA). As shown in Fig 3.4., neither CA nor UDCA induced the expression of CXCL8, at any of the given concentrations. DCA was able to induce CXCL8 protein expression at a variety of concentrations.

DCA-mediated dose-response curve in CRC cell lines. To determine the range of DCA that would induce the expression of CXLC8, a dose-response curve analysis was performed. As shown in Fig. 3.5.A., DCA induced the expression of CXCL8 through a range of concentrations from 150 μM to 500 μM in the HT29-parental cell line. As shown in Fig. 3.5.B., DCA induced the expression of CXCL8 through a range of concentrations from 200 μM to 400 μM in the HCT116 cell line.

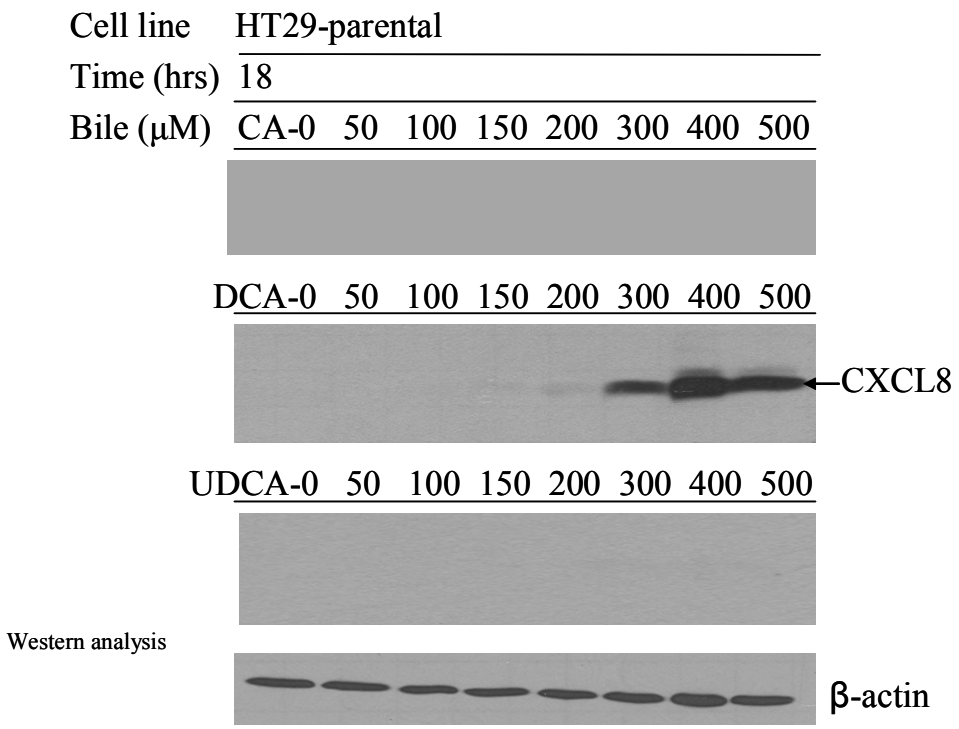


Figure 3.4. Bile acid-mediated induction of CXCL8. The HT29-parental cell line was plated, serum starved, exposed to various concentrations of different bile, and then lysed. Total cell lysate was separated by SDS-PAGE and antibody directed against CXCL8 was used to measure the effects of bile acid exposure.

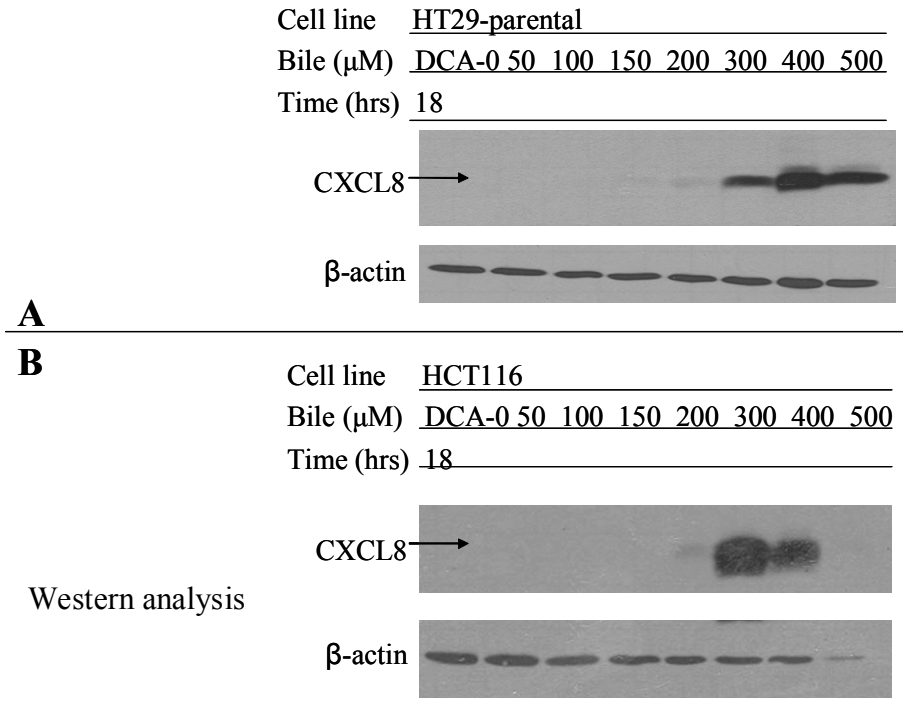


Figure 3.5. DCA-mediated CXCL8 dose-response. CRC cell lines were plated, serum starved, exposed to DCA for 18 hours and then lysed. Total cell lysate was separated by SDS-PAGE and the blot was probed with antibody directed against CXCL8.

DCA increases CXCL8 steady state protein in multiple CRC cell line models. To determine if DCA-mediated CXCL8 expression could be generalized, multiple cell lines were exposed to DCA. As shown in Fig. 3.6., cell lines in both the CIN-pathway (HT29) and the MIN-pathway (HCA7 and HCT116) increased the expression of CXCL8 when exposed to 300 μM DCA.

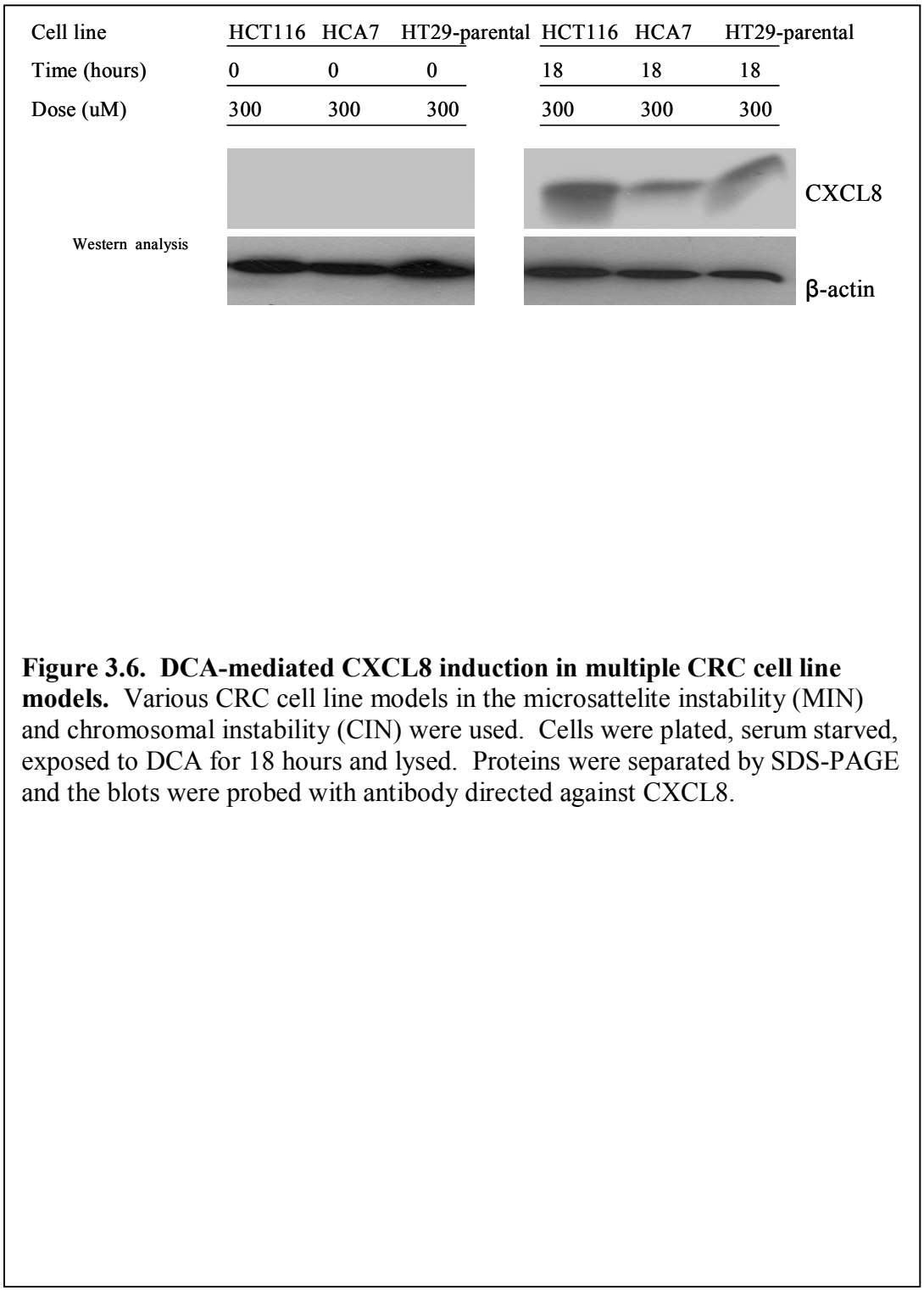


Figure 3.6. DCA-mediated CXCL8 induction in multiple CRC cell line models. Various CRC cell line models in the microsattelite instability (MIN) and chromosomal instability (CIN) were used. Cells were plated, serum starved, exposed to DCA for 18 hours and lysed. Proteins were separated by SDS-PAGE and the blots were probed with antibody directed against CXCL8.

The purpose of the study was to determine the role of bile acid-mediated inflammation in CRC cell lines. The aims were to demonstrate that DCA could activate intracellular signaling pathways that were consistent with inflammation. In addition, the aims were to identify inflammatory molecules that are mediated by DCA. Finally, the aims were to determine whether the inflammatory molecules were sensitive to various bile acids or specific to DCA.

NF-κB is a key mediator of inflammation in carcinogenesis [41, 61, 62, 84, 85]. DCA has been shown to activate NF-κB [27, 30, 94]. Consistent with the report by Mulbauer, *et al*, the negative regulator IκB-α was phosphorylated [67]. The specific serine residues, Ser-32 and Ser-36 on IκB-α are a novel finding. The phosphorylation of IκB-α is consistent with the marked degradation of IκB, by phosphorylation and then ubiquitination [84]. Although there does not appear to be an appreciable amount of IκB-α degradation, the 2 hour time course may not have been sufficient to detect the changes. While NF-κB may be associated with apoptosis [27], the resistance to apoptosis and the activation of survival genes may play a more significant role in colon carcinogenesis [30, 94, 95].

The activation of the promoter-reporter systems is consistent with NF-κB activation. It validates the finding that DCA is an important bacterial derived, luminal risk factor that may play a role in colon carcinogenesis through NF-κB activation.

While activation of NF-κB may be necessary, it is not sufficient. Multiple inflammatory molecules have NF-κB binding sites in their promoter regions [92, 93]. When the HT29-parental cell line was exposed to DCA, only CXCL8 was induced. This was surprising given the finding that bile acids induce reactive nitrogen species [96]. It was also unexpected as Nos2 knockout mice have shown attenuated deoxycholic acid-induced colitis [97]. Yet, further analysis revealed that in *in vitro* models, DCA-resistant cell lines demonstrated an increase in the NOS-2 expression, not the DCA-sensitive cell lines [98]. Other stimuli have been shown to induce TNF-α and IL-1-β, but in other model systems [92, 93].

DCA-mediated induction of CXCL8 was consistent with previous reports [67]. It was surprising to find that neither CA nor UDCA increased CXCL8 protein expression through a range of concentrations. Upon further analysis, the finding resonates with the proposed mechanism of intracellular signaling. DCA has been shown to activate pathways that include NF-κB as well as AP-1 and C/EBP [27-29, 91, 99]. By contrast, neither CA nor UDCA activate these pathways with the same results [17, 23, 27, 28].

In summary, it was determined that DCA, not CA nor UDCA, is permissive for characteristics associated with inflammation through the activation of NF-κB and production of CXCL8.

CHAPTER 4:

FULL LENGTH *APC* SUPPRESSES DCA-MEDIATED CXCL8

INTRODUCTION:

The Wnt pathway regulates many normal cellular processes. Some of the normal processes include regulation of morphology, proliferation, migration, cellular adhesion, and remodeling [100]. Shown in Table 4.1., is a partial list of genes activated by Wnt signaling. Both *APC* and β-catenin are integral members of the Wnt signaling family. β-catenin is regulated by *APC,* through the phosphorylation, ubiquitination and degradation of β-catenin [101]. Over 80% of sporadic CRC harbor somatic mutations in the APC gene or mutations of its binding partner β-catenin [9]. *APC* mutations are thought to be transforming events in sporadic CRC [34] found in clinical samples [35] and in genetic models of CRC [32]. Loss of function of the *APC* tumor suppressor gene, through inactivation of both alleles, is an initiating event in familial and sporadic cases of CRC [31-33, 102]. *APC* mutations have led to activation of survival genes that increase the proliferation of the cell [36, 82].

The proinflammatory cytokine IL-8 (also named CXCL8) has been identified as a β-catenin-Tcf-4 target in hepatoma [10] and a Wnt/β-Catenin target in endothelial [7] cell lines, but not in colorectal cancer cell lines. Increased CXCL8 has been associated with growth advantages of malignant cells [103]. *In vitro* data demonstrate that CXCL8 has been implicated in angiogenesis [68], as an autocrine growth factor [69], migration [70], and epithelial-mesenchymal-transition (EMT) [71] in models of the colon.

c-myc	human colon cancer	up
Cyclin D	human colon cancer	up
Tcf-1	human colon cancer	up
LEF1	human colon cancer	up
PPAR delta	human colon cancer	up
c-jun	human colon cancer	up
fra-1	human colon cancer	up
uPAR	human colon cancer	up
MMP-7	human colon cancer	up
Axin-2	human colon cancer	up
Nr-CAM	human colon cancer	up
ITF-2	human colon cancer	up
Gastrin	human colon cancer	up
CD44	human colon cancer	up
Eph/ephrin-B	human colon cancer	up/down
BMP4	human colon cancer	up
claudin-1	human colon cancer	up
Survivin	human colon cancer	up
VEGF	human colon cancer	up
FDF18	human colon cancer	up
Met	human colon cancer	up
endothelin-1	human colon cancer	up
c-myc binding protein	human colon cancer	up
CXCL8	**endothelial cells**	

Table 4.1. Genes activated by Wnt signaling. Genes involved in various aspects of normal cellular functions are regulated by the Wnt pathway. *Adapted from [1]*

The chemokine super-family can be subdivided into 2 main families, based on conserved cysteine residues; CXC and CC with 2 smaller families; CX_3C and C [2, 56-58, 104]. The CXC family is further divided into glutamate-leucine-arginine positive (ELR+) or glutamate-leucine-arginine negative (ELR-). CXCL8 is and ELR+ chemokine the binds to 7-transmembrane, g-coupled receptors, CXCR1 or CXCR2 [105, 106].

Increased amounts of the bacterial-derived, luminal risk factor, DCA is a risk factor for CRC [22] and can induce CXCL8 production [67]. CXCL8 is regulated through DNA promoter elements; AP-1, C/EBP and NF-κB as well as mRNA stability factors [107-109]. DCA alters intracellular signaling pathways [27, 91] through the expression of activator protein-1 (AP-1) [28] as well as nuclear factor-kappa B (NF-κB) [30] and CAAT/enhancer-binding protein (C/EBP) [29].

RESULTS:

DCA induces CXCL8 mRNA and protein. The effects of DCA on CXCL8 expression were assessed in HT29-parental and HCT116 cell models. Shown in Fig. 4.1.A, steady state CXCL8 mRNA and protein were elevated within 1.5 and 4.5 hours of DCA treatment respectively. In Fig. 4.1.B., mRNA and protein levels were increased by 6 hours of treatment with 300 μM DCA and were sustained for 24 hours. Similar results were found in HCT116 in Fig. 4.1.C. This effect was not seen when the cells were treated with the primary bile acid precursor, cholic acid, or with the tertiary bile acid, ursodeoxycholic acid, previously shown in Fig. 3.4.

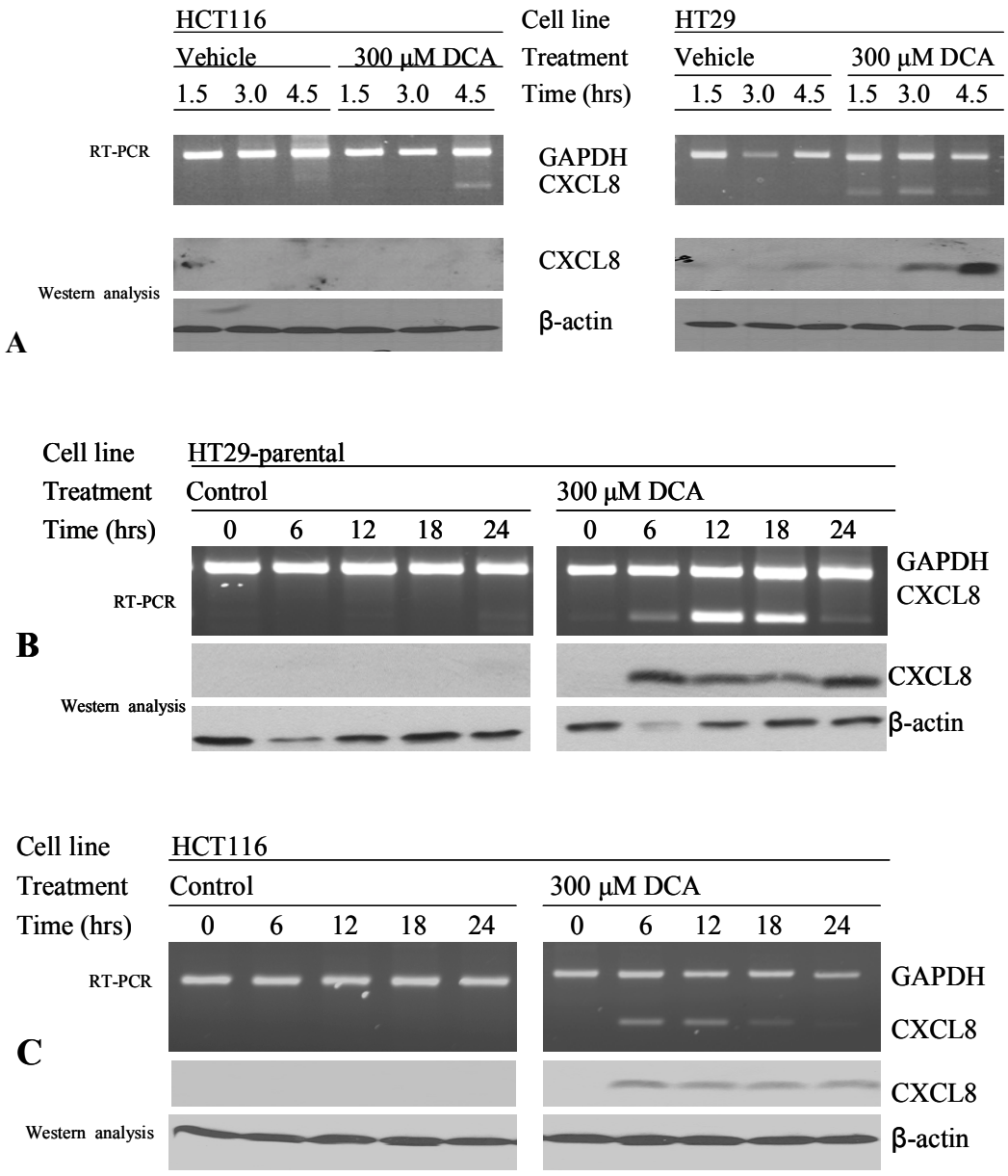


Figure 4.1. DCA-mediated CXCL8 induction. Cells were plated, serum starved, and exposed to DCA for the times indicated. Cells were harvested for either RNA or protein extraction. Primers or antibody were directed against CXCL8 to quantify induction. (A) DCA induces CXCL8 mRNA before protein. (B) CXCL8 mRNA is elevated in HT29-parental cell lines. (C) CXCL8 mRNA and protein is elevated in the HCT116 cell line

Full length *APC* suppresses DCA-mediatedCXCL8 mRNA and protein. To determine the influence of *APC* status on the expression of CXCL8 in colonocytes, HT29-APC and HT29-β-Gal cells were utilized. The HT29-APC cell line has a stably transfected functional *APC* gene under zinc control, whereas the HT29-β-Gal cell line is a transfection control with the β-galactosidase gene. As seen in Fig. 4.2.A., the HT29-APC cells treated with 300 μM $ZnCl_2$ for 3 hours induced full length *APC* but not the HT29-β-Gal cells. For this experiment, the HT29-parental cell line served as a negative control for wild type *APC* and the HCT116 cell line served as a positive control for wild type *APC* also seen in Fig 4.2.A. To determine the effect of *APC* influenced inflammation by DCA, HT29-APC and HT29-β-Gal cells were pretreated with zinc or control for 3 hours. Cells were then treated with either 300 μM DCA, or control for 18 hours. The HT29-APC cells differ in CXCL8 expression by their *APC* status. Mutant *APC* was permissive for the DCA-mediated CXCL8 production, but wild type *APC* was restrictive for the DCA-mediated CXCL8 production at both the mRNA level and protein level, as shown in Fig. 4.2.B. The HT29-β-Gal cells did not significantly alter the steady state CXCL8 mRNA or protein in response to DCA treatment.

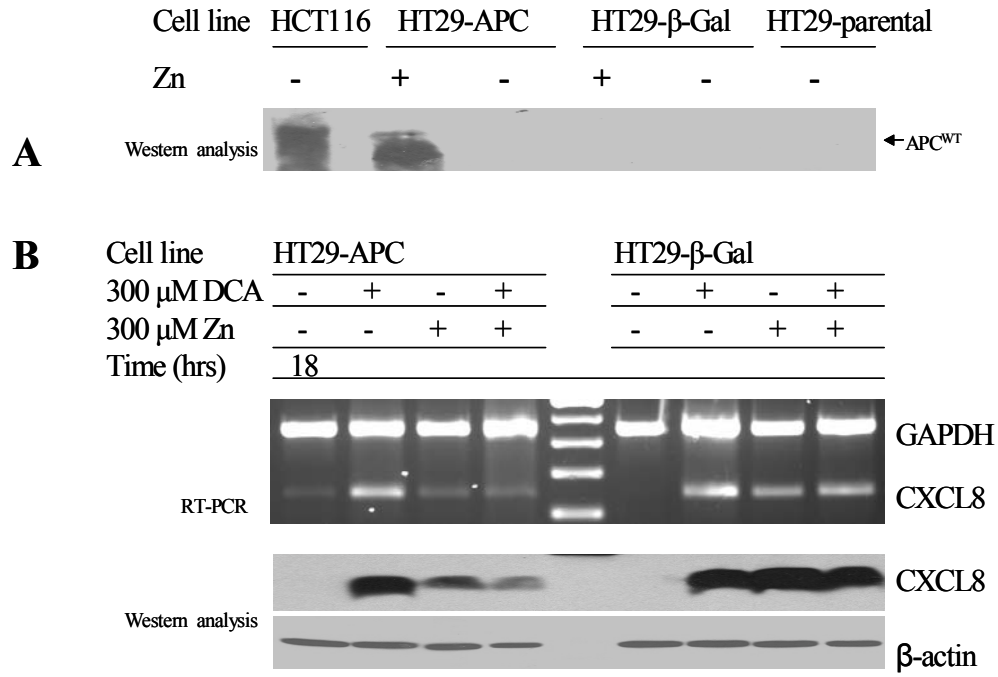


Figure 4.2. Full length *APC* suppresses CXCL8 mRNA and protein. HT29-APC and HT29-β-Gal cells display different characteristics with various treatments. (A) The HT29-APC and HT29-β-Gal cells were plated, serum starved, exposed to zinc for 3 hours and lysed. The proteins were separated by SDS-PAGE and the blot was probed with antibody directed against *APC*. HT29-parental served as a negative control for full length *APC* and HCT116 served as a positive control for *APC*. (B) The HT29-APC and HT29-β-Gal cells were plated, serum starved and exposed to zinc (or control) for three hours and then exposed to DCA (or control) for 18 hours. The cells were either trypsinized or lysed for mRNA or protein collection. Primers or antibody were directed against CXCL8 for detection.

Full length *APC* suppresses DCA-induced CXCL8 promoter activity. To determine the mechanism of increased CXCL8 expression, promoter-reporter assays were performed. Shown in Fig. 4.3.A., is a representation of the CXCL8 5'-promoter region. Shown in Fig. 4.3.B., the HCT116, HT29-parental and HT29-APC (without zinc) cell lines had a 2.5 fold induction of the CXCL8 promoter activity after the addition of 300 μM DCA ($p<0.00001$). Shown in Fig. 4.4.A. the HT29-parental cell line had a 2.5 fold induction with 300 μM DCA. Conversely, in Fig. 4.4.B., the HT29-APC cell line had greater than 80% suppression in promoter activity by wild type *APC* when compared to the mutant *APC* controls ($p<0.01$). The HT29-β-Gal cells had a roughly 40% decrease in promoter activity when treated with Zn and DCA.

To further investigate CXCL8 induction by DCA, HT29-parental cells were transfected with promoter-reporter constructs containing the promoter region from -1481, +44 (WT) or with promoter-reporters with point mutations for: AP-1, C/EBP, and NF-kB. Shown in Fig. 4.5., DCA-mediated luciferase activity was decreased by 80%, 75% and 95% for AP-1, C/EBP and NF-κB mutated reporter constructs compared to the WT promoter (-1481, +44). The reduction in luciferase activity by the promoter-mutation constructs indicated that CXCL8 expression was dependent upon the activation of these transcription factors. Similar results were found for the HCT116 cell line.

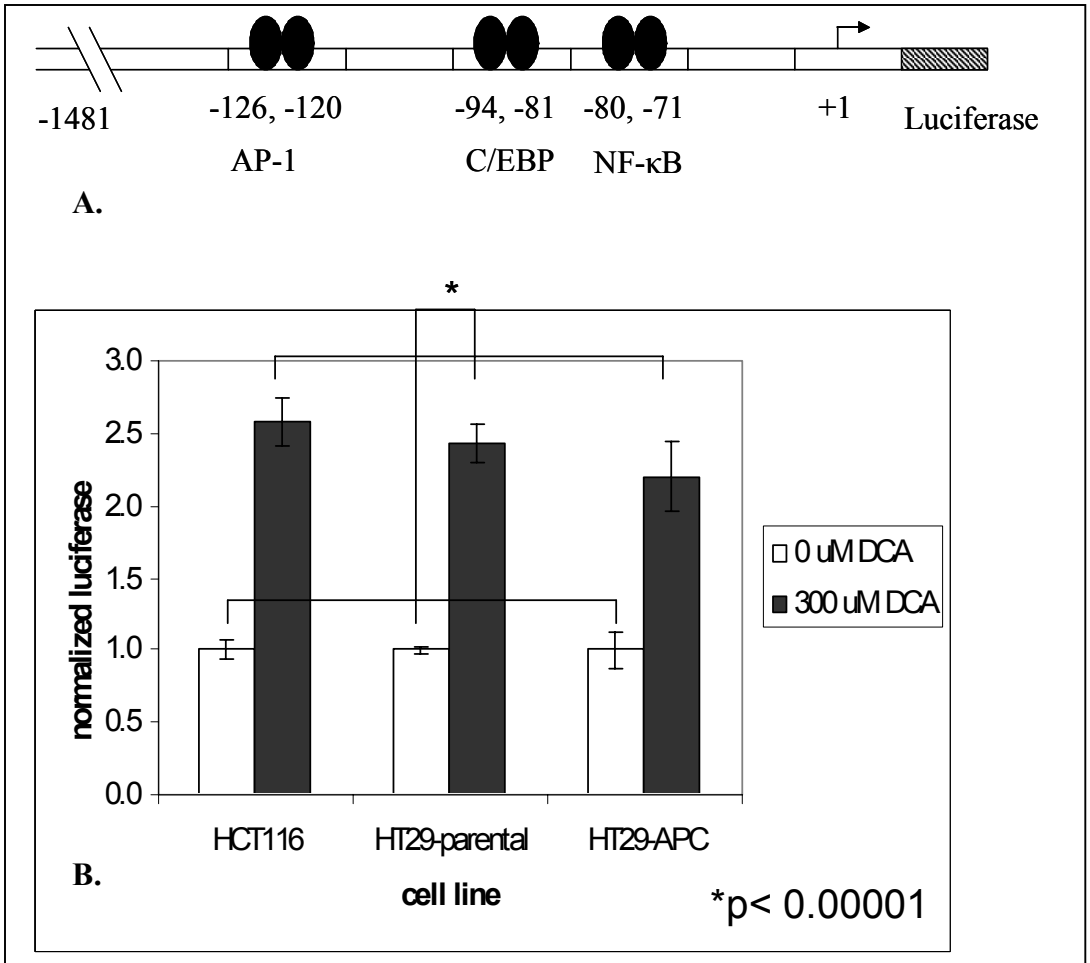
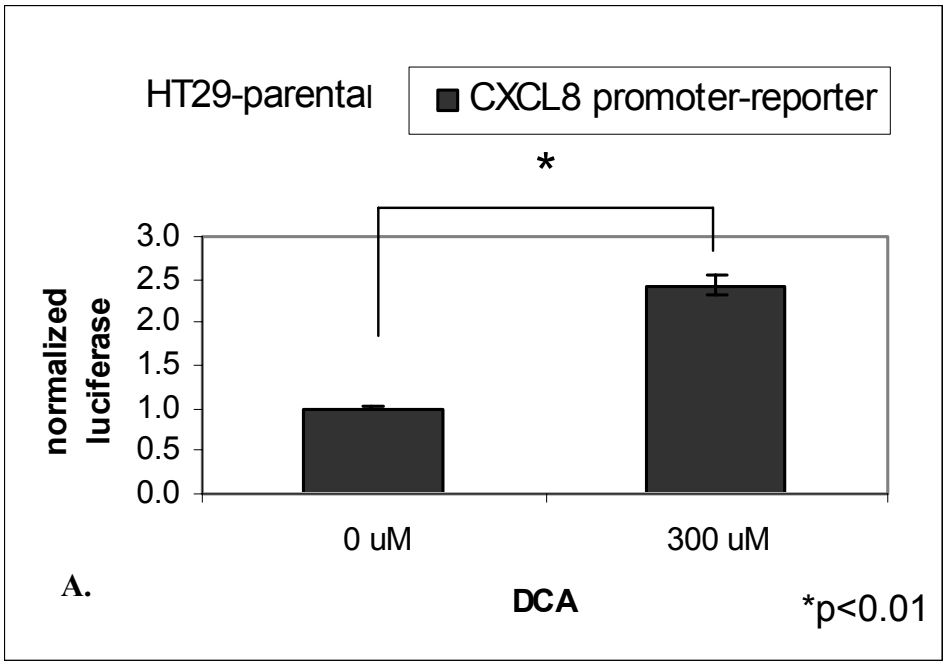


Figure 4.3. CXCL8 promoter and DCA-mediated promoter-reporter activity. (A) A schematic representation of the CXCL8 5'-promoter with transcription factor binding sites for: AP-1, C/EBP, and NF-κB. (B) Addition of DCA to cell models transfected with the CXCL8 promoter-reporter plasmid, mediates a 2.5 fold induction of the reporter.



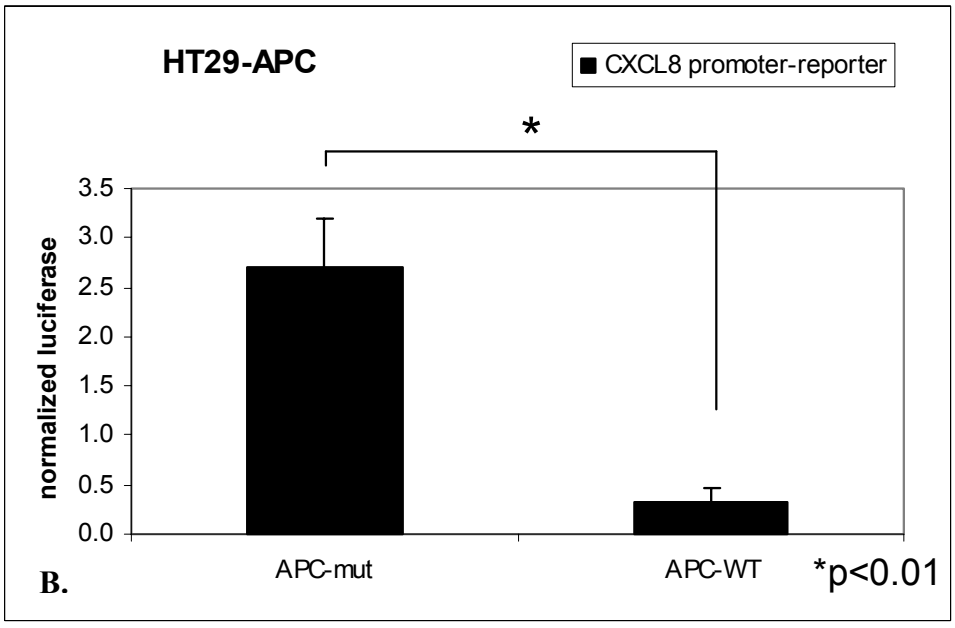
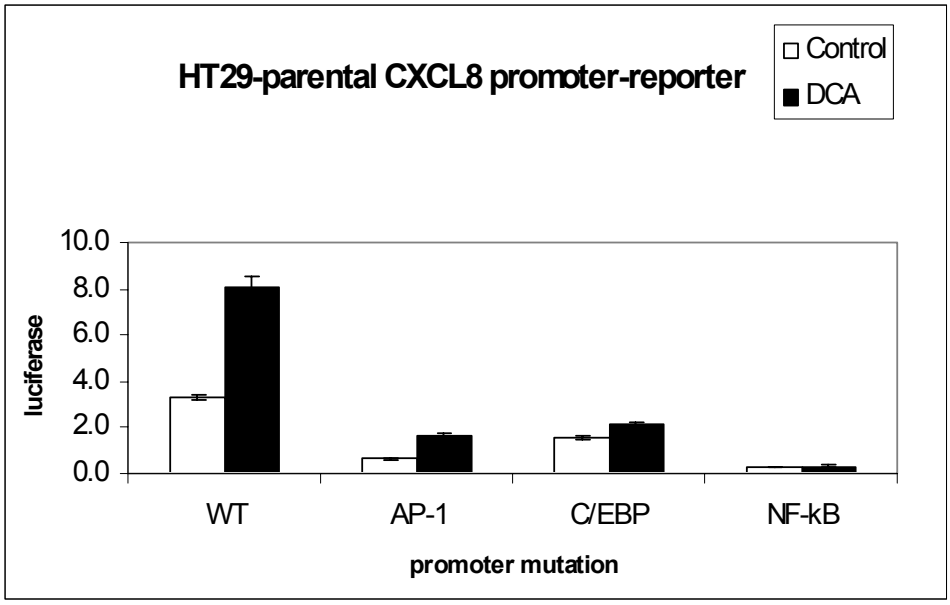


Figure 4.4. ***APC*** **mediates DCA induction of CXCL8.** Cells were plated, transfected, and exposed to DCA. (A) HT29-parental cells were exposed to control or DCA for twenty four hours. (B) The HT29-APC cells were pre-treated with zinc or control for three hours and then exposed to DCA or control for twenty four hours.



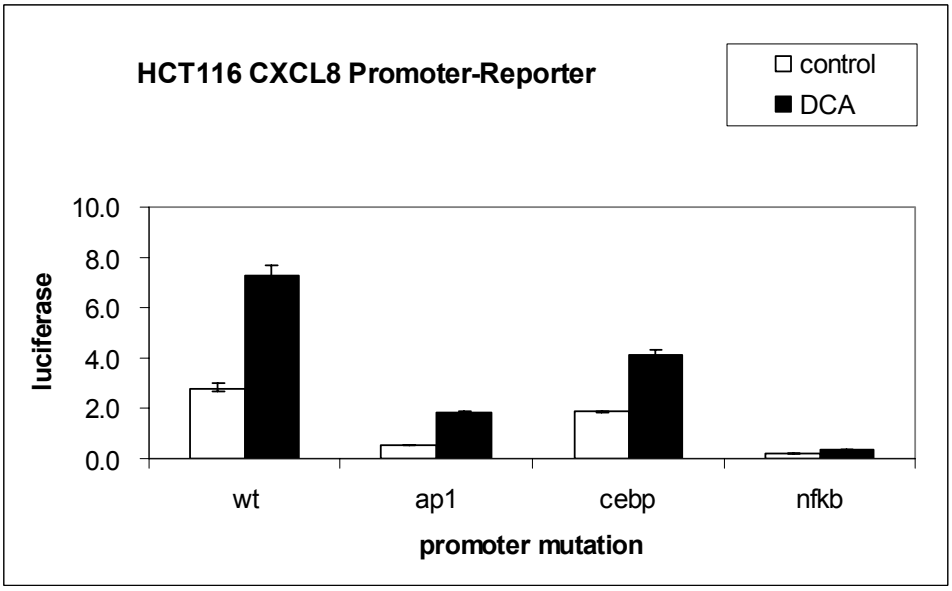


Figure 4.5. CXCL8 promoter-reporter studies in CRC cell lines. Cells were plated, transfected, exposed to control or DCA and then lysed. Luciferase activity was normalized for transfection efficiency. (A) HT29-parental cells (B) HCT116 cells. Abbreviations: wt, wild-type promoter; ap1, activator protein-1; cebp, CAAT enhancer binding protein.

DCA-mediated activation of transcription factors. HT29-parental cytoplasmic and nuclear fractions were isolated after two hours treatment with 300μM DCA. The blot was transferred to nitrocellulose, and then probed with antibodies directed against the transcription factors as seen in Fig. 4.6. The AP-1 transcription factor is a heterodimer of Jun and Fos subunits. As seen in the first row and eighth column, the Fos family member c-Fos, increased nuclear localization 2 hours after DCA treatment. Downstream of mutant *APC* is nuclear accumulation of β-catenin. Shown in the second row and eighth column, DCA incubation did not appear to change the localization of β-catenin. The NF-κB transcription factor is a heterodimer of p50 and p65 subunits. The evidence in the third and fourth rows, eighth columns showed that nuclear localization of the p50 and p65 subunits did not significantly alter 2 hours after exposure to DCA. The loading control for these experiments was β-actin, fifth row.

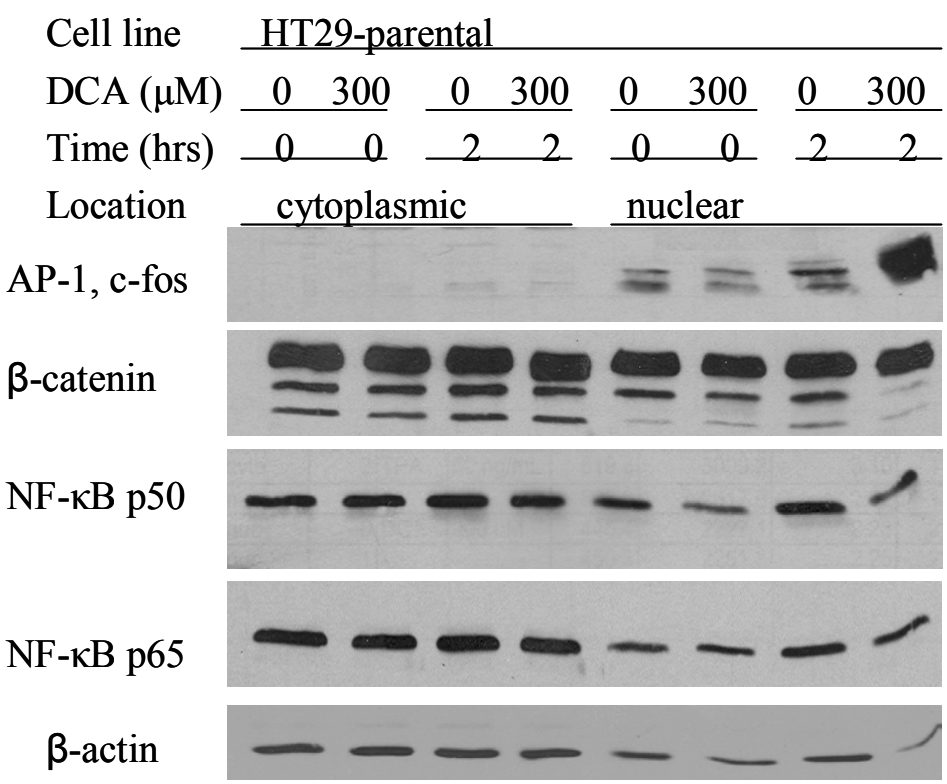


Figure 4.6. DCA-mediated activation of transcription factors. The HT29-parental cell line was plated, serum starved, exposed to control or DCA for two hours and the trypsinized. Nuclear and cytoplasmic fractions were collected with the proteins separated by SDS-PAGE. The blots were probed with antibodies directed against the transcription factor subunits

Chromatin Immunoprecipitation (ChIP) of the CXCL8 5'-promoter. ChIP was performed to determine binding of the transcription factors to the CXCL8 5'-promoter. The HT29-parental cell line was treated with 300 μM DCA or control for 8 hours and then formalin fixed. To elucidate transcription factor proteins that were bound to the genomic DNA complex, antibodies directed against c-Fos, β-catenin, NF-κB p50 or p65 were incubated with sheared DNA fragments. Isolated complexes were digested with proteinase and purified, resulting in fragments of DNA to which the transcription factors bound. Primers were designed to amplify 240 bp upstream and 73 bp downstream from the start of CXCL8 transcription, for a 313 bp product. This region corresponds with transcription factor binding sites, previously shown in Fig. 4.3.A. Amplification of the 313 bp product indicated that the transcription factor was bound to the chromatin. As shown in Fig. 4.7., the PCR reactions amplified a 313 bp product for the Input DNA template for which served as a control for both the DCA treated and control treated cells. β-catenin pull-down amplified a product in both the control and DCA-treated HT29-parental cells, indicating that it was bound to the promoter region at a basal level. The AP-1 family member, c-Fos and the NF-κB family members p50 and p65 did not amplify a product in the control group. When the HT29-parental cells were exposed to DCA, c-Fos and p65 amplified a significant amount of the 313 bp product, indicating that CXCL8 gene expression is influenced by both AP-1 and NF-κB.

DCA-mediated secretion of CXCL8 protein. To determine whether the CXCL8 protein was secreted into the media, cells were treated with control or DCA. Media was collected at the given time points. Proteins were precipitated by TCA and then separated by SDS-PAGE. Western analysis was performed on proteins, using antibody directed against CXCL8. As shown in Fig. 4.8., CXCL8 was detected after 6 hours after the HT29-parental cell line was exposed to 300 μM DCA.

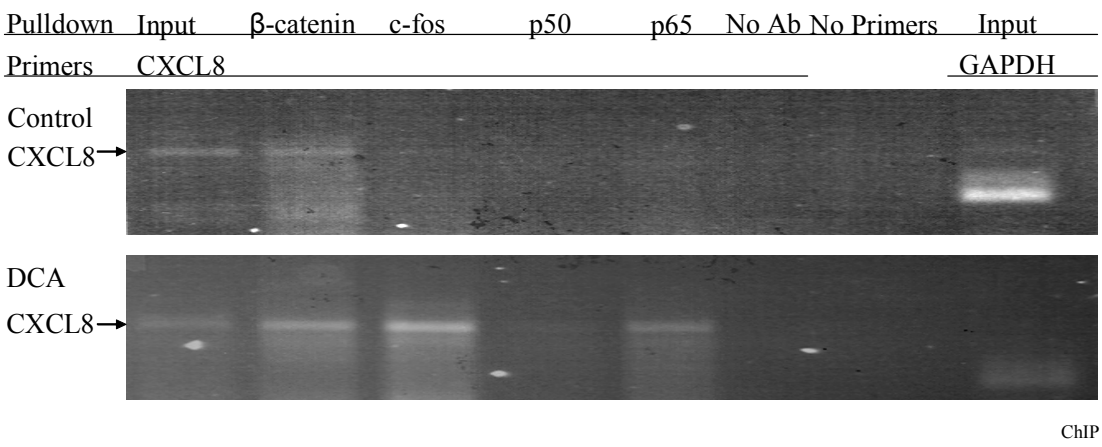


Figure 4.7. Chromatin Immunoprecipitation of the CXCL8 promoter region. HT29-parental cells were plate, serum starved and exposed to control or DCA for 8 hours. Formaldehyde was used to cross-link chromatin complexes, enzyme was used to shear the chromatin and antibodies directed against the transcription factor subunits were used to isolate fractions. Primers were deigned to yield a 313 bp product for the CXCL8 promoter region of interest. The GAPDH primers amplified a 166 bp product.

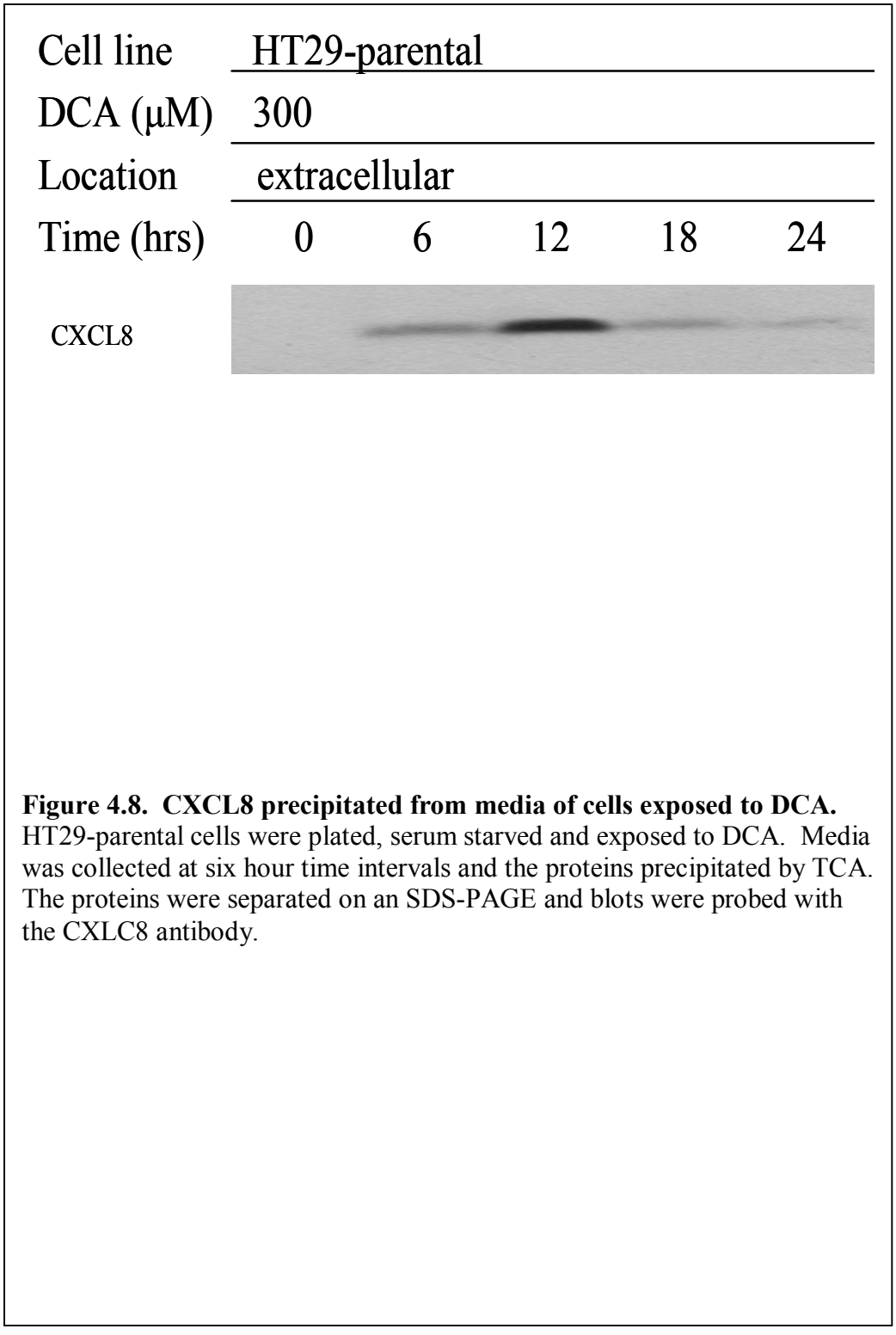


Figure 4.8. CXCL8 precipitated from media of cells exposed to DCA. HT29-parental cells were plated, serum starved and exposed to DCA. Media was collected at six hour time intervals and the proteins precipitated by TCA. The proteins were separated on an SDS-PAGE and blots were probed with the CXLC8 antibody.

SUMMARY:

The purpose of this study was to determine the role of DCA-mediated inflammation in carcinogenesis. DCA has been implicated in colorectal carcinogenesis, but the mechanisms for this action are been poorly understood [15, 20-23, 26]. The data shown here demonstrates that DCA induction of CXCL8 is dependent on *APC* status. Wild type *APC* suppresses DCA-mediated CXCL8 gene expression. Mutant APC is permissive for DCA-mediated CXCL8 gene expression. The increased expression of CXCL8 increases MMP-2 production and invasion by HT29-parental cells. CXCL8 neutralizing antibody limits the invasive phenotype of HT29-parental cells when exposed to DCA. Wild type *APC* limits DCA-mediated invasion. Therefore, the potential mechanism of DCA-mediated, CXCL8-dependent, inflammation occurs in transformed cells with mutant *APC*.

The present study confirms the role of bile acid-meditated CXCL8 production and expands upon them. Others had shown that DCA-dependent CXCL8 expression was permissive with mutant *APC* [67]. The data presented here places DCA-dependent CXCL8 expression in the context of physiologically relevant concentrations and times of DCA exposure. This study further excludes the primary and tertiary bile acids from induction of CXCL8. Increased CXCL8 expression also occurs as a result of Wnt signaling, through activation of the Wnt receptor [7] or inactivation of the negative regulator of the pathway GSK-3β [10]. The data shown here demonstrates that the

HT29-parental cell line with a mutant *APC* as well as the HCT116 cell line with a mutant *β-catenin*, does not show appreciable CXCL8 production when treated with control alone. While Wnt signaling may be necessary for CXCL8 production, it is not sufficient. Furthermore, the data shown here demonstrate that wild type *APC* is protective for DCA-mediated CXCL8 expression at both the steady state levels of mRNA and protein. The proposed mechanism of DCA-mediated CXCL8 production involves downstream signaling by both DCA and mutant *APC*.

A potential mechanism of DCA-mediated increase in steady state CXCL8 mRNA has been identified, although not fully explained [67]. The data presented here confirm the importance of DCA-mediated increased CXCL8 mRNA production. It also expands the role of DCA to show direct activation of the 5'-promoter by transcription factors. The proximal 200 base pairs of the CXCL8 promoter regulates gene expression shown by promoter analysis studies in multiple models [3, 108-112]. Promoter reporter studies in the HT29-parental cells demonstrated that DCA increased the promoter activity using the full-length CXCL8 5'-promoter (-1481, +44). This is consistent with data published by others indicating that DCA can activate the transcription factors AP-1, C/EBP, and NF-κB [28-30] The data are also consistent with the role of these transcription factors binding to the 5'-promoter to regulate CXCL8 expression in other models [3, 103, 113]. In addition to the DCA-activated transcription factors, the regulation of CXCL8 expression is influenced by *APC* status.

Use of the HT29-APC cell line demonstrated that wild type *APC* suppressed DCA mediated CXCL8 promoter reporter activity. This is consistent with reports that identified increased CXCL8 expression a result of Wnt signaling [7, 10]. The finding that wild type *APC* is suppresses DCA-mediated CXCL8 is novel.

Promoter reporter studies with mutated transcription factor binding sites, demonstrated the significance of AP-1, C/EBP and NF-κB in DCA-mediated CXCL8 expression. Luciferase activity was significantly decreased by mutations in the AP-1 and C/EBP binding sites of the CXCL8 5'-promoter. Luciferase activity was abolished by mutations in the NF-κB binding site of the same region. The absence of CXCL8 reporter activity suggested that NF-κB is necessary for expression and is consistent with previous studies [3, 67]. AP-1 and C/EBP mutations also decreased luciferase activity and suggest the mechanism of CXCL8 regulation involves AP-1, C/EBP and NF-κB. [108, 109].

Transcription factors are often found in the cytoplasm and activated when they translocate to the nucleus. Data shown here demonstrates that DCA exposure induces the AP-1 family member, c-Fos, translocation to the nucleus and is consistent with published data [28]. We show here that c-Fos binds to the CXCL8 5'-promoter when cells were treated with DCA. While c-Fos cannot bind to DNA by itself, it may be a surrogate for AP-1 activation. Further analysis of c-Jun and Jun D [3, 28] may be a reasonable heterodimeric partner for the activation of AP-1. The ChIP also reveals that treatment with DCA allows for the binding of p65 and to a lesser extent p50 to the CXCL8 5'-

promoter. The level of nuclear β-catenin may not be significantly altered by DCA. We also demonstrate that β-catenin binds to the 5'-promoter in the absence or presence of DCA. Therefore we conclude that increased CXCL8 expression is through the combined effect of bacterial derived luminal risk factors and genetic risk factors that drive the expression if CXCL8.

CHAPTER 5:

CXCL8 IS PERMISSIVE FOR INVASION BY HUMAN CRC

INTRODUCTION:

DCA alone has been published to stimulate migration [89]. DCA, at varying concentrations, has been established to induce CXCL8 production [67]. Increased CXCL8 has been associated with human cancer [103]. CXCL8 has been shown to allow for a migratory phenotype in multiple model systems [70, 79]. CXLC8 has been identified as an integral part of EMT in colon carcinoma cells [71]. Increased CXCL8 has also been shown to up-regulate the expression and activity of matrix metalloproteinase-2 (MMP-2) in other cell models [65, 78, 79, 114]. These features are consistent with increased malignancy and therefore EMT provides a potential mechanism by which carcinoma cells may acquire a more aggressive phenotype [77].

Degradation of the extracellular matrix by matrix metalloproteinase has been the proposed mechanism by which MMP-2 exerts its effects [4, 80]. The HT29-parental cell line has been published to have integrin families of receptors that migrate on laminin [115]. Integrins are adhesion proteins that promote attachment and migration through the extracellular matrix surrounding a cell and are potential targets in cancer therapy [116].

The purpose of this study was to determine the effects of CXCL8 production in CRC. The aims were to establish a potential mechanism for DCA mediated migration, establish if CXCL8 exposure could induce MMP-2 in CRC cell lines, and to ascertain whether CXCL8 could invade through the MMP-2 sensitive laminin.

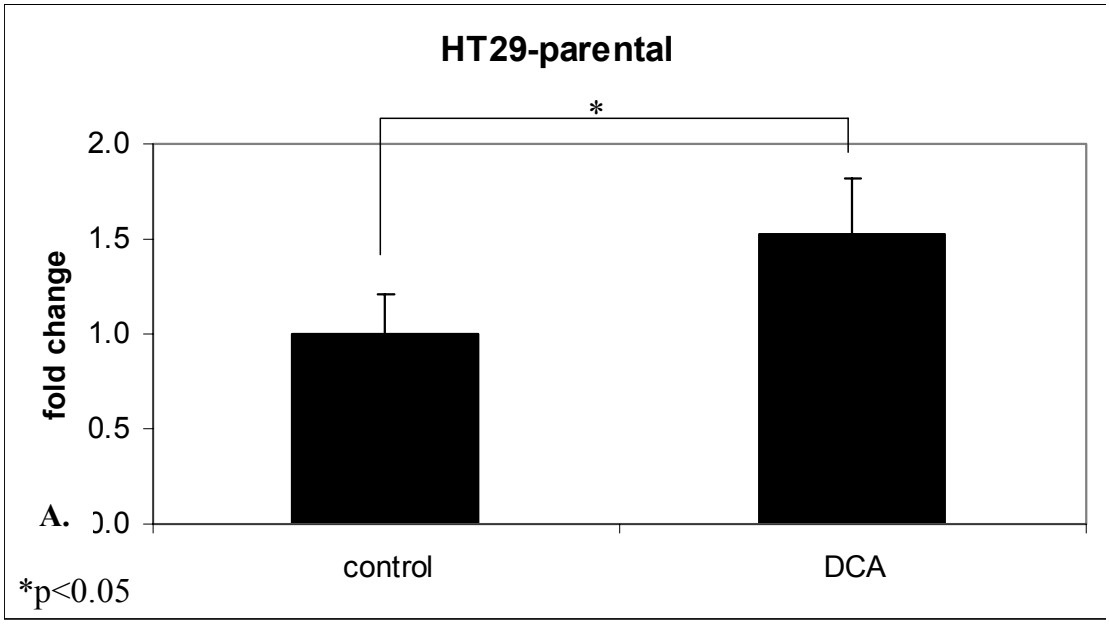
RESULTS:

Effects of *APC* status on DCA-mediated invasion. To determine the effects of *APC* status on invasion, the HT29-APC cells were plated on laminin-coated filters. The HT29-APC cells were exposed to 300 μM $ZnCl_2$ or vehicle for 3 hours. HT29-APC cells were then incubated on laminin coated filters and dosed with 300 μM DCA for 24 hours. As seen in Fig. 5.1.A., mutant *APC* was permissive for DCA-mediated invasion. As shown in Fig. 5.1.B., wild type *APC* was restrictive for DCA mediated-invasion ($p<0.05$).

CXCL8 effects on invasiveness of colon cancer cells. The effects of CXCL8 were determined by invasion assays. DCA has been shown to increase migration in CRC cell lines [89]. HT29-parental cells have alpha-3 and beta-1 integrins that suggest an ability to migrate or invade on laminin [115]. HT29-parental cells were incubated on laminin coated filters for 48 hours. The HT29-parental cells were incubated with 2.0 μg/mL CXCL8 or control. Invasion was measured by colorimetric assay. As seen in Fig. 5.2.A., HT29-parental cells treated with CXCL8 had 30% more invasion ($p<0.01$), when compared to the control. As seen in Fig. 5.2.B., invasion by HT29-parental cells treated

with DCA and CXCL8 antibody was limited by more than 30% when compared to HT29-parental cell treated with DCA and IgG ($p<0.01$).

CXCL8-mediated MMP-2 production. Western analysis was performed on total cell lysate from HT29-parental cells treated with CXCL8. Proteinases digest elements of the basement membrane and thereby aid in invasion. As shown in Fig. 5.3., matrix metalloproteinase-2 (MMP-2), a transcriptional target of CXCL8 was detected in the cell lysate 36 hours after cells were dosed with 2.0 μg/mL CXCL8.



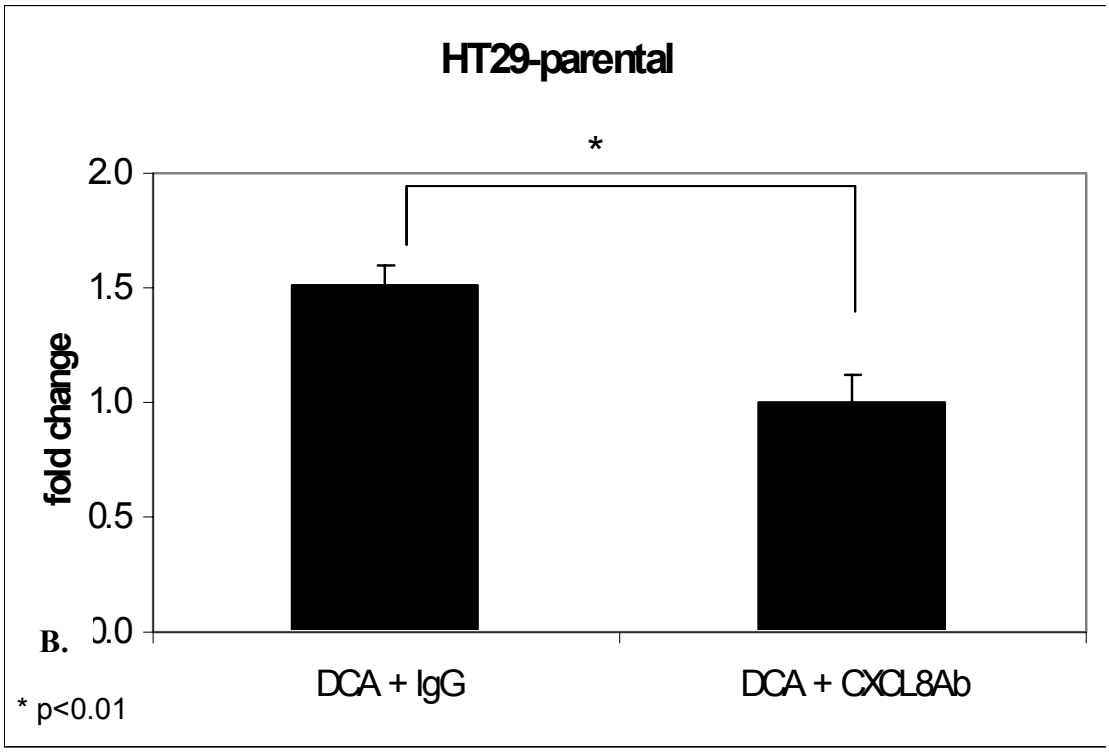
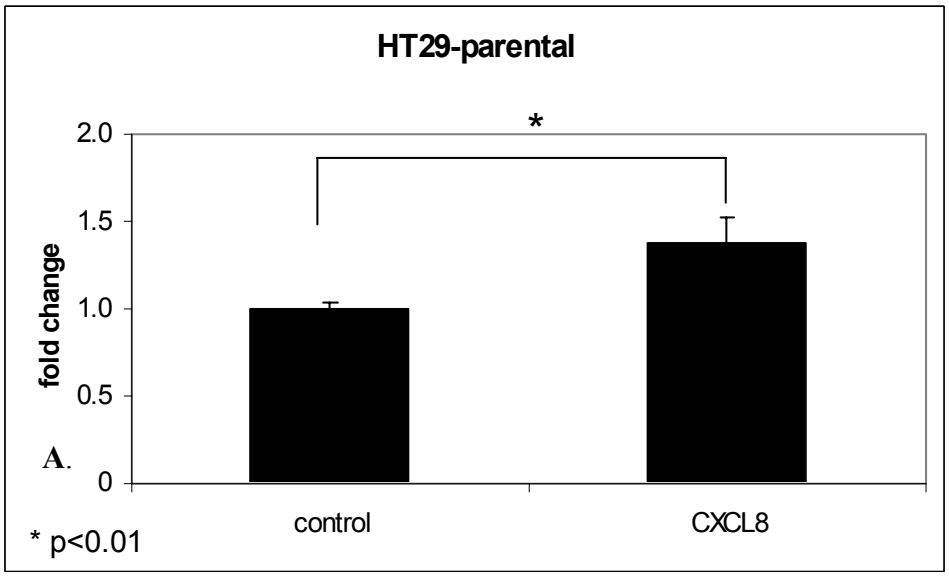


Figure 5.1. Mutant *APC* and CXCL8 are permissive for invasion. HT29-parental cells were plated on laminin coated filters and allowed to invade. (A) Cells were treated with control or DCA. (B) Cells were treated with DCA and IgG or DCA and CXCL8 antibody.



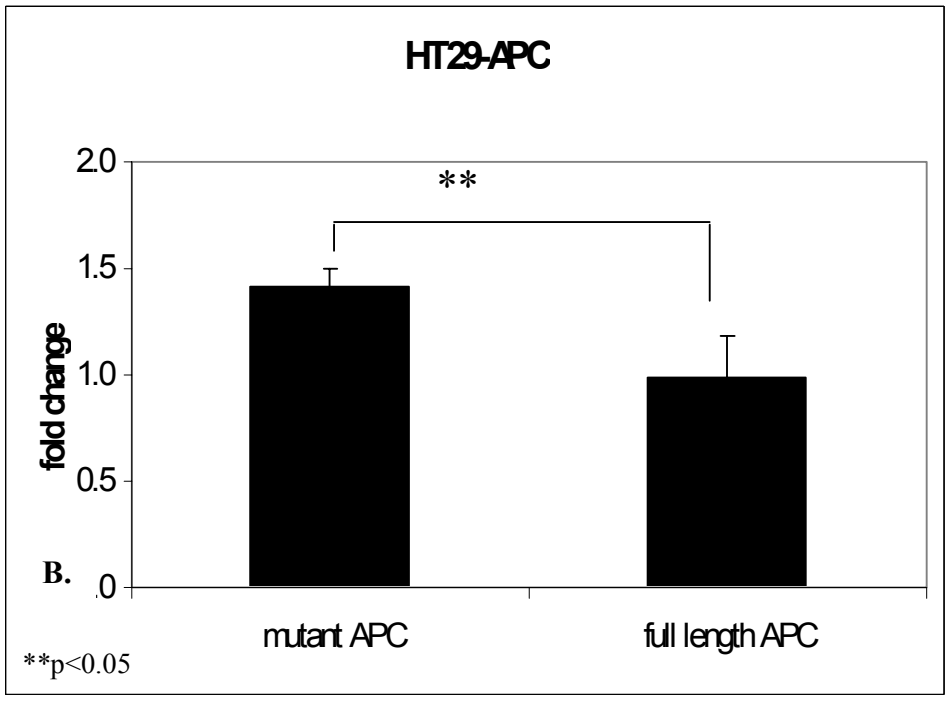
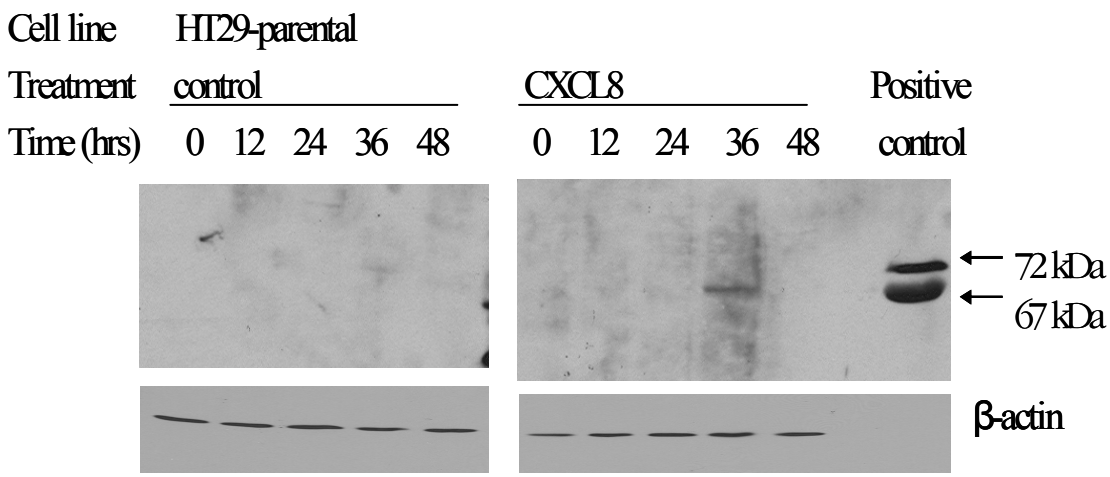


Figure 5.2. CXCL8 and *APC* status mediate invasion. Cells were plated on laminin coated filters and allowed to invade. (A) HT29-parental cells were exposed to control or CXCL8. (B) HT29-APC cells were exposed to zinc for three hours and then to DCA. Cells were allowed to invade for twenty four hours.



Western analysis

Figure 5.3. CXCL8-mediated MMP-2 expression. HT29-parental cells were plated, serum starved and exposed to CXCL8 for the given time period. Cells were lysed, proteins were separated by SDS-PAGE and antibody directed against MMP-2 was used.

SUMMARY:

The purpose of this study was to determine the effects of CXCL8 production in CRC. The aims were to establish a potential mechanism for DCA mediated migration. We hypothesized that CXCL8 exposure could induce MMP-2 in CRC cell lines. Finally, the aims also included whether CXCL8 could invade through the MMP-2 sensitive laminin.

We show here that exposure of HT29-parental cells to DCA is sufficient to increase invasion on laminin coated filters. It is consistent with the ability of DCA to increase *in vitro* migration in Caco-2 CRC cell line [89]. It is also consistent with the integrin expression profile of the HT29-parental cell line [115].

We show here that the HT29-parental cells treated with DCA and a CXCL8 antibody, neutralized invasion. This is consistent with DCA to induce CXCL8 [67]. It is also consistent with CXCL8-mediatied migration in other models [65, 78, 79, 103].

We show here that invasion was suppressed in the presence of wild type *APC* when cells were treated with DCA as compared to the mutant *APC* cells. This is consistent with the roles of DCA to produce CXCL8 and CXCL8 to increase migration and invasion [65, 67, 78, 79, 89, 103].

CXCL8 is an inducible cytokine that can act in autocrine and paracrine loops. CXCL8 acts as an autocrine growth factor [69] and suggests a role for other effects by an autocrine loop. The increased expression of CXCL8 in CRC cell lines is consistent with increased expression of chemokines and patient tumor burden [43, 59, 117-119].

Increased CXCL8 has also been associated with metastasis, through mouse models [120]. One potential mechanism is through the production of proteinases that may aid in invasion through the extracellular matrix surrounding a cell. Increased MMP-2 production was found in response to CXCL8 exposure in HT29-parental and is consistent with reports of activation of the 5'-promoter of MMP-2 by CXCL8 [78, 79]. MMP-2 is activated by membrane-type matrix metalloproteinase-1 (MT1-MMP) and inhibited by tissue inhibitor metalloproteinase-2 (TIMP-2) [121]. The evidence presented here does not exclude the possibility for CXCL8 to up-regulate the expression of MT1-MMP. The evidence presented here does not exclude the possibility for CXCL8 to down-regulate the expression of TIMP-2.

Epithelial-mesenchymal transition (EMT) is an essential component of embryonic development, tissue remodeling, wound repair and is implicated in cancer progression [75]. Features of EMT are consistent with a loss of polarity and loss of adhesive constraints as well as morphology that is appropriate for migration [76]. CXCL8 expression, and its subsequent influence on cellular phenotypes, is consistent with features of EMT. CXCL8 has been identified as a potential factor in EMT [71]. The data

presented here are consistent with the potential role of CXLC8 in EMT to affect invasion through the production of metalloproteinases [78, 80].

CHAPTER 6:

CXCL8 EXPRESSION IN HUMAN CRC TISSUE

INTRODUCTION:

Pro-inflammatory cytokines, including CXCL8, can be detected in the serum and cancerous tissue of colorectal cancer patients [59, 117, 122-124]. The elevated circulating levels of CXCL8 in CRC patients has been associated with increased metastatic lesions [74, 125]. Elevated expression of CXCL8 in cancerous tissue has also been associated with increased angiogenesis in the surrounding normal tissue [72].

Single nucleotide polymorphisms (SNPs) have been identified in the CXCL8 gene with increased risk for development of adenomatous polyps [126]. Increased CXCL8 expression in the normal tissue of humans with colon cancer [127]. Patients with inflammatory bowel disease (IBD) also have elevated cytokine levels, including CXCL8, that may lead to chronic tissue injury and increase the risk of developing CRC [44, 45, 49, 128]. There has not been a thorough investigation of CXCL8 expression in colorectal adenomas that related the status of *APC*.

The purpose of this study was to determine the expression of CXLC8 based upon β-catenin, as a marker of Wnt signaling. The aims of the study were to assess the expression of CXCL8 and the localization of β-catenin in normal colonic epithelium, adenomatous polyps and adenocarcinoma of the colon.

RESULTS:

CXCL8 expression and β-catenin localization in normal human colonic epithelium. CXLC8 expression was determined by immunohistochemistry (IHC). HT29-parental cells were treated with 300 μM DCA or control. Cell pellets were made by trypsinizing, centrifuging the samples and resuspending the cell pellets in formalin. As shown in Fig. 6.1., the DCA treated cell pellet shows positive staining that is consistent with the mRNA and protein data. Patient samples were previously collected for: tumor tissue, adjacent and normal mucosa. The samples were then exposed to CXCL8 antibody, as described in the Materials and Methods section. As shown in Fig. 6.2., the normal colonic mucosa did not express detectable quantities of CXCL8, nor did β-catenin localize to the nucleus.

CXCL8 expression and β-catenin localization in human adenomatous colon polyp. CXCL8 expression in adenomatous polyp was detectable. As shown in Fig. 6.3., CXCL8 expression was found in the colonic epithelium. CXCL8 positively correlated with nuclear β-catenin, indicating that CXCL8 expression may occur in transformed epithelium.

CXCL8 expression and β-catenin localization in human colonic adenocarcinoma. CXCL8 expression in the colonic adenocarcinoma was detectable. As shown in Fig. 6.4., the expression of CXCL8 did not correspond to epithelial cells with nuclear localization of β-catenin. Instead, CXCL8 expression may correspond with endothelial cells and neutrophils as shown in Fig. 6.5.

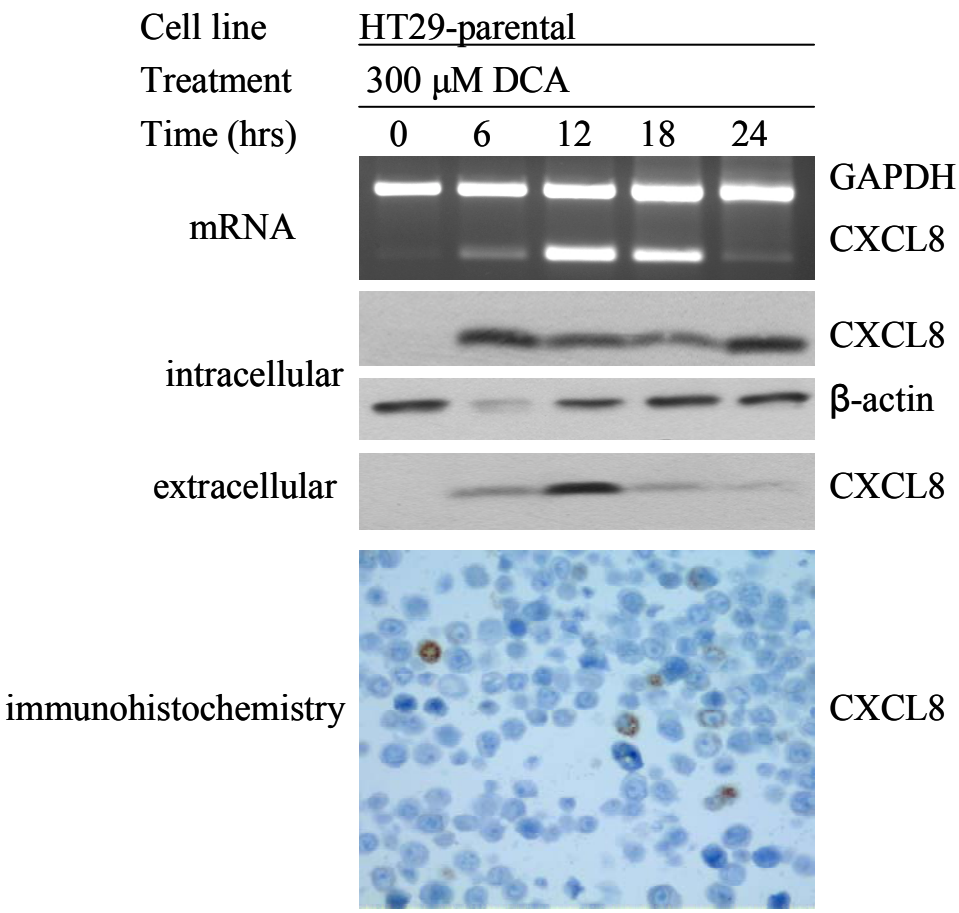
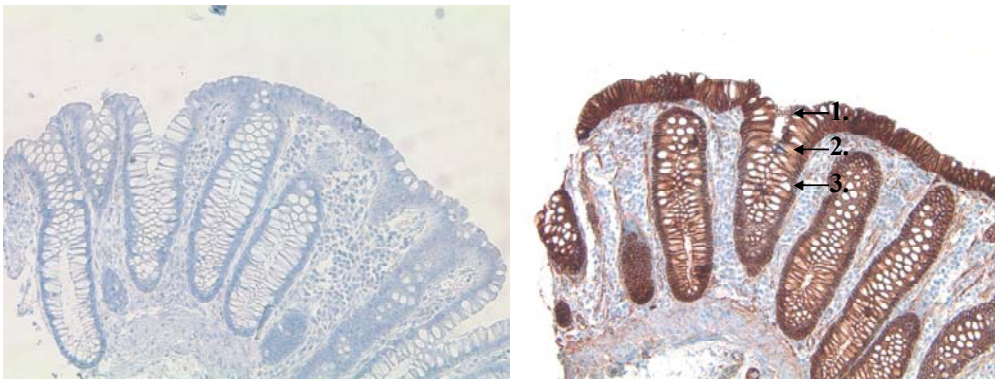
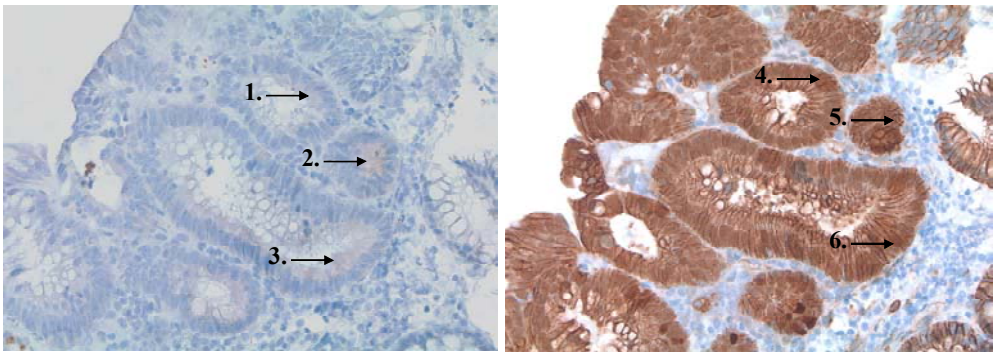


Figure 6.1. DCA-mediated CXCL8 expression in HT29-parental cells. Cells were treated with DCA for the time indicated and prepared for various CXCL8 quantifications



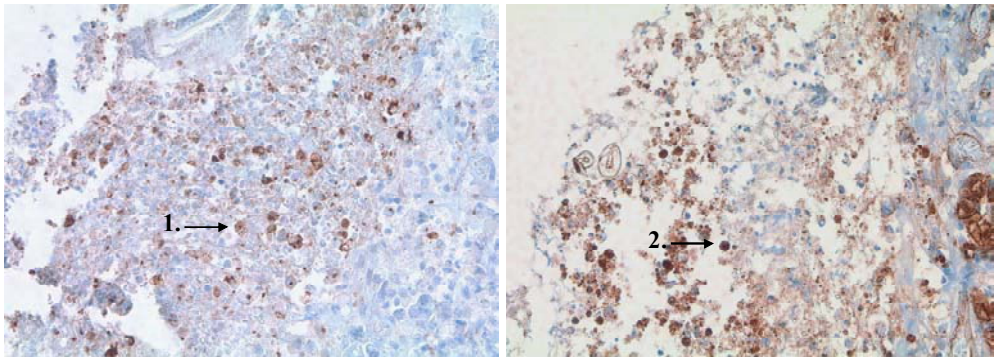
A. CXCL8 B. β-catenin

Figure 6.2. CXCL8 and β-catenin immunohistochemistry in normal colonic epithelium. Formalin fixed tissue blocks were serial cut in three micrometer sections and mounted on glass slides. Antibodies directed against (A.) CXCL8 or (B.) β-catenin were used to detect the presence of CXCL8 protein as well as localization of the β-catenin protein. In the normal colonic epithelium, (B.) β-catenin localizes to membranous (1.) and cytoplasmic (2.) regions, but not nuclear (3.) regions, as indicated by the arrows.



A. CXCL8 B. β-catenin

Figure 6.3. CXCL8 and β-catenin immunohistochemistry in a human adenomatous polyp. Formalin fixed tissue blocks were cut in three micrometer serial sections and mounted on glass slides. Antibodies directed against (A.) CXCL8 or (B.) β-catenin were used to detect the presence of CXCL8 protein as well as localization of the β-catenin protein. Staining for (A.) CXCL8 corresponded with (B.) nuclear localization of β-catenin, as indicated by arrows (1-3) and (4-6) respectively.



A. CXCL8 B. β-catenin

Figure 6.4. CXCL8 and β-catenin immunohistochemistry in a human colon adenocarcinoma. Formalin fixed tissue blocks were cut in three micrometer serial sections and mounted on glass slides. Antibodies directed against (A.) CXCL8 or (B.) β-catenin were used to detect the presence of CXCL8 protein as well as localization of the β-catenin protein. Staining for (A.) CXCL8 did not corresponded with (B.) nuclear localization of β-catenin, as indicated by arrows 1 and 2 respectively.

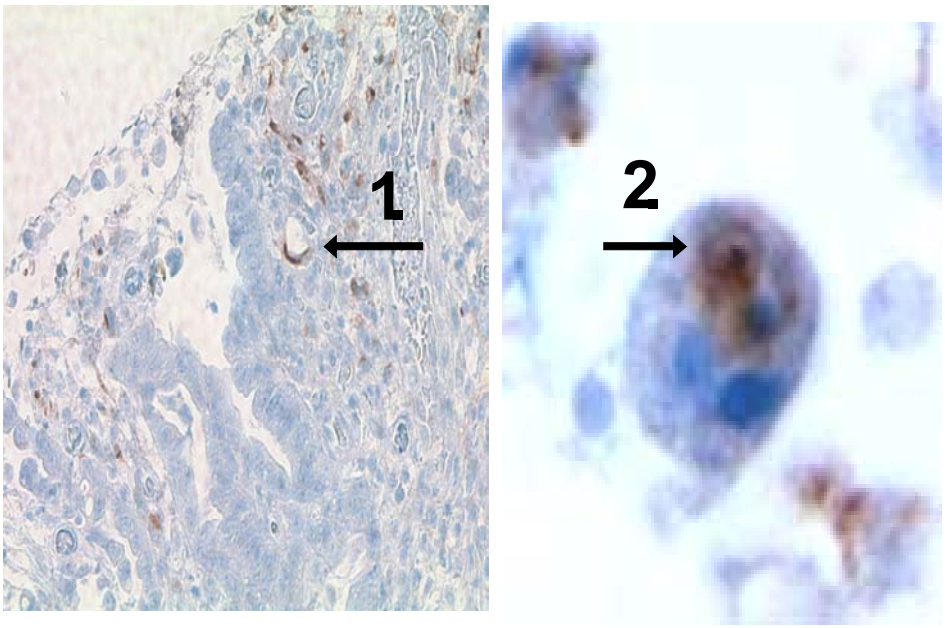


Figure 6.5. CXCL8 immunohistochemistry in a human colon adenocarcinoma. Formalin fixed tissue blocks were cut in three micrometer serial sections and mounted on glass slides. Antibodies directed against CXCL8 was used to detect the presence of CXCL8 protein. Staining for CXCL8 may have corresponded with endothelial cells and neutrophils as indicated by arrows 1 and 2 respectively.

SUMMARY:

The purpose of this study was to determine the expression of CXLC8 based upon β-catenin, as a marker of Wnt signaling. On of the aims of the study was to assess the expression of CXCL8 and the localization of β-catenin in normal colonic epithelium. We found that membranous and cytoplasmic β-catenin staining correlated well with the absence of CXCL8 expression in the normal colonic epithelium. The absence of CXCL8 in the normal colonic epithelium has been previously reported by others [123].

Another of the aims was to determine if adenoumatous polyps produced CXCL8, and if so did it correlate with nuclear β-catenin. Shown in the data is CXCL8 expression in adenomatous polyps that corresponds to the nuclear localization of β-catenin. It is a novel finding. It fits well into the regulation of CXCL8 gene expression by the Wnt signaling pathway [7, 10].

The final specific aim was to determine the expression of CXCL8 in the colonic adenocarcinoma tissue. While we did not find elevated CXCL8 expression in the epithelial, we did find increased CXCL8 expression in the adenocarcinoma. Elevated CXCL8 in the endothelial cells and neutrophils is consistent with the role of CXCL8 in angiogenesis and neutrophils recruitment, as previously reported [59, 72, 123, 124, 129]. Elevated CXCL8 in these cells types is also consistent with increased microvessel density as well as risk of metastatic lesions [73, 74, 125]. Paracrine effects cannot be excluded

from the discussion. CXCL8 has been hypothesized to play a role in the maintenance of neovasculature in colon carcinoma [73].

CXCL8 has also been described as a chemoattractant for neutrophils which may potentially be involved in tissue remodeling during carcinogenesis [37, 63, 104, 130]. Neutrophil production of matrix metalloproteinase-9 (MMP-9) has been identified in human colon cancer specimens and hypothesized to be involved in extracellular proteolysis during carcinogenesis [131]. CXCL8 and nuclear β-catenin staining were not associated with normal epithelium but were associated with transformed epithelium (Fisher's exact $p=0.0029$, $p=0.0006$).

CXCL8 was not appreciated in the normal or the adjacent tissue. CXCL8 was appreciated in the adenomatous polyps by the epithelial cells. CXCL8 was also found in the endothelial cells and neutrophils. There may be multiple interpretations of the data. First, previous reports of CXCL8 in colonic tissue used snap-frozen tissue [59, 117] or used other antibodies [72, 124] that may affect the signal of CXCL8 in human tissue. Yet CXCL8 was detected in both the adenomatous polyps as well as the stromal cells in the adenocarcinoma cells. One potential interpretation of the data is that the CXCL8 produced in the early lesions was a ligand for the activation signaling pathways that lead to increased cell growth. Further in the carcinogenic sequence, the adenocarcinoma may have outgrown the need for the ligand and outgrown its dependence on the activation of pathways leading to increased cell growth.

The production of CXCL8 may also be related to stromal derived factors. Up-regualtion of the COX-2 enzyme and its down stream effects may also play a role in the regulation of CXCL8, as prostaglandin E_2 (PG E_2) can stimulate CXCL8 [132]. CXCL8 production is initiated in the epithelium in the adenomatous polyps and may contribute to carcinogenesis through the recruitment of blood vessels and cells involved in tissue remodeling.

CHAPTER 7:

DISCUSSION AND PERSPECTIVE

The transformation from normal to cancerous tissue begins with the dysregulation of ordinary cellular processes. Both proto-oncogenes and tumor suppressor genes mediate the balance of cellular processes through proliferation and apoptosis. In the presence of an oncogenes, or loss of a tumor suppressor gene, ordinary cellular processes of proliferation and apoptosis become dysregulated. These now are the transformed cells from which cancer arises. The studies in this dissertation, sought to understand how mutation of the *APC* tumor suppressor gene could yield a selection advantage to CRC cells.

In addition to genetic risk factors for cancer, factors from the environment may also contribute. The high fat content of the Western diet increases bile acid concentrations in the luminal intestinal tract. Conversion of primary bile acids to secondary bile acids, by enteric bacteria of the colon exposes the epithelium to carcinogens.

The excessive exposure of the colonic epithelium to secondary bile acids may lead to chronic inflammation of the tissue. CXCL8 is a mediator of inflammation that is induced by DCA. Yet functional *APC,* a tumor suppressor gene, can suppress this effect. It suggests that DCA-mediated inflammation occurs in the transformed epithelium.

In the context of mutation in the tumor suppressor *APC* and the presence of the bacterial derived DCA, the highly proliferative colonic epithelial cells produce excessive inflammation via the chemotactic cytokine, CXCL8. Shown in Fig. 7.1. is a potential mechanism by which DCA and *APC* may regulate CXCL8 expression.

Mutant *APC* was permissive for the chemokine, CXCL8. A wild-type *APC* suppressed the ability of DCA to induce CXCL8 protein, RNA and in promoter reporter assays. One of the novel findings in these studies was the presence of β-catenin at the CXCL8 5'-promoter even in the absence of DCA exposure. Another novel finding was the importance of both AP-1 and NF-κB activation by DCA to mediated increased CXCL8 expression. Regulation of CXCL8 expression underscores the importance of risk factors from genetic and luminal sources in colorectal carcinogenesis. It is crucial to understand the mechanism of regulation as it may provide a possible target for chemoprevention or chemotherapies.

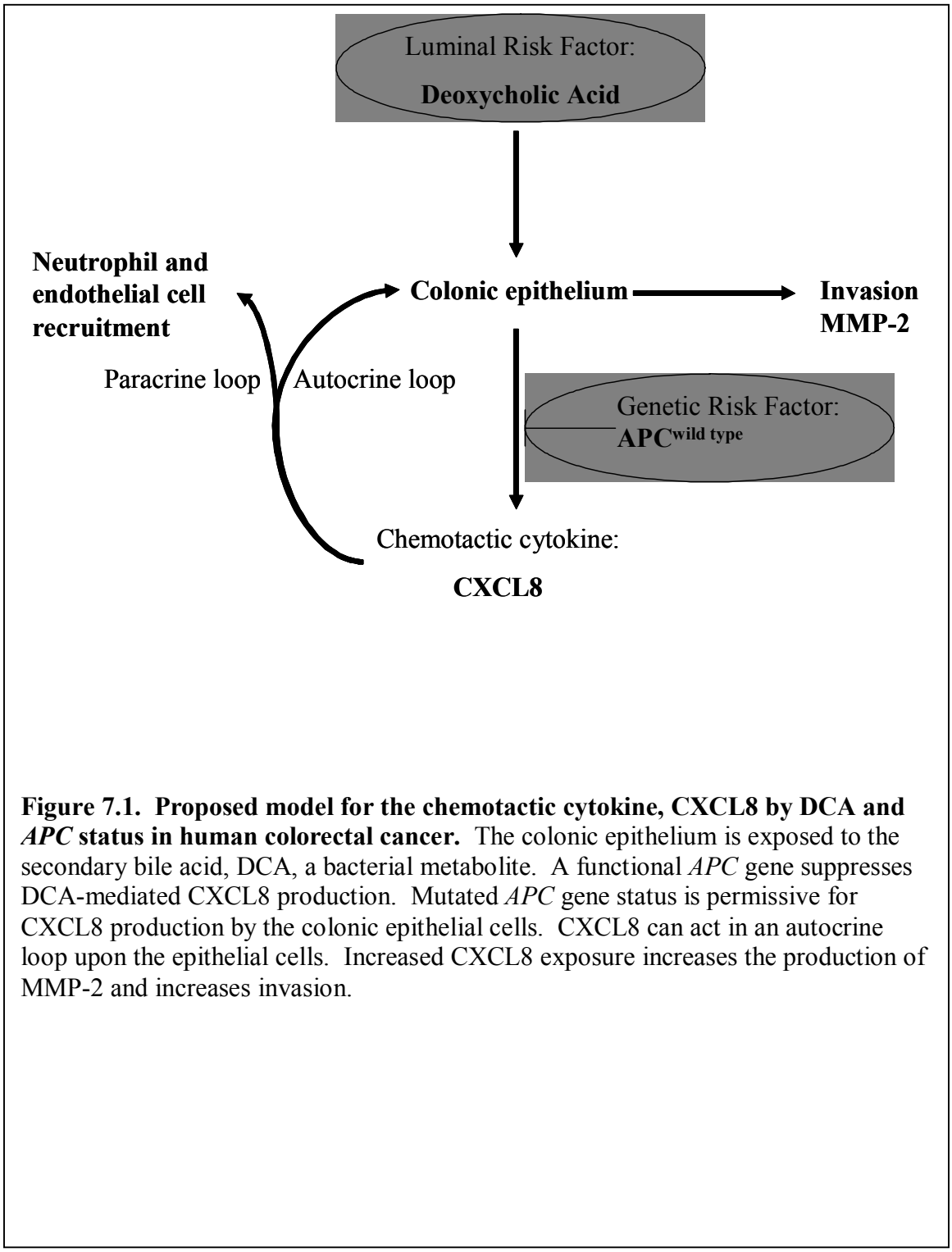


Figure 7.1. Proposed model for the chemotactic cytokine, CXCL8 by DCA and *APC* status in human colorectal cancer. The colonic epithelium is exposed to the secondary bile acid, DCA, a bacterial metabolite. A functional *APC* gene suppresses DCA-mediated CXCL8 production. Mutated *APC* gene status is permissive for CXCL8 production by the colonic epithelial cells. CXCL8 can act in an autocrine loop upon the epithelial cells. Increased CXCL8 exposure increases the production of MMP-2 and increases invasion.

The increased expression of CXCL8 was mediated by DCA and mutant APC. The studies in this dissertation show evidence of an invasive phenotype, based on CXCL8 exposure. Antibody directed against CXCL8 blocked DCA-dependent invasion. In addition, wild-type *APC* suppressed DCA-mediated invasion, when compared to the mutant *APC*. Exposing cells to CXLC8 could increase invasion as well as increase the production of a proteinase capable of digesting components of the extracellular matrix. These findings demonstrate the potential importance of CXCL8 in colon carcinogenesis and are consistent with features of EMT. EMT is a proposed mechanism by which cancer cells may localize at distant sites. The findings of this dissertation place into context the ability of both DCA and CXCL8 to contribute to features of EMT, as shown in Table 7.1.

Another novel finding was CXCL8 expression localized to the epithelium in the adenomatous polyp, but not in the adenocarcinoma. While the adenomatous polyps may not invade, the literature cited indicates that CXCL8 may act as an autocrine growth factor. CXCL8 staining in the adenocarcinoma was found in cells that may potentially be endothelial cells and neutrophils. It suggests that the role of CXCL8 in carcinogenesis may be two-fold. First, as functional *APC* was suppressive for DCA-mediated CXCL8, the data suggest that chronic inflammation may occur in the transformed epithelium but not the normal. Second, the data suggest that CXCL8 may play a more significant role in colon carcinogenesis through the recruitment of blood vessels and neutrophils which help to remodel the tumor environment.

- **Proteins that increase in abundance**
  - N-cadherin
  - Vimentin
  - Fibronectin
  - Snail1 (Snail)
  - Snail2 (Slug)
  - Twist
  - Goosecoid
  - FOXC2
  - Sox10
  - MMP-2
  - MMP-3
  - MMP-9
  - Integrin αvß6
- **Proteins whose activity increases**
  - ILK
  - GSK-3ß
  - Rho
- **Proteins that accumulate in the nucleus**
  - ß-catenin
  - Smad-2/3
  - NF-κß
  - Snail1 (Snail)
  - Snail2 (Slug)
  - Twist
- **In vitro functional markers**
  - Increased migration
  - Increased invasion
  - Increased scattering
  - Elongation of cell shape
  - Resistance to anoikis

Table 7.1. Markers for epithelial-mesenchymal transition. Both DCA and mutant *APC* can directly, or indirectly, affect markers that are consistent with EMT. *Adapted from [4]*

This research sought to validate the expression of CXCL8 in human tissue. The evidence presented here demonstrates that CXCL8 is found in CRC tissue. In addition, the production of CXCL8 in the adenomatous polyps is novel as well. The nuclear localization of β-catenin that correlates with the expression of CXLC8 is also novel. It suggests that increased CXCL8 is an early event in carcinogenesis.

The questions asked in this dissertation sought to understand the interaction between genetic risk factors as well as risk factors from the environment. The experiments shown identify a potential mechanism by which mutant *APC* and DCA can affect inflammation. Excessive CXCL8 has been further shown to increase the malignant phenotype through proteinase production and invasion. To translate these findings, the data may be interpreted that a low fat diet would reduce the risk of metastasis. The data suggest that chemotherapies directed against CXCL8 or its receptors, may be an effective strategy to reduce tumor blood vessel growth. In a cancer prevention model, preventing AP-1 and NF-κB activation by DCA by small molecule inhibitors may be a useful approach to reduce CXCL8 production in the pre-malignant stages of carcinogenesis. The potential therapeutic intervention for highly expressing CXCL8 tumors in CRC may be through its neutralization [64]. A humanized anti-CXCL8 antibody inhibits angiogenesis, tumor growth, metastasis and MMP activity in pre-clinical models [65, 66]. The expectation would be a reduction in tumor burden of CRC patients based on these parameters.

APPENDIX

HUMAN SUBJECTS APPROVAL

Appendix 1. Institutional Review Board Approval Letter

THE UNIVERSITY OF ARIZONA.

Human Subjects
Protection Program

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30 July 2007

Nathaniel Rial, Doctoral Student
Advisor: M. Peter Lance, MD
Department of Cancer Biology
PO Box 245024

BIO A07.046 CXCL8 PROTEIN DETERMINATION IN HUMAN TISSUE SAMPLES

Dear Mr. Rial:

We received your research proposal as cited above. The procedures to be followed in this study pose no more than minimal risk to participating subjects and have been reviewed by the Institutional Review Board (IRB) through an Expedited Review procedure as cited in the regulations issued by the U.S. Department of Health and Human Services [45 CFR Part 46.110(b)(1)] based on their inclusion under *research category 5*. The requirement for *obtaining informed consent* has been waived for this study since the research involves no more than minimal risk, the waiver will not adversely affect subjects' rights and welfare, the research could not practicably be carried out without the waiver [and whenever appropriate, the subjects will be provided with additional pertinent information after participation], as allowed by 45 CFR46.116(d).

Although full Committee review is not required, a brief summary of the project procedures is submitted to the Committee for their endorsement and/or comment, if any, after administrative approval is granted. This project is approved with an **expiration date of 30 July 2008.** Please make copies of the attached IRB stamped consent documents to consent your subjects.

The Institutional Review Board (IRB) of the University of Arizona has a current ***Federalwide Assurance*** of compliance, ***FWA00004218***, which is on file with the Department of Health and Human Services and covers this activity.

Approval is granted with the understanding that no further changes or additions will be made to the procedures followed without the knowledge and approval of the Human Subjects Committee (IRB) and your College or Departmental Review Committee. Any research related physical or psychological harm to any subject must also be reported to each committee.

A university policy requires that all signed subject consent forms be kept in a permanent file in an area designated for that purpose by the Department Head or comparable authority. This will assure their accessibility in the event that university officials require the information and the principal investigator is unavailable for some reason.

Sincerely,

Brenda J Wittman, MD, MPH
Chair, Biomedical Committee
UA Institutional Review Board (IRB)

BW:mm
cc: Department/College Review Committee

Arizona's First University – Since 1885.

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