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THE SHEDDASE ACTIVITY OF ADAM10/ADAM17 ON CXCL16
INCREASES PROLIFERATION AND SURVIVAL OF COLORECTAL
CANCER CELLS

by

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A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Biology
in the College of Sciences
at the University of Central Florida
Orlando, Florida

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ABSTRACT

CXCL16 is an interferon-inducible chemokine of the CXC-subfamily and functions as an adhesion molecule, when membrane bound, and a chemoattractant when soluble. Upregulation of cell associated CXCL16 (cCXCL16) in colorectal cancer is associated with increased tumor infiltrating lymphocytes and good prognosis. ADAM10 and ADAM17 are metalloproteinases responsible for cleaving CXCL16, releasing soluble CXCL16 (sCXCL16) and contributing to proliferation and migration of mesangial cells, in kidney inflammatory disease. We hypothesize that cCXCL16 is a substrate for ADAM10 and ADAM17 cleavage in colorectal cancer, releasing sCXCL16, which mediates cell proliferation. To this end, we first identified CXCL16 in the human colon carcinoma cell line, RKO, by immunohistochemistry. cCXCL16 was found in the membrane, cytoplasm and nucleus. We treated RKO, in vitro, with an inflammatory cytokine mix containing 1.4 nM rhIFN γ , 2.0 nM rhTNF α and 2.0 nM rhIL1 β to increase the cleavage of cCXCL16 to sCXCL16. Overnight incubation with the cytokine mix significantly ($P=.004$) increased the release of sCXCL16 compared to normal conditions. To confirm that a metalloproteinase is responsible for the cleavage of cCXCL16, we used a broad spectrum metalloproteinase inhibitor, GM6001, in combination with inflammatory stimulation, in cell culture. We assayed the supernatant using ELISA for sCXCL16. GM6001 at 100 μ M decreased sCXCL16 to levels indistinguishable from the background. Using siRNA, we knocked down the expression of ADAM10 and ADAM17, independently, to determine if the activity of each on cCXCL16 was mediated by inflammatory stimulation. It was shown that ADAM10 constitutively cleaved cCXCL16, and ADAM17 cleavage activity was induced by inflammatory stimulation. To determine if sCXCL16 increased colorectal cancer cell (CRC) proliferation

through ligand-receptor binding, we treated cells with a range of rhCXCL16 from 3.125-100 ng/mL. rhCXCL16 did not increase RKO proliferation at doses up to 100 ng/mL. We used GM6001, to inhibit the cleavage of cCXCL16 into sCXCL16 then performed an ATPase assay and 6 day cell cycle analysis, under inflammatory stimulation. Increased cleavage of cCXCL16 induced by inflammatory stimulation with the cytokine mix containing 1.4 nM rhIFN γ , 2.0 nM rhTNF α and 2.0 nM rhIL1 β , increased RKO proliferation and reduced apoptosis. We conclude that ADAM10 and ADAM17 cleavage of cCXCL16 to sCXCL16 is increased by ADAM17 activation with inflammatory stimulation. The cleavage of the extracellular portion from cCXCL16 is associated with increased proliferation and decreased apoptosis of colorectal cancer cells.

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LIST OF ACRONYMS/ABBREVIATIONS

ADAM10	a Disintegrin and Metalloprotease 10
ADAM17	a Disintegrin and Metalloprotease 17
APS	ammonium persulfate
ATCC	American Type Culture Collection
BCA	bicichoninic acid
β -ME	2-mercaptoethanol
BMB	bromophenol blue
BSA	bovine serum albumin
cDNA	copy deoxyribonucleic acid
CXCL16	CXC chemokine ligand 16
cCXCL16	cell associated CXCL16
sCXCL16	soluble CXCL16
DAB	Diaminobenzidine
ddH ₂ O	double-distilled water
DPBS	Dulbecco's phosphate buffered saline
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCl	hydrochloric acid

HRP	horseradish peroxidase
ICC	immunocytochemistry
LiCO ₃	lithium carbonate
mRNA	messenger ribonucleic acid
N	Normal
NaOH	sodium hydroxide
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-2
qRT-PCR	quantitative reverse transcription- polymerase chain reaction
SDS	sodium dodecyl sulfate
siRNA	small-interfering ribonucleic acid
TBST	Tris-buffered saline with Tween-20
tcRNA	total cellular ribonucleic acid
TEMED	N,N,N',N''-tetramethylethylenediamine

INTRODUCTION

Inflammation marks the transition between the innate and adaptive immune response, when macrophages and neutrophils secrete cytokines and chemokines responsible for moderating cellular activities and recruiting cells expressing the appropriate receptors ¹. Antigen presenting cells (APCs) travel back to lymph nodes and the splenic red pulp where B cells are activated and T cells expressing antibodies specific to peptides found on the surface of APCs, are cloned. Inflammation has been associated with cancer since Virchow observed leukocytes in neoplastic tissue 150 years ago ². These tumor infiltrating leukocytes are recruited through ligand-receptor interactions directed by chemokine gradients. In 2003, it had been determined that almost 25% of all cancer cases worldwide were attributable to chronic infection and inflammation, according to B. Stewart and P. Kleihues World Cancer, Report (2003). CXCL16 is an inflammatory chemokine and known participant in the transition between the innate and adaptive immune response by recruiting CD4⁺, CD8⁺ T cells and NKT cells, which express the cell surface receptor CXCR6 ^{3,4}. The known biological functions of CXCL16 appear to be mitigated by post-translational proteolytic processing. While expressed as a membrane-bound protein, CXCL16 acts as an adhesion molecule, forming tight junctions through ligand-receptor binding. In this manner, CXCL16 expressed on dendritic cells supports the adhesion of T cells in splenic red pulp ⁵. After cell surface shedding into soluble form, CXCL16 is chemotactic for cells involved in the immune response, vascular endothelial cells ⁶ and human mesangial cells ⁷, expressing CXCR6. CXCL16 has been found up-regulated in inflammatory disease and cancer and positively correlated to increased leukocyte trafficking, aggressiveness and metastasis of tumor

cells^{7, 4, 8-10}. Cell associated CXCL16 has been associated with good prognosis and increased tumor infiltrating lymphocytes in colorectal cancer⁴ suggesting that CXCL16 expression may be prognostic for patient survival; however the role of CXCL16 in cancer progression is not fully understood.

ADAM10 and ADAM17 are α -secretases that shed cell surface molecules by proteolytic cleavage. This cleavage releases c-terminal fragments (CTFs) that are further processed by β - and γ -secretases. ADAM10 and ADAM17 shed CXCL16 from the cell membrane into the soluble form releasing CTFs of unknown function¹¹. In some inflammatory proliferative diseases and cancers the sheddase activity of ADAM10 and ADAM17 on CXCL16, specifically, has been linked to increased tumor cell invasiveness and migration, as well as T cell chemotaxis, proliferation, and migration^{7, 8, 10}. In addition, CXCL16 promotes chemotaxis, proliferation and tube formation in human vascular endothelial cells⁶. Cell associated CXCL16 has been shown to decrease and inhibit proliferation in human mammary and colon carcinomas and pancreatic ductal adenocarcinomas, whereas soluble CXCL16 (sCXCL16) has been shown to increase proliferation^{10, 12}. Inhibiting the expression of CXCL16, ADAM10 and ADAM17 has been shown to arrest mesangial cell proliferation under inflammatory stimulation⁷.

Stimulation of pancreatic ductal cells with exogenous sCXCL16, under normal conditions, does not increase cell proliferation¹⁰. Also, inhibiting the expression of CXCL16, ADAM10 and ADAM17 or CXCL16 alone, under normal conditions, does not significantly affect proliferation⁷. Mouse colon cancer cells already expressing CXCL16 do not show a notable increase in proliferation when stimulated with exogenous sCXCL16¹². These data suggest that the function of CXCL16 in cancer cell proliferation, under inflammatory

stimulation, is determined by the shedding of sCXCL16 from the cell membrane. Therefore, we tested ADAM10 and ADAM17 sheddase activity as a mediating event in human colon cancer cell proliferation, using *in vitro* studies with the human colon cancer cell line RKO. Our results suggest that the increase in colon cancer cell proliferation is not the result of autocrine signaling through ligand-receptor binding of sCXCL16, but through intercellular signaling by the CTF also released by ADAM10 and ADAM17 shedding. The release of sCXCL16 has the added effect of decreasing apoptosis. Our data suggest that CXCL16 may be a novel target for reducing proliferation and increasing apoptosis in colon cancer.

MATERIALS AND METHODS

Identification of cell-associated CXCL16

Materials

Human colon carcinoma cell line, RKO, was purchased from ATCC, and cultured in vented cap T-75 tissue culture flasks (NUNC) in EMEM supplemented with 10% heat inactivated FBS (GIBCO/Life Technologies) and 1% Penicillin-Streptomycin (GIBCO/Life Technologies) in a humidified incubator with 5% CO₂ at 37° C. Cells expressing CXCL16 were identified by ICC using an anti CXCL16 (C-20) goat polyclonal IgG antibody (Santa Cruz). Immunocytochemistry was performed on cells fixed to precleaned glass microscope slide cover slips (VWR) using 4% paraformaldehyde solution.

Reagents and solutions

4% Paraformaldehyde solution

While 80 mL of autoclaved distilled water was heated to 60°C, 4 grams of paraformaldehyde was added as solution stirred. The solution was covered and allowed to come up to temperature. 10 N NaOH was added drop wise until solution cleared: approximately 10 drops. 4 mL of 25X PBS was added to bring volume to 100 mL. pH was adjusted to 7.0 using HCl. Solution was sterilized using vacuum filtration and stored in a light sensitive bottle.

Peroxidase quencher

Peroxidase quencher was made in PBS with a final concentration of 3% peroxide and 1% sodium azide.

PBST

2 mL Tween-20 was dissolved in 800 mL PBS. The pH was adjusted to 7.2, and then the solution was autoclaved.

SDS

.5 grams SDS was added to 50 mL PBS and the solution was stirred until clear.

10% BSA

5 grams of BSA was dissolved in PBS to a final volume of 50 mL.

0.1% LiCO₃

.05 grams of LiCO₃ was dissolved in 50 mL autoclaved distilled water.

Fixed cell preparation

250,000 cells were plated on glass cover slips placed in each well of a 6 well plate. Cells were grown to confluence in 2 mL EMEM full growth media until cells reached confluence.

Immunocytochemistry

All steps were performed with cover slips in 6 well plates. Full growth media was removed and cells were washed and incubated for 1 minute; two times with ice cold PBS. Cells were fixed for 10 minutes in 1 mL of 4% paraformaldehyde, then washed and incubated for 2 minutes; three times with PBST. Cells were then dehydrated by 5 minute incubations with increasing concentrations of methanol (25%, 50%, 75% and 100%). Antigen retrieval was performed by washing fixed cells three times, in PBS for 5 minutes each, then incubating in 1% SDS for 5 minutes and finally washing three times for 5 minutes in PBS. Peroxidase activity was quenched with a 15 minute incubation in peroxidase quencher. Fixed cells were blocked in 10% BSA for 30 minutes, and then blocking agent was removed. Cells were incubated for one hour at room temperature in primary antibody specific for an epitope on the internal portion of CXCL16 (Santa Cruz) at a 1:50 concentration. Primary antibody was removed after one hour and cells were washed three times for five minutes in PBS. Cells were incubated for 1 hour at room temperature in secondary antibody, anti-goat-HRP (Santa Cruz) at a 1:100 concentration. Secondary antibody was removed after one hour and cells were washed three times for 5 minutes in PBS. Color was developed with a 5 minute incubation in DAB. After incubation cells were washed 2 times in tap water. Counter staining was performed with a 5 minute incubation in hematoxylin. After incubation the hematoxylin solution was removed. The cells were washed two times in tap water, dipped in 3% acid-alcohol (Sigma), washed one time in tap water then dipped in LiCO_3 . Fixed cells were then dehydrated by incubating for three times in 100% methanol for 5 minutes then incubating three times in 100% xylene for 5 minutes. Glass cover slips were mounted on precleaned glass microscope slides (Fisher Scientific). Digital images

were taken using a camera (Leica DFC300 FX) mounted on a light microscope (Nikon Eclipse E400).

Induced increase in soluble CXCL16 in cell culture

Materials

The inflammatory cytokine mix ⁷ containing 1.4 nM rhIFN γ (R & D Systems), 2 nM rhTNF α (R & D Systems) and 2 nM rhIL1 β (R & D Systems). Conditioned media was assayed for soluble CXCL16 using a premade Quantikine Human CXCL16 ELISA kit (R & D Systems), according to the manufacture's instructions.

Cell culture preparation

RKO cells were plated in 100 cm tissue culture dishes (BD Biosciences) at the concentration of 4 million cells in 5 mL of full growth media. The cells were allowed to adhere in a humidified incubator with 5% CO₂ at 37°C overnight. After incubation the media was removed, the cells were washed two times with DPBS, and the media was changed to include the inflammatory cytokine mix (1.4 nM rhIFN γ , 2 nM rhTNF α and 2 nM rhIL1 β). Cells were allowed to incubate in a humidified incubator with 5% CO₂ at 37°C for either 15 minutes or overnight. Control cells were plated, incubated 15 minutes or overnight in full growth media that did not include the inflammatory cytokine mix. After incubation, the conditioned media was removed, centrifuged to remove any debris then assayed for soluble CXCL16.

ELISA

All samples were assayed in quadruple. A Quantikine Human CXCL16 ELISA was purchased from R & D Systems (Minneapolis, MN). All buffers and reagents were brought to room temperature before assay was performed. The CXCL16 standard was reconstituted with 1 mL autoclaved distilled water to a stock concentration of 100 ng/mL. Serial dilutions were made using the provided Calibrator Diluent RD5R to final working concentrations of 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.315 ng/mL and 1.156 ng/mL. The Calibrator Diluent was used as the zero standard. 100 mL of the provided 100µl Assay Diluent RD1W was added to each well. 50 µl of each standard and sample was added to individual wells. After 2 hours incubation at room temperature, the solutions were removed and the wells were washed four times with 300 µl of the provided wash buffer. After the fourth wash 200 µl of the provided conjugate was added to each well and incubated for two hours at room temperature. After the incubation the solutions were removed and the plate was washed four times with 300 µl of the provided wash buffer. After the fourth wash 200 µl of the provided substrate solution was added to each well in incubated for 30 minutes at room temperature protected from light. After the incubation 50 µl of the provided stop solution was added to each well and the absorbance intensity was read using a microplate reader (FLUOstar OPTIMA).

Optimization of GM6001 broad spectrum metalloproteinase inhibitor

Materials

The broad spectrum metalloproteinase inhibitor, GM6001 (Sigma) was used to inhibit ADAM10 and ADAM17 proteolytic activity. The inflammatory cytokine mix containing 1.4 nM rhIFN γ (R & D Systems), 2 nM rhTNF α (R & D Systems) and 2 nM rhIL1 β (R & D Systems) was used to sCXCL16 concentrations in the cell culture supernatant. Conditioned media was assayed for soluble CXCL16 using a premade Quantikine Human CXCL16 ELISA kit (R & D Systems), according to the manufacture's instructions.

Determining sCXCL16 concentration using ELISA

250,000 RKO cells were plated in 6 well plates in full growth media and incubated overnight in a humidified incubator at 37°C in 5% CO₂, to allow cells to adhere. After incubation, the media was removed and the cells were washed 2 times with DPBS. Cultures were then incubated in increasing concentration of GM6001 (Sigma): 25 μ M, 50 μ M, 100 μ M, 200 μ M and 400 μ M for 15 minutes, then 2 mL of full growth media with the addition of the inflammatory cytokine mix was added to the wells and the plates were incubated overnight in a humidified incubator at 37°C in 5% CO₂. After incubation, culture media was removed and centrifuged to remove debris and assayed for sCXCL16 using a premade Quantikine Human CXCL16 ELISA kit (R&D Systems) as previously described. All conditions were performed in triplicate. All samples were assayed in duplicate.

Determining the role of ADAM10 and ADAM17 in cCXCL16 cleavage into sCXCL16

Materials

siRNA duplexes were purchased from Invitrogen (Carlsbad, CA). The sequences are as follows: ADAM10 5' AGA CAU UAU GAA GGA UUA UTT 3'; ADAM17 5' GAG AAG CUU GAU UCU UUG CTT 3'; and a scrambled control 5' AGG UAG UGU AAU GCG CUU GTT 3'. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and using electroporation with a GenePulser Xcell with Shockpod (BioRad).

Optimization of knockdown

500,000 cells were treated with differing concentrations of siRNA duplexes. Transfections were carried out using Lipofectamine 2000 (Invitrogen). Different picomole concentrations of siRNA were diluted in 25 μ l of Opti-MEM I Medium, and mixed gently. 10 μ l of Lipofectamine 2000 was diluted into 25 μ l of Opti-MEM I Medium (Invitrogen) and incubated at room temperature for 5 minutes. After incubation, the diluted Lipofectamine 2000 was added to 6 well plates with the diluted RNAi complexes, mixed gently, and incubated for 15 minutes at room temperature. After incubation, cells were added to the well and the media volume was brought up to 1 mL using Opti-MEM I Medium. Cells were incubated overnight in a humidified incubator at 37°C with 5% CO₂. After overnight incubation, the media was removed, the cells were washed 2 times with DPBS and 1 mL of EMEM full growth media was added. After overnight incubation in a humidified incubator at 37°C with 5% CO₂ the media was

collected for use in ELISA. This procedure was repeated with the exception that cells were transfected by electroporation at 300V and 960 μ F with a GenePulse Xcell (BioRad). ELISA was performed on cell culture supernatant to assay for sCXCL16 using a premade Quantikine Human CXCL16 ELISA kit (R&D Systems) as previously described. Effective knockdown was determined through RNA analysis using qRT-PCR of cDNA reverse transcribed from tcRNA extracted from cell pellets.

sCXCL16 after ADAM10/ADAM17 knockdown under non-inflammatory and inflammatory conditions

500,000 cells for each condition were electroporated at 300V and 960 μ F with predetermined concentrations of siRNA duplexes for ADAM10, ADAM17 and equivalent concentration of scramble siRNA duplexes. Cells were plated in 6 well plates in 1 mL media and incubated overnight in a humidified incubator at 37°C with 5% CO₂. After overnight incubation, the media was removed, the cells were washed 2 times with DPBS and 1 mL of fresh media was added. This procedure was repeated with the addition that the fresh media contained the inflammatory cytokine mix including: 1.4 nM rhIFN γ (R & D Systems), 2 nM rhTNF α (R & D Systems) and 2 nM rhIL1 β (R & D Systems). Each condition was plated in triplicate. sCXCL16 was quantified using a premade Quantikine Human CXCL16 ELISA kit (R & D Systems) as previously described. Knockdown was determined using qRT-PCR, as previously described. Wild type RKO cell culture was used as a control. GAPDH was used as the endogenous gene expression control.

Comparison of proliferation in sCXCL16 versus cCXCL16

Materials

Proliferation was measured using the ATPase assay, Cell Titer Glo (Promega). Luminescence intensity was measured using a microplate reader (FLUOstar OPTIMA). GM6001 (Sigma) was used to inhibit ADAM10 and ADAM17 activity to increase cCXCL16.

Growth curve for optimal plating density

RKO cells were plated, in quadruple, in white walled 96 well plates (Lonza) at 500 cells/well, 1500 cells/well, 4500 cells/well, 13,500 cells/well and 40,500 cells/well, in 100 µl of full growth media. Each cell concentration was plated for a reading at a 0 time point to establish a baseline, 2 days, 4 days and 6 days. 100 µl of reconstituted cell titer glo reagent was added to each well and incubated protected from light at room temperature to ensure complete lysis and allow optimal luminescence intensity. The plate was incubated in a humidified incubator at 37°C in 5% CO₂ in between time points.

Measuring the effect of cCXCL16 on proliferation

EMEM full growth media was made including 100 µM GM6001. Cells were trypsinized from culture, pelleted and resuspended in 1 mL DPBS with the addition of 100 µM GM6001, allowed to sit for 15 minutes then counted. 1000 cells were plated in wells of a 96 well plate using 100 µl of full growth media containing 100µM GM6001. Full growth media containing GM6001 was plated without cells as a background luminescence control. 100 µl of cell titer glo

reagent was added to the wells then incubated for 15 minutes at room temperature.

Luminescence was quantified on a microplate reader (FLUOstar OPTIMA). Each experimental condition was plated in triplicate. Each condition was assayed on days 1 - 7. These experimental conditions were repeated with the additional treatment of the inflammatory cytokine mix.

Measuring the effect of sCXCL16 on proliferation

Cells were trypsinized from culture, pelleted, resuspended in 1 mL DPBS and counted. 1000 cells were plated in wells of a 96 well plate using 100 μ L of full growth media. Full growth media was plated without cells as a background luminescence control. 100 μ l of cell titer glo reagent was added to the wells then incubated for 15 minutes at room temperature. Luminescence was quantified on a microplate reader (FLUOstar OPTIMA). Each experimental condition was plated in triplicate. Each condition was assayed on days 1 - 7. These experimental conditions were repeated without the additional treatment of the inflammatory cytokine mix.

Measuring the effect of exogenous sCXCL16 on proliferation

Aliquots of EMEM full growth media including 100 μ M GM6001 were made with the following concentration of rhCXCL16 to simulate stimulation of cells by exogenous soluble CXCL16: 3.125 ng/mL, 6.25 ng/mL, 12.5 ng/mL, 25 ng/mL, 50 ng/mL and 100 ng/mL. One aliquot contained no rhCXCL16 for use as a control. Cells were trypsinized from culture, pelleted and resuspended in 1 mL DPBS with the addition of 100 μ M GM6001, allowed to sit for 15 minutes then counted. 1000 cells were plated in wells of a 96 well plate using 100 μ l of the media aliquots containing rhCXCL16 100 μ l of media only was plated as a background control.

100 µl of cell titer glo reagent (Promega) was added to the wells then incubated for 15 minutes at room temperature. Luminescence was quantified on a microplate reader (FLUOstar OPTIMA). Each experimental condition was plated in triplicate. Each condition was assayed on days 1 – 7.

Determining the effect of sCXCL16 vs. cCXCL16 on proliferation and apoptosis under inflammatory conditions

Materials

FACS Caliber (BD Biosciences) was used to perform the cell cycle analysis

Reagents and solutions

PI stain

To make 50 mL, 59 mg sodium citrate, 2.5 mg propidium iodide and 50 µl Triton X were mixed. Autoclaved deionized water was added for a total volume under 50 mL. The solution was brought to pH 7.8 and then 150 µl of RNase 1 was added. Autoclaved deionized water was added to a total volume of 50 mL.

Staining protocol

Cells were fixed in 70% ethanol. After fixing, cells were washed in PBS and PI stain was added in the dark while vortexing.

Proliferation under inflammatory stimulation determined by ATPase assay

1,000 cells were plated in wells of a 96 well plate in 100 μ l of EMEM full growth media with the addition of the inflammatory cytokine mix containing: 1.4 nM rhIFN γ (R & D Systems), 2 nM rhTNF α (R & D Systems) and 2 nM rhIL1 β (R & D Systems). Cells were incubated for 15 minutes with the metalloproteinase inhibitor, GM6001, and then plated in wells of a 96 well plate in 100 μ l of EMEM full growth media with the addition of GM6001 and the inflammatory cytokine mix. Full growth media and full growth media with GM6001 were plated with no cells for use as a background luminescence control. For 6 days proliferation was determined by adding 100 μ l of cell titer glow reagent, allowing the cells to incubate at room temperature for 15 minutes and reading luminescence intensity with a microplate reader (FLUOstar OPTIMA). Each condition was read daily for 6 days.

Apoptosis analysis under inflammatory stimulation determined by cell cycle analysis

1 million cells were plated in each well in a 6 well plate with EMEM full growth media with the addition of the inflammatory cytokine mix containing: 1.4 nM rhIFN γ (R & D Systems), 2 nM rhTNF α (R & D Systems) and 2 nM rhIL1 β (R & D Systems). Cells were incubated in a humidified incubator at 37°C with 5% CO $_2$. Cells were trypsinized and collected for PI staining and cell cycle analysis by flow cytometry, every day for 6 days, using a FACS Caliber (BD Biosciences) The experimental conditions were repeated with the addition of the metalloproteinase inhibitor, GM6001.

RESULTS

CXCL16 expression in RKO

CXCL16 is one of two known chemokines which have a membrane bound form functioning as a strong adhesion molecule for cells expressing the receptor, CXCR6⁵. Ectodomain shedding produces a soluble form of CXCL16 which is a strong chemoattractant¹¹. CXCL16 was identified in the Colo205, LS174T, SW480, and T84 colon cancer cell lines as well as human tissues resected from colon tumors by RT-PCR and immunohistochemistry using an antibody recognizing an unidentified epitope¹. In this study, the transcription of CXCL16 in human tumor samples was compared to transcription in the normal mucosa, grouped as weak, intermediate and strong expressers using staining intensity with immunohistochemistry. Transcription levels were then compared to staining intensity of CD4⁺ and CD8⁺ tumor infiltrating lymphocytes. Colon tumor cells had higher transcription of CXCL16 than normal mucosa and the CXCL16-strong stain intensity group had a higher incidence of TIL (tumor infiltrating lymphocytes). CXCL16 was found to be associated with the cell membrane and the cytoplasm. CXCL16 was also identified in a sample of 23 human rectal patients using RT-PCR, western blotting and immunohistochemistry using an antibody recognizing an epitope on the chemokine domain. These data showed no significant difference in transcription between tumor cells and normal cells of the same patient, and western blotting and immunohistochemistry showed decreased CXCL16 in tumor tissue and lysate compared to the normal tissue of the same patient¹⁶.

We wanted to determine if CXCL16 was expressed in the human colon cancer cell line RKO. Understanding that CXCL16 is shed from the cell membrane, we used an antibody targeting an epitope in the cytoplasmic domain. γ -secretases process the surface molecules, Notch and E-Cadherin, which generate C-terminal fragments functioning as signal transmitters¹¹. C-terminal fragments of CXCL16 have been found in the normal embryonic kidney cell line, HEK293¹¹, suggesting that CXCL16 may require similar processing for functional activation or perform a similar functional role as Notch or E-Cadherin. To further elucidate the potential for CXCL16, not just as an adhesion molecule and soluble chemoattractant, but possibly as an intracellular signaling molecule, we performed immunocytochemistry on RKO cells fixed to glass cover slips, with an anti-CXCL16 antibody targeting a cytoplasmic epitope, to identify the areas of the cell containing CTFs (c-terminal fragments) of CXCL16.

We identified CXCL16 on the cell membrane, in the cytoplasm, and in the nucleus of RKO cells, as indicated by red staining (Figure 1). Protein that has performed its function or is nonfunctional is normally targeted for degradation. These data suggest that the C-terminal fragments of CXCL16 may perform a functional role in colorectal tumor cells. Due to the identification of CXCL16 in RKO further studies are possible.

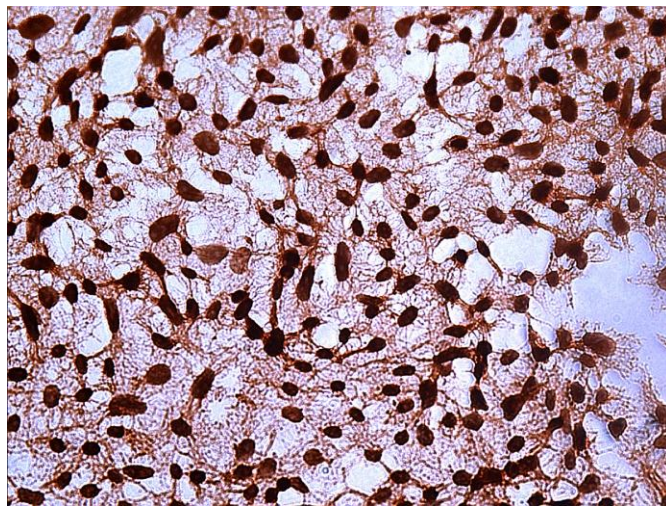


Figure 1. Immunocytochemistry of RKO cells stained using anti-CXCL16 antibody. CXCL16 can be found associated with the cell membrane, cytoplasm and nucleus of RKO cells in culture.

Inflammatory stimulation increases sCXCL16

Treating cells *in vitro* with inflammatory stimulation including TNF α and IFN γ increases the release of sCXCL16 in astrocytes, glioma cells, endothelial cells from human umbilical vein and smooth muscle cells, and human mesangial cells^{7, 8, 17}. To determine if CXCL16 is shed from the membrane of human colorectal cells, we treated RKO cells with a cytokine mix containing 2.0 nM IL-1 β , 2.0 nM TNF α and 1.4 nM IFN γ ⁷. We used two incubation times to determine if sCXCL16 increased over time under constant stimulation. We used ELISA to test the concentration of CXCL16 in the cell culture supernatant after an incubation of 15 minutes and overnight incubation. Soluble CXCL16 increases with stimulation in as little as 15 minutes after the initial assault, however there was a significant increase of sCXCL16 (P=.004) compared to the unstimulated control after overnight stimulation (<24 hr) (Figure 2). CXCL16 is an IFN γ – inducible chemokine, which suggests that inflammatory stimulation would increase the transcription of CXCL16 making more available for sheddase activity. However, post-transcriptional modifications, evidenced by the shedding of CXCL16, may be induced by this same mechanism. Understanding the transcriptional and post-transcriptional mechanisms involved when inflammatory stimulation increases sCXCL16 may suggest one or more targets to mediate this activity.

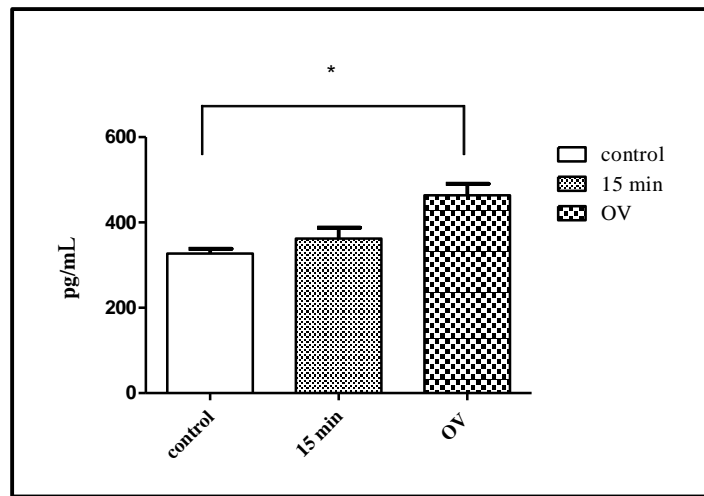


Figure 2. sCXCL16 concentrations in RKO cell culture supernatants after 15 minute or overnight (OV) incubations with the inflammatory cytokine mix containing 1.4 nM rhIFN γ , 2.0 nM rhTNF α and 2.0 nM rhIL1 β . ELISA was performed to measure the amount (pg/mL) of sCXCL16 in RKO cell supernatant.

*** indicates P=.004 using Welch's t-test between the control and overnight cell culture populations.**

GM6001 optimal biological dose as determined by ELISA

ADAM10 and ADAM17 are responsible for shedding of CXCL16 in human mesangial cells⁷. To investigate the role of ADAM10 and ADAM17 on the shedding of CXCL16 in human colorectal cancer cells we confirmed that shedding of CXCL16 is achieved through metalloproteinase activity. GM6001, a broad spectrum metalloproteinase inhibitor, obtained lyophilized from Sigma, inhibits proteolysis of some metalloproteinases, including ADAM10 and ADAM17⁷. To measure the efficacy of GM6001 to inhibit metalloproteinase cleavage of cCXCL16 into sCXCL16 on RKO, cells were incubated with GM6001, reconstituted in DMSO, with concentrations ranging from 25 μ M to 400 μ M under inflammatory stimulation. After an initial 15 minute incubation in GM6001, cells were cultured overnight in media including GM6001 with the addition of inflammatory cytokine stimulation. The supernatants were collected and assayed for sCXCL16 concentrations using ELISA. Full growth media was used as a control to identify baseline absorbance. GM6001 at 100 μ M was determined to be optimal for metalloproteinase inhibition by causing a decrease in sCXCL16 to levels indistinguishable from background absorbance levels (Figure 3). Although higher doses of inhibitor, up to 400 μ M concentration, showed a trend in decreased detectable levels of sCXCL16, this also increased the volume of DMSO introduced into cell culture, thereby increasing the risk of toxicity to the cells.

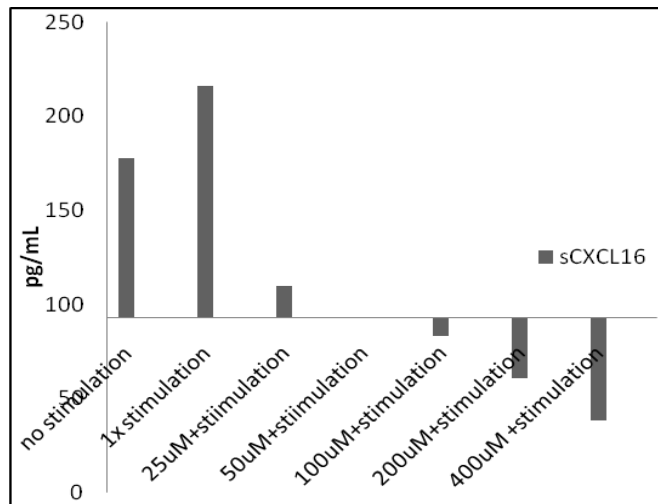


Figure 3. sCXCL16 concentrations in cell culture supernatants as determined by ELISA. RKO cells were incubated overnight in full growth media in the presence of GM6001 (25 μ M - 400 μ M) and inflammatory stimulation with 1.4 nM rhIFN γ , 2.0 nM rhTNF α and 2.0 nM rhIL1 β .

ADAM10 is responsible for constitutive shedding of sCXCL16

ADAM10 has been associated with the constitutive cleavage of cell membrane adhesion molecules including CXCL16^{17 18} and ADAM17 has been associated with inflammatory induced cleavage^{8, 17}. To determine the roles of ADAM10 and ADAM17 in shedding CXCL16 from RKO cells we knocked down the expression of each metalloproteinase with siRNA oligonucleotides targeting ADAM10 (labeled A10) and ADAM17 (labeled A17). Control cells were transfected with scrambled oligonucleotides in concentrations comparable to those used for ADAM10 and ADAM17, 750 pmol/mL (SCR 750) and 1500 pmol/mL (SCR 1500), respectively. We measured CXCL16 proteolysis using sandwich ELISA targeting sCXCL16 in the cell culture media. We assayed the degree of knockdown by qRT-PCR with primers specific for ADAM10 and ADAM17 then determined the percentage of gene expression as compared to the control using the formula $100 - [(1 - 2^{-\Delta\Delta CT}) \times 100]$. After overnight incubation without inflammatory stimulation the knockdown of ADAM10 decreased sCXCL16 to levels indistinguishable from background absorbance levels, although ADAM17 was still being expressed (Figure 4 A). ADAM10 expression was 38.6% of the control expression level (Figure 4 C). Under non-inflammatory conditions ADAM17 expression was knocked down to 44.34% of control levels, as ADAM10 continued to be expressed (Figure 4 C). Although the level of knock down for ADAM17 was similar to that shown for the independent knock down of ADAM10, the decrease of sCXCL16 was not comparable to that in the ADAM10 knockdown experiment, as shown in Figure 4 A. These data suggest that under non-inflammatory conditions ADAM10

shedase activity is necessary and sufficient to account for sCXCL16 in comparison to ADAM17.

Although we attempted to repeat these studies under inflammatory conditions, we did not achieve the level of knockdown for either ADAM10 (2%) or ADAM17 (24%), (Figure 4 D), compared to the scrambled controls, SCR 750 and SCR 1500, respectively. It is possible that inflammatory conditions induce the transcription of ADAM10 and ADAM17 at a rate which overwhelmed the siRNA ability to induce degradation of the mRNA. However, the slight decrease in sCXCL16, as shown in Figure 4 B, suggests that ADAM17 does participate in shedding sCXCL16 under inflammatory conditions. These data suggest that ADAM10 constitutive cleavage is not sufficient to account for all sCXCL16 released under inflammatory conditions.

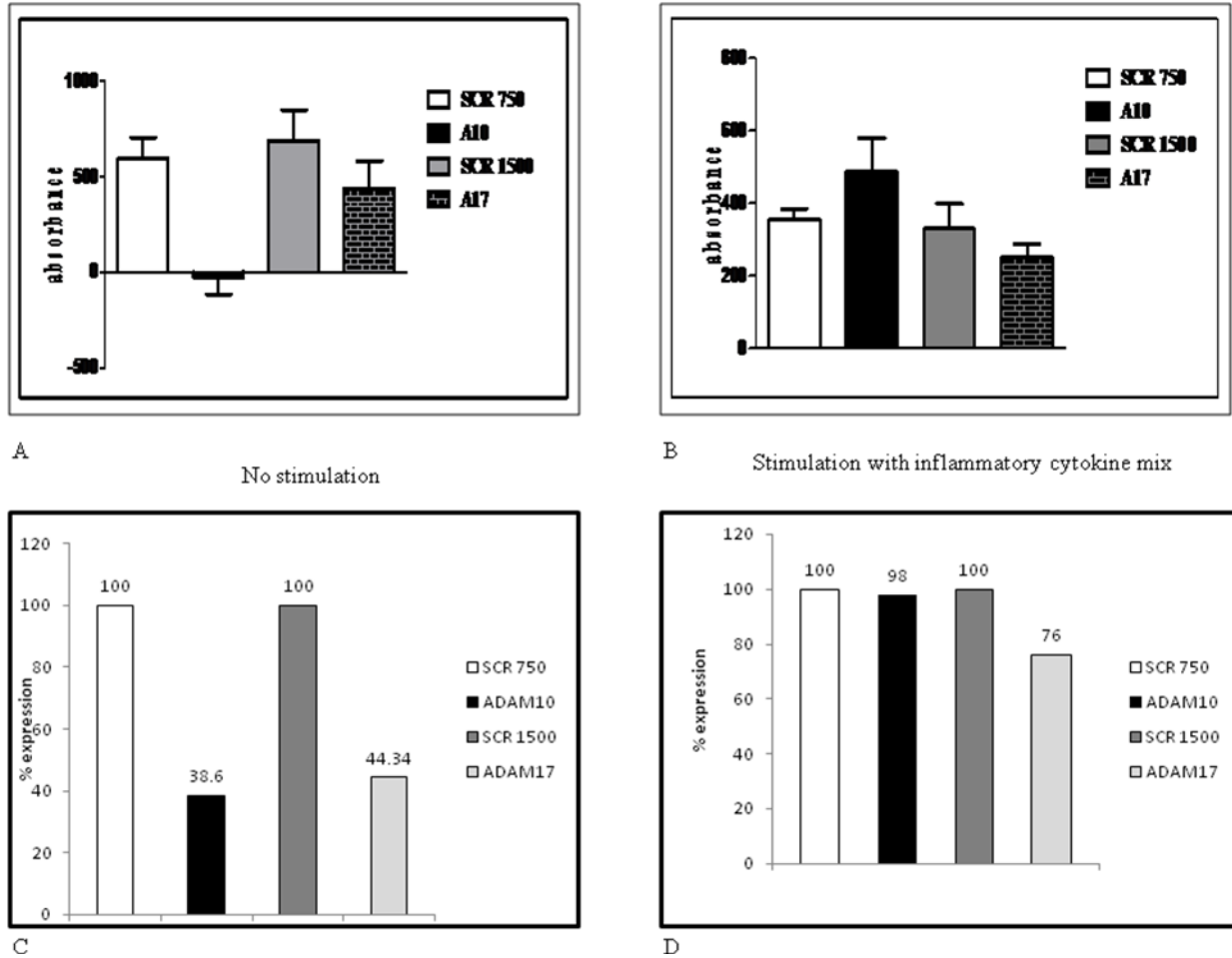


Figure 4. sCXCL16 concentrations in cell culture supernatants determined by ELISA after siRNA-knockdown of ADAM10 or ADAM17 under normal (A) and inflammatory (B) stimulation. Percent (%) expression compared to the controls were determined by qRT-PCR of mRNA levels after knockdown as determined by the formula $1 - [(1 - 2^{-\Delta\Delta CT}) \times 100]$ under normal (C) and inflammatory (D) stimulation.

Student t-test determined there is no significant difference in sCXCL16 concentrations between experimental conditions and control $P > 0.05$.

Optimal plating density for RKO cells

To identify the effect of CXCL16 on the proliferation of RKO cells, we measured cell viability as determined by ATP concentration. Increased ATP levels suggest increased cellular metabolic activity and cell number. However, limiting factors for cells in culture include growth factor and nutrient resources, as well as growth surface area, as many cells maintain contact inhibition. We tested for the optimal plating density for RKO cells in 96 well plates to ensure the results of our proliferation studies were not biased due to the limiting factors named above. Cells were plated at the lowest density of 500 cells up to 40,500 cells. Cells were cultured in 100 μ l full growth media, with no media changes for one week. We determined the optimal plating density to be between 50 and 1500 cells per well (Figure 5). Cell culture concentrations of 1000 cells per well in a 96 well plate would allow for testing of inflammatory stimulation on proliferation, over five days, without the deleterious effects of over-confluence and lack of media resources for cells. These data allow us to extrapolate optimal densities for other cell growth areas using the formula: $(\text{growth area cm}^2 / .32 \text{ cm}^2) \times 1000 = \text{optimal plating density}$.

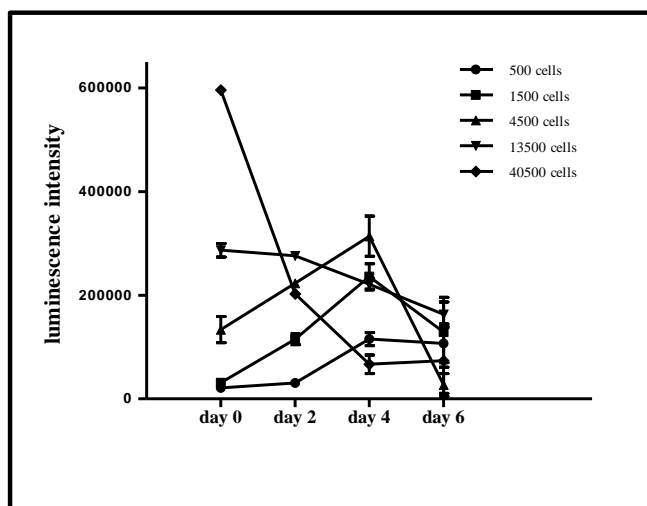


Figure 5. Cell viability assay to determine optimal plating density of RKO cells in 96 well plates. Cells were plated at different densities and on day 0, 2, 4 and 6, cell viability was measured by Cell Titer Glo Luminescent Cell Viability Assay (cell number correlates with luminescent output, RLU).

Exogenous sCXCL16 does not increase RKO cell proliferation

The shedding of cell surface molecules is a regulatory mechanism necessary to mediate biological activities such as juxtacrine, autocrine and paracrine signaling and releasing cytoplasmic domains for intracellular signaling. CXCL16 recruits CXCR6-expressing CD4⁺ and CD8⁺ T cells ⁴, and is associated with increased proliferation of murine astroglial and microglial CXCR6-expressing cells⁸. CXCL16 activity occurs with a concentration of 10 ng/mL, as measured by chemotaxis of CXCR6 expressing JURKAT cells and CXCR6 expressing CD4⁺, CD8⁺, and NKT cells ³. To test the effect of CXCL16 on cell proliferation through ligand-receptor binding, RKO cells were incubated overnight with purchased recombinant human CXCL16 (rhCXCL16) at concentrations ranging from 3.125 ng/mL – 100ng/mL and ATP cell viability assay was performed. Stimulation of RKO cells with exogenous sCXCL16 in the form of recombinant human CXCL16, did not affect the growth rate and viability of the cells (Figure 6). These data suggest that any effect CXCL16 may have on colon cancer cell proliferation does not operate in the receptor-ligand binding mechanisms defining autocrine, paracrine or juxtacrine signaling. It is possible that CXCL16 shedding from the cell membrane releases the cytoplasmic portion of CXCL16 which is then involved in intracellular signaling.

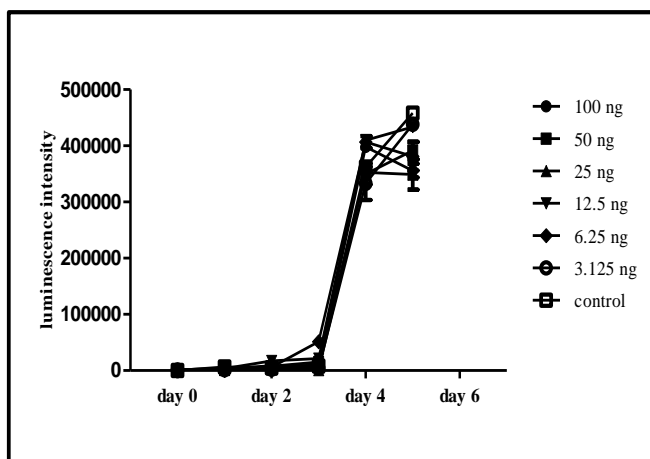


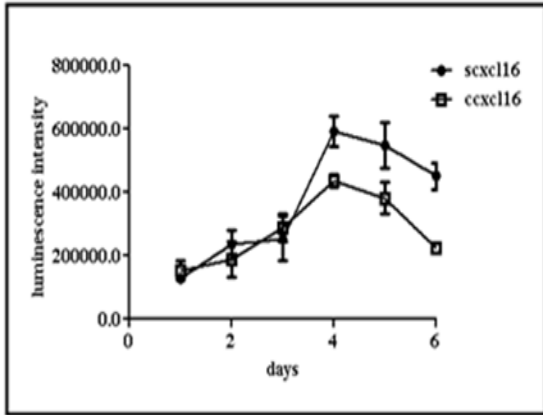
Figure 6. Proliferation measured by ATPase assay of RKO cells after treatment with increasing concentrations of exogenous sCXCL16.

sCXCL16 increases proliferation and decreases apoptosis compared to cCXCL16 under inflammatory conditions

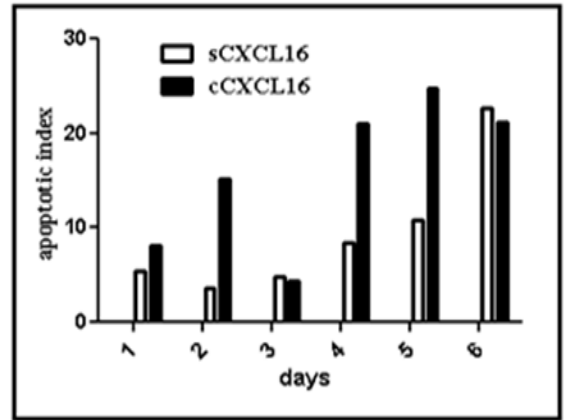
We assessed the ability of CXCL16 to affect RKO cell proliferation through intracellular signaling. The shedding of cell surface CXCL16 is catalyzed by inflammatory stimulation that includes IFN γ , TNF α and IL-1 β . Notably, although TNF α and IFN γ alone increase the release of sCXCL16, stimulation with these inflammatory cytokines in concert significantly increases sCXCL16 concentrations within the same time window¹³. Shedding of the extracellular portion of cell surface molecules is necessary to release the intracellular portion¹¹. All cultures were treated with the inflammatory cytokine mix containing 1.4 nM rhIFN γ , 2.0 nM rhTNF α and 2.0 nM rhIL1 β . Cell viability, as measured by ATP assay, was assessed every 24 hours for 6 days. To further distinguish the shedding of CXCL16 involvement in proliferation from any treatment effects attributable to inflammatory stimulation we performed our proliferation studies with the additional condition of metalloproteinase inhibition, using the established optimal dose (100 μ M) of GM6001. Cells releasing sCXCL16 showed greater proliferation than cells retaining cCXCL16, although Student's t-test determined no significant difference, $P > 0.05$ (Figure 7 A). As treatment with increased exogenous sCXCL16 did not increase proliferation in RKO cells, our data suggest that CXCL16 plays a role in RKO cell proliferation through intracellular signaling.

To further clarify the association of shedding CXCL16 with proliferation and thereby a possible role in tumor growth, we repeated the above experiment using cell cycle analysis as a means to measure proliferation and apoptosis. Cell cycle analysis was conducted by flow cytometry of PI stained cells. Experimental conditions were compared to control cell cultures

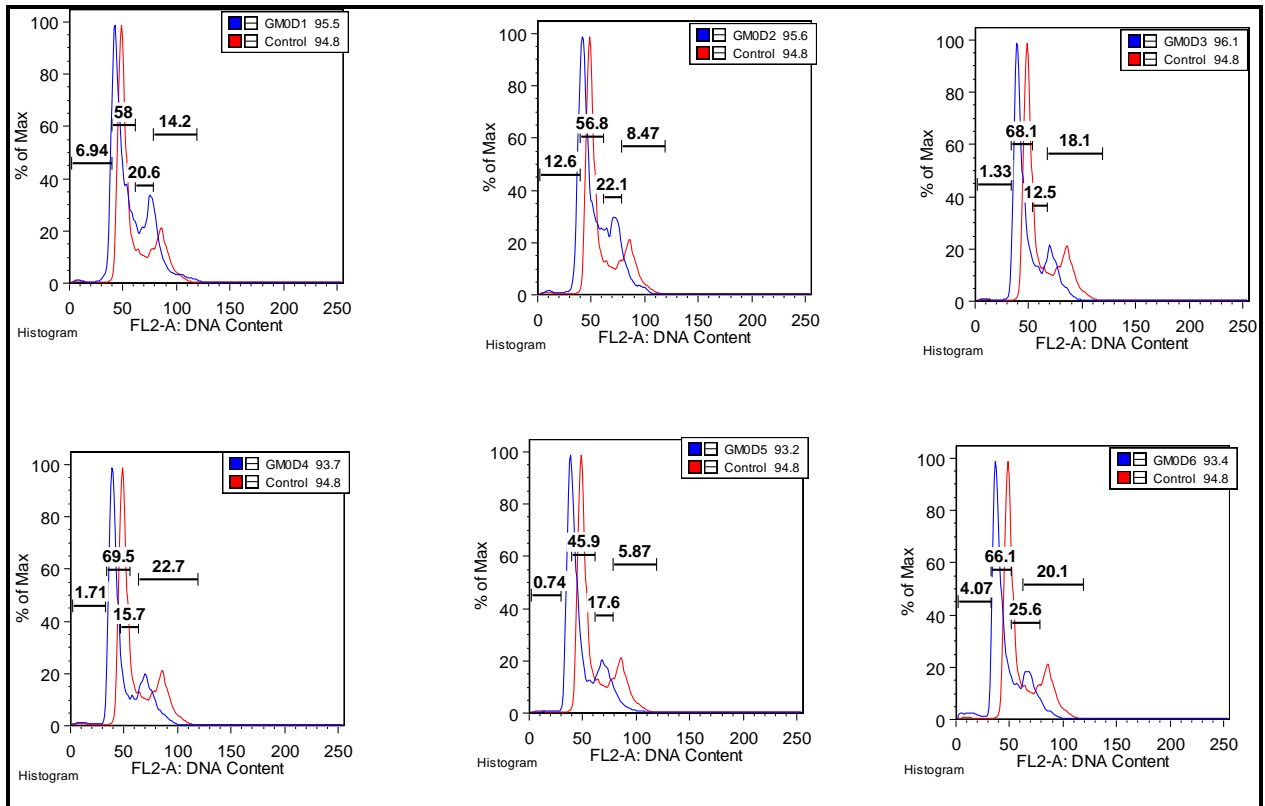
grown under non-inflammatory conditions and without metalloproteinase inhibition. Control cell cycle analysis is represented in red. Experimental conditions are represented in blue. The portion of the cell cycle analysis representing apoptotic cells (0 – 50 on the x-axis), is larger for cells maintaining cCXCL16 compared to cells shedding sCXCL16 (Figure 7 C and D, respectively). Graphical comparison of the apoptotic indices is represented in Figure 7 B. Cell cycle analysis supported our ATP cell viability results and indicated that shedding of CXCL16 from the cell membrane of RKO cells was associated with increased proliferation, as shown by an increase in the number of cells in the population in G₂ (Figure 7 D, portion of histogram with a mean of 100 on the x-axis). These data suggest that CXCL16 is an important mediator of colon cancer cell survival and proliferation through inflammatory signaling pathways.



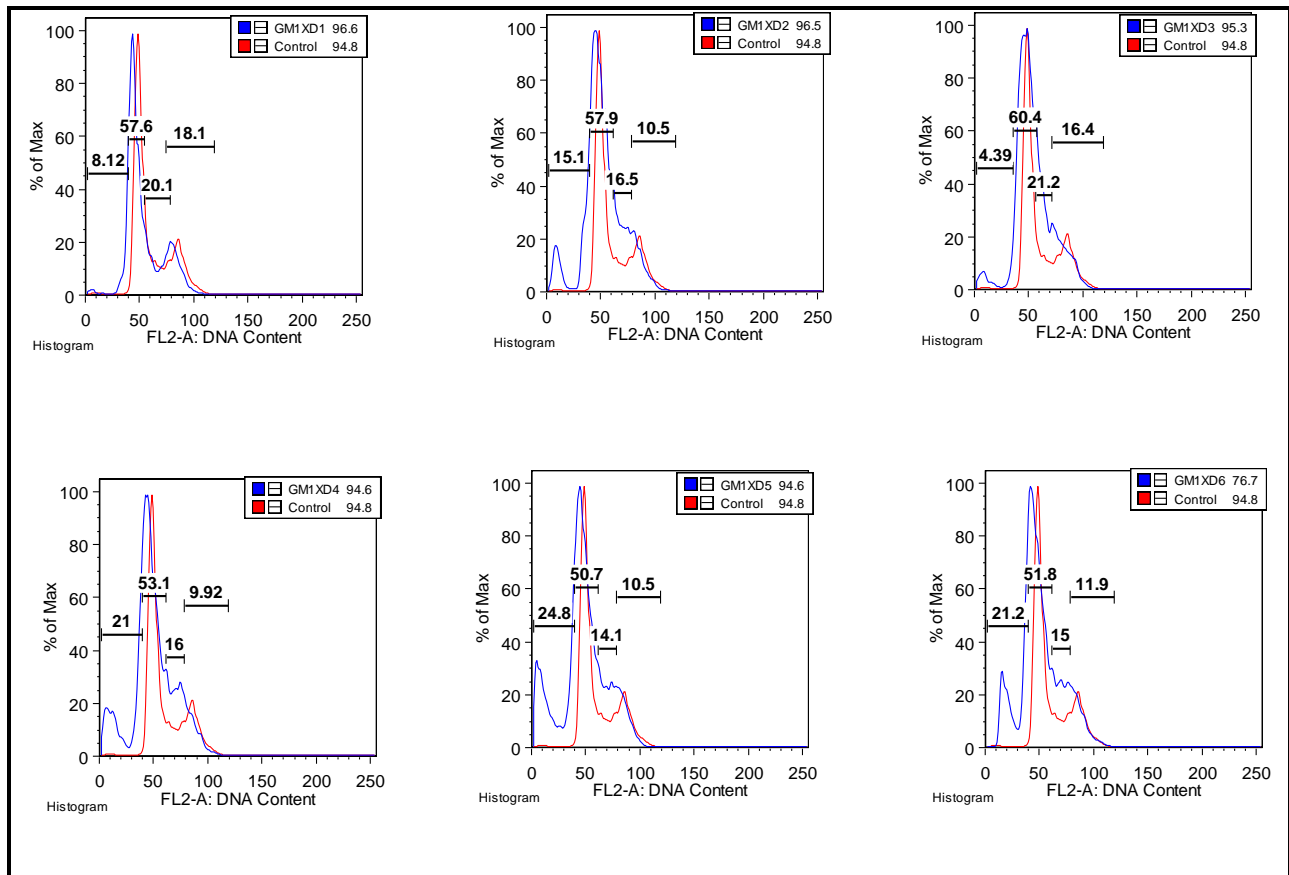
A



B



C



D

Figure 7. ATPase assay and cell cycle analysis of RKO cells (A) ATPase assay showing sCXCL16 is associated with increased RKO cell proliferation compared to cCXCL16. (B) Apoptotic index of cell cycle analysis shows that cCXCL16 is associated with increased RKO cell death compared to sCXCL16. (C) Cell cycle analysis of RKO cells grown in inflammatory conditions while under GM6001 metalloproteinase inhibition and assayed every 24 hours for 6 days after PI staining and measured using flow cytometry. (D) Cell cycle analysis of RKO cells grown under inflammatory conditions and assayed every 24 hours for 6 days after PI staining and measured using flow cytometry.

Student t-test determined there is no significant difference in experimental conditions for proliferation or apoptosis $P > 0.05$.

CONCLUSION

We have shown that CXCL16 is involved in the proliferation and survival of colorectal cancer cells. ADAM10 constitutively cleaves CXCL16, however inflammatory stimulation promotes the increased release of sCXCL16 through increased ADAM17 proteolysis. The shedding of cell surface CXCL16 is necessary to initiate functional activity related to increased proliferation and survival under inflammatory conditions. As increased proliferation and decreased apoptosis is not evidenced with increased soluble CXCL16 alone, our data suggest these effects do not result from ligand-receptor signaling. CXCL16 is constitutively cleaved by ADAM10, however increased cleavage through ADAM17 proteolysis suggests a threshold must be reached to overwhelm competing signals or that CXCL16 is only one among other molecules up-regulated by inflammatory stimulation working in concert to increase survival and growth.

Although it is known that CXCL16 is expressed as a cell surface molecule with an extracellular, transmembrane and cytoplasmic domain¹³, we attempted to detect cell associated fragments of CXCL16, specifically by targeting an epitope on the cytoplasmic domain. C-terminal fragments (CTFs) released after the cell surface portion of CXCL16 is shed are not immediately degraded and have been detected in cell lysates¹¹. As cells degrade proteins that are no longer useful, we speculated that the CXCL16 CTFs previously identified may function in intracellular signaling. Our immunocytochemical analysis revealed that CXCL16 CTFs are found in the cytoplasm and associated with the nucleus thereby suggesting further analysis of CXCL16 biological activity may be revealing.

ELISA analysis of sCXCL16, after inflammatory stimulation with a cytokine mix containing IL-1 β , TNF α and IFN γ , determined that inflammation increases CXCL16 shedding, and sCXCL16 concentrations increase with continued inflammatory stimulation (Figure 2). It has been shown that ADAM10 and ADAM17 are responsible for the shedding of sCXCL16 from the cell surface^{7, 11, 17, 19}. Using siRNA to knockdown the expression of ADAM10 and ADAM17 under normal and inflammatory conditions, we found that a decrease in ADAM10 expression to less than half of normal expression levels (Figure 4 C) precluded the detection of sCXCL16 (Figure 4 A), supporting the role of ADAM10 in constitutive cleavage of cell associated CXCL16. Knockdown of ADAM17 to less than half of normal expression levels (Figure 4C) did not significantly decrease sCXCL16 levels (Figure 4 A), indicating that ADAM17 does not have a significant role, if any, in the cleavage of CXCL16 under normal conditions. The knockdown experiments under inflammatory stimulation were not conclusive. However, less than 30% knockdown of ADAM17 (Figure 4 D) from normal expression levels did result in a slight decrease in sCXCL16 (Figure 4 B). These data in conjunction with our ELISA data of increased sCXCL16 with inflammatory stimulation, as well as data from previous studies that determined ADAM17 cleaves human mesangial cells under inflammatory stimulation⁷, suggest ADAM17 participate in CXCL16 cleavage in colorectal cancer cells under inflammatory stimulation.

Autocrine, paracrine and juxtacrine signaling occurs through ligand-receptor binding. The chemotactic and cell adhesion functions of CXCL16 occur through paracrine²⁰ and juxtacrine signaling^{5, 21, 22}, respectively. We determined that CXCL16 signaling in proliferation does not occur through ligand-receptor binding in the human colon cancer cell line RKO. Stimulation of RKO with increasing concentrations of sCXCL16 in the form of recombinant human CXCL16

(rhCXCL16) did not increase proliferation (Figure 6), although we confirmed the natural ligand, CXCR6, is expressed by RKO cells, using RT-PCR and western blot analysis (data not shown). However, increased sCXCL16 associated with increased cleavage of cell associated CXCL16 (cCXCL16), induced by inflammatory stimulation, positively correlated with RKO cell proliferation (Figure 7A) and decreased apoptosis (Figure 7 B). The extracellular domain of CXCL16, which contains a chemokine domain chemotactic for cells expressing the receptor CXCR6^{3,5,721}, is shed from the cell membrane due to proteolytic cleavage attributed to the metalloproteinases ADAM10 and ADAM17^{7,8,11,17,19}, as metalloproteinase inhibition prevents cCXCL16 proteolysis into sCXCL16. In addition, inhibition of cCXCL16 shedding into sCXCL16 with broad spectrum metalloproteinase inhibitors precludes proliferation and the anti-apoptotic effect of increased sCXCL16 associated with inflammatory stimulation.

CXCL16 has been shown to increase proliferation of human umbilical vein endothelial cells through ligand receptor binding, although this effect is not as significant when cells are stimulated with bFGF⁸. However, knockdown of CXCR6 in human colon carcinoma cells showed significantly increased proliferation¹². While stimulation of wild type colon carcinoma cells with soluble CXCL16 showed slight increase in proliferation, pancreatic tumor cells with knocked down expression of endogenous CXCL16 showed a 50% increase in proliferation when stimulated with exogenous soluble CXCL16¹². We cannot ignore that these data suggest that there is another signaling mechanism, other than CXCL16-CXCR6 axis signaling, by which CXCL16 functions to increase proliferation. Using iPSort, we performed an analysis of the CXCL16 protein sequence using the FASTA sequence and found a nuclear localization signal characterized by a leucine rich repeat (LRR) domain, in the cytoplasmic region. In addition we

performed preliminary studies to determine the intercellular localization of any CXCL16 CTF. After performing subcellular fractionation on RKO cells in culture, we performed western blot analysis using an antibody recognizing an epitope on the cytoplasmic domain of CXCL16. We found CXCL16 in the chromatin associated nuclear fraction. Although these data are preliminary, they provide insight into the roles of CXCL16 CTF, after further processing. These data are also suggestive that CXCL16 may be involved in multiple signaling pathways. CXCL16 has been found upregulated in brain and pancreatic cancers, where it is not found in normal tissue of this type^{8, 12}. In addition to being found upregulated in colon cancer cells^{4, 16}, CXCL16 has been found to be a prognostic factor in prostate cancer⁹. These data highlight CXCL16 as an interesting target for studies in colorectal cancer progression inclusive of cancer progression as a whole. We speculate that involvement of CXCL16 on increased proliferation of cancer cells may include intercellular signaling by CXCL16 CTFs. Studies to determine the involvement of CXCL16 on other proliferative pathways would further elucidate the role of CXCL16 and highlight the multifunctional capacity of CXCL16 as a representative of the chemokine family of immune molecules, as a whole.

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