Direct Inhibition of Elastase Activity by Indole-3-Carbinol Triggers a CD40-TRAF Regulatory Cascade That Disrupts NF-κB Transcriptional Activity in Human Breast Cancer Cells

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Cancer Res 2010;70:4961-4971. Published OnlineFirst June 8, 2010.
Direct Inhibition of Elastase Activity by Indole-3-Carbinol Triggers a CD40-TRAF Regulatory Cascade That Disrupts NF-κB Transcriptional Activity in Human Breast Cancer Cells

Ida Aronchik¹, Leonard F. Bjeldanes², and Gary L. Firestone¹

Abstract

Treatment of highly tumorigenic MDA-MB-231 human breast cancer cells with indole-3-carbinol (I3C) directly inhibited the extracellular elastase-dependent cleavage of membrane-associated CD40, a member of the tumor necrosis factor (TNF) receptor superfamily. CD40 signaling has been implicated in regulating cell survival, apoptosis, and proliferation, as well as in sensitizing breast cancer cells to chemotherapy, and is therefore an important potential target of novel breast cancer treatments. The I3C-dependent accumulation of full-length unprocessed CD40 protein caused a shift in CD40 signaling through TNF receptor–associated factors (TRAF), including the TRAF1/TRAF2 positive regulators and TRAF3 negative regulator of NF-κB transcription factor activity. Because TRAF1 is a transcriptional target gene of NF-κB, I3C disrupted a positive feedback loop involving these critical cell survival components. siRNA ablation of elastase expression mimicked the I3C inhibition of CD40 protein processing and G1 cell cycle arrest, whereas siRNA knockdown of TRAF3 and the NF-κB inhibitor IκB prevented the I3C-induced cell cycle arrest. In contrast, siRNA knockdown of PTEN had no effect on the I3C control of NF-κB activity, showing the importance of CD40 signaling in regulating this transcription factor. Our study provides the first direct in vitro evidence that I3C directly inhibits the elastase-mediated proteolytic processing of CD40, which alters downstream signaling to disrupt NF-κB–induced cell survival and proliferative responses. Furthermore, we have established a new I3C-mediated antiproliferative cascade that has significant therapeutic potential for treatment of human cancers associated with high levels of elastase and its CD40 membrane substrate. Cancer Res; 70(12); 4961–71. ©2010 AACR.

Introduction

The development of effective breast cancer therapies requires the ability to efficiently target a wide range of cell phenotypes, including steroid-sensitive and steroid-insensitive tumors, with reduced side effects especially during prolonged treatments. Considerable epidemiologic evidence shows that frequent consumption of Brassica genus vegetables, such as broccoli, bok choy, and cabbage, is associated with a lower incidence of human cancers at various sites, including reproductive cancers (1). Also, supplementation of these vegetables into the diets of experimental animals causes a striking reduction in incidence and multiplicity of carcinogen-induced and spontaneous mammary epithelial tumors (2, 3). These studies implicate the existence of specific bioactive phytochemicals that represent a largely untapped source of chemotherapeutic agents for treatment of human cancers. One such promising compound is indole-3-carbinol (I3C), a phytochemical derived by hydrolysis from glycosinolates in Brassica genus vegetables.

There is compelling evidence that I3C has potent anti-tumorigenic and proapoptotic properties in human breast cancer cells and in animal models of mammary tumorigenesis with negligible levels of toxicity (4, 5). Furthermore, out of a broad spectrum of analyzed phytochemicals, I3C emerged as one of the few that tested positive as chemopreventive in a panel of short-term bioassays relevant to carcinogen-induced DNA damage, tumor initiation and promotion, and oxidative stress (6). We and others have established that I3C treatment of human reproductive cancer cells modulates specific transcriptional, signal transduction, and metabolic cascades that lead to a cell cycle arrest, apoptosis, downregulation of migration, and regulation of hormone receptor signaling (3, 4, 7). Key downstream targets of I3C signaling that are consistently observed in different human cancer cell types include transcription factors such as Sp1 and NF-κB and their target genes (7, 8); the p53 tumor suppressor protein (9); specific cell cycle components such as the G1 acting CDKs, CDK inhibitors, cyclin E, and cyclin D (10, 11); signal transduction components such as Akt, RhoA kinase (12), and protein kinases, estrogen and

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doi: 10.1158/0008-5472.CAN-09-3349
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androgen receptors (13); and various apoptosis-related genes (14, 15).

The pleiotropic antiproliferative responses to I3C suggest the existence of individual indole target proteins that link and regulate specific sets of cell signaling networks. Consistent with this concept, using the highly invasive and hormone-independent MDA-MB-231 human breast cancer cell line, which represents a model of late-stage breast carcinoma, we originally discovered that I3C acts as noncompetitive allosteric inhibitor of the serine protease human neutrophil or leukocyte elastase. Our studies identified elastase as a first known direct protein target of I3C directly involved in antiproliferative pathways in human cancer cells (16). Elastase is a prognostic factor for late-stage breast cancer (17, 18), and tumor levels of elastase correlate with poor prognosis and survival rate for patients (19), demonstrating that the direct inhibition of elastase activity is a biologically significant target of I3C.

Elastase is highly expressed in human breast cancer cells (20) and can be detected in different intercellular compartments, such as cytoplasmic azurophilic granules and intercellular membranes, as well as released extracellularly and localized to the cell surface (21). The availability of compartment-specific substrates plays a key role in understanding the cellular cascades that can be modulated by regulating elastase activity. The I3C inhibition of intracellular elastase leads to the accumulation of the full-length 50 kDa cyclin E protein, which is an elastase substrate. The disruption of cyclin E processing results in loss of the low molecular weight forms of the protein that confer enzymatic hyperactivity to CDK2 complex (16). However, elastase also cleaves extracellular matrix components and other cell surface molecules, such as cytokine receptors (22), which suggests that I3C also may modulate elastase-dependent signaling networks by inhibition of its extracellular form.

One such intriguing substrate for extracellular elastase is CD40, a 44 kDa membrane protein and member of the tumor necrosis factor (TNF) receptor superfamily that is activated by binding to its ligand CD40L to the full-length receptor (23, 24). Activation of CD40 stimulates recruitment of TNF receptor–associated factors (TRAF) to the intracellular domain of CD40 (25). Two important TRAF-interacting kinases are NF-κB inducing kinase (NIK) and IκB kinase (IKK), which are primarily involved in phosphorylation of NF-κB and its intracellular inhibitor IκB, respectively (26). Binding of TRAF1 and TRAF2 to CD40 upregulates nuclear translocation and transcription factor activity of NF-κB, whereas binding of TRAF3 plays an inhibitory role in NF-κB activation (27).

The NF-κB transcription factor plays a critical role in development and progression of breast cancer (28, 29), as well as in proliferation and survival of breast cancer cells (30). Previous investigations have correlated the loss of NF-κB transcriptional signaling with indole-mediated antiproliferative pathways (8); however, the precise mechanism of NF-κB inactivation by indoles remains elusive. In this study, we have established for the first time that the I3C inhibition of extracellular elastase cleavage of plasma membrane CD40 modulates signaling through its associated TRAF molecules and thereby leads to NF-κB inactivation. Most significantly, we have identified the direct mechanistic link between I3C inhibition of elastase enzymatic activity and the inactivation of NF-κB transcription factor activity in highly invasive human breast cancer cells. Our study further implicates this natural phytochemical as a highly promising therapeutic for human cancers associated with high levels of elastase.

Materials and Methods

Materials, cell culture, flow cytometry, immunofluorescence, and fluorescence-activated cell sorting

MDA-MB-231 cells were obtained directly from American Type Culture Collection and kept at low passage throughout the study. The purchased materials are detailed in the Supplementary Materials and Methods. Culturing of MDA-MB-231 and MCF7 human breast cancer cells, flow cytometry, and immunofluorescence were performed as previously described (16, 31) and the fluorescence-activated cell sorting (FACS) procedure was based on Chemicon Direct Staining Protocol for Flow Cytometry. These methods are detailed in the Supplementary Materials and Methods. For elastase treatment of intact cells, the cells were seeded at 50% confluency. After 24 hours, 600 nmol/L elastase was added directly to the cell culture medium in the presence or absence of 200 μmol/L I3C, the cells were incubated for 24 hours at 37°C, and the level of CD40 protein was monitored by Western blot analysis as described below.

In vitro cleavage of the CD40 chimera protein

Purified human neutrophil elastase was preincubated with vehicle control dimethyl sulfoxide (DMSO) or with 500 μmol/L I3C at 4°C for 2 hours. The CD40-IgG chimera (R&D Systems), which contains the CD40 NH2-terminal residues 21–193 fused to a IgG1 fragment (CD40ch), was then added to the reactions to a final molar ratio of elastase to CD40ch of 1:100. For control reactions, either no elastase was added or the CD40ch substrate was omitted. The reactions were incubated at 37°C for 1.5 hours and terminated by the addition of SDS polyacrylamide gel loading buffer. The reaction products were electrophoretically fractionated on 8.5% SDS polyacrylamide gels, and the protein bands were visualized after transfer to a polyvinylidene difluoride (PVDF) membrane by Ponceau red staining. A set of PVDF membranes was used for the Edman degradation analysis performed by the HHMI Mass Spectrometry Laboratory at the University of California, Berkeley.

Computer-aided molecular binding simulations

The structures of elastase and CD40 were obtained from the Protein Data Bank (PDB, http://www.rcsb.org/pdb/home/home.do), PDB files 1b0f and 1CDF, respectively. The structures of elastase and CD40 were loaded into Hex Protein Docking Server (http://www.csd.abdn.ac.uk/hex_server/), and the modeling of binding was performed using shape as...
the restrictive parameter. The results of each modeling run were visualized using Maestro software (Schrödinger).

**Reverse transcriptase-PCR assay**

Total RNA was extracted using RNeasy extraction kit for mammalian cells (Qiagen) and spectrophotometrically quantified at 260 nm. Reverse transcriptase reactions were carried out using RT-MLV (Invitrogen) as described in the Supplementary Materials and Methods, and cDNA was used for PCR reactions using the following primers at 10 pmol/L:

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>CD40</td>
<td>CAGAGTTCACTGA</td>
</tr>
<tr>
<td>Traf1</td>
<td>AACGGATGAGCC</td>
</tr>
<tr>
<td>Traf2</td>
<td>GCTTGGCTTCTAT</td>
</tr>
<tr>
<td>Traf3</td>
<td>GCTGCAAGAAAGCT</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>TACTACCGC</td>
</tr>
<tr>
<td>GADPH</td>
<td>TGAACGGGAA</td>
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<tr>
<td>GCTCAGTG</td>
<td>GTTTGCTGTA</td>
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**Western blot analysis**

The levels of CD40, elastase, Traf1, cyclin D1, and Hsp90 proteins were analyzed by Western blot analysis as previously described (16) and detailed in the Supplementary Materials and Methods.

**siRNA transfections**

Transfections were carried out according to Qiagen HiPerFect transfection protocol as we previously described (16) and detailed in Supplementary Materials and Methods.

**NF-κB reporter plasmid transfection, green fluorescent protein cell sorting, and luciferase assays**

MDA-MB-231 cells were seeded on 60-cm plates and treated with indicated siRNA and I3C as described above. Twenty-four hours after the addition of I3C, the cells were transfected with p65 NF-κB response element luciferase reporter plasmid with internal green fluorescent protein (GFP) marker used for transfection efficiency tracking according to Qiagen SuperFect transfection protocol guidelines. Luciferase assay was carried out as described by us previously (13) and detailed in the Supplementary Materials and Methods.

**Results**

**In silico structural analysis of elastase interactions with CD40, and in vitro I3C inhibition of CD40-IgG chimera cleavage by elastase**

A previous study suggested that extracellular elastase proteolytically cleaves plasma membrane–associated CD40 (23); however, no structural or direct experimental information is known about the functional interactions between the molecules. In *silico* modeling of this bimolecular interaction was used to predict potential elastase proteolytic cleavage sites within the CD40 molecule. Analysis of the residues in CD40 calculated to be localized within 7 Å of the elastase catalytic site, and therefore relevant for biological interactions, revealed potential cleavage sites at residues Val91, Ala115, and Val138 within the extracellular CD40 terminus, which is involved in the CD40 ligand binding (Fig. 1A and color version in Supplementary Results; ref. 32).

Based on the *in silico* information, an *in vitro* processing assay was developed using a CD40-IgG chimera (CD40ch) containing the human CD40 NH2-terminal residues 21 to 193, which includes the predicted elastase cleavage sites, fused to a human IgG1 fragment (see diagram in Fig. 1B) as an elastase substrate. CD40ch was incubated with or without added purified human neutrophil elastase with a molar ratio of elastase to CD40ch of 1:100. After incubation at 37°C for 1.5 hours, the resulting proteins were electrophoretically fractionated in SDS-polyacrylamide gels and the protein bands were visualized after transfer to a PVDF membrane by Ponceau red staining. In the absence of added elastase, the intact CD40ch electrophoretically migrated as a 60 kDa protein (Fig. 1C, lane 1). The addition of pure elastase caused CD40ch to be proteolytically processed into several specific products, a 36/34 kDa protein doublet and a 19 kDa protein fragment (Fig. 1C, lane 2). Subsequent Edman degradation analysis revealed that the two higher molecular weight fragments were the products of chimera cleavage at the EGRM motif sequence in the linker region (upper doublet band) and inside the IgG sequence at HT(C) PP (lower doublet band), respectively. Importantly, the lower molecular weight fragment contained the NH2-terminal sequence of the CD40 molecule, and the size of the fragment closely correlated with the calculated weight of the first 138 amino acid fragment of CD40, including site-specific glycosylation of the molecule. This result strongly suggests that Val138 in the NH2-terminal portion of CD40 is the primary elastase cleavage site.

To directly determine whether I3C inhibits elastase cleavage of CD40, elastase was preincubated with vehicle control DMSO or with 500 μmol/L I3C before the addition of CD40ch. The elastase cleavage of CD40 was completely inhibited by exposure to I3C shown by an intact 60 kDa CD40ch in the indole-containing lane (Fig. 1C, lane 3 versus lane 2). Taken together, these results show for the first time that CD40 can be proteolytically cleaved by elastase *in vitro*, and that this cleavage is abrogated by the I3C inhibition of elastase activity.

**I3C increases the level of total and cell surface–associated unprocessed CD40 protein in human breast cancer cells**

I3C-responsive and estrogen-insensitive MDA-MB-231 human breast cancer cells, which represent a model for late-stage breast cancer, were used to test whether I3C treatment disrupts CD40 protein processing in a cellular context because this cell line expresses high levels of elastase (16). Cells were treated with increasing doses of I3C for 48 hours,
and full-length CD40 protein levels were assessed by Western blot analysis using antibodies that target the NH2-terminal domain of CD40, which is present only in the unprocessed form of the protein. As shown in Fig. 2A (top left panel), I3C induced a dose-dependent increase in the accumulation of full-length CD40 protein. Densitometry analysis of these Western blot data shows that accumulation of unprocessed CD40 protein in the presence of 100 μmol/L I3C was significantly greater compared with lower indole concentrations, and reached the maximal level by 200 μmol/L I3C (Fig. 2A, top right graph). Therefore, for the remainder of this study, we used 200 μmol/L I3C unless stated otherwise. MDA-MB-231 cells were then treated with or without 200 μmol/L I3C over a 72-hour time course, and as also shown in Fig. 2A (bottom panels), I3C caused a significant accumulation of the uncleaved 44 kDa CD40 protein within the first 24 hours of indole treatment, which was maximal by 48 hours. Estrogen-sensitive MCF7 human breast cancer cells, which have a phenotype representative of early-stage breast cancer, showed the same I3C response (Supplementary Results, Fig. S1, panel A). As also shown in Fig. 2A (bottom panels), reverse transcriptase-PCR (RT-PCR) analysis using CD40-specific oligonucleotide primers showed that I3C had no effect on the level of CD40 mRNA throughout the time course.

To assess whether the I3C-induced accumulation of the 44 kDa CD40 may have been due to an inhibition of its proteasome-mediated degradation, cells were treated with or without 200 μmol/L I3C for 48 hours, and then exposed to either 10 μmol/L of the MG-132 proteasome inhibitor or to DMSO vehicle control for the last 4 hours of incubation. As shown in Fig. 2B, Western blot analysis revealed that MG-132 had no effect on accumulation of unprocessed CD40 protein in I3C-treated or untreated cells, suggesting that the I3C-induced accumulation of unprocessed CD40 protein cannot be attributed to an inhibition of proteasome activity. The ability of excess elastase to override the I3C inhibition of CD40 processing was examined in MDA-MB-231 cells after the addition of elastase and/or I3C directly to the growth medium. As shown in Fig. 2C, 24-hour exposure to exogenous elastase cleaved the CD40 protein produced in either I3C-treated or untreated cells, resulting in the loss of the 44 kDa band. As expected, addition of I3C alone increased the level of uncleaved CD40. Taken together, these results suggest that the I3C inhibition of elastase activity is responsible for the increased levels of the 44 kDa CD40 protein in indole-treated cells.

To analyze the level of cell surface-localized CD40 protein during a time course of treatment with 200 μmol/L I3C, intact cells were incubated with CD40 NH2 terminus–specific antibodies followed by staining with the specific secondary antibodies conjugated to fluorescent probe Alexa Fluor 488. The amount of cell surface–labeled CD40 was quantified using FACS analysis. As shown in Fig. 2D (left panel), I3C induced a significant increase in number of CD40-positive cells, which was maximal by 48 hours of treatment. The histograms representing the fluorescence peak shift in I3C-treated cells compared with untreated cells are shown in the Supplementary Results, Fig. S2.

I3C-treated and untreated MDA-MB-231 cells were further analyzed by indirect immunofluorescence using antibodies targeting the NH2 terminus of the CD40 molecule. As shown in Fig. 2D (top images), treatment with I3C strongly enhanced plasma membrane localization of CD40. CD40L is a highly specific high-affinity ligand that binds to the NH2-terminal region of CD40 (32), and our in vitro processing data indicate that elastase cleavage of CD40 at Val138 should eliminate the CD40L binding site within the cell.
surface receptor. Consistent with I3C-inducing accumulation of the uncleaved CD40, immunofluorescence revealed that I3C treatment strongly stimulated colocalization of CD40L at the cell surface, which is indicative of its binding to CD40 (Fig. 2D, bottom images).

**siRNA ablation of cellular elastase levels mimics the I3C-stimulated accumulation of the uncleaved CD40 protein at the cell surface**

To functionally test the role of elastase in the I3C-induced accumulation of the uncleaved cell surface–localized CD40 protein, elastase expression in MDA-MB-231 cells was knocked down by transfection of the elastase-specific siRNA. A control set of transfections was carried out with scrambled siRNA. Transfected cells were treated for 48 hours with either 200 μmol/L I3C or with the DMSO vehicle control. Transfection with the elastase-specific siRNA efficiently downregulated elastase production, whereas treatment with scrambled siRNA had no effect on elastase protein levels (Fig. 3A, left). Western blot analysis revealed that siRNA ablation of elastase expression resulted in an accumulation of the 44 kDa uncleaved CD40 protein (Fig. 3A, right). These results were also observed using MCF-7 cells (Supplementary Results, Fig. S1, panel B). Indirect immunofluorescence of intact cells showed that either siRNA ablation of elastase expression or inhibition by I3C similarly increased levels of cell surface–localized CD40 (Fig. 3B). The effect of a combination of I3C and elastase-specific siRNA resembled that of either of the individual treatments. As a complementary approach, FACS analysis showed that treatment with I3C or expression of elastase-targeting siRNA (or combination of both) induced accumulation of approximately the same level of cell surface CD40 protein (Fig. 3C).

**I3C disrupts the elastase-dependent stimulation of NF-κB transcription factor activity and nuclear localization**

In human breast cancer cells, CD40 signaling regulates NF-κB transcription factor activity, which in turn controls transcription of genes involved in cell cycle progression and cell...
survival (30, 33). NF-κB must be localized to the nuclei to be accessible to its gene targets (34), and the subcellular localization of the p65 subunit of NF-κB was examined in elastase-specific siRNA-transfected MDA-MD-231 cells treated with or without 200 μmol/L I3C compared with cells transfected with scrambled siRNA. Immunofluorescence microscopy showed that in cells transfected with scrambled siRNA, I3C caused the redistribution of p65 away from the nucleus to the cytosol (Fig. 4A, top panels). Ablation of elastase expression by siRNA mimicked the effects of I3C in preventing the nuclear localization of NF-κB, and the combined treatment showed no additive effects (Fig. 4A, bottom). Thus, the loss of elastase-dependent cleavage of CD40 by either I3C inhibition of elastase activity or by siRNA knockdown of elastase expression resulted in the inactivation of NF-κB. This I3C effect is not a cell line–specific phenomenon because a similar result was observed using MCF-7 cells (Supplementary Results, Fig. S1, panel C).

To show that I3C inhibits NF-κB–mediated gene expression in an elastase-dependent manner, the effects on cyclin D1 gene expression, a known NF-κB target gene in human breast cancer cells (35), was assessed by RT-PCR. As shown in Fig. 4B (gels and bar graphs of densitometry), treatment with I3C or elastase-specific siRNA effectively downregulated cyclin D1 transcripts. Total NF-κB activity was monitored by transfection of a NF-κB–responsive DNA element linked to a luciferase reporter plasmid and GFP maker for transfection efficiency control. As shown in Fig. 4C, either I3C treatment or siRNA knockdown of elastase strongly inhibited NF-κB transcriptional activity. Interestingly, siRNA knockdown of PTEN expression, a phosphatase known to reduce AKT activity in cancer cells (36), did not prevent I3C downregulation of total NF-κB activity. Thus, in contrast to observations in other cell systems (8, 37), our results in human breast cancer cells directly show that the PTEN/Akt signaling cascade is not involved in I3C-mediated downregulation of NF-κB transcriptional activity.

**I3C disrupts a NF-κB–mediated positive feedback loop involving the CD40 signaling component TRAF1**

In human breast cancer cells, the TRAF1, TRAF2, and TRAF3 molecules, which directly interact with the intracellular COOH terminus of CD40, mediate NF-κB function by regulating its activating kinases IKK and NIK (25, 38). TRAF3 has a negative effect on NF-κB activation, whereas TRAF1 and TRAF2 cooperate to activate this transcription factor (see Fig. 5A, diagram; ref. 39). In turn, NF-κB drives TRAF1 gene expression (39). To test the possibility that I3C downregulates
NF-κB activity by disrupting a positive feedback involving TRAF1, MDA-MD-231 cells were treated with varying concentrations of I3C for 48 hours, and expression of individual TRAF transcripts was examined by RT-PCR analysis. As shown in Fig. 5B (top panels), I3C strongly downregulated TRAF1 expression but had no effect on TRAF2 or TRAF3. Western blot analysis confirmed that the loss of TRAF1 transcripts resulted in a significant loss of TRAF1 protein levels (Fig. 5B, bottom panels). The protein levels of TRAF2 and TRAF3 remained unaffected throughout the treatment (data not shown). In addition, as shown in Fig. 5C, RT-PCR analysis revealed that knockdown of elastase expression effectively downregulated TRAF1 transcript levels, which mimicked the I3C effect.

**siRNA knockdown of TRAF3 and IκB expression prevents I3C-induced cell cycle arrest of MDA-MD-231 cells**

NF-κB activity can be negatively regulated by CD40-dependent TRAF3 signaling or by interaction with the NF-κB-specific inhibitor IκB (40, 41). To directly test whether TRAF3 and IκB are involved in I3C-induced G1 cell cycle arrest, expression of these molecules and of CD40 was knocked down by transfection of siRNA specific for each NF-κB regulator (efficiency of knockdowns shown in Fig. 6A), and the effects on the cell cycle were examined by flow cytometry. For comparison, cells were also subjected to siRNA knockdown of elastase expression (efficiency described in Fig. 3A) or transfected with scrambled siRNA. As shown in Fig. 6B, flow cytometry analysis of propidium iodide–stained DNA revealed that siRNA ablation of elastase induced a G1 cell cycle arrest similar to that induced by I3C treatment, which is consistent with our previous observations (16). Interestingly, siRNA knockdown of CD40 expression mimicked the I3C growth arrest, which was not further enhanced by I3C treatment. Consistent with an important role in controlling NF-κB signaling through CD40-TRAF interactions, siRNA knockdown of the negative regulators of NF-κB, TRAF3, or IκB prevented I3C from inducing a G1 cell cycle arrest. Thus, maintaining expression of TRAF3 and IκB is required for I3C to mediate its antiproliferative effects through the modulation of CD40 processing and signaling.

**Discussion**

In human breast cancer cells and other reproductive cancers, disruption of NF-κB transcription factor activity by antiproliferative indoles is a biologically significant downstream effect because of the critical role NF-κB target genes play in cell cycle progression and cell survival.
responses (29, 42). Previous studies correlated the inactivation of NF-κB with treatment by I3C and related indoles (8, 43); however, the precise cellular mechanism remained elusive because the direct indole target protein was unknown. By extending our studies demonstrating that I3C is a non-competitive inhibitor of elastase enzymatic activity (16), we have uncovered a novel indole-regulated cellular cascade in which the I3C-elastase interaction modulates the protein processing and signaling of CD40, a member of TNF receptor superfamily, which then triggers the disruption in NF-κB nuclear localization resulting in the downregulation of NF-κB transcription factor activity and altered expression of its target genes. Our studies provide the first direct in vitro and cellular evidence for the elastase-mediated proteolytic processing of CD40, and establish in human breast cancer cells that elastase-dependent CD40 signaling is an essential regulator of NF-κB induced cell survival and proliferation.

We propose that I3C inhibition of extracellular elastase prevents the proteolytic cleavage of cell surface CD40 that results in the stable accumulation of its full-length form (see diagram in Supplementary Results, Fig. S3). Furthermore, we propose that this I3C effect alters the balance between the TRAF1 and TRAF2 activation of NF-κB and the TRAF3-mediated downregulation of NF-κB transcription factor activity, thereby disrupting a positive feedback loop between NF-κB and TRAF1. These findings implicate the critical involvement of CD40-mediated signaling through its associated TRAF molecules in the I3C-induced cell cycle arrest of human breast cancer cells.

CD40 expression and activation is associated with reduced cell proliferation and survival in many types of carcinoma cells and tissues (44, 45). Activation of CD40 by its CD40L ligand induces apoptosis of human reproductive cancer cells, including breast cancer cells (46), and CD40-transfected HeLa cervical carcinoma cells (47). Reduced CD40 signaling is associated with a greater risk of residual cancer after the preoperative chemotherapy with trastuzumab and concomitant paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide in HER-2-overexpressing breast cancer (48). CD40 activation has been investigated for its therapeutic potential in development of novel treatments for several types of malignancies (49). Current experimental approaches include the subcutaneous administration of recombinant CD40L protein (50, 51), intravenous single-dose injections of autologous CD40L-transfected cells (52–54), and injection of CD40-activated cells for plasma cell leukemia treatment (55). However, some of these treatments are accompanied by considerable side effects, such as transient liver toxicity in case of recombinant CD40L treatment. Notably, most of the currently tested treatments aim to increase ligation or expression of CD40, whereas they do not address endogenous CD40 protein stability.

In breast cancer cells, elastase is present at high levels and associated with higher grade of the disease progression and poor prognosis for the patients (17, 19). We have shown that I3C inhibition of elastase results in accumulation of full-length CD40 protein levels in the cell membrane, which leads to amplification of the antiproliferative signaling cascade downstream of CD40. A therapeutic prospective of CD40 activation is to potentiate the antitumor immune response. Transfection of CD40-negative tumor cell lines with CD40 cDNA conferred responsiveness to CD40L and anti-CD40 antibody, and the presence of CD40 on the surface of carcinoma lines was found to be an important factor in the generation of tumor-specific T-cell responses (36). Although I3C modulation of antitumor immune responses in humans has not been investigated, an animal model study established that high-dose daily I3C diet produces activation of T cell–mediated delayed hypersensitivity responses (57). It is tempting to consider that these responses result, in part, from the I3C modulation of CD40 signaling cascades. Conceivably, the indole control of elastase-dependent processing and modulated signaling of CD40 may be useful to address the complex pathogenesis of pulmonary diseases.
such as chronic obstructive pulmonary disease and cystic fibrosis, where elastase is often upregulated and has been shown to cleave CD40 and other surface molecules (58). The disruption of antigen presentation by CD40-positive dendritic cells and initiation of proper immune responses to bacterial infections also contribute to pathogenesis of these diseases (58). An important future direction will be to determine whether the I3C inhibition of elastase activity that leads to modulation of CD40 signaling and NF-κB function can form the preclinical basis for developing new therapeutic strategies for specific human disorders where elastase plays an important role in the etiology of the disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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