Cancer Research

Annexin 1 Induced by Anti-Inflammatory Drugs Binds to NF-κB and Inhibits Its Activation: Anticancer Effects *In vitro* and *In vivo*

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Abstract

Annexin A1 (ANXA1), a mediator of the anti-inflammatory action of glucocorticoids, is important in cancer development and progression, whereas NF-κB regulates multiple cellular phenomena, some of them associated with inflammation and cancer. We showed that glucocorticoids and chemopreventive modified nonsteroidal anti-inflammatory drugs, such as nitric oxide–donating aspirin (NO-ASA) and phospho-aspirin, induced ANXA1 in cultured human colon and pancreatic cancer cells. ANXA1 associated with NF-κB and suppressed its transcriptional activity by preventing NF-κB binding to DNA. The induction of ANXA1 by glucocorticoids was proportional to their anti-inflammatory potency, as was the suppression of NF-κB activity, which was accompanied by enhanced apoptosis and inhibition of cell growth mediated by changes in NF-κB–dependent cell signaling. The proposed novel mechanism was operational in the intestinal mucosa of mice treated with dexamethasone or NO-ASA. ANXA1-based oligopeptides displayed the same effects as ANXA1 on NF-κB. One such tripeptide (Gln-Ala-Trp) administered to nude mice inhibited the growth of SW480 human colon cancer xenografts by 58% compared with control ($P < 0.01$). Our findings reveal that ANXA1 is an inducible endogenous inhibitor of NF-κB in human cancer cells and mice, provide a novel molecular mechanism for the action of anti-inflammatory agents, and suggest the possibility of mechanism-driven drug development. *Cancer Res; 70(6); 2379–88. ©2010 AACR.*

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) have emerged as important agents for the prevention of several human cancers (1). A derivative of aspirin, nitric oxide–donating aspirin (NO-ASA), consisting of aspirin and a NO-donating moiety covalently attached to it, prevents various cancers in preclinical models and displays anti-inflammatory properties (2). Inhibition of NF-κB by NO-ASA seems important for its chemopreventive effect; NO-ASA inhibits NF-κB in various cancer cell lines and animal models of cancer (3).

NF-κB plays a role in autoimmune responses, cell proliferation, and apoptosis and represents a plausible link between inflammation and carcinogenesis (4, 5). NF-κB is sequestered inactive in the cytoplasm bound to IκB proteins, an interaction that regulates its activity. Multiple stimuli activate NF-κB signaling, which consists of translocation of NF-κB to the nucleus where it binds to κB binding sites in the enhancer or promoter regions of target genes, regulating their transcription (6, 7). The NF-κB signal transduction pathway is dysregulated in various human cancers (8, 9). In most such cases, NF-κB is constitutively active and resides in the nucleus, whereas in others the enhanced NF-κB activity is due to changes in the IκB kinase pathway. The sustained NF-κB activation not only protects cancer cells from apoptotic cell death but may also enhance their proliferation.

While studying the mechanism by which NO-ASA suppresses NF-κB activation, we noted that NO-ASA induces the expression of Annexin A1 (ANXA1), a 37-kDa protein originally identified as a mediator of the anti-inflammatory effect of glucocorticoids (10). ANXA1 has diverse functions, including the regulation of cell division, apoptosis, and cell growth. Although there is no proof that *ANXA1* is a disease-causing gene, it is clear that altering its expression or the localization of the protein it encodes can contribute to the pathogenesis of inflammatory diseases and cancer (11, 12). The induction of endogenous ANXA1 is part of the mechanism of action of glucocorticoids such as dexamethasone (13, 14). Dexamethasone-induced apoptosis and anti-inflammatory responses are associated with inhibition of NF-κB. Here, we show that ANXA1 is required for the inhibition of NF-κB activity by anti-inflammatory drugs, propose a novel mechanism of NF-κB inhibition, and show that an ANXA1-based peptide suppresses the growth of colon cancer xenografts in nude mice.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-09-4204

Materials and Methods

Reagents. NO-ASA was synthesized by us; all others were from Sigma-Aldrich. The NH_2-terminal peptides of ANXA1, Ac2-26 and Ac2-12, were from Phoenix Pharmaceuticals. ANXA1 short peptides were synthesized by GenScript Corp. Monoclonal antibodies (mAb) were from Cell Signaling or Santa Cruz Biochemical.

Cell lines. All cell lines were from the American Type Culture Collection and were grown according to their specifications.

MTT assay. The assay was done using a kit from Sigma according to the recommended protocol.

Apoptosis. The ELISA method (Roche) was used according to the manufacturer's protocol.

Cell fractionation. Cell fractions were obtained as described (15). Briefly, cells treated with the test drug were harvested, washed, and resuspended in buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L $MgCI_2$, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF)] with a protease inhibitor cocktail (Sigma) and incubated on ice for 15 min. Cell lysates were spun at 5,000 rpm three times. The supernatants were the cytoplasmic extracts. Nuclear pellets were washed with buffer A and resuspended in buffer C [20 mmol/L HEPES (pH 7.9), 450 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 25% glycerol] with the protease inhibitor cocktail and incubated on ice for 30 min. Nuclear extracts were cleared by centrifugation.

Immunoblotting. Immunoblotting was done following standard protocols. β-Actin was used as the loading control.

NF-κB activity. NF-κB activity was measured using a kit from Panomics and following the manufacturer's protocol. Nuclear extracts were incubated in a plate coated with NF-κB probe. The primary antibody against NF-κB was incubated for 1 h, followed by horseradish peroxidase–conjugated secondary antibody for 1 h.

siRNA or cDNA clone transfections. siRNA (Santa Cruz) was transfected according to product protocol, and after 24 h, the transfected cells were treated with NO-ASA for 3 h. Whole-cell lysates were used for protein determination and NF-κB activity measurement. cDNA clones (OreGen) were transfected into BxPC-3 cells using Lipofectamine according to product protocol (Invitrogen) and incubated for 24 h. Whole-cell lysates were used to determine the protein level and NF-κB activity. PCMV6XL5 was used as control vector.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSA) were carried out according to the manufacturer's protocol (Panomics). p65 double-stranded oligonucleotide probe: 5-CATCGGAAATTTCCGGAAATTT-CCGGAAATTTCCGGC-3.

Coimmunoprecipitation. Cell extracts were incubated overnight with agarose-conjugated anti-p65 antibody (Santa Cruz). The precipitate was washed, dissolved in 2× Laemmli buffer, boiled, and separated by SDS-PAGE and then detected by immunoblotting.

Confocal microscopy. Cells exposed to NO-ASA for 2 h were fixed, permeabilized, blocked, and incubated with mouse monoclonal IgG2b anti-ANXA1 (Santa Cruz) and rabbit monoclonal IgG anti-p65 (Cell Signaling) at room temperature for 1 h. After washes, cells were incubated with Alexa555-conjugated antimouse IgG (Molecular Probes) and Alexa488-conjugated antirabbit IgG for 1 h at room temperature. Images were acquired with a Zeiss LSM 510 META NLO two-photon laser scanning image confocal microscope. Colocalization scores were generated by the Colocalization Macro Program.

Mice. *Min* and C57BL/6J mice (The Jackson Laboratory) were treated with 100 mg/kg NO-ASA or 10 mg/kg/d dexamethasone i.p. once a day for 7 d, and then were euthanized; small intestinal mucosa was harvested by scraping. We evaluated the induction of ANXA1 and the interaction between ANXA1 and NF-κB p65 in mucosal whole-cell lysates. We determined NF-κB activity in the nuclear fraction. We established xenografts in 6-wk-old female BALB/c nude mice (The Jackson Laboratory) by s.c. injecting 2×10^6 SW480 cells in 100 μL of PBS. Tumor volume (V) was determined using the formula $V = L \times W(L + W/2) \times 0.56$ (L, length; W, width; ref. 16).

Statistical analyses. We used the two-tailed unpaired Student t test. Differences were considered significant at $P < 0.05$.

Results

NO-ASA and glucocorticoids induce ANXA1 in human cancer cells. Initially, we assessed the effect of NO-ASA, conventional ASA, other NSAIDs, and various glucocorticoids on the expression of ANXA1 in human pancreatic and colon cancer cell lines. As shown in Fig. 1, NO-ASA induced the expression ANXA1 in a concentration- and time-dependent manner. In the cytoplasm, the induction of ANXA1 was rapid and its levels were maximal at 3 hours, remaining relatively stable for at least 8 hours. In the nucleus, ANXA1 became detectable at 2 hours, peaked sharply at 3 hours, and declined rapidly thereafter, likely indicating a time-dependent transport process. A similar effect was observed in HT-29 human colon cancer cells. Dexamethasone, the synthetic corticosteroid with the highest anti-inflammatory potency (17), also induced ANXA1 in BxPC-3 cells, but only in the nuclei; its cytoplasmic levels did not show any significant change. The same effect was observed in HT-29 cells (data not shown). We also evaluated phospho-aspirin (structurally similar to NO-ASA, bearing in the place of the NO-donating moiety diethyl phosphate), which has strong anticancer properties (16, 18). Phospho-aspirin induced ANXA1, similar to NO-ASA (data not shown). However, conventional ASA up to 5 mmol/L, cortisone up to 100 μmol/L, and six additional conventional NSAIDs, each at 1 mmol/L for 6 hours, failed to induce the expression of ANXA1 in BxPC-3 cells (Supplementary Fig. S1).

We investigated the effect on ANXA1 expression of seven glucocorticoids, representing a broad range of anti-inflammatory potencies (17). They included (anti-inflammatory potency in parentheses) cortisol (1), cortisone (0.8), prednisone (4), triamcinolone (5), fludrocortisone (10), betamethasone (25), and dexamethasone (25). BxPC-3 cells were treated for

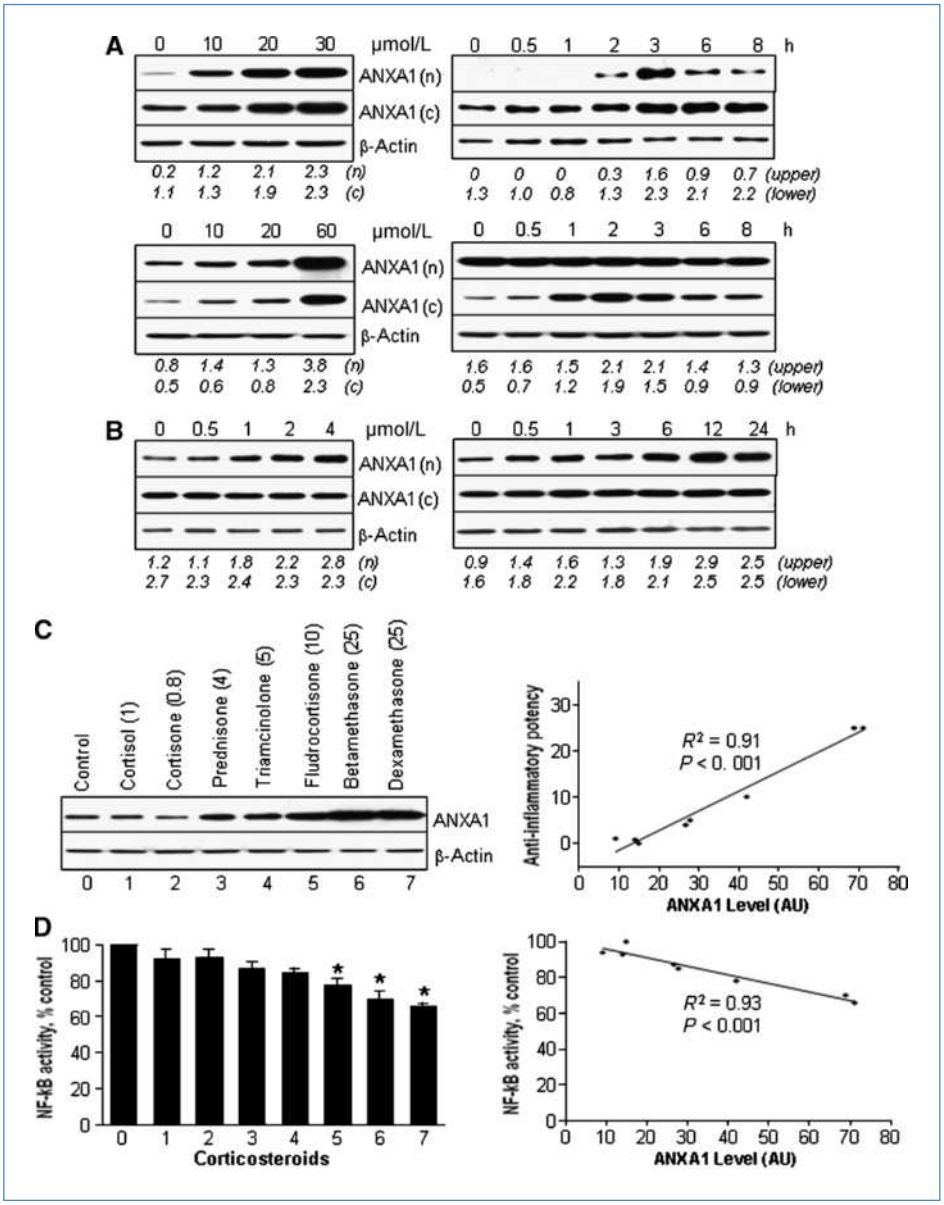

Figure 1. NO-ASA and glucocorticoids induce ANXA1 in human cancer cells. The expression of ANXA1 was determined by immunoblotting in the nuclei (n) and cytoplasm (c); β-actin was the loading control. NO-ASA induced the expression of ANXA1 in BxPC-3 pancreatic (A, top) and HT-29 colon cancer cells (A, bottom). Dexamethasone was studied in BxPC-3 cells (B). The concentrations in the time-response experiments were 30 μmol/L NO-ASA and 4 μmol/L dexamethasone. The numbers in italics below the immunoblots represent the band intensity compared with that of the corresponding loading control. C, BxPC-3 cells were treated for 6 h with glucocorticoids, each at 4 μmol/L; ANXA1 was evaluated by immunoblotting. The anti-inflammatory potency of these glucocorticoids, shown in parentheses, correlates with the levels of ANXA1, which they induce. ANXA1 levels, determined by densitometry, are expressed in arbitrary units (AU). D, the effect of these glucocorticoids on the NF-κB activity of these cells was determined in parallel by ELISA. Columns, mean (n = 3); bars, SEM. The numbers in the abscissa, reflecting those in C, denote the corresponding glucocorticoids. The association between NF-κB activity and ANXA1 levels as determined in C and D is statistically significant.

6 hours with these compounds, each at 4 μmol/L, applied individually. The induction of ANXA1 was proportional to their relative anti-inflammatory potency, with dexamethasone having the greatest effect (Fig. 1C and D). Indeed, the anti-inflammatory potency of these glucocorticoids and the corresponding cellular levels of ANXA1 were significantly correlated (R^2 = 0.91; P < 0.001).

ANXA1 is required for the inhibition of NF-κB by anti-inflammatory agents. We investigated whether ANXA1 mediates the inhibition of NF-κB by NO-ASA and dexamethasone. BxPC-3 and HT-29 cells were treated with NO-ASA or dexamethasone for 3 hours, and NF-κB activity was determined using an ELISA assay. Both NO-ASA and dexamethasone inhibited the activity of NF-κB in both cell lines (Fig. 2). As shown in Fig. 1, all of the glucocorticoids that we studied inhibited NF-κB activity in a manner that paralleled their anti-inflammatory potency. Of interest, NF-κB activity was inversely correlated to the level of ANXA1 that was induced by them (R^2 = 0.93; P < 0.001), further suggesting that ANXA1 mediates the NF-κB inhibitory effect of these compounds.

To directly assess this possibility, we knocked down the expression of ANXA1 in BxPC-3 cells using an *ANXA1*-specific siRNA (Fig. 2C). NO-ASA at 20 μmol/L had only a marginal effect on NF-κB activity (10% reduction); in control cells, treated with vehicle or nonspecific siRNA, 20 μmol/L NO-ASA suppressed NF-κB activity by 40%. Dexamethasone had a similar effect (data not shown). The siRNA against ANXA1 enhanced NF-κB activity compared with controls

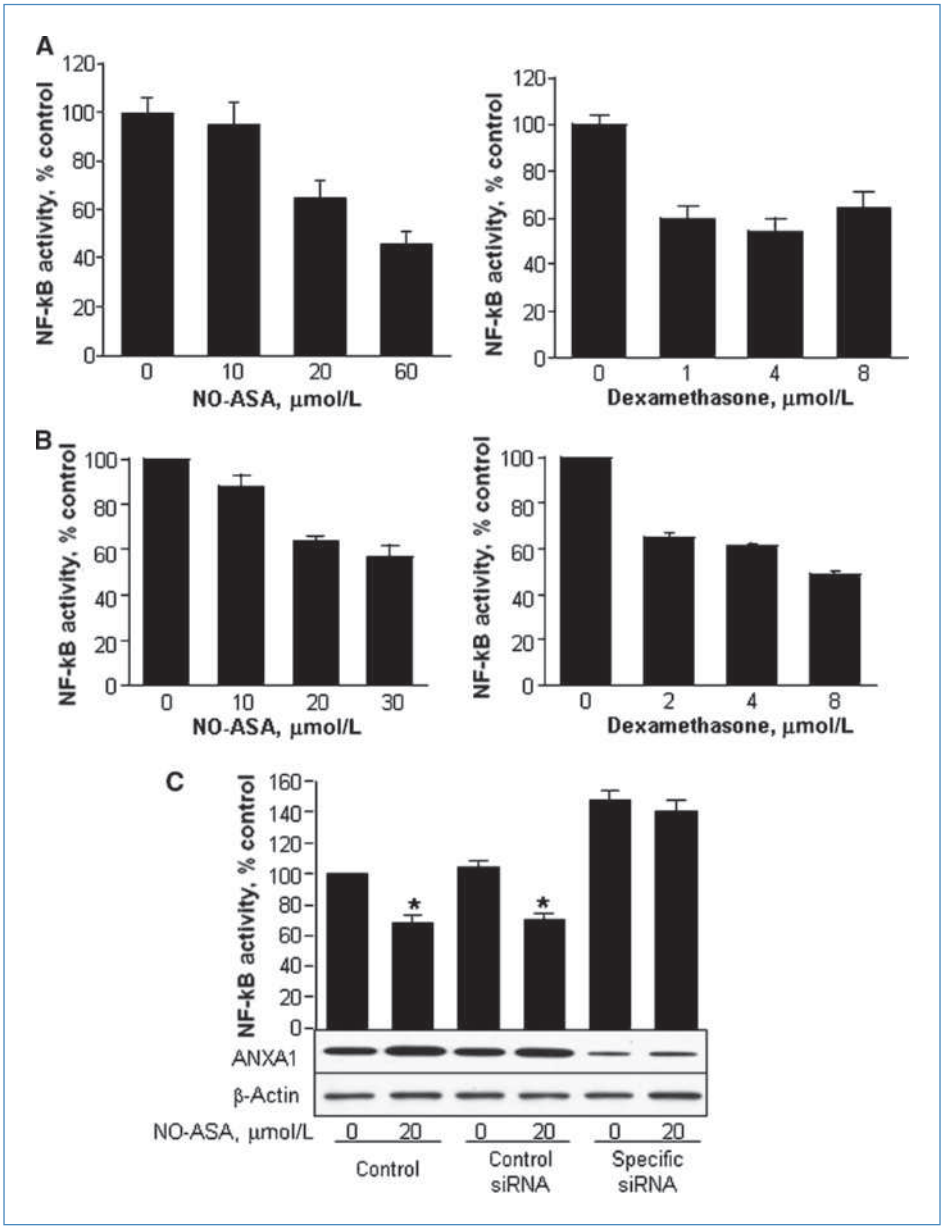

Figure 2. ANXA1 mediates the inhibition of NF-κB activity by NO-ASA in human cancer cells. HT-29 human colon (A) or BxPC-3 pancreatic (B) cancer cells were treated for 3 h with NO-ASA or dexamethasone. NF-κB activity was determined by ELISA. C, the expression of ANXA1 in BxPC-3 cells was knocked down by siRNA. NO-ASA reduced NF-κB activity in nontransfected cells and those transfected with nonspecific siRNA. When the expression of ANXA1 was reduced by siRNA, NO-ASA had no effect on NF-κB activity. Compared with the two controls, NF-κB activity was enhanced when ANXA1 expression was suppressed. Columns, mean (n = 3); bars, SD. *, $P < 0.05$.

(nonspecific siRNA and not transfected cells), suggesting a baseline inhibitory effect on NF-κB activity by ANXA1.

ANXA1 is required for the induction of apoptosis by anti-inflammatory agents in human cancer cells. NO-ASA inhibits the growth of human cancer cell lines, predominantly through enhanced apoptosis (19). As Fig. 3A confirms, NO-ASA inhibited the growth of HT-29 cells (IC_{50} = 18.8 μmol/L), inducing apoptosis vigorously (up to 4.5-fold over baseline). Similar results were obtained with BxPC-3 cells (IC_{50} = 9.8 μmol/L). In both cell lines, NO-ASA inhibited the expression of Bcl-2, a NF-κB–dependent antiapoptotic gene, and of the apoptosis-related proteins survivin, c-IAP-1, c-IAP-2, and TRAF-1 in BxPC-3 cells.

To assess the role of ANXA1 in the cell growth inhibitory effect of these compounds, we knocked down its expression by siRNA (Fig. 3B). Knocking down the expression of ANXA1 completely abrogated the apoptosis induced by either NO-ASA or dexamethasone. This finding suggests that ANXA1 is a key player in the proapoptotic effect of these anti-inflammatory agents. This notion is reinforced by the finding that with overexpression of ANXA1 by transfecting *ANXA1* cDNA into BxPC-3 cells, cell growth was decreased by ~60% compared with controls. A control plasmid showed no such effect (Fig. 3C).

ANXA1 directly binds to the NF-κB p65 subunit. We obtained several lines of evidence indicating that to inhibit

the activity of NF-κB, ANXA1 associates physically with the NF-κB dimer. First, we used 96-well plates of an ELISA NF-κB assay in which double-stranded oligomers containing the κB recognition sequence (5′-CATCGGAAATTTCCG-GAAATTTCCGGAAATTTCCGGC-3′ and its complementary strand) were immobilized on the walls of the reaction wells. Nuclear extracts from BxPC-3 cells treated for 3 hours with or without 20 μmol/L NO-ASA were reacted with these κB oligomers. NF-κB dimers bound to the κB oligomers were recognized by anti-p65 or anti-p50 antibodies through a color reaction dependent on a secondary antibody. When, instead of the anti-p65 or anti-p50 antibodies, we used an anti-ANXA1 mAb that did not cross-react with either p50 or p65, we obtained a positive reaction (recognition of the protein bound to the κB oligomers); a nonspecific isotypic antibody gave a negative result (Fig. 4A). These findings suggest either that ANXA1 is associated with the NF-κB dimer or that it cross-binds to the κB oligomers.

To clarify this finding, we immunoprecipitated the nuclear protein fraction of BxPC-3 cells treated with NO-ASA as above

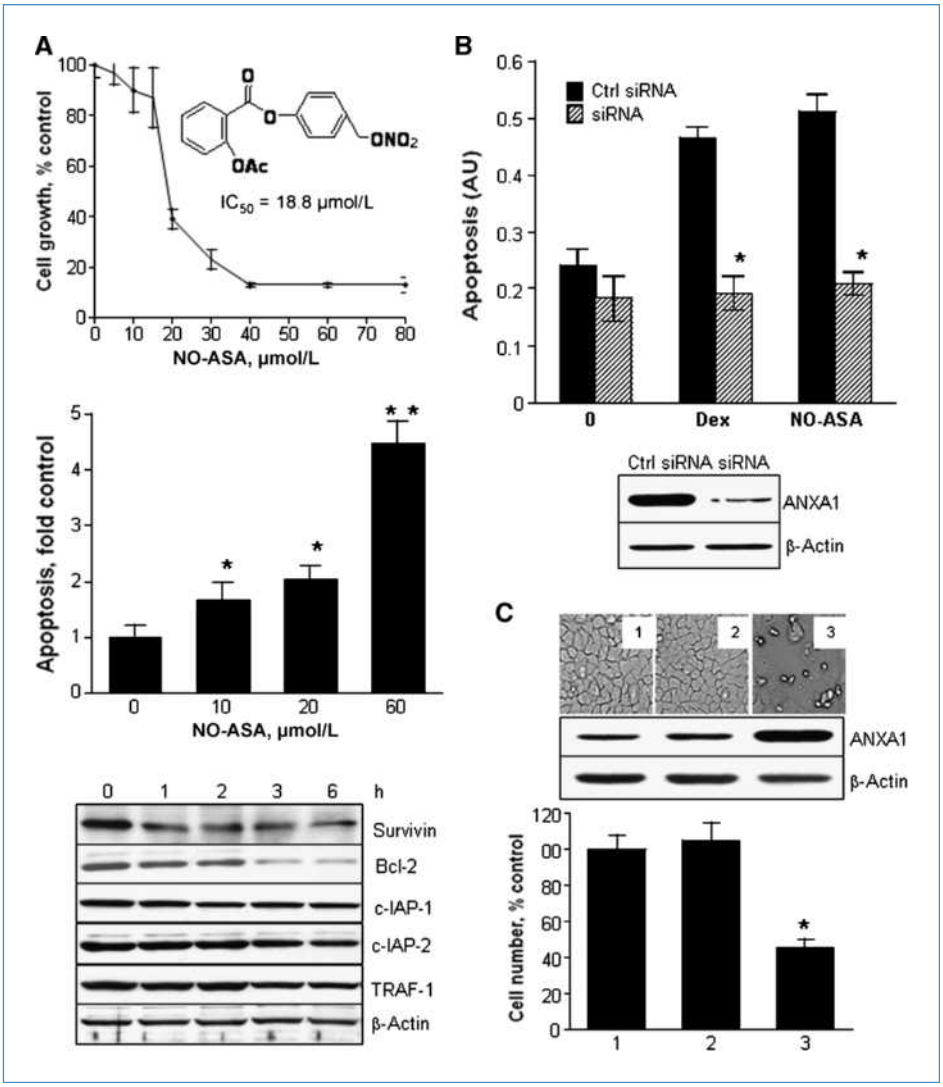

Figure 3. ANXA1 mediates the apoptosis and cell death induced by anti-inflammatory agents. A, NO-ASA inhibited the growth of HT-29 human colon cells (top). The chemical structure of NO-ASA is shown. NO-ASA enhanced HT-29 cell apoptosis in a concentration-dependent manner (middle). This effect was associated with the suppressed expression of several NF-κB–dependent antiapoptotic genes (bottom), determined by immunoblotting over a 6-h observation period; BxPC-3 cells were treated with 30 μmol/L NO-ASA. *, $P < 0.04$; **, $P < 0.001$, compared with control. B, the expression of ANXA1 was knocked down in HT-29 cells using *ANXA1*-specific siRNA, as described in Materials and Methods, but not in cells transfected with control siRNA. The cells were treated for 3 h with 30 μmol/L NO-ASA or 4 μmol/L dexamethasone, and apoptosis was determined using an ELISA assay. *, $P < 0.001$, compared with control. C, BxPC-3 cells were transfected with an *ANXA1* cDNA or with empty vector, whereas controls were not transfected. The expression status of ANXA1 was evaluated by immunoblotting (middle), confirming its overexpression by the *ANXA1* cDNA construct. Cell death was induced only in the cells overexpressing ANXA1, as shown in photomicrograph #3 at the top (the numbers in each image correspond to that inside each column of the graph). Cell growth was determined by MTT assay, as described in Materials and Methods. Columns, mean ($n = 3$); bars, SD. *, $P < 0.01$.

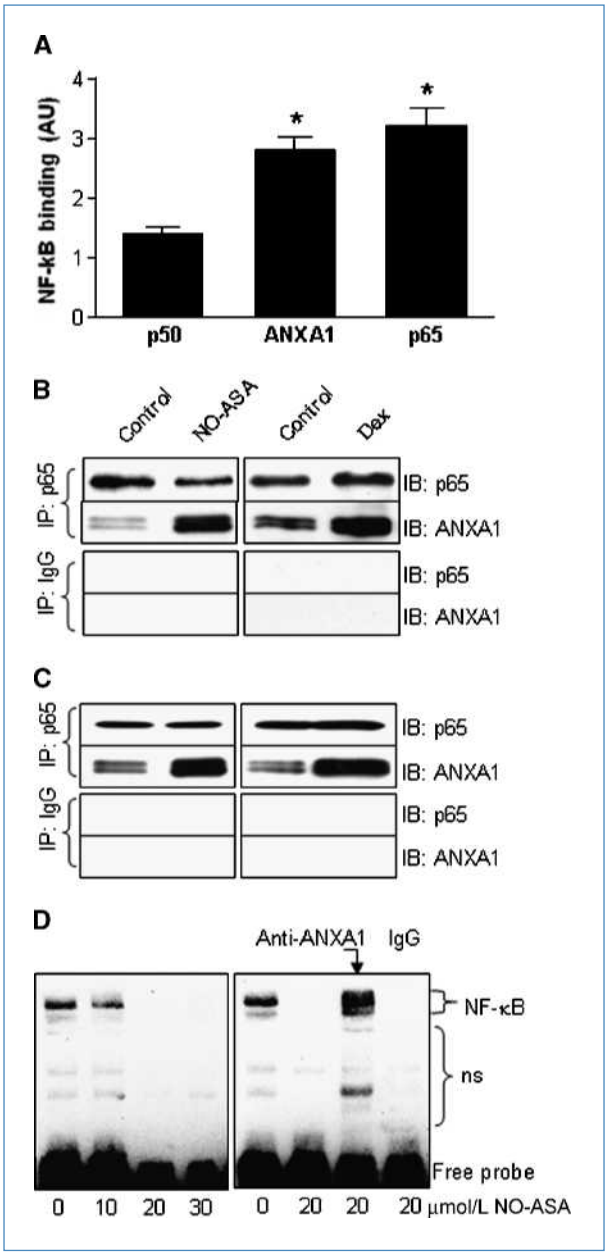

Figure 4. ANXA1 directly binds to NF-κB p65 subunit in human cancer cells. A, nuclear proteins from BxPC-3 cells treated for 3 h with 20 μmol/L NO-ASA were incubated with κB oligomers immobilized on the wall of the reaction wells of 96-well plates. NF-κB dimers bound to these oligomers were recognized by anti-p65 or anti-p50 antibodies through a color reaction. An anti-ANXA1 mAb (middle column) that did not cross-react with either p50 or p65 generated a positive reaction. Columns, mean ($n \geq 3$); bars, SEM. *, $P < 0.001$, compared with control. B, total protein lysates of BxPC-3 pancreatic cancer cells treated with either 30 μmol/L NO-ASA or 4 μmol/L dexamethasone were immunoprecipitated with either an antibody against the p65 subunit of NF-κB or a nonspecific isotypic IgG antibody (control) and immunoblotted (IB) as indicated. C, the same study as in B was done on HT-29 colon cancer cells, giving similar results. D, left, EMSA from BxPC-3 cells treated with up to 30 μmol/L NO-ASA for 3 h. NO-ASA inhibited the binding of NF-κB to the DNA probe. Right, in this EMSA, the nuclear protein extract was reacted with an anti-ANXA1 mAb or with nonspecific isotypic IgG (control) before being reacted with the κB probe. ns, nonspecific binding.

using an anti-p65 mAb. Immunoblotting with the anti-ANXA1 mAb revealed the presence of markedly increased amounts of ANXA1 in the NO-ASA–treated cells compared with controls. Immunoprecipitation with an isotypic nonspecific mAb failed to precipitate ANXA1 (Fig. 4B). HT-29 cells gave similar results (Fig. 4C). Finally, we performed an EMSA using nuclear extracts from BxPC-3 cells treated with NO-ASA (Fig. 4D). As expected (3), NO-ASA markedly suppressed the binding of NF-κB to the κB probe. When, however, the nuclear extract from NO-ASA–treated cells was reacted with an anti-ANXA1 mAb during the nuclear protein extract-κB probe binding step, the binding of NF-κB to the κB oligomer was restored, as evidenced by a strong NF-κB band in the EMSA. A nonspecific control IgG antibody had no such effect. This finding suggests that ANXA1 is associated with the NF-κB dimer and prevents its binding to the κB binding site. The induction of ANXA1 by NO-ASA and its physical association with NF-κB were also shown in HT-29 human colon cancer cells (data not shown).

Confocal microscopy studies indicated that ANXA1 physically associated with the NF-κB p65 subunit. In BxPC-3 cells, we examined whether p65 and ANXA1 colocalized following treatment with NO-ASA. In untreated cells, the two proteins colocalized minimally, if at all (Fig. 5A). In response to a 2-hour treatment with NO-ASA, there was a marked concentration-dependent colocalization of p65 and ANXA1. When cells were treated with 20 μmol/L NO-ASA, colocalization was more pronounced in the nuclei (Fig. 5A), consistent with the enhanced nuclear ANXA1 levels detected by immunoblotting (Fig. 1). Dexamethasone generated similar results (Supplementary Fig. S2). We were unable to document binding of ANXA1 to p50, the other subunit of the NF-κB dimer in these cells. Whether such an interaction occurs below the detection power of our methods remains unclear.

In* Min *mice, NO-ASA and dexamethasone induce ANXA1, which binds to the p65 subunit of NF-κB and inhibits its activation. To assess whether the changes in cancer cell lines occur *in vivo*, we evaluated the effect of NO-ASA and dexamethasone on NF-κB activity in the intestinal mucosa of *Min* mice and the corresponding wild-type mice C57BL/6J. Heterozygous *Min* mutants spontaneously develop tumors in the intestine and represent a useful model of intestinal carcinogenesis (20). As previously reported, NO-ASA inhibits intestinal carcinogenesis in *Min* mice (21). Mice were treated with 100 mg/kg NO-ASA or 10 mg/kg dexamethasone i.p. once a day for 1 week and then were euthanized. Protein lysates from the scraped intestinal mucosa were evaluated for NF-κB activity.

As shown in Fig. 5B, NO-ASA and dexamethasone suppressed NF-κB activity in *Min* mice by 64.3% and 60.8%, respectively ($P < 0.01$). There was a modest (35.6% and 38.8%) but significant ($P < 0.05$) reduction of NF-κB activity in wild-type mice in response to these agents. To confirm the physical association between ANXA1 and p65, we used an anti-p65 mAb to precipitate protein lysates from the intestinal mucosa. Immunoblotting established the presence of ANXA1 in the precipitates (Fig. 5C). In addition, confocal microscopy showed colocalization of ANXA1 and p65 in mucosal cells dispersed from intestinal mucosal scrapings,

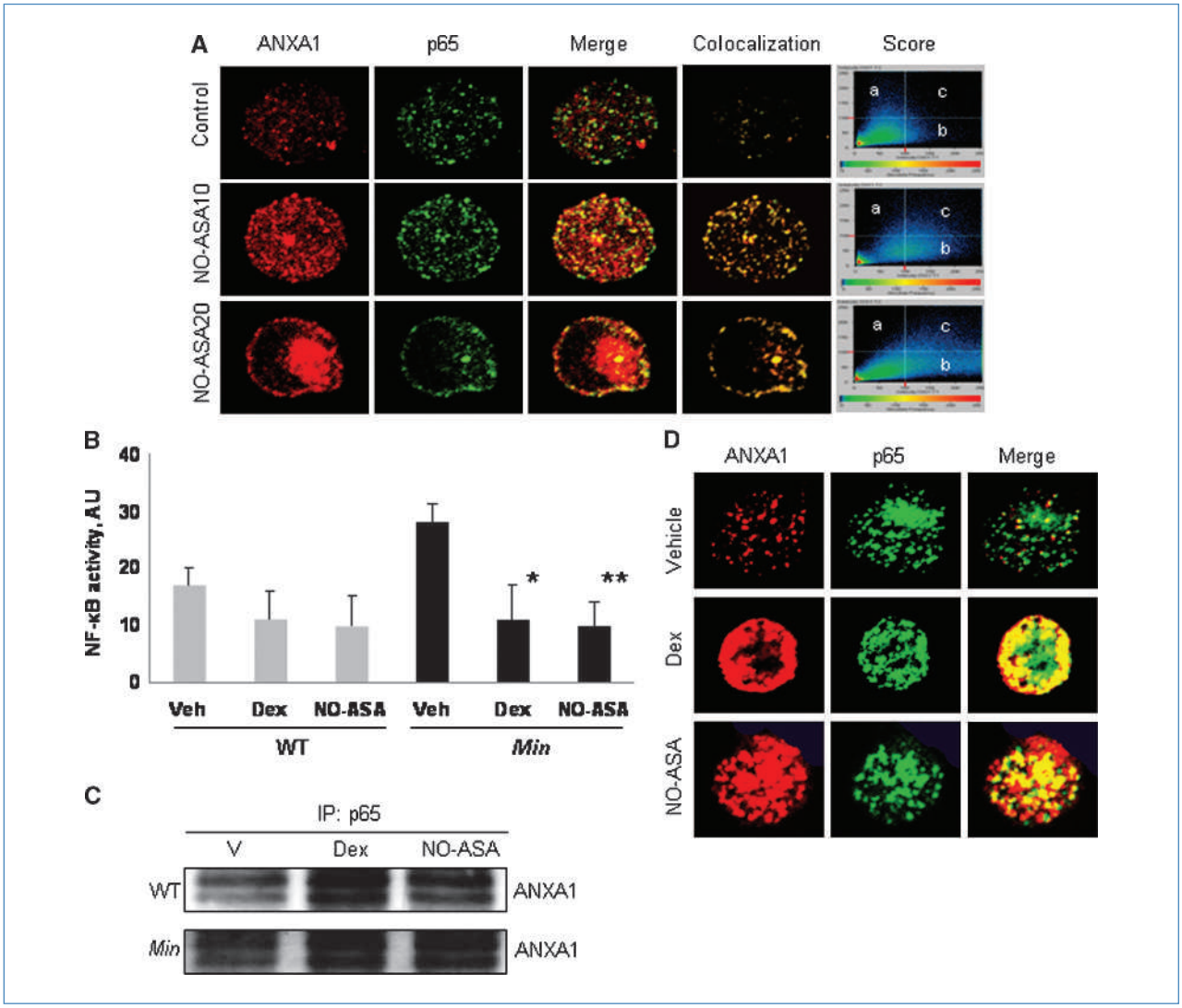

Figure 5. ANXA1 colocalizes with the p65 subunit of NF-κB in response to NO-ASA treatment. A, BxPC-3 cells were treated for 2 h with 10 μmol/L NO-ASA (NO-ASA10) or 20 μmol/L NO-ASA (NO-ASA20), fixed with 4% paraformaldehyde, and reacted with mouse anti-ANXA1 mAb and rabbit anti-p65 mAb, which do not cross-react with each other, followed by Alexa555 (antimouse; red fluorescence)- and Alexa488 (antirabbit; green fluorescence)-conjugated secondary antibodies, and then examined by confocal microscopy. Colocalization of the two proteins generates yellow fluorescence. Score graphs show the fluorescence intensity of p65 alone (a), ANXA1 alone (b), or of both when colocalized (c). B, *Min* mice (n = 6) and C57BL/6J wild-type (WT) mice (n = 6) were treated with vehicle (Veh) or with 100 mg/kg NO-ASA or 10 mg/kg dexamethasone i.p., 26 and 2 h before sacrifice. NF-κB activity was determined by ELISA in protein extracts from small intestinal epithelial cells of these mice. Columns, mean; bars, SD. *, $P < 0.05$; **, $P < 0.01$. C, protein cell lysates from small intestinal epithelial cells from each animal were immunoprecipitated using an anti-p65 mAb and immunoblotted using an anti-ANXA1 mAb. Representative examples from wild-type and *Min* mice are shown. D, single small intestinal epithelial cells from *Min* mice were analyzed by confocal microscopy. Yellow color, colocalization of ANXA1 and p65.

confirming their physical association in the intestinal mucosa of these mice (Fig. 5D).

ANXA1-derived peptides: inhibition of NF-κB activity and suppression of xenograft tumor growth. The NH_2-terminal sequence of ANXA1 can reproduce the anti-inflammatory actions of the full-length protein (22). Therefore, we treated BxPC-3 cells for 3 hours with two commercially available NH_2-terminal fragments of ANXA1, Ac2-26 or Ac2-12, at 30 μmol/L each. They decreased NF-κB activity by 25% and 30%, respectively ($P < 0.05$ for both; data not shown). Similar results were obtained in SW480 cells (data not shown). Consequently, we synthesized a series of peptides based on the NH_2-terminal sequence of the ANXA1 protein. As shown in Fig. 6, three of six such peptides inhibited NF-κB activity in SW480 cells; the most potent was the tripeptide Ac-Gln-Ala-Trp (Ac = acetyl), designated QW-3. Their NF-κB inhibitory activity was accompanied by enhanced apoptosis. For example, treatment of SW480 cells with 30 μmol/L QW-3 for 3 hours decreased NF-κB activity by 40% and enhanced apoptosis 1.7-fold over untreated controls

(data not shown). A similar effect was observed in BxPC-3 pancreatic and MCF-7 breast cancer cells (data not shown).

Finally, we investigated the effect of QW-3 on the growth of subcutaneous xenografts of SW480 human colon cancer cells in nude mice. Starting when the average tumor volume was about 750 mm^3, nude mice ($n = 18$) were treated with 80 μg of QW-3 i.p. once a day for 12 days. Compared with the control group ($n = 18$; vehicle alone), QW-3 suppressed tumor growth, its effect being statistically significant on day 8 ($P < 0.05$) and day 12 ($P < 0.01$), reducing tumor volume by 48.0% and 58.1%, respectively (Fig. 6C). The mice tolerated this treatment well without any evidence of distress or toxicity, including no change in their body weight compared with controls.

Discussion

Our study provides a novel mechanism for the regulation of NF-κB. This mechanism integrates three seemingly disparate components: (*a*) NF-κB, the master regulator of multiple cellular phenomena, some of which are associated with inflammation and cancer; (*b*) ANXA1, a member of a superfamily whose members regulate several functions in the cell; and (*c*) anti-inflammatory drugs, including glucocorticoids and the newer modified NSAIDs; glucocorticoids are used clinically as strong anti-inflammatory compounds, and modified NSAIDs hold promise as chemopreventive agents.

The mechanism that our results document is the following: Most glucocorticoids and modified NSAIDs induce the expression of ANXA1, which associates physically with NF-κB and suppresses its transcriptional activity by rendering the NF-κB dimer incapable of binding to DNA. These steps have been clearly documented. The induction of ANXA1 is selective, occurring only in response to glucocorticoids and modified NSAIDs such as NO-ASA and phospho-aspirin, but not in response to conventional NSAIDs. A second feature of this induction is that it is proportional to the pharmacologic potency of the inducing agent. This was clearly established in the case of glucocorticoids, the class of ANXA1 inducers for which such analysis was possible (their anti-inflammatory potency and ANXA1 induction were almost perfectly correlated). This may also be true of NSAIDs, if we include the two modified aspirins, which are much more potent than conventional aspirin (16, 18, 19).

The induction of ANXA1 is accompanied by its physical association with NF-κB, in particular with its p65 subunit. The binding of ANXA1 to NF-κB has been documented amply *in vitro* (cell protein extracts), in cultured cells, and in intestinal epithelial cells of mice. It is noteworthy that our data indicate that ANXA1 binds only to the p65 subunit of NF-κB; we were unable to document an interaction with the p50 subunit. Ongoing studies are attempting to address the reason for such preferential interaction and to identify the region critical for the physical association of the two molecules. It is unclear where in the cell the association of ANXA1 and NF-κB occurs. Based mainly on our confocal microscopy studies, it seems that this binding occurs in the cytoplasm and that the complex translocates to the nucleus; such translocation, however, seems quite limited in dexamethasone-treated cells. Thus, it is conceivable that the cellular distribution of the ANXA1/NF-κB complex may differ depending on the agent inducing it.

The binding of ANXA1 to NF-κB inhibits the activation of NF-κB. It is, however, unclear how the binding of ANXA1 to

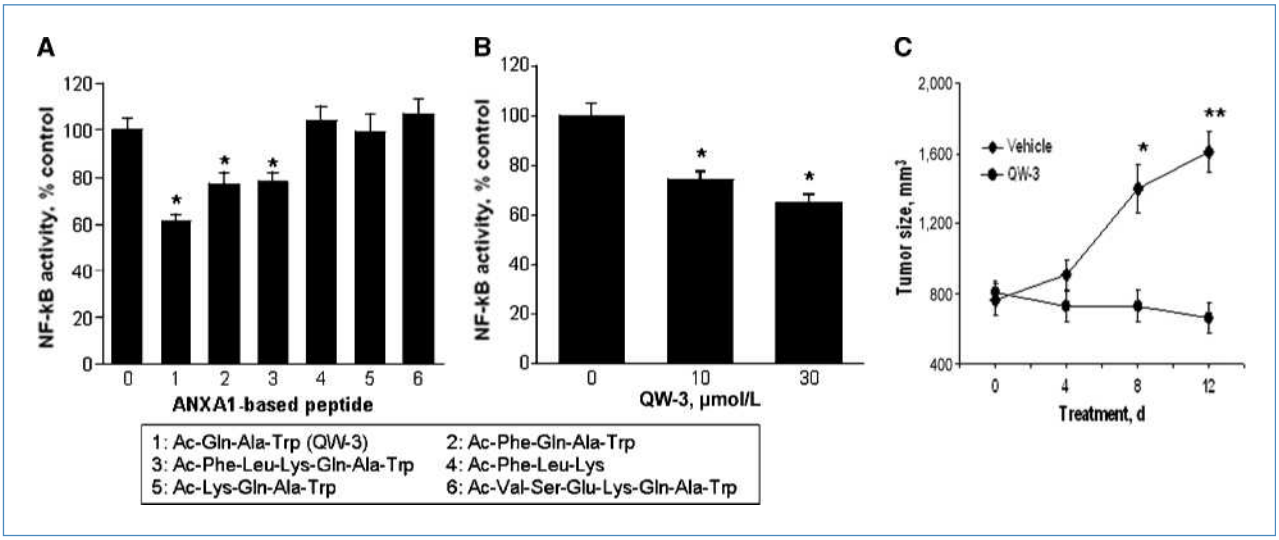

Figure 6. NH_2-terminal peptides of ANXA1 inhibit NF-κB and the growth of SW480 human colon cancer cell xenografts in nude mice. A, six peptides, based on the NH_2-terminal sequence of ANXA1, each 30 μmol/L for 3 h, inhibited NF-κB activity in SW480 cells. The amino acid sequences of the peptides are shown in the box (Ac = acetyl); the numbers correspond to those in the abscissa. *, $P < 0.01$, compared with control. B, peptide QW-3 (#1 in box) inhibited NF-κB activity in BxPC-3 cells. *, $P < 0.01$, compared with control. C, QW-3 suppresses the growth of subcutaneous xenografts of SW480 human colon cancer cells in nude mice. Starting on day 0, mice ($n = 18$) were daily injected i.p. with 80 μg of QW-3 (dissolved in 0.5% DMSO in PBS; injected volume, 100 μL). Control mice ($n = 18$) were given vehicle (identical to test peptide solvent). Points, mean; bars, SEM. *, $P < 0.05$; **, $P < 0.01$.

p65 inhibits the activation of NF-κB. It is conceivable that the presence of ANXA1 in the NF-κB dimer prevents the binding of NF-κB to its target DNA sequence either through steric hindrance or through a conformational change in p65 that alters its DNA binding site. Whatever the mechanism, this inhibition, although not complete under our experimental conditions, is nevertheless functionally important. This was documented by *in vitro* and *in vivo* cellular changes. For example, the induction of ANXA1 and its interaction with NF-κB in cultured cells derived from two human cancers (colon and pancreatic) enhanced apoptotic cell death; disruption of any of these steps abrogated cell death and rescued cell growth. Furthermore, the inhibition of NF-κB activation by ANXA1 changed several NF-κB–dependent signaling molecules (survivin, Bcl-2, etc.), confirming the involvement of the expected pathway (23).

Our findings extend previous observations that the NH_2-terminal portion of ANXA1 mediates some of its known biological effects. Indeed, not only did we show that a 12- and a 22-amino-acid peptide inhibited NF-κB activation but we were also able to design even shorter effective peptides, with a tripeptide (QW-3) being the most potent. Several tripeptides are known to be very effective biologically, with perhaps the best known examples being glutathione and the thyrotropin-releasing hormone. QW-3 inhibited the activation of NF-κB in cultured cells (as well as their growth).

The novel interaction between ANXA1 and NF-κB may help explain several prior observations on the effect of glucocorticoids and ANXA1-derived peptides in aspects of inflammation and related phenomena. For example, it is known that NF-κB activity is persistently increased during neutrophil-mediated inflammatory disorders (24). On the other hand, ANXA1, peptides based on its NH_2-terminal sequence, and glucocorticoids (but not conventional NSAIDs) inhibit various neutrophil-mediated phenomena such as inflammation and ischemia reperfusion injury (25); also, glucocorticoids suppress NF-κB in neutrophils (26). It is conceivable that the sequence anti-inflammatory agent→induction of ANXA1→inhibition of NF-κB may explain these effects (and the lack of effect by conventional NSAIDs).

Besides its inhibitory effect on cell growth, QW-3 inhibited the growth of colon cancer cells grown as xenografts, achieving, in effect, cytostasis (xenograft volume remained stable during the period of observation). Although the overall significance of this observation for cancer control is at present unclear, it nonetheless suggests that the link between ANXA1, NF-κB, and tumor growth is worth exploring further. Indeed, annexins have long been considered significant players in tumor development and progression (12). Our findings suggest the potential for drug development not only for cancer but also for the control of inflammation-related diseases. ANXA1-derived peptides or small molecules with similar activities may bypass the considerable side effects of conventional anti-inflammatory agents, including glucocorticoids, or may have significant anticancer properties.

Whereas ANXA1 mediates some of the effects of potent glucocorticoids and of the potent modified NSAIDs, it is uncertain whether its inhibitory action on NF-κB is the main (or only) mediator of their pharmacologic effects. Our data, limited as they are to cell culture studies, establish a very strong association between the anti-inflammatory potency of glucocorticoids, induction of ANXA1, and NF-κB inhibition. It is thus tempting to speculate that the ability of a compound to induce ANXA1 may determine its anti-inflammatory potency. If this is proved to be the case, one may predict that defects in ANXA1 may be responsible for some cases of steroid resistance, a well-described and clinically significant phenomenon (27).

In conclusion, our findings reveal that ANXA1 is an endogenous inhibitor of NF-κB that can be induced in human cancer cells and mice by potent anti-inflammatory glucocorticoids and modified NSAIDs. ANXA1 inhibits the activation of NF-κB by binding to its p65 subunit. Oligopeptides based on the NH_2-terminal sequence of ANXA1 have similar effects, and one of them inhibits strongly the growth of tumor xenografts. This novel molecular mechanism for the action of anti-inflammatory agents suggests an area for mechanism-driven drug development.

Disclosure of Potential Conflicts of Interest

Z. Zhang and B. Rigas have a patent pending regarding the use of Annexin A1-derived peptides in the treatment of cancer and other inflammation-related conditions. The other authors declared no potential conflicts of interest.

Grant Support

NIH grants CA92423 and CA101019.

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Received 11/24/2009; revised 01/12/2010; accepted 01/12/2010; published OnlineFirst 03/09/2010.

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