Apoptosis Caused by Chemotherapeutic Inhibition of Nuclear Factor-κB Activation

Debajit K. Biswas, Katherine J. Martin, Cliona McAlister, Antonio P. Cruz, Edgard Graner, Sun-chun Dai, and Arthur B. Pardee

Dana-Farber Cancer Institute, Department of Cancer Biology, Boston, Massachusetts 02115

Abstract

Both the protein kinase C (α/β) inhibitor Go6976 and expression of dominant-negative nuclear factor (NF)-κB inhibitor mutants: (a) blocked the growth and caused regression of a mammary tumor in syngeneic mice; (b) inhibited epidermal growth factor (EGF)-induced activation, nuclear translocation, and DNA-binding activity of NF-κB; and (c) caused apoptosis of EGF-stimulated cultured mammary tumor cells. cDNA microarray analysis revealed that these treatments reversed the expression changes of a subset of genes altered by EGF treatment. These included: up-regulation of proapoptotic genes of the tumor necrosis factor (TNF) pathway, death-associated protein (DAP) kinase, p53, and p21/Waf1; and down-regulation of inhibitors of apoptosis: inhibitor of apoptosis (IAP)-1 and X-IAP, TNF receptor-associated factor (TRAF)-2, and factors OX40 and 4-1BB. These results and our previous studies suggest the practicality of a target-directed chemotherapy for EGF-responsive breast cancers, by blocking NF-κB activation and thereby reinstating apoptosis.

Introduction

Advanced breast cancers do not respond well to therapy, and thus represent an important focus for new drug discovery. They tend to be ER− and many express elevated levels of EGFRs that correlate with increased levels of active NF-κB (1, 2, 4–7). Their gene expression is altered to favor uncontrolled growth. We found that Go6976 (5 μM), a low-molecular-weight PKC (α/β) inhibitor, blocked NF-κB activation and dramatically inhibited the proliferation of ER− breast cancer cells in a syngeneic mouse model and provided a potential effective cancer therapy (1, 2). Active NF-κB is a general designation for heterodimeric complexes of different rel family proteins (8, 9). The basal level of active NF-κB is minimal in most cell types, except in B-lymphocytes wherein NF-κB was originally discovered and shown to activate the light-chain gene expression (8). In inflammatory diseases and many cancers, the level of active NF-κB is elevated, leading to increased cell proliferation and resistance to apoptosis (2, 3, 5). Exposure of cells to a variety of molecules including inflammatory cytokines, mitogens, growth factors, infectious agents, irradiation, and stress-related agents leads to the activation of NF-κB (9–11), typically through interaction with their respective receptors and the generation of signals that are transmitted via cascades of interacting kinases that converge on Ikk. EGF causes NF-κB activation via phosphorylation of the cytoplasmic NF-κB inhibitory protein IκB to cause its release from a complex with NF-κB and degradation, and subsequent translocation of active NF-κB into the nucleus (4, 5, 9, 12). NF-κB induces cell cycle regulatory genes such as cyclin D1 that stimulates cell proliferation (4, 5, 13, 14) and also antiapoptotic genes such as survivin (15). It down-regulates the expression of proapoptotic genes such as p53 and NIAp. In this study, we assessed the validity of NF-κB as a target for an apoptosis–based therapy of EGF-responsive ER− breast cancers. This was performed with two inhibitors of NF-κB activation: the small molecule PKC (α/β) inhibitor Go6976 and dnIkBmut. These agents modified expressions of genes related to apoptosis and specifically produced a high rate of apoptosis of the cancer cells.

Materials and Methods

Materials. The growth conditions of mouse mammary adenocarcinoma cells (CSMLO) and stable transfectants expressing dnIkBmut in rich medium (R DMEM supplemented with 10% FBS) and in basal medium [DCC (phenol red-free DMEM supplemented with 10% dextran-coated charcoal-treated FBS, without added glu-tamine)] were described (1, 2). Hydrocortisone, insulin, DTT, DMSO, and phenylmethylsulfonyl fluoride were obtained from Sigma Chemicals (St. Louis, MO).

Mouse Tumor. The anti-tumorigenic activity of Go6976 was monitored in a mouse tumor system, with some modifications of the previous protocol (2). For tumor inhibition studies, Go6976 was initially injected at 48 h after implantation of cells and then once a week. For tumor regression studies, the drug was injected at 7 weeks. Body weight and tumor volumes (16) were monitored weekly. Histological examinations of the tumor tissue and vital organs (liver, lung, and spleen) from treated and control animals were done on H&E-stained sections processed in core facilities of the Institute (1, 2).

Double Immunofluorescence Assay. The active p50/p65 NF-κB complex in the nuclei of CSMLO cells was determined by the double immunofluorescence technique as described previously (17). Cells were grown on coverslips in indicated media. After fixation and permeabilization, they were incubated with a mixture of anti-p50 (SC-7178X) rabbit polyclonal and anti-p65 (SC-8008X) mouse monoclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1 h at room temperature. The p65 subunit was identified with the primary monoclonal antibody and a secondary Fluorescin (FITC)-conjugated goat antimouse IgG that generated green fluorescence. Similarly the p50 subunit was stained with a rabbit polyclonal primary antibody and a Rhodamine-Red-conjugated goat antirabbit IgG generating red fluorescence. Fluorescence microscopy was performed on a Zeiss Axioplan 2 (MOT) and images were captured on a Zeiss Axiocam HRC camera using OPENLAB software. The images were superimposed using Adobe Photoshop software. The p50- and p65-associated merged-red-and-green-fluorescence signals generated the brownish yellow signal in the complex observed in the nuclei of cells.

EMSA. The preparation of nuclear extracts (18), and 32P-labeled NF-κB oligonucleotide binding activity of active NF-κB was determined by EMSA as described previously (1, 2). The 32P-DNA-protein complex was detected as a retarded radioactive band by autoradiography of the dried gel. The p50/p65 complex was characterized by supershift of the radioactive band using anti-p50 and p65 antibodies (1, 2). Quantification of the 32P-DNA-NF-κB complex was carried out by densitometric scanning of the retarded band followed by...
integration of the signals with the program Multianalyst Ver.1.0.2. (Molecular Dynamics).

Apoptosis. Apoptosis was assayed (a) by AnnexinV-FITC binding to the externalized phosphatidyl serine. Apoptosis was monitored with the assay kit and protocol provided by the supplier (Oncogene Research Products, Cambridge, MA). The apoptotic cell numbers were determined by flow cytometry using core facilities of this Institute; and (b) by the enzymatic in situ labeling of apoptosis-induced DNA strand breaks involving DNA polymerase and TdT tailing reactions to produce the TUNEL technique using a kit and the protocol provided by the supplier (Roche Molecular Biochemicals, Indianapolis, IN).

Gene Expression Profile by Microarray Analysis. Total cellular RNA, prepared by using the Qiagen RNEasy kit (Valencia, CA) following the protocol provided by the supplier, was reverse-transcribed into cDNA with mouse mammary tumor virus reverse transcriptase (Promega, Madison, WI) in the presence of [α-32P]dCTP (NEN, Boston, MA). The labeled cDNA probe was hybridized to a membrane containing 96 specific cDNA fragments of apoptosis-related genes, under conditions described by the supplier (Mouse Apoptosis GEArray Q Series, Catalogue No. MM-002N; SuperArray Inc., Bethesda, MD). Membranes were exposed to phosphorimaging screens for 3 days. Signal intensities for individual array features within equal-size ellipses were quantified by using ImageQuant software (Molecular Dynamics). Mean signals were calculated from quadruplicate measurable spots. Signal intensities for each membrane were normalized to the median signal of that membrane. A single median BKG value was determined from an entire set of membranes being compared and was substituted for all BKG values. Median local “BKG” was subtracted, and signals <2-fold above background were considered too low to measure accurately. Signals for individual genes were normalized to the mean expression level of that gene in the set of membranes being compared. Data were filtered to remove genes with consistently low signals, and those not related to apoptosis (pUC-18, β-actin, RPL13-A, cyclophilin A, and GAPDH). Only genes with marked expression changes (exceeding 4-fold) were included in the analysis. Data sets were logarithmically transformed, and hierarchical cluster analysis was performed using publicly available software written by M. Eisen, Stanford University (http://rana.stanford.edu/clustering). Average link- age hierarchical clustering was performed using an uncentered correlation in the gene-clustering dimension.

Results

Antitumorigenic Effect of Go6976. We previously reported strong antitumorigenic activity of Go6976 on ER− CSMLO cells implanted s.c. in syngeneic mice (2). In this report, we extend and confirm these findings by (a) administering Go6976 via an alternative route, i.v. instead of locally; (b) injecting a dose (0.6 mg/kg body weight/week) approximately five times less than that applied previously; (c) including a larger number of animals; and (d) extending the study for a longer period of time. Treatment of animals with Go6976
was initiated either 48 h (Fig. 1A) or 7 weeks (Fig. 1B) after the implantation of tumor cells and continued weekly for 16 consecutive weeks. The former treatment completely blocked tumor formation in 92% of the animals, whereas the latter caused essentially complete regression of established tumors. Tumor regression is particularly significant because it shows that the compound caused cell death rather than merely cessation of growth. Histopathological examination of the treated residual tumor revealed a loss of vascularization, which typically is extensive in untreated tumors, suggesting antiangiogenic activity of this compound, as shown earlier (2). Go6976 treatment of control non-tumor-bearing animals did not cause any obvious toxic side effects nor cause any pathological damage to the vital organs liver, lung, and spleen (data not shown). These and the previously reported results that dnIkkβ-mut-expressing cells did not form tumors in syngeneic mice (2) establish a role of activated NF-κB in mammary epithelial cell carcinogenesis and qualify Go6976 as a potentially effective anticancer agent for ER+ mammary epithelial carcinoma.

Modulation of the Level of Active NF-κB. We proposed that ER+ cells are resistant to apoptosis because of their high level of active NF-κB (1, 2). Active NF-κB was measured first by a double immunofluorescence assay that specifically identifies the p50/p65 complex in the nucleus (Fig. 1, C and D). The nuclei with p50/p65 complex in a total of ~200 DAPI-stained cells were counted. In cells grown in rich medium (Fig. 1C, Row R), this fraction was 45–52%; in cells grown in DCC medium (Fig. 1C, Row DCC), this fraction was 15–18%; and in cells grown in DCC medium supplemented with EGF (Fig. 1C, Row DCC + EGF), this fraction was 34–39%. In Go6976 (5 μM)-treated cells, the active p50/p65 NF-κB complex-containing population was in the order of 5–10% and was undetectable in dnIkkβ-expressing transfectant (B-1-1) grown in rich medium.

Active NF-κB was also determined by EMSA, which reflects the functional state of this complex by the ability to interact with its response element (Fig. 1E). Elevated active NF-κB was detected in CSMLO cells grown in rich medium (Fig. 1E, Lanes 1 and 2) and was greatly reduced in cells grown in basal (DCC) medium containing stripped serum (Lanes 3 and 4). NF-κB was partly reactivated by treatment of these cells with EGF (Lanes 5 and 6). This activation was detected as early as 15 min, attained a maximum at about 4 h, and remained the same for at least 12 h (data not shown). The EGF-induced activation of NF-κB of cells grown in DCC was blocked by simultaneous treatment with 5 μM Go6976 (Lanes 7 and 8). This treatment also blocked cell cycle progression, leading to the accumu-
loration in G2-M and a sub G1-G0 apoptotic population (data not shown). Immunofluorescence assay and EMSA were complementary in regard to the NF-kB status in control and treated cells. These results with ER- mouse cells and previous ones with human breast cancer cells (MDA-MB-231; Refs. 1 and 2) demonstrate that NF-kB is activated by EGF and is blocked by Go6976 in both systems.

**Role of NF-kB in Apoptosis.** An early event of apoptosis was monitored by Annexin binding, based on surface exposure of phosphatidylserine, a component of the cell inner membrane, an early reversible event (Fig. 2A). The fraction of apoptotic cells in DCC (8%) was decreased to 1.7% by EGF addition. The inhibitory effect of EGF on apoptosis was reverted by Go6976 to 8.2%. These results showed an inverse correlation between the active state of NF-kB and apoptosis. The low-molecular-weight inhibitor blocked an antiapoptotic activity presumably mediated via NF-kB, thereby reverting the cancer cells to an apoptotic state. This correlation was substantiated by monitoring apoptosis in transfectants of CSMLO cells in which NF-kB activation was stably blocked by the expression of dnIkBa-mut. A greater number of apoptotic cells were detected in the dnIkBa-mut transfectant (B-1-1) by both apoptosis assays. EGF alone or EGF plus Go6976 treatment did not alter the number of apoptotic cells in dnIkBa-mut expressing cells.

The TUNEL assay is based on the irreversible terminal process of DNA fragmentation. It showed a similar pattern as did the Annexin binding assay, with EGF alone and EGF plus Go6976-treated CSMLO cells (Fig. 2B), although the percentage of apoptotic cells was smaller. This differential in the level of apoptosis may be explained on the basis of the irreversible and reversible nature of late and early apoptotic cells, respectively, as determined by these two assays. A stable vector transfectant control (V-1-5) showed a similar apoptosis profile to the parent cells, measured by both Annexin (Fig. 2A) and TUNEL assay (Fig. 2B). These results demonstrate an inverse correlation of the level of active NF-kB with the apoptotic state and tumorigenic potential of these cells.

**Apoptosis-related Gene Expression Profiles.** To investigate transcriptional events after the activation or inhibition of NF-kB, gene expression profiling was performed. Changes in the expressed levels of 100 apoptosis-related genes were assessed using SuperArray Q10 arrays. Profiles were compared for (a) cells in the absence of EGF (Fig. 3A, Lane 1) relative to cells in the presence of EGF (Lane 2); (b) cells treated with Go6976 (plus EGF) for the indicated period (Lanes 3–6) relative to untreated (plus EGF) cells (Lane 2); and (c) three independent clones expressing dnIkBa-mut (Lanes 8–10) relative to vector control plasmid-expressing cells (Lane 7).

The reproducibility of the assay was confirmed by the similarity of expression changes among the four different Go6976 time points and the three different dnIkBa-mut clones. To accommodate the variability inherent in the microarray procedure, only the 65 genes with marked expression changes, defined here as alterations of at least 4-fold in at least two different conditions, are shown (Fig. 3). Cluster analysis was used to group genes with related expression patterns.

On withholding EGF, the expression levels of 27 genes were increased, and the expression levels of 11 were decreased. Consistent with the apoptotic phenotype of these cells, the increased genes generally had proapoptotic functions, e.g., DAP kinase (ref), CD27L, bcl-10, and bak (7), whereas decreased genes tended to have an antiapoptotic function, e.g., IAP-1, apaf-1, flash, and TRAF family member associated NF-kB activator (I-TRAF). TRAIL receptor and TNF-a do not appear to fit this theme, because they are proapoptotic genes that were down-regulated without EGF. After treatment of EGF-stimulated cells with Go6976, expression changes were essentially the same as those seen in the absence of EGF. Expression was modulated as early as 2 h posttreatment and did not change significantly thereafter. Again, these gene expression changes are consistent
with the ability of Go6976 to induce apoptosis by inhibiting the action of EGF. Cells that expressed different dnIkβ mutations showed expression changes in a subset of the genes altered in the absence of EGF or treatment with Go6976. We refer to the subset of genes altered by both Go6976 and dnIkβ mutations as the “coregulated genes” (Fig. 3).

Two clusters of genes (Fig. 3, I and III) were strongly coregulated by treatment with Go6976 and dnIkβ-mutation (Fig. 3). A strongly up-regulated cluster included the highly apoptotic genes RIP, DAP-kinase, Nip3, CD40, and p53, as well as the cell cycle inhibitor p21/Waf-1 and two antiapoptotic genes bcl-w and TNFSF11 (RANKL). Genes in this up-regulated cluster were generally also increased in the absence of EGF. A strongly down-regulated cluster included the genes X-linked IAP, IAP-1, TRAIL, TRAIL-R, bak, bad, TNF-α, TRAF-2, and TRAF-6. For the most part, EGF did not alter the expression of these genes. A weakly up-regulated cluster included the apoptotic genes TRAF-4, TNFR1, CD40L, CARD, and TNF-β, which were coregulated by the absence of EGF, and treatment with Go6976 and dnIkβ-mutation. Because dnIkβ-mut expression specifically inhibits Ikβ and, thereby, NF-κB activation, these coregulated genes are likely to be involved in controlling apoptosis via the NF-κB signaling pathway. As an inhibitor of PKC (α/β), which function not only to activate NF-κB but also in additional apoptosis-related pathways, it was anticipated that Go6976 would affect a relatively broad group of apoptosis genes. In contrast to the coregulated genes, the two inhibitors regulated the expression of other genes differently. These genes (Fig. 3, clusters II and IV) were up-regulated by Go6976 and down-regulated by dnIkβ-mut (Fig. 4), and were down-regulated by Go6976 and up-regulated by dnIkβ-mut. These results identify genes involved in the antagonistic role of NF-κB on apoptosis, either by stimulating antiapoptotic and/or by inhibiting proapoptotic genes.

Discussion

We have demonstrated potent tumor prevention and regression on systemic application of Go6976, a low-molecular-weight inhibitor of PKC (α/β), and more specific blocker of NF-κB activation with dnIkβ-mut. These antitumor effects were correlated with decreased NF-κB. Active NF-κB has been reported in many human breast cancers, more specifically in ER- breast cancer patients with high levels of EGFRs. We have demonstrated that EGF-EGFR interaction mediates signal transmissions that involve phosphatidylinositol 3′ kinase and PKC (α/β), resulting in activation of Ikβ and NF-κB (2). Active NF-κB is a critical inhibitor of cell death (apoptosis) in many types of cancer cells, as well as of cell proliferation (2, 3, 5–7). These properties qualify increased NF-κB as a prime mediator in the multistage carcinogenesis process. Several mechanisms are implicated in the antiapoptotic role of NF-κB (6, 19–21).

An antiapoptosis-related gene expression profile appeared, as determined by microarray analysis, that correlates with the blocking of apoptosis after activation of NF-κB by EGF. These changes were eliminated by Go6976 and, more specifically, by transfection with dnIkβ-mut. The expression of genes in cells treated with these two inhibitors of NF-κB can be broadly grouped on the basis of their known apoptotic and antiapoptotic roles. For example, the activation of NF-κB and up-regulation of TNF-α are mutually controlled via an auto-up-regulatory loop. Interaction of TNF-α with its receptor induces either antiapoptotic or apoptotic effects in different cell systems (5–7), which may be attributable to a balance between the active and inactive state of cellular regulatory molecules. Among others, the well-defined proapoptotic gene p53 and p21 were up-regulated by these inhibitors (22). Antiapoptotic genes such as IAP1, gadd45, TNF-α, and TRAF2/TRAF6 were down-regulated after treatment of the cells with inhibitors of NF-κB activation. The enhanced apoptosis and reduced cell cycle progression observed after the inhibition of NF-κB activation may also be related, at least partly, to the down-regulation of survivin.

From a broader perspective, cancer therapy could take advantage of the altered homeostasis of cancer cells relative to normal cells. In the light of the hypothesis of “gene addiction” and “gene hypersensitiviy” proposed by Weinstein (23), the balance between up-regulated and down-regulated genes before and after activation and inactivation of a multifaceted key cellular regulatory molecule is different in cancer versus normal cells. Early in carcinogenesis, a cell with imbalanced regulation is produced; counteraction of apoptosis-related genes leads to misregulated cell proliferation, as in atypical hyperplasia and ductal carcinoma, has been proposed (24). A different balance is restored in later stages through mutations of additional genes. Examples have been proposed; in particular, cancer cells must develop a resistance against apoptosis (25), of which the best known mechanism is the inactivation of proapoptotic p53 (22). These defenses can provide ideal targets for a chemotherapy that destroys the antiapoptotic mechanism. Activated NF-κB is such a target, the removal of which by an inhibitor can reverse the specific antiapoptosis of cancer cells. As an example of practicality of this approach, Go6976, which selectively kills cancer cells in culture and in mice, is the first potentially applicable chemotherapeutic agent that restores apoptosis via blocking the activation of NF-κB. Proapoptotic therapy is novel, differing from classical therapeutics directed against cell proliferation.

Acknowledgments

We thank Dr. Shridar Ganesan and Christine Merrese, Department of Cancer Biology, Dana-Farber Cancer Institute for their assistance with immunofluorescence detection of active NF-κB.
References


Apoptosis Caused by Chemotherapeutic Inhibition of Nuclear Factor-κB Activation


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/2/290

Cited articles
This article cites 21 articles, 6 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/2/290.full#ref-list-1

Citing articles
This article has been cited by 18 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/2/290.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/63/2/290.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC)
Rightslink site.