Activation of Nuclear Factor κB through the IKK Complex by the Topoisomerase Poisons SN38 and Doxorubicin: A Brake to Apoptosis in HeLa Human Carcinoma Cells

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ABSTRACT

The transcription factor nuclear factor (NF) κB is involved in the regulation of cell survival. NFκB is activated in many malignant tumors and seems to play a role in the resistance to cytostatic treatments and escape from apoptosis. We have studied the effects on NFκB activation of two topoisomerase poisons and DNA damaging agents that are used in chemotherapy: SN38 (7-ethyl-10-hydroxycamptothecin), the active metabolite of CPT11, and doxorubicin. In HeLa cells, both drugs activate NFκB using a preexisting pathway that requires a functional IκB-specific kinase complex, IκB-specific kinase activation, IκB-κ phosphorylation, and degradation. Blocking NFκB activation by stable expression of a mutant super-repressor IκB-κ molecule sensitized HeLa cells to the apoptotic actions of drugs and tumor necrosis factor. RNase protection assay analysis demonstrate that NFκB is involved in the regulation of a complex pattern of gene activation and repression during the cellular response of HeLa cells to topoisomerase poisons. The blockade of NFκB activation seems to shift the death/survival balance toward apoptosis.

INTRODUCTION

Different natural or chemical compounds are used for cytotoxic treatment of cancer. They act at various levels to block metabolic functions or cell division to finally induce apoptosis of cancerous cells. Taxanes (such as paclitaxel) operate at the level of the cytoskeleton to stabilize microtubules of the mitotic spindle (1), whereas Vinca alkaloids (such as vinblastine and vincristine) induce their depolymerization (2). Antimetabolites such as 5-fluorouracil or methotrexate prevent DNA synthesis (3). Other anticancer drugs such as doxorubicin, camptothecin, CPT11, and its active metabolite, SN38, target topoisomerases to cause DNA damage (4). All these compounds have in common the activation of the transcription factor NFκB (5), which, besides well-known functions in the control of immunity and inflammation (6), now seems to be an important player in the regulation of cell survival through the induction of antiapoptotic genes (7). A constitutive NFκB activity has been observed in many cancer types and was shown to contribute to cell survival and protection against apoptosis induced by DNA-damaging agents (reviewed in Ref. 8). In addition, the blockade of NFκB activation increased the sensitivity of malignant cells to the proapoptotic effect of TNF or, more dramatically, to treatments with drugs or radiation (9). The failure to activate NFκB in response to ionizing radiation results in cell apoptosis (10). Therefore, NFκB has been proposed as a therapeutic target to increase the efficiency of actual treatments of cancer (11). In most cells, Rel/NFκB proteins are rendered inactive in the cytoplasm by inhibitory subunits of the IκB family and activated by a large number of stimuli arising from membrane receptors (i.e., TNF, IL-1; reviewed in Ref. 5). After receptor engagement, IKKs (IKKα and IKKβ) that reside in a high-molecular-weight, multiprotein complex, the IKK signalsome, are activated to phosphorylate IκB molecules on two NH2-terminal serines (32 and 36 for IκB-α). This phosphorylation targets IκB-α for polyubiquitination and subsequent degradation in situ by the 26S subunit of the proteasome to liberate NFκB. NFκB subunits then can translocate to the nucleus to activate target gene expression (reviewed in Ref. 12).

NFκB is activated by UV (13) or γ-ray irradiation (14) or cytotoxic drugs (5). However, it is not clear whether these conditions use a signaling pathway(s) from the nucleus to the cytoplasm or directly generate cytoplasmic events. Topoisomerases are essential for cell survival and crucial for every aspects of DNA metabolism. They are a target of choice for several drugs used in various cancer treatments. Topoisomerases control the degree of supercoiled DNA (15), and whereas type I is crucial for transcription (16), type II is necessary for DNA replication (17). In both situations, the enzymes create transient single-strand breaks in DNA, and the poisons prevent the religation of the strand breaks. SN38, a powerful active metabolite of CPT-11, targets topoisomerase I activity (18), whereas doxorubicin and daunorubicin of the anthracycline family are topoisomerase type II poisons (17).

The aim of the present study was to define the mechanisms of NFκB activation by the topoisomerase poisons SN38 and doxorubicin in HeLa cells. Studying the effect of topoisomerase poisons on NFκB activation and dissecting this signaling pathway is a prerequisite to a better understanding of the effects of DNA damage on cellular functions and to the identification of crucial cellular steps, the targeting of which could help to increase the efficiency of cancer treatments. We show here that SN38 and doxorubicin activate NFκB in a pathway that requires a functional IKK complex, IKK activation, IκB-α phosphorylation, and degradation. This activation of NFκB protects cells from the apoptotic effects of the drugs. Moreover, we have studied the effects of SN38 and doxorubicin on the expression of several genes that are important for the control of the balance between survival and death.

MATERIALS AND METHODS

Antibodies, Plasmids, and Reagents. Anti-IκB-α rabbit polyclonal (C15), anti-p50 rabbit polyclonal, anti-p65 rabbit polyclonal, and anti phospho-IκB antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).
The IKK-expressing vectors were kindly provided by Frank Mercurio (19). SN38 was kindly provided by Rhône Poulenc Rorer. TNF was from PeproTech (Rockey Hill, NJ). Doxorubicin was from Pharmacia & Upjohn SA (St-Quentin-en-Yvelines, France), PMA was from Sigma Chemical Co.-Aldrich (L’Isle d’Abeau, FR).

**Cell Culture.** HeLa cells were maintained in DMEM containing 50 units/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM glutamine, and 10% FCS (Life Technologies, Inc., Cergy Pontoise, France). The 70Z/3 murine pre-B cell line and its NEMO-defective variant have been described previously (20) and were maintained in RPMI 1640 supplemented with 10% FCS and 50 μM β-mercaptoethanol.

**Luciferase Assays.** HeLa cells were transiently transfected by the classical calcium phosphate method, as described previously (21) with 0.5 μg of a luciferase reporter gene controlled by a minimal thymidine kinase promoter and six reiterated κB sites (κB6 thymidine kinase luc). Forty-eight h after transfection, cells were stimulated for 18 h as indicated.

Cells were harvested and lysed in 200 μl of lysis reporter buffer (Promega). Soluble extracts were assayed for luciferase activity on a luminometer. Luciferase activity was normalized to protein amount.

**EMSA.** Total cellular extracts were prepared in Totex lysis buffer [20 mM HEPES (pH 7.9), 350 mM NaCl, 20% glycerol, 1% NP40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 10 μg/ml aprotinin]. Supernatants from 15,000 × g, 15 min centrifugation, were collected. For mobility shift assay, a NFκB probe was used consisting of a synthetic double-stranded oligonucleotide containing the κB binding site of the immunoglobulin promoter (5’-GATCCAGGGACTTTCCAGT-3’). The end-labeled probe (T4 kinase) was incubated with Totex extracts (10 μg) for 20 min at 37°C. Complexes were separated by electrophoresis on a 5% nondenaturing polyacrylamide gel in 0.5 × Tris-borate EDTA. Dried gels were subjected to autoradiography.

For supershift assay, antibodies against p65 or p50 or non immune serum were added 10 min before the labeled probe. Western blot Analysis. After stimulation, HeLa cells were solubilized in lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 20 mM EDTA, 100 μM NaF, 10 mM Na3VO4, 1 mM PMSF, and 0.5% NP40] and incubated for 30 min at 4°C. Lysates were centrifuged at 10,000 × g for 10 min at 4°C, and supernatants were adjusted with concentrated SDS sample buffer. Proteins were separated by SDS/PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA) in a Tris (20 mM), glycine (150 mM) and methanol (20%) buffer at 55 V for 4 h at 4°C.

IκB-α was detected with polyclonal antibodies (Santa Cruz Biotechnology) in saturation buffer [50 mM Tris (pH 7.5), 50 mM NaCl, 0.15% Tween 20, and 5% skim milk] and with a secondary peroxidase-conjugated antirabbit antibody. Proteins were visualized with Amersham enhanced chemiluminescence detection.

**Immunoprecipitation and Kinase Assay.** Whole extracts from 1.2 × 10⁶ HeLa cells treated or not as indicated were prepared in lysis buffer and precleared by incubation with nonimmune serum and protein A-Sepharose. Then lysates were incubated for 3 h at 4°C with a 1:100 dilution of anti-IKKB antiserum and IPs were collected by adding 15 μl of a 50% slurry protein A-Sepharose/PBS for 1 h at 4°C and centrifugation. IPs were washed twice in lysis buffer 0.5% NP40, once in STOP and once in kinase buffer [20 mM HEPES (pH 7.5), 10 mM MgCl2, 100 μM Na3VO4, 20 mM β glycerophosphate, 2 mM DTT, and 50 mM NaCl complete without EDTA protease inhibitors cocktail]. Samples were incubated for 30 min at 30°C in kinase buffer with 2.5 μM [γ-32P]ATP and 0.5 μg of recombinant IκB-α as substrate. Reactions were stopped by the addition of SDS sample buffer, and samples were boiled and resolved on 10% SDS-PAGE. Dried gels were autoradiographed on X-OMAT AR films (Kodak, Rochester, NY).

**Determination of Subdiploid DNA Content.** HeLa cells were harvested and subjected to centrifugation. Cell cycle analysis was performed by quantifying the DNA content using propidium iodide staining, according to Vindelov et al. (22).

**Generation of HeLa Expressing IκB-α WT or IκB-αSA.** The cDNA for WT IκB-α or serines 32 and 36 to alanines IκB-α mutant was a kind gift of Patrick A. Bauerle and were subcloned in pEF6/myc-His A (Invitrogen, Carlsbad, CA). HeLa cells were transfected with 10 μg of plasmid using the Tfx 20 reagent (Promega, Madison, WI). Forty-eight h after transfection, cells were split into fresh medium containing 10 μg/ml of blasticidin until blasticidin-resistant colonies emerged. The expression of transfected IκB-α was monitored by Western blot using anti-myct antibodies, and the inhibitory effect of the mutated protein on NFκB activation was checked by EMSA.

**Measurement of Caspase Activity.** Cells were stimulated as indicated and then incubated for 30 min at 4°C in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM EDTA, 0.2% Triton X-100, 1 mM PMSF, and 10 μg/ml aprotinin]. Lysates were cleared at 10,000 × g for 15 min at 4°C. One hundred μg of protein were incubated in a 96-well plate with 0.2 mM Ac-DEVD-pNA for various times at 37°C. The caspase activity is measured at 410 nm in the presence or absence of 1 μM of N-acetyl-Asp-Glu-val-Asp-CHO. The specific caspase activity is expressed in Ac-pNA cleavage or released absorbance.

**RPA.** After stimulation, cells were harvested, and total RNA was isolated using the TriPure reagent (Boehringer, Mannheim, Germany). Multiprobe template sets (hAP0-1c, hAP02c, and hAP05) were from PharMingen (San Diego, CA). The [γ-32P]-labeled riboprobes were hybridized for 18 h with 5 μg of RNA. The hybridized RNA was digested with RNase, and remaining RNase-protected probes were purified, resolved on denaturing polyacrylamide gels, imaged on a STORM 840 (Molecular Dynamics, Amersham Pharmacia Biotech, Freiburg, Germany), and quantified using the ImageQuant software (Molecular Dynamics). The expression of each mRNA species was calculated after equalization according to the expression level of the two housekeeping genes L32 and GAPDH.

**RESULTS**

**Activation of NFκB by SN38 and Doxorubicin.** The effect of the DNA-damaging agents, SN38 and doxorubicin, on NFκB activation, were analyzed on HeLa cells. After different treatments with the drugs, HeLa cells were harvested, and the binding of NFκB to DNA was visualized by EMSA. No activation of NFκB could be detected in unstimulated cells (Fig. 1, A, B, and D, Lanes 1, and C, Lane 2), SN38 treatment for 2 h induced a dose-dependent activation of NFκB (Fig. 1A, Lanes 2–6), with a maximum at 10 μM (Lane 5). The activation was slightly lower than that observed after TNF stimulation (Lane 7). At 10 μM, SN38 induced a rapid activation of NFκB, which was detectable after 1 h (Fig. 1B, Lane 3) and lasted until 3 h (Lane 5). The upper complex corresponding to NFκB binding was competed by an excess of κB-unlabeled probe, but not by an unrelated double-strand oligonucleotide (data not shown).

Treatment with doxorubicin also induced a dose- and time-dependent activation of NFκB (Fig. 1, C and D), with a maximal effect at 5 μM (Fig. 1C, Lane 5) after 2 h (Fig. 1D, Lane 4).

The ability of SN38 and doxorubicin to induce NFκB-dependent gene activation was then examined in luciferase assays. HeLa cells were transfected with a reporter construct in which the luciferase gene is placed under a 6-fold repetition of a κB binding site and treated for 18 h with different concentrations of SN38 or doxorubicin. Luciferase accumulation was measured as indicated in “Materials and Methods.” As shown in Fig. 1E, SN38 and doxorubicin induced κB-transcription of the luciferase gene in a dose-dependent manner. Taken together, the results showed that SN38 and doxorubicin are powerful activators of NFκB in HeLa cells.

**Effect of CHX on NFκB Activation.** NFκB activation by SN38 and doxorubicin could be either a direct mechanism or mediated through the secondary production of NFκB activators such as TNF-α and IL1β. To distinguish between these possibilities, protein neosynthesis was blocked by pretreatment of HeLa cells with CHX before drug stimulation. As shown in Fig. 1E, CHX had no effect on NFκB activation induced by two doses (5 and 10 μM) of SN38 (compare Lanes 3 and 5 with Lanes 2 and 4) nor by two doses (1 and 5 μM) of doxorubicin (compare Lanes 7 and 9 with Lanes 6 and 8). Thus, activation of NFκB by topoisomerase inhibitors does not require synthesis of a protein intermediate.
Serines 32 and 36 on IκB-α Are Required for SN38 and Doxorubicin-induced NFκB Activation. Engagement of the classical NFκB activation pathway requires phosphorylation of IκB-α on serines 32 and 36 with subsequent degradation to allow the release of active NFκB.

Luciferase assays were performed in the presence of plasmids coding for WT IκB-α or a serines 32–36 to alanines-mutated, dominant-negative version of IκB-α. As shown in Fig. 2A, expression of WT IκB-α only slightly decreased SN38- or TNF-induced activation, but had no effect on doxorubicin-induced activation. By contrast, the double serine mutant of IκB-α totally inhibited TNF, SN38, or doxorubicin-induced NFκB activation.

SN38 and Doxorubicin-induced Degradation of IκB-α. We then measured the half-life of IκB-α after drug treatment. HeLa cells were treated or not with CHX for 1 h before stimulation by topoisomerase poisons. The cellular levels of IκB-α were visualized by Western blotting. SN38 induced a rapid decrease in IκB-α levels after 1 h that lasted for 3 h (Fig. 2B, top panel, Lanes 2–4). The decrease was
amplified by CHX pretreatment which prevented IκB-α resynthesis (top panel, Lanes 6–8). The same results were observed with doxorubicin, except that degradation of IκB-α was slower and delayed compared with SN38. Degradation was visible only after 2 h (bottom panel, Lane 3). CHX pretreatment strongly amplified the effect of doxorubicin (bottom panel, Lanes 6–8).

Involvement of IKKs in Drug-induced NFκB Activation. Phosphorylation and degradation of IκB-α is regulated by two specific kinases, IKKα and IKKβ (12). We investigated whether these kinases could be involved in NFκB activation induced by SN38 and doxorubicin. HeLa cells were left untreated or stimulated with the DNA-damaging drugs for 15 min. The IKK complex was then precipitated from cell lysates with specific IKKβ antibodies, and IPs were subjected to in vitro kinase assay in the presence of recombinant IκB-α (0.5 μg). The results displayed on Fig. 2C show that SN38 (Lane 4) and, to a lesser extent, doxorubicin (Lane 6) induced phosphorylation of IKKβ (top panel) as well as of IκB-α (bottom panel). No kinase activity could be detected in unstimulated cells (Lane 2) nor in precipitates with nonimmune serum (Lanes 1, 3, and 5). The stimulatory conditions used (SN38 and doxorubicin) did not affect the amount of cellular IKKα, IKKβ, and NEMO proteins (three bottom panels).

Phosphorylation of IκB-α was then studied using a phosphospecific IκB-α antibody. Cells were treated or not with the proteasome inhibitor ALLN before stimulation with SN38 doxorubicin or TNF for the indicated times. ALLN was used to stabilize the phosphoform of IκB-α. In the presence of ALLN, SN38 and doxorubicin as well as TNF-induced phosphorylation of IκB-α that was detectable after 30 min and remained stable over 2 h (Fig. 2D). The levels of total IκB-α decreased upon stimulation and were slightly stabilized by ALLN pretreatment.

Activation of NFκB by Drugs Requires a Functional IKK Complex. The IKK are part of a high-molecular weight protein complex whose integrity and functionality is guaranteed by a scaffold component NEMO/IKKγ. In a gene reporter experiment, cDNAs for dominant-negative forms of IKKs mutated within the ATP acceptor site (IKKαKM and IKKβKM) were transfected in HeLa cells, and NFκB activation was measured in a luciferase assay. Expression of IKKαKM or IKKβKM totally inhibited NFκB activation induced by SN38, doxorubicin or, as control, TNF (Fig. 3A). These experiments highlight the importance of the IKK proteins in the effect of topoisomerase poisons.

Then, we used a murine pre-B cell line 70Z/3 and its variant 1.3E2, which is defective for NEMO expression. As a consequence, this variant displays a profound impairment of NFκB activation (20). This is shown on Fig. 3B. Stimulation by PMA (Lanes 2 and 10) leads to NFκB activation in 70Z/3 cells but not in the 1.3E2 variant (Lanes 6 and 15). Similarly, SN38 and doxorubicin induced a dose-dependent NFκB activation in the parental (respectively, Lanes 3, 4, and 11–13) but not in the NEMO-deficient variant (Lanes 7, 8, and 16–18).

Altogether, these results demonstrate that the DNA-damaging drugs SN38 and doxorubicin require an intact and functional IKK complex for activation of NFκB.

Regulation of Drug-induced Apoptosis by NFκB. In some but not all tumor cell lines, activation of NFκB prevents apoptosis. We have stably transfected HeLa cells with WT or dominant-negative forms (IκB-αSA, mutated on the two regulatory NH2-terminal series 32 and 36) of IκB-α. The stable expression of IκB-αSA but not of WT IκB-α completely blocked NFκB activation by various stimuli (TNF, IL1, PMA, SN38, and doxorubicin) as observed in EMSA experiments (data not shown). We then studied the sensitivity of stable clones to drug-induced apoptosis. Cell death was visualized by quantification of either caspase 3 activity and of the number of cells with a DNA content <2 n (sub-G1 population). As shown on Fig. 4A, stimulation of WT IκB-α-expressing cells with SN38, doxorubicin, and TNF for 8 h lead to increases in caspase 3 activity (respectively...
Fig. 5. RPA analysis of the expression of life-and-death-regulatory genes. HeLa cells expressing either WT IκB-α or IκB-αSA super-repressor were stimulated with 10 μM SN38 (S), 10 μM doxorubicin (D), or 10 ng/ml TNF (T) for the indicated hours (S1, SN38 for 1 h). RNA were prepared and analyzed by RPA. The protected and labeled RNA were resolved on a denaturing polyacrylamide gel. Each mRNA species was quantified and its expression level calculated as a function of the expression of L32 and GAPDH species. All data are presented as the mean ± SD. The statistical significance of the differences between control cells and cells treated with the drugs were determined by Student’s t test. *, P < 0.2; **, P < 0.1; ***, P < 0.05. The Y axis represents the relative mRNA expression in arbitrary units.
1.4-, 2.5-, and 1.8-fold) that were potentiated by expression of the dominant inhibitor IκB-αSA (2.2-, 3-, and 2.9-fold). The blockade of NFκB activation also potentiated the number of apoptotic cells visualized as sub-G1 cells after stimulation by SN38 and TNF (Fig. 4B). The apoptotic effect of doxorubicin could not be evaluated by this method, likely because of an interference of the drug with propidium iodide staining.

These results show that in HeLa cells, the activation of NFκB by drugs or TNF decreased the efficiency of these compounds to induce cell death.

Expression of Life and Death-regulatory Genes by DNA-damaging Drugs. The main effect of these drugs is to kill cancerous cells by apoptosis. We thus analyzed by RPA their effects on the expression of several genes that are involved in the control of cell death or survival. To evaluate the contribution of NFκB to the actions of the drugs, we used the stable transfectants of HeLa that express either WT or a dominant-negative form of IκB-α (IκB-αSA). After stimulation of HeLa cells, RNA was prepared and hybridized to specific probes. The relative expression of the mRNA species, expressed in arbitrary units, was calculated after quantification of each band and normalization according to the expression levels of the housekeeping genes L32 and GAPDH. SN38 and doxorubicin induced a rapid decrease (1.3–2-fold) in the expression of the antiapoptotic genes coding for Bcl-w, Bcl-xl, Mcl-1 (Fig. 5). Used – and doxorubicin induced a rapid decrease (1.3–2.5-fold) that were potentiated by expression of the IκB-α repressor. TNF could no longer stimulate Bcl-w, Bcl-xl, and Mcl-1 gene expression. Moreover, in these conditions, SN38 rather had a up-regulatory effect (1.2-fold) on Bcl-w and Bcl-xl expression. TNF had no significant effect on the expression of the genes for the proapoptotic mitochondrial proteins Bad, Bax, and Bak. On the contrary, SN38 and doxorubicin slightly decreased their expression levels (1.3–1.4-fold). In the absence of NFκB activation, these mRNA species were either up-regulated (1.1-fold) by SN38 or still decreased (1.3–1.5-fold) by doxorubicin. The drugs were found to stimulate the expression of the genes for the inhibitors of apoptosis c-IAP-1, c-IAP-2, XIAP, and TRPM-2, although with a lower efficiency compared with TNF (average, 1.1–1.4-fold versus 1.1–1.5-fold). When NFκB was blocked, the drugs had a rather inhibitory effect (1.4–2.2-fold). In particular, the high-level expression of TRPM2 completely collapsed with a basal level decreased by 2.8-fold compared with WT IκB-α-expressing cells, and no stimulatory effects were observed.

Finally, SN38 and doxorubicin had an inhibitory effect on caspase 8 expression (respectively, 1.5- and 1.7-fold). In the IκB-αSA super repressor-expressing cells both drugs slightly stimulated expression of caspase 8 (1.1-fold), SN38 and doxorubicin stimulated transcription of the caspase 9 gene (3-fold) in an NFκB-dependent manner. Although TNF had no effect on caspase 8 expression and other caspases tested (caspases 1–7 and 10, not shown), its small activatory effect on caspase 9 expression (1.2-fold) was potentiated in the absence of NFκB activation (2.8-fold). Student’s t test analysis shows that the differences are statistically significant, with P values between (∗, P < 0.2; ** P < 0.1; and *** P < 0.05).

On the whole, the results show that NFκB is involved in a complex pattern of gene activation and repression during the cellular response of HeLa cells to topoisomerase poisons. The blockade of NFκB activation seems to shift the death/survival balance toward apoptosis.

DISCUSSION

Topoisomerase B is essential for cell survival and proliferation and represent key targets for numerous drugs used in actual treatments of cancers. In the present study, we have analyzed the signaling pathway that leads to NFκB activation after production of DNA damage by the topoisomerase poisons SN38 and doxorubicin. Both compounds induced a rapid and dose-dependent activation of NFκB. This is the consequence of stimulation of the IKK kinases that phosphorylate and inactivate the inhibitory subunit IκB-α. Drug-induced NFκB activation measured by binding of the factor to DNA or activation of transcription could be blocked by the dominant-negative form of the IKK or by a dominant-negative mutant of IκB-α. Moreover, disruption of the integrity of the IKK complex by the absence of the NEMO component prevents activation of NFκB by the drugs. These results demonstrate that topoisomerase-targeting agents activate NFκB through mobilization and stimulation of the classical IKK complex.

The primary site of action of DNA-damaging drugs is in the nucleus. It has been shown by other investigations that camptothecin requires binding to topoisomerase I (23) whereas mitoxantrone, an anthranceridine, one, must bind topoisomerase II (24) to activate NFκB. We have excluded an indirect action of SN38 and doxorubicin through the synthesis of an autocrine NFκB activator, because their effects could not be blocked by a protein synthesis inhibitor. Therefore, the two compounds likely mobilize a preexisting signaling pathway that starts from the nucleus at the level of DNA strand breaks to end up in the cytosol at the IKK complex. Several proteins have been proposed to map in the NFκB-signaling pathway mobilized by DNA damage. One interesting candidate is ATM, a member of the high molecular weight PI3-kinase-like family, which is mutated in ataxia-telangiectasia patients (25), characterized by immunodeficiency and extreme radiosensitivity (26). NFκB activation by ionizing radiation is defective in ataxia-telangiectasia cell lines (27, 28) or in tissues or from ATM null (−/−) mice (28). Activation of NFκB subsequent to DNA damage was also shown to depend on ATM expression (29).

DNA-PK has also been implicated in the activation of NFκB after DNA damage, however the results seem more conflicting. On one side, no NFκB activation could be detected after irradiation in DNA-PK-negative cell lines derived from a human glioma biopsy (30). On the contrary, recently it has been reported clearly that separate invalidation of the three subunit of DNA-PK, the catalytic subunit PKCs and the DNA binding subunits Ku 70 and Ku 80 did not prevent NFκB activation by irradiation (28). It remains possible that the type (single- or double-strand breaks) or by the extent of the lesions to the DNA, together with the production or not of reactive oxygen intermediates, could have different requirements for ATM versus DNA-PK.

NFκB is now recognized as an important player in the regulation of cell survival. Inactivation of the p65 NFκB (relA) gene results in embryonic death after massive liver apoptosis (31). Many malignant tumors display constitutive NFκB activation that allows malignant cells to escape growth regulation by apoptosis (32). This constitutive NFκB activation is caused by genetic alterations affecting nfkβ/ikb genes or to unrestrained IKK stimulation (reviewed in (8)). The survival power of NFκB could be demonstrated in various cell lines by the expression of a super-repressor mutant form of the inhibitory subunit IκB-α, which become more susceptible to apoptosis induced by TNF, cytotoxic drugs, or γ-rays (9, 31, 33). Using HeLa cells stably transfected with a dominant negative mutant of IκB-α, we could confirm that activation of NFκB by SN38 and doxorubicin lowered the efficiency of the drugs to induce apoptosis. This phenomenon has also been described in vivo, inasmuch as the expression of the IκB-α repressor in a mouse model of tumor formation with injected human fibrosarcoma cells decreased the chemo-resistance to CPT and SN38 of the resulting tumors (11). In some models however, NFκB does not regulate or even promotes apoptosis (34–36). It would be most important to elucidate which cellular event regulates the cellular response to NFκB modulation.

NFκB controls a wide set of genes, among which several are interesting candidates for regulators of apoptosis (5). Differential
screening approaches on various cellular models have allowed the identification of several antiapoptotic genes (37–40): TRAF1 and TRAF2 adapters likely act by inducing a constitutive signaling to NF-κB to strengthen transcription of the antiapoptotic gene (38); c-IAP-1 and c-IAP-2 bind and block caspase activation (41); Bel-2 and Bfl1 of the Bcl-2 family control mitochondrial homeostasis and prevent release of cytochrome c and subsequent activation of caspase 9 (42).

HeLa cells expressing the IκB-αSA repressor were an exquisite model to study the implication of NFκB on gene expression during drug-induced DNA damage. An RPA analysis of several genes involved in cell death/survival was performed in the present study and revealed the existence of a complex network of gene activation and repression. Interestingly, SN38 and doxorubicin seemed to exert both pro- and antiapoptotic actions. Proapoptotic effects result, at least in part, from the down-regulation of molecules Bcl-w, Bclx-l, Mcl-1 and drug-induced DNA damage. An RPA analysis of several genes in- doxorubicin failed to up-regulate effects of the drugs on gene expression that could account for the identified chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibitor of apoptosis B. Science (Wash. DC), 274: 8029–8036, 1997.


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