Anti-Cancer Drugs 1998, 9, pp. 703–714

Preclinical report

Acquisition of cellular resistance to 9-nitro-camptothecin correlates with suppression of transcription factor NF-κB activation and potentiation of cytotoxicity by tumor necrosis factor in human histiocytic lymphoma U-937 cells

Sanjaya Singh, Uma Raju, John Mendoza,[1] Panayotis Pantazis[1] and Bharat B Aggarwal

Cytokine Research Laboratory, Department of Molecular Oncology, University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. Tel: (+1) 713 792-3503; Fax: (+1) 713 794-1613. [1]The Stehlin Foundation for Cancer Research at St Joseph Hospital, Houston, TX 77003, USA.

Resistance of tumor cells to chemotherapeutic agents is a major problem in cancer therapy. Continuous exposure of human histiocytic lymphoma U-937 cells to 9-nitro-camptothecin (9NC), an inhibitor of the nuclear DNA topoisomerase I, induces resistance to this drug. Because of the involvement of the nuclear factor NF-κB in the expression of several growth regulatory genes, we examined the activation of this transcription factor in 9NC-resistant U-937 cells. We found that resistance to increasing concentrations of 9NC correlated with resistance to tumor necrosis factor (TNF)-dependent activation of NF-κB. However, the constitutive synthesis of NF-κB proteins remained unaffected. Cellular resistance was not unique to TNF, as other activators of NF-κB, including interleukin-1, phorbol ester and hydrogen peroxide, also had no effect. There was no difference between 9NC-sensitive and -resistant cells in the activation of NF-κB by okadaic acid. Other transcription factors, including AP-1 and Oct-1, were not affected in the resistant cells. When examined for the inhibitory subunit of NF-κB (IκBα), resistant cells showed a faster rate of resynthesis than the control. Interestingly, although 9NC resistance correlated with resistance to TNF-dependent NF-κB activation, TNF-dependent cytotoxicity in these cells was enhanced by several hundred fold despite a significant decrease in the number of TNF receptors. In conclusion, our results suggest that NF-κB activation may play a role in tumor cell killing by 9NC but not by TNF. [© 1998 Lippincott Williams & Wilkins.]

Key words: 9-Nitro-camptothecin, cellular resistance, cytotoxicity, NF-κB, tumor necrosis factor.

Introduction

Like the parental natural product camptothecin (CPT), the semisynthetic derivative 9-nitro-camptothecin (9NC) interferes with the action of the nuclear enzyme topoisomerase I (Topo I) involved in the cleavage-repair mechanism of the chromatin DNA. As a result, 9NC-treated human cells are either arrested at the boundary of the S/G_2 phase or die by apoptosis while traversing the S phase. The differential response of the cells to the 9NC action, i.e. induction of cytostasis or cytotoxicity, mechanistically remains unclear, but it appears that, in addition to Topo I, other parameters can regulate the response of the cells to 9NC. Activities of 9NC against normal and malignant cells *in vitro* and *in vivo* have been recently reviewed.[1,2] Results of a phase I clinical study have been recently reported on cancer patients that orally received 9NC.[3]

Resistance of tumors to 9NC is an undesirable but possible outcome in patients that are treated with 9NC. In this context, we have extensively studied the activity and synthesis of Topo I in a series of sublines of human leukemia U-937 cells with increasing resistance to 9NC.[4–7] Comparison of parental cells and cells with low 9NC resistance showed that the cells have similar proliferation rates *in vitro*; express similar levels of Topo I mRNA; synthesize proteins of identical molecular weight; exhibit similar Topo I catalytic activity in the absence of 9NC, whereas the Topo I activity from the resistant cells is about 10-fold more resistant than from the parental cells; differ in the extent of Topo I methylation, with the resistant Topo I exhibiting hypermethylation; differ in the

This research was supproted in part by a grant from The Clayton Foundation for Research and funds from the Stehlin Foundation for Cancer Research.

Correspondence to BB Aggarwal

nucleotide sequence of Topo cDNAs at position 361, resulting in the presence of serine or phenylalanine in Topo I of the parental or resistant cells, respectively; differ in the steady-state synthesis of c-*jun* mRNA, which is undetectable in the parental cells but readily detectable in the resistant cells; and both induce tumors following xenografting in nude mice[4,5] (unpublished results). Development of higher 9NC resistance by the cells is accompanied by a decrease in the proliferation rate; decreased synthesis of Topo I; increased synthesis of Topo II and, consequently, increased sensitivity to Topo II-directed etoposide; expression of c-*fms* mRNA, which is undetectable in the parental cells; significant synthesis of the apoptosis-promoting protein Bax; the appearance of morphological and functional features of more mature cells; and loss of the ability to induce tumors when xenografted in nude mice[6,7] (unpublished data). Finally, acquisition of 9NC resistance does not correlate with the presence of P-glycoprotein that has been associated with drug resistance in several proliferative diseases of the hematopoietic cells (reviewed in Nooter and Sonneveld[8]).

The nuclear factor (NF)-κB is a pleiotropic transcription factor present in its inactive state in the cytoplasm but in active state in the nucleus (for references, see Miyamoto and Verma[11]). The cytoplasmic retention is mediated by a family of inhibitory proteins, termed IκB, which mask the nuclear localization signals present on the p65 subunit of NF-κB. The nuclear translocation is induced by several agents of which some are DNA damaging agents including tumor necrosis factor (TNF), interleukin (IL)-1, oxidative stress (H_2O_2), UV light and phorbol esters.[11,12] Upon activation of the NF-κB, a large number of cellular and viral genes are induced, including those involved in regulation of cell growth.[11] For instance, activation of the NF-κB is required for expression of genes that regulate cell growth including TNF, IL-1, gro (also referred to as melanoma growth stimulating activity), p53, c-*myc*, A20 and *ras*.[13-17] In addition, cell cycle progression from G_0 to G_1 is also dependent on NF-κB activation,[18] and this may involve both p53 and c-*myc* expression.[19] Although NF-κB is activated during the cell cycle, TNF activates NF-κB with equal efficiency in both cycling and growth-arrested cells.[20] It should be noted that TNF alone inhibits cell growth and induces DNA strand breaks (for references, see Baloch *et al.*[9,10]), while it induces differentiation of 9NC-resistant U-937 cells that synthesize mutated Topo I.[4]

Due to the role of a nuclear factor NF-κB in the expression of several genes that regulate cell growth, in the present report we have examined this transcription factor in U-937 cell sublines resistant to various concentrations of 9NC. We found that as the cells become increasingly more resistant to 9NC, there is a decline in the activation of NF-κB by a wide variety of DNA damaging agents. This is not due to a decrease in protein synthesis but rather to rapid resynthesis of the IκBα. The resistance of U-937 cells to 9NC does not appear to affect other transcription factors including AP-1 and Oct-1. Although 9NC resistance correlated with a decrease in TNF-induced NF-κB activation, the TNF-dependent cytotoxicity in these cells was enhanced in spite of a decrease in the number of TNF receptors, suggesting that NF-κB activation may play a role in the cell killing ability of 9NC but not of TNF.

Experimental procedures

Materials

Penicillin, streptomycin, RPMI 1640 medium and fetal calf serum were obtained from Gibco (Grand Island, NY). Glycine, NaCl and bovine serum albumin were obtained from Sigma (St Louis, MO), and polyethylene glycol (PEG 400) from Aldrich (Milwaukee, WI). Hydrogen peroxide, okadaic acid (OA) and phorbol myristate acetate (PMA) were obtained from Calbiochem (San Diego, CA). Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was kindly provided by Genentech (South San Francisco, CA). Antibody against IκBα and double-stranded oligonucleotides having AP-1 and Oct-1 consensus sequences were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 9NC was semisynthesized and purified (over 99% purity) at the Stehlin Foundation according to the regulations of the Food and Drug Administration.

Cell lines

The development and characterization of U-937 cell sublines resistant to 0.2 μM 9NC (U-937/RC-0.2), 1 μM 9NC (U-937/RC-1) and 10 μM 9NC (U-937/RC-10) has been described previously.[4-7]

Electrophoretic mobility shift assays (EMSA)

Aliquots of 2×10^6 cells/ml were treated separately with different concentrations of NF-κB activators at 37 C and then nuclear extracts were prepared as

described.[21] Briefly, cells were lysed in a buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, 0.5 mg/ml benzamidine and 12.5 μl of 10% NP-40. The nuclear pellet was extracted in 25 μl ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin and 0.5 mg/ml benzamidine). After centrifugation, the supernatant (nuclear extract) was either used immediately or stored at −70 C for later use. The protein content was determined by the method of Bradford.[22]

EMSA were performed by a 15 min incubation at 37 C of 4 μg of nuclear extract (NE) with ^{32}P-end-labeled double-stranded NF-κB oligonucleotide, the sequence of which was derived from the HIV long-terminal repeat (LTR) (5′-TTGTTACAA**GGGACTTTC**CGCTG**GGGACTTTC**CAGGGAGGCGTGG-3′).[23] The incubation mixture included 2–3 μg of poly(dI-dC) in a binding assay buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% NP-40, 5% glycerol and 50 mM NaCl).[24,25] The DNA–protein complex formed was resolved on a 4.5% native polyacrylamide gel in a buffer containing 50 mM Tris, 200 mM glycine, pH 8.5, and 1 mM EDTA.[26] A double-stranded mutated oligonucleotide, 5′-TTGTTACAA**CTCACTTTC**CGCTG**CTCACTTTC**CAGGGAGGCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also assessed by competition with the unlabeled oligonucleotide and by gel supershift with antibodies specific to NF-kB proteins as described.[27]

The EMSAs for AP-1 and Oct-1 were performed as described for NF-κB using ^{32}P-end-labeled double-stranded oligonucleotide. Specificity of binding was determined routinely by using an excess of unlabeled oligonucleotide for competition as described earlier.[28] Visualization and quantitation of radioactive bands were carried out by a phosphorimager (Molecular Dynamics, Sunnyvale, CA) using 'Image-quant' software.

Western blotting for IκBα

After the NF-κB activation reaction described above, postnuclear extracts were resolved on 10% SDS–polyacrylamide gels and the proteins were electrotransferred to nitrocellulose filters. Presence of IκBα was detected with a rabbit polyclonal antibody against IκBα followed by chemiluminescence (ECL; Amersham, Amersham, UK) as described.[28]

Receptor binding assays

Receptor binding assays were carried out as described previously.[29,30] TNF was labeled with Na^{125}I using the Iodogen method.[29] The specific activity of the labeled TNF ranged from 20 to 30 mCi/mg.

Binding assays were performed in flexible 96-well plates precoated with 0.2 ml of FBS for 24 h at 4 C. The binding medium (RPMI 1640) contained 10% FBS. Cells (0.4–0.5 $\times 10^6$/0.1 ml) were incubated with [^{125}I]TNF in the absence (total binding) or in the presence of 100 nM unlabeled ligand (non-specific binding) for 1 h at 4 C. The cells were washed three times with ice-cold medium (PBS containing 0.1% BSA) at 4 C and the cell-bound radioactivity was determined in a γ-counter (Cobra-Auto-Gamma, Packard Instruments, Meriden, CT). All determinations were performed in triplicate. Specific binding of the ^{125}I-labeled TNF was calculated by subtracting non-specific binding from the total binding. Inhibition of specific binding was calculated from the specific binding obtained from the untreated cells (100%).

Cytotoxicity assays

The sensitivities of the parental and 9NC-resistant U-937 cells to 9NC or to TNF was determined by the amount of [^{3}H]thymidine incorporated by the cells. For this, cells (5×10^3/well) were plated in 0.1 ml of the medium (DMEM plus 10% FBS) in 96-well Falcon plates. After incubation overnight in a CO_2 incubator at 37 C, the medium was removed and different concentrations of 9NC or TNF were layered in 0.1 ml of the fresh medium for 3 days. [^{3}H]Thymidine (6.7 Ci/mmol; DuPont NEN Medical Products, Wilmington, DE) was added to each well (0.5 μCi/well) 6 h prior to cell harvesting. Thereafter, the culture medium was removed, the wells were washed twice with phosphate-buffered saline (PBS) and the cells were detached by the addition of a solution containing trypsin (0.5%) with EDTA (5.3 mM). The cell suspension was then harvested with the aid of a PHD cell harvestor (Cambridge Technology, Watertown, MA) and lysed with distilled water. Radioactivity bound to the filter was measured in a liquid scintillation counter (Model 1600 TR; Packard Instruments).

Results

In the present report we have further characterized sublines of the human histiocytic lymphoma U-937 cells that have acquired various levels of resistance to

the cytotoxic activity of the anticancer drug, 9NC. The cells were examined for NF-κB activation, IκB synthesis, presence of functional TNF receptors and TNF-dependent cytotoxicity. When cell viability was examined by thymidine incorporation after treatment for 72 h with different concentrations of 9NC, there was a decrease in thymidine uptake with increasing concentration of 9NC in wild-type (WT) cells but no significant decrease in the uptake was found in 9NC-resistant (RC-0.2, RC-1 and RC-10) cells (Figure 1).

TNF-dependent NF-κB activation is suppressed in 9NC-resistant cells

Because NF-κB controls the expression of several growth regulatory genes, we examined the activation of this transcription factor in U-937 cells resistant to various concentrations of 9NC. For this, cells were activated with TNF (0.01 and 0.1 nM) for 30 min at 37 °C and then examined for NF-κB activation by EMSA. The results in Figure 2(a) show that 9NC-sensitive (WT) cells responded to TNF with activation of NF-κB. The band observed in the gel shift assay was due to NF-κB proteins, as competition with unlabeled oligonucleotide eliminated the band, it did not compete with mutated oligonucleotide, and it was supershifted by incubating with antibodies against the p50 and p65 subunits (data not shown). In contrast to WT, 9NC-resistant cells were unresponsive to TNF-induced NF-κB activation in parallel with the degree of 9NC resistance. Thus the U-937/RC-10 cells, which are resistant to 10 μM 9NC, showed no activation of NF-κB in response to TNF. Treatment of the cells with 9NC neither induced NF-κB activation by itself nor affected TNF-induced NF-κB activation (data not shown).

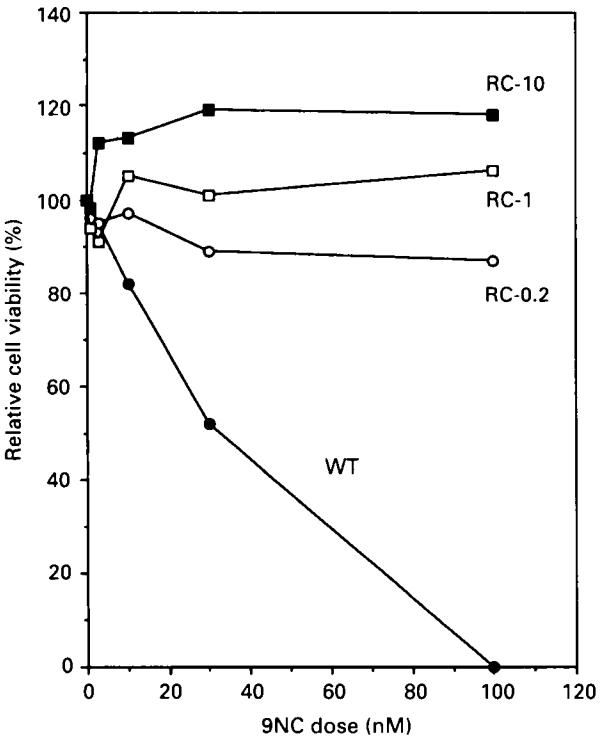

Figure 1. Effect of different concentrations of 9NC on the viability of 9NC-sensitive and -resistant cells. Cells (5×10^3) were plated overnight and then incubated with the drug in 0.1 ml for 72 h at 37 °C. During the last 24 h, the cells were pulse-labeled with thymidine, harvested and counted as described in Experimental procedures. All determinations were in triplicate. The variations between triplicates were 10% or less.

It is possible that TNF did not induce NF-κB activation in the 9NC-resistant cells because the NF-κB proteins, i.e. the p50-p65 complex, are not expressed in these cells. To examine this possibility, the cytoplasmic extracts from both sensitive and resistant cells were treated with deoxycholate (8%), which dissociates the p50-p65 complex from IκBα, and then examined for DNA binding by EMSA. The results of this experiment, shown in Figure 2(b), indicate that there was no difference in the expression of these proteins between sensitive and resistant cells. Thus, lack of NF-κB activation in 9NC-resistant cells was not due to downmodulation of NF-κB protein expression.

Suppression of NF-κB activation in 9NC-resistant cell is not limited to TNF

Besides TNF, the activation of NF-κB can also be induced by other DNA damaging agents including IL-1, PMA, H_2O_2 and OA. The signal transduction pathway that leads to the NF-κB activation induced by these agents may differ. Therefore, we examined the activation of the transcription factor by these various agents in 9NC-resistant and parental cells. The results, shown in Figure 3, indicate that like TNF, 9NC-resistant cells were also resistant to NF-κB activation induced by IL-1, PMA and H_2O_2. Further, the resistance to these agents was more pronounced than that to TNF, since cells resistant to even low concentrations of 9NC were completely refractory. Interestingly, however, there was no difference between sensitive and resistant cells in their responsiveness to OA-induced NF-κB activation. These results suggest that OA, an inhibitor of serine/threonine phosphatases, activates NF-κB by a mechanism that is different from that of other agents tested and that this mechanism is fully intact in all the 9NC-resistant cells.

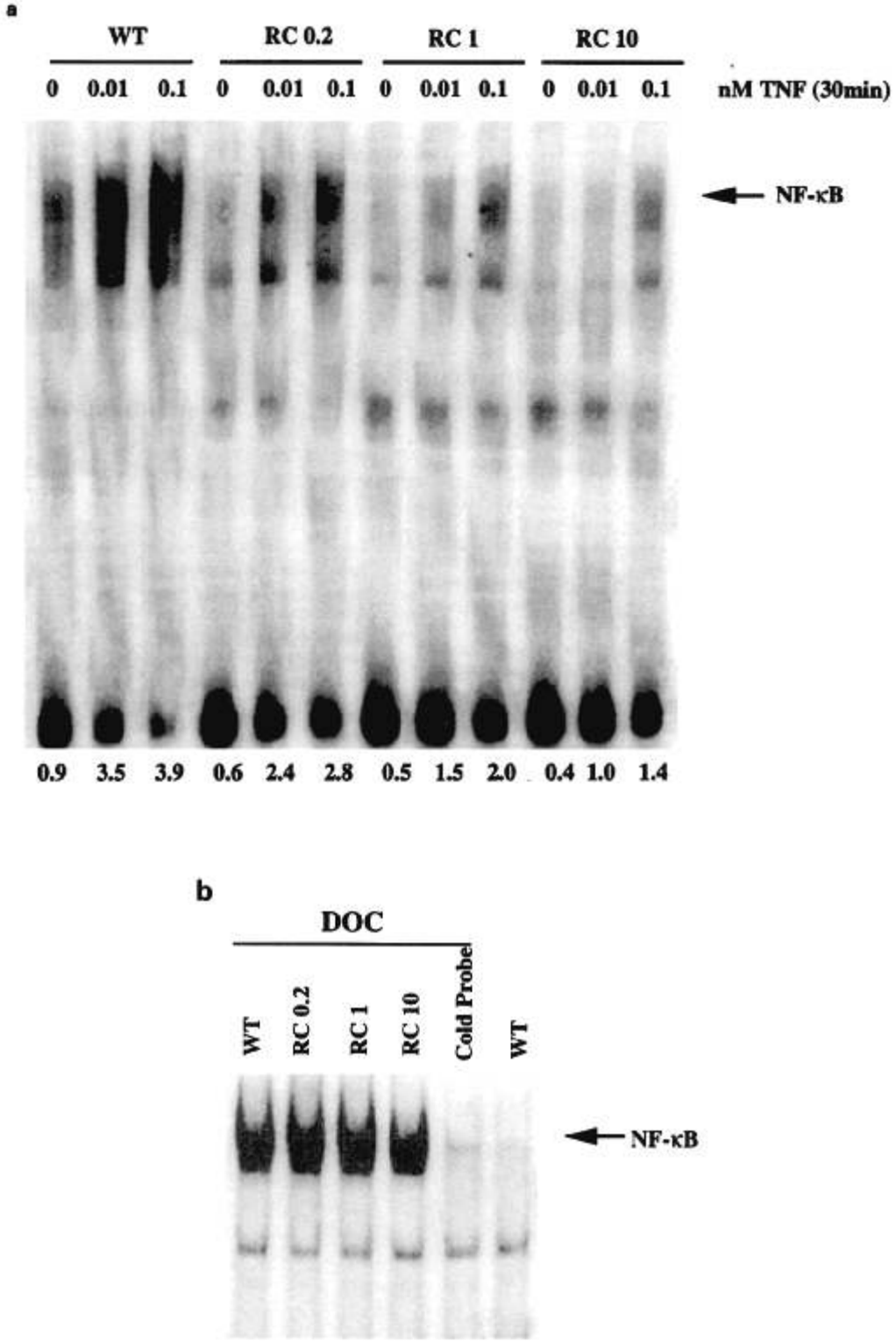

Figure 2. Correlation of 9NC resistance with TNF-dependent NF-κB activation in U-937 cells (a) wild-type (WT), and cells resistant to 9NC concentrations of 0.2 μM (RC-0.2), 1 μM (RC-1) and 10 μM (RC-10) were incubated with either media or 0.01 or 0.1 nM TNF at 37°C for 30 min. After these treatments nuclear extracts were prepared and assayed for NF-κB as described in Experimental procedures. The arbitrary units at the bottom represent the relative amounts of the radioactivity present in the retarded bands. (b) Expression of p50–p65 NF-κB complex in 9NC-sensitive and -resistant U937 cells. Cytoplasmic extracts were treated with 0.8% DOC for 5 min on ice and then assayed for NF-κB as described in Experimental procedures.

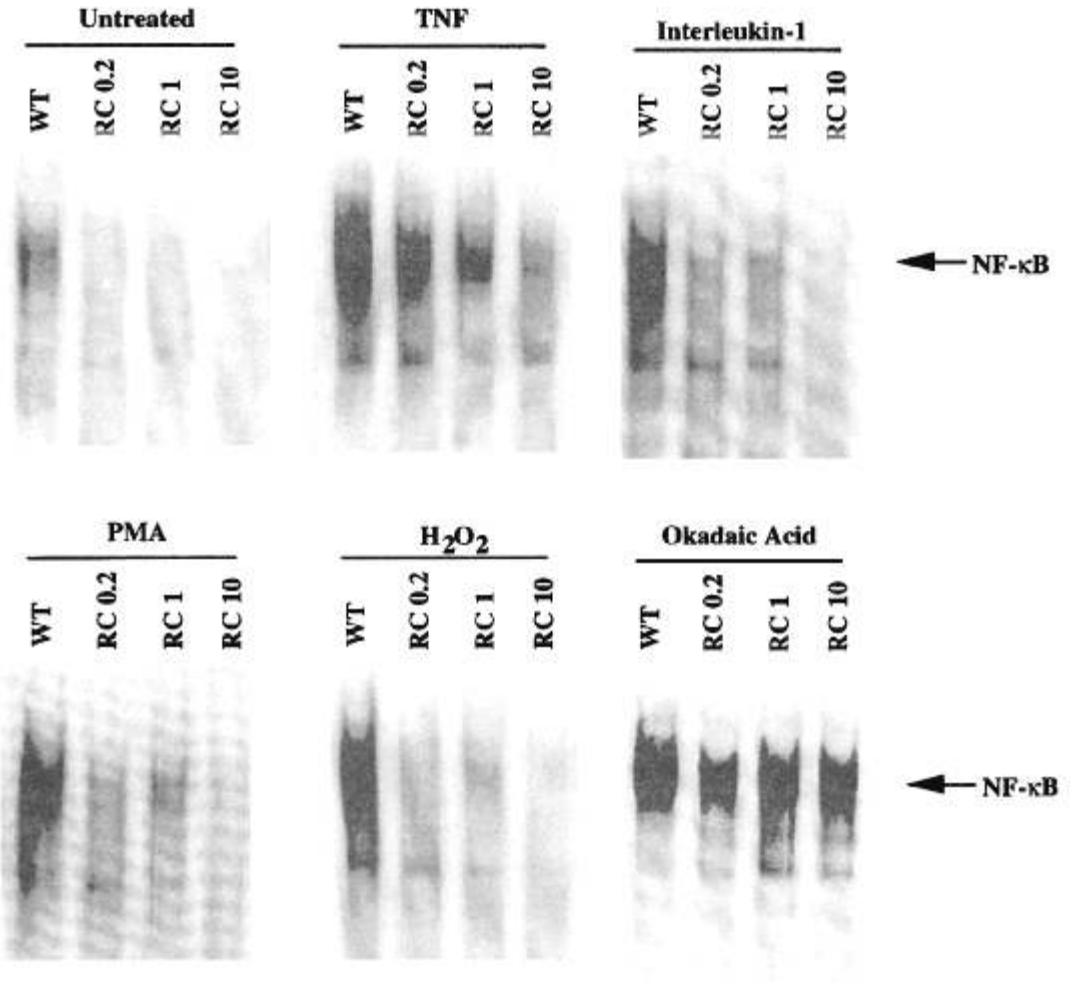

Figure 3. Activation of NF-κB by TNF, PMA, IL-1, H_2O_2 and OA in 9NC-sensitive and -resistant U-937 cells. Cells (2×10^6/ml) were incubated for 30 min at 37 C with TNF (0.1 nM), PMA (25 ng/ml), IL-1 (100 ng/ml), H_2O_2 (50 μM) and OA (600 nM), and then tested for NF-κB activation as described in Experimental procedures.

Oct-1 and AP-1 are not modulated in 9NC-resistant cells

It is possible that 9NC resistance correlates with the downregulation of not only NF-κB activation but also other transcription factors. Therefore, we examined the binding of Oct-1 and AP-1 in 9NC-sensitive and -resistant cells. As Figure 4(a) shows, there was no significant difference in the level of either of these transcription factors between control and 9NC-resistant cells.

It is known that AP-1 can be induced by both TNF and PMA in different cell types, but in contrast to NF-kB activation, it takes several hours of treatment. Therefore, we examined the effect of 9NC-resistance on the induction of AP-1 by TNF and PMA (Figure 4b). Interestingly, 1 h treatment with TNF showed a slight decrease in the levels of AP-1 as compared to the control in both sensitive and resistant cells. After 16 h treatment, however, both TNF and PMA induced AP-1 in 9NC-sensitive cells (WT) and this induction was not significantly different from that observed in 9NC-resistant cells (RC-10). These results thus suggest that 9NC resistance modulates neither consititutive nor inducible levels of AP-1.

IκBα resynthesis is faster in 9NC-resistant cells than sensitive cells

The degradation of IκB is a critical event for activation of NF-κB. We have so far shown that although 9NC-

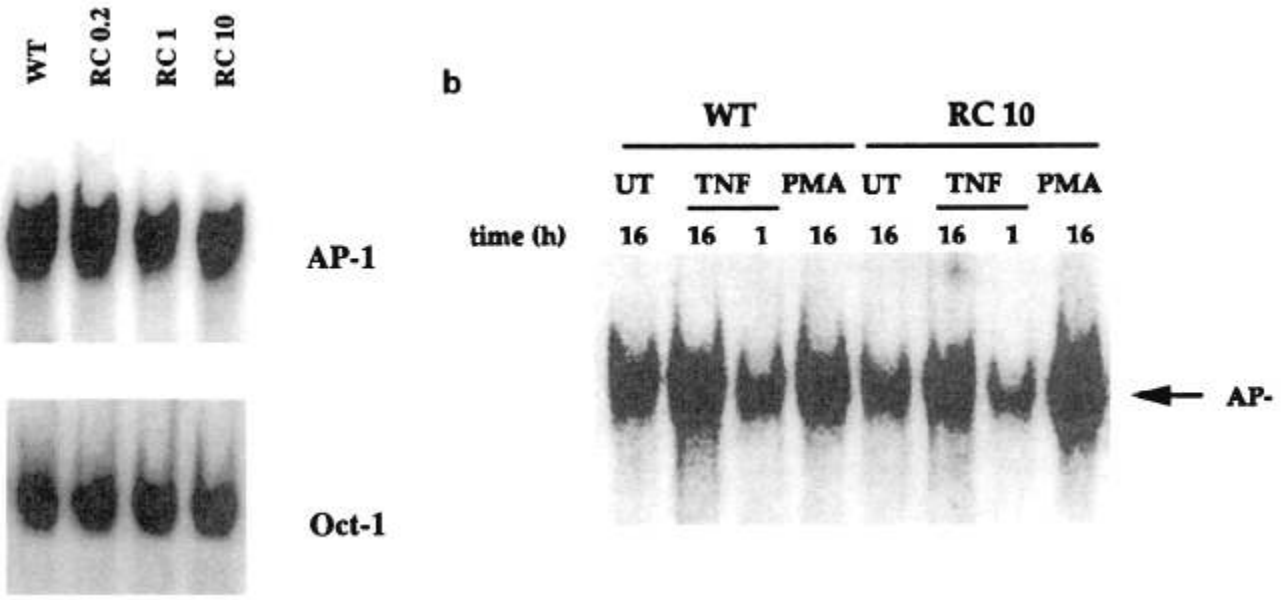

Figure 4. (a) Expression of AP-1 and Oct-1 transcription factors in 9NC-sensitive and -resistant cells. Nuclear extracts were prepared from cells and used for EMSAs of AP-1 and Oct-1 transcription factors as described. (b) Effect of TNF and PMA on the expression of AP-1 transcription factors in 9NC-sensitive and -resistant cells. Nuclear extracts were prepared from cells treated with either TNF (0.1 nM) or PMA (100 ng/ml) for either 1 or 16 h and then used for EMSA of AP-1 transcription factors as described. UT, untreated

resistant cells express NF-κB proteins, the activation of NF-κB is impaired. This impairment could result from either lack of degradation or rapid resynthesis of IκBα protein. To examine these possibilities, the cells were treated with TNF for different times, and then cytoplasmic extracts were prepared and examined for IκBα by Western blot analysis. The results shown in Figure 5 (upper panel) indicate that TNF causes the disappearance of IκBα in both sensitive and resistant cells within 15 min. By 30 min IκBα is resynthesized/reappears, only in 9NC-resistant cells but not in sensitive cells. These results demonstrate that an accelerated rate of resynthesis of IκBα prevents the activation of NF-κB. This mechanism is similar to that recently reported for dexamethasone, which also inhibits NF-κB activation.[31,32] As a control, we confirmed in our system the faster rate of resynthesis of IκBα (Figure 5, upper panel) and inhibition of NF-κB activation (Figure 5, lower panel) induced by dexamethasone.

TNF-mediated cytotoxicity is enhanced in 9NC-resistant cells

The resistance of U-937 cells to 9NC-induced cytotoxicity correlates with their resistance to TNF-induced NF-κB activation. It is not clear, however, whether these cells are also resistant to TNF-dependent cytotoxicity. To examine this, we treated the sensitive and resistant cells with different concentrations of TNF for 72 h and then examined their viability by thymidine incorporation. The results shown in Figure 6 indicate that 9NC-resistant cells were approximately 100 000-fold more responsive to TNF than the sensitive cells and the extent of response correlated with the degree of resistance to 9NC. These results suggest that NF-κB activation may have a role in 9NC-dependent cell killing but not in TNF-mediated cell killing and that the factor or an intermediate responsible for resistance of cells to 9NC may also contribute to the enhanced sensitivity of the cells to TNF.

Enhancement of TNF-mediated cytotoxicity in 9NC-resistant cells is not due to TNF receptors

Since TNF-induced NF-κB activation is downmodulated, whereas TNF-induced cytotoxicity is enhanced in 9NC-resistant cells, it is possible that these effects are mediated through modulation of TNF receptors. To explore this possibility, we examined the cell surface expression of TNF receptors by ligand binding. The

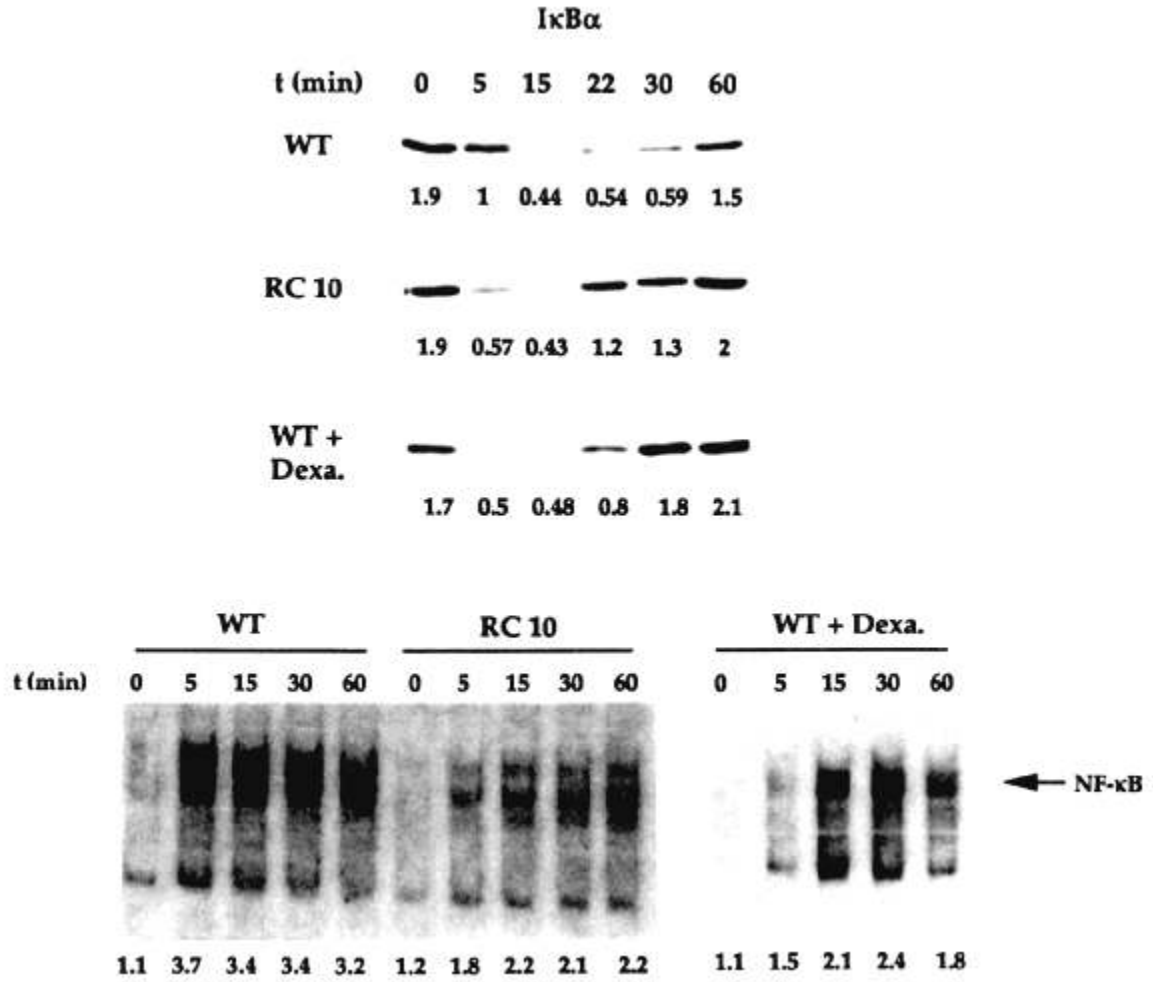

Figure 5. Degradation and resynthesis of IκBα in 9NC-sensitive and -resistant cells. Cells (2×10^6/ml) were incubated for different times with TNF (0.1 nM) and then assayed for IκBα (upper panel) in cytosolic fractions by Western blot analysis as described in Experimental procedures. For the dexamethasone (Dexa) experiment, 9NC-sensitive cells (WT) were pretreated with 2 μM dexamethasone for 1 h at 37 C and then exposed to TNF (0.1 nM) for different times. Nuclear extracts prepared from the same cells were used for NF-κB activation (lower panel) as described in Experimental procedures. Numbers at the bottom show quatitation as arbitrary units.

result of this experiment, shown in Figure 7, indicate that specific binding of TNF is reduced by almost 50% in the 9NC-resistant cells (RC-10) as compared to the 9NC-sensitive (WT) cells and that this decrease is inversely proportional to the level of resistance to the drug. Thus, the decrease in the number of TNF receptors appears to correlate with the decrease in sensitivity of these cells to TNF for NF-κB activation but not with TNF-dependent cytotoxicity.

Discussion

Resistance of tumor cells to chemotherapeutic agents is a major problem in cancer biology. CPT and its analogs are potent anticancer drugs. Continuous exposure of tumor cells to 9NC may lead to acquisition of resistance presumably by qualitative and/or quantitative alterations in Topo I, but other mechanisms exist including factors that regulate the cell cycle and apoptosis. Since the nuclear factor NF-κB controls the expression of several genes involved in apoptosis, we examined the activation of this factor in 9NC-sensitive and -resistant U-937 cells. Our results show that cellular resistance to increasing concentrations of 9NC correlates with the decreased ability of TNF to induce activation of NF-κB without changes in the constitutive synthesis of NF-κB proteins. Other activators of NF-κB, including IL-1, PMA

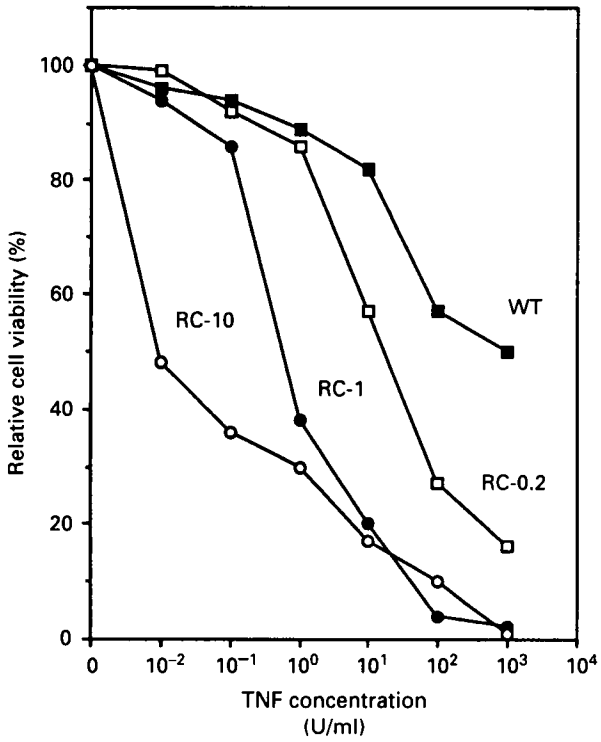

Figure 6. Effect of different concentrations of TNF on the viability of 9NC-sensitive and -resistant cells. Cells (5×10^3) were plated overnight and then incubated with TNF (0–1000 U/ml) in 0.1 ml for 72 h at 37°C. During the last 24 h, the cells were pulse-labeled with thymidine, harvested and counted as described in Experimental procedures. All determinations were in triplicate. The variations between triplicates were 10% or less.

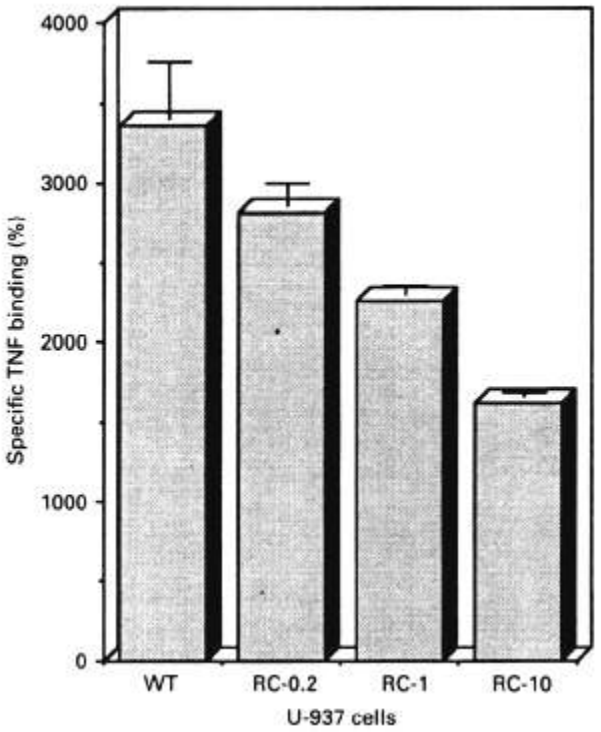

Figure 7. Specific binding of TNF to 9NC-sensitive and -resistant cells. Cells (0.5×10^6) were incubated with labeled TNF (0.2×10^6 c.p.m.) in 0.1 ml volume either in the presence (non-specific binding) or absence (total binding) of 100 nM of unlabeled TNF for 1 h at 4°C. Cells were then washed and the cell bound radioactivity was determined as described in Experimental procedures. All determinations were made in triplicate.

and H_2O_2, also had no affect. There was no difference between the sensitive and resistant cells in the activation of NF-κB by OA or with respect to other transcription factors including AP-1 and Oct-1. The suppression of NF-κB activation correlated with a faster rate of resynthesis of the inhibitory subunit of NF-κB (IκBα) as compared to the control cells. Our results also show that acquisition of 9NC resistance correlated with the decreased ability of TNF to induce NF-κB activation without inhibition of the TNF-mediated cytotoxicity. In fact, the TNF-mediated cytotoxicity was enhanced in spite of a decrease in the number of TNF receptors on these cells.

Our results show that although the basal level of expression of NF-κB proteins is similar between sensitive and resistant cells, there was a difference in the activation of this nuclear factor. Our results differ from a report showing a selective loss of NF-κB DNA binding proteins in chemically selected subclones of the CEM cell line.[33] How cellular resistance to 9NC desensitizes the cells to NF-κB-activating agents is unclear. The activation of NF-κB has been shown to be mediated through reactive oxygen intermediates, serine/threonine protein kinase, protein tyrosine kinase, protein tyrosine phosphatase, ubiquitin-dependent proteases and ceramide (see Miyamoto and Verma[11] and references therein).[28] The signal transduction cascade leading to the activation of NF-κB activation is not understood. Accordingly, the 9NC-resistant cells may be deficient in one or more of these intermediates required for NF-κB activation. Certain chemotherapeutic agents such as 1-β-D-arabinofuranosylcytosine (ara-C) and doxorubicin are known to induce ceramide production required for NF-κB activation and apoptosis.[34,35] Although both ara-C and 9NC can induce differentiation of leukemic cells,[4,6,34] it is not known whether the 9NC activities are also mediated through production of ceramide. It is possible that lack of NF-κB activation is linked to lack of ceramide production in the 9NC-resistant cells.

The 9NC-resistant cells were insensitive to a wide variety of NF-κB-activating agents, but they were sensitive to OA, a specific inhibitor of the serine/threonine protein phosphatase. How OA activates NF-κB is not clear, but the mechanism by which TNF activates NF-κB has been shown to be different from that of OA.[36] Both reactive oxygen intermediate (ROI)-independent and -dependent activations of NF-κB by OA have been reported.[37,38] It is also possible that the resistant cells can still produce ROI but this alone is not sufficient for other activators of NF-κB. However, this possibility is rather unlikely considering that the 9NC-resistant cells were also unresponsive to H_2O_2.

Irrespective of the agent, NF-κB activation requires phosphorylation and ubiquitination that results in degradation of IκBα (for references, see Miyamoto and Verma[11]). Our results show that IκBα is still degraded in 9NC-resistant cells treated with NF-κB activators but the rate of resynthesis of IκB is accelerated. This mechanism is similar to that described for glucocorticoids, which also suppress NF-κB activation by increasing the stability of the mRNA encoding IκBα.[31,32]

The decrease in sensitivity of 9NC-resistant cells to TNF-mediated NF-κB activation correlated with an almost 50% decrease in the number of cell surface TNF receptors. It is unlikely that this decrease in the number of TNF receptors was responsible for the decreased responsiveness to TNF, because only 25% of the total receptors present on U-937 cells are required for full activation of NF-κB.[39] In addition, despite low receptor levels, the TNF-mediated cytotoxicity was enhanced in 9NC-resistant cells as described below.

That resistance to cell killing by 9NC correlates with resistance to NF-κB activation raises the question of to what extent activation of NF-κB is essential for cell killing. The answer appears to depend on the type of agent. For instance, sindbis virus-induced apoptosis of cells requires activation of NF-κB and it is blocked by Bcl-2, an anti-apoptotic protein.[40] Our results show that although TNF-mediated NF-κB activation is abolished in 9NC-resistant cells, TNF-induced cytotoxicity is enhanced by several thousand-fold. These results suggest that NF-κB activation is not required for TNF-mediated cell killing and it is in agreement with a previous report.[41] These results also suggest that the same factor responsible for 9NC-resistance is involved in the increased TNF sensitivity. Unlike some other chemotherapeutic agents, cellular resistance to 9NC or CPT is not associated with overexpression of multidrug resistance (MDR) protein[5] (reviewed in Pantazis[1,2]). Similarly, MDR does not play a role in the susceptibility of cells to TNF.[42] In contrast, however, overexpression of cells with manganese superoxide dismutase (SOD) leads to TNF resistance.[43] The downmodualtion of SOD may increase sensitivity to TNF but decrease the sensitivity to 9NC. Treatment of sensitive U-937 cells with 9NC induces a transient expression of the early response gene c-*jun*.[44] TNF also induces expression of c-*jun* in leukemic cells.[45] The c-*jun* gene is a member of a multigene family of transcription factors (reviewed in Mitchell and Tjian[46]) and is the major component of the transcription factor AP-1.[47,48] It is possible that c-*jun* is differentially expressed in the 9NC-resistant cells and this sensitizes the cells to TNF-mediated cytotoxicity. Our present results show that both 9NC-resistant and -sensitive cells constitutively express comparable levels of c-*jun*/AP-1. Human breast adenocarcinoma MCF-7 cells resistant to adriamycin (doxorubicin) do not exhibit altered c-*jun* expression in response to stress.[49] In contrast to 9NC resistance, adriamycin-resistant MCF-7 cells are resistant to the cytotoxic effects of TNF but sensitive to the TNF-dependent NF-κB activation and both correlate with the overexpression of SOD.[42]

TNF has been shown to synergize with CPT for cytotoxic activity against tumor cells[9,10] and enhancement of Topo I activity by TNF has been suggested as the mechanism of synergism.[50] Our results demonstrate that the 9NC-resistant cells become highly sensitive to TNF. Although the mechanism of sensitization of the 9NC-resistant cells to TNF is unclear, TNF and other cytokines may be useful in combination therapies for the treatment of tumors resistant to 9NC and other Topo I-targeting agents. Our results also provide an important correlation between drug resistance and desensitization for NF-κB activation. As NF-κB activation is critical for HIV replication,[11] our results suggests that 9NC-resistant cells will also be resistant to HIV-1 infection as demonstrated for chemically selected subclones of the CEM cell line which selectively loses NF-κB DNA binding proteins.[33]

References

1. Pantazis, P. Preclinical studies of water-insoluble camptothecin congeners: cytotoxicity, development of resistance and combination treatments. *Clin Cancer Res* 1995; **1**: 1235.
2. Pantazis, P. The water-insoluble camptothecin analogues: promising drugs for the effective treatment of hematological malignancies. *Leuk Res* 1995; **19**: 775–88.
3. Natelson EA, Giovanella BC, Verschraegen CF, *et al.* Phase I clinical and pharmacological studies of 20-(*S*)-camptothecin and 20-(*S*)-9-nitrocamptothecin as anticancer. *Ann NY Acad Sci* 1998; in press.

4. Pantazis P, Mendoza JT, DeJesus A, Rubin E, Kufe D, Giovanella BC. Partial characterization of human leukemia U-937 cell sublines resistant to 9-nitrocamptothecin. *Eur J Hematol* 1994; **53**: 135.
5. Rubin E, Pantazis P, Bharti A, Toppmeyer D, Giovanella B, Kufe D. Identification of a mutant human topoisomerase I with intact catalytic activity and resistance to 9-nitrocamptothecin. *Biol Chem* 1994; **269**: 2433.
6. Pantazis P, Mendoza J, DeJesus A, Early J, Shaw J, Giovanella BC. Development of resistance to 9-nitrocamptothecin by human leukemia U-937 cells *in vitro* correlates with altered sensitivities to several anticancer drugs. *Anti-Cancer Drugs* 1994; **5**: 473.
7. Pantazis P, Vardeman D, Mendoza J, *et al.* Sensitivity of camptothecin-resistant human leukemia cells and tumors to anticancer drugs with diverse mechanisms of action. *Leuk Res* **19**: 43.
8. Nooter, K, Sonneveld P. Clinical relevance of P-glycoprotein expression in hematological malignancies. *Leuk Res* 1994; **18**: 233.
9. Baloch Z, Cohen S, Coffman FD. Synergistic interactions between tumor necrosis factor and inhibitors of DNA topoisomerase I and II. *J Immunol* 1990; **145**: 2908.
10. Verschraegen CF, Natelson EA, Giovanella BC, *et al.* A phase I clinical and pharmacological study of oral 9-nitrocamptothecin, a novel water-insoluble topoisomerase I inhibitor. *Anti-Cancer Drugs* 1998; **9**: 36–44.
11. Miyamoto S, Verma IM. REL/NF-κB/IκB story. *Adv Cancer Res* 1995; **66**: 255.
12. Devary Y, Rosette C, DiDonato JA, Karin M. NF-κB activation by ultraviolet light not dependent on a nuclear signal. *Science* 1993; **261**: 1442.
13. Anisowicz, A, Messineo M, SW Lee, Sager R. An NF-κB-like transcription factor mediates Il-1/TNF-α induction of gro in human fibroblasts. *J Immunol* 1991; **147**: 520.
14. Duyao M P, Buckler AJ, Sonenshein GE. Interaction of an NF-κB-like factor with a site upstream of the *c-myc* promoter. *Proc Natl Acad Sci USA* 1990; **87**: 4727.
15. La Rosa FA, Pierce JW, Sonenshein GE. Differential regulation of the *c-myc* oncogene promoter by the NF-κB Rel family of transcription factors. *Mol Cell Biol* 1994; **14**: 1039.
16. Wu H, Lozano G. NF-κB activation of p53: a potential mechanism for suppressing cell growth in response to stress. *J Biol Chem* 1994; **269**: 20067.
17. Sarma V, Lin Z, Clark L, *et al.* Activation of the B-cell surface receptor CD40 induces A20, a novel zinc finger protein that inhibits apoptosis. *J Biol Chem* 1995; **270**: 12343.
18. Baldwin A S J, Azizkhan JC, Jensen DE, Beg AA, Coodly LR. Induction of NF-κB DNA-binding activity during the G_0-to-G_1 transition in mouse fibroblasts. *Mol Cell Biol* 1991; **11**: 4943.
19. Rosnitzky D, Kimchi A. Deregulated *c-myc* expression abrogates the interferon and interleukin-6 mediated G_0/G_1 cell cycle arrest but not other inhibitory responses in M1 myeloblastic cells. *Cell Growth Different* 1991; **2**: 33–41.
20. Duckett CS, Perkins ND, Leung K, Agranoff AB, Nabel GJ. Cytokine induction of nuclear factor κB in cycling and growth-arrested cells. *J Biol Chem* 1995; **270**: 18836.
21. Schreiber, E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 1989; **17**: 6419.
22. Bradford, MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–54.
23. Nabel G, Baltimore D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* 1987; **326**: 711.
24. Collart M A, Baeuerle P, Vassalli P. Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four κB-like motifs and of constitutive and inducible forms of NF-κB. *Mol Cell Biol* 1990; **10**: 1498.
25. Hassanain HHW, Dai W, Gupta SL. Enhanced gel mobility shift assay for DNA-binding factors. *Anal Biochem* 1993; **213**: 162.
26. Singh H, LeBowitz JH, Baldwin AS, Jr, Sharp PA. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. *Cell* 1988; **52**: 415.
27. Chaturvedi M, LaPushin R, Aggarwal BB. Tumor necrosis factor and lymphotoxin: qualitative and quantitative differences in the mediation of early and late cellular responses. *J Biol Chem* 1994; **269**: 14575.
28. Singh S, Aggarwal BB. Protein tyrosine phosphatase inhibitors block tumor necrosis factor-dependent activation of the nuclear transcription factor NF-κB. *J Biol Chem* 1995; **270**: 10631.
29. Higuchi M, Aggarwal BB. Microtiter plate radioreceptor assay for tumor necrosis factor and its receptors in large numbers of samples. *Anal Biochem* **204**: 53.
30. Higuchi M, Aggarwal BB. Inhibition of ligand binding and antiproliferative effects of tumor necrosis factor and lymphotoxin by soluble forms of recombinant p60 and p80 receptors. *Biochem Biophys Res Commun* 1992; **182**: 638.
31. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin ASJ. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. *Science* **270**: 283.
32. Auphan N, DiDonato JA, Rosette C, Halmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. *Science* 1995; **270**: 286.
33. Qian J, Bours V, Manischewitz J, Blackburn R, Siebenlist U, Golding H. Chemically selected subclones of the CEM cell line demonstrate resistance to HIV-1 infection resulting from a selective loss of NF-κB DNA binding proteins. *J Immunol* 1994; **152**: 4183.
34. Strum JC, Small GW, Pauig SB, Daniel LW. 1-β-D-arabinofuranosylcytosine stimulates ceramide and diglyceride formation in HL-60 cells. *J Biol Chem* 1994; **269**: 15493.
35. Bose R, Verheij M, Haimovitz-Friedman A, Scotto K, Fuks Z, Kolesnick R. Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. *Cell* 1995; **82**: 405.
36. Sun S, Maggirwar SB, Haraj. Activation of NF-κB by phosphatase inhibitors involves the phosphorylation of IκBα at phosphatase 2A-sensitive sites. *J Biol Chem* 1995; **270**: 18347.
37. Suzuki, YJ, Mizuno M, Packer L. Signal transduction for nuclear factor-κB activation: proposed location of anti-oxidants-inducible step. *J Immunol* 1994; **153**: 5008.

38. Schmidt KN, Traenckner EB-M, Meier B, Baeuerle PA. Induction of oxidative stress by okadaic acid is required for activation of transcription factor NF-κB. *J Biol Chem* 1995; **270**: 27136.
39. Chan H, Aggarwal BB. Role of tumor necrosis factor receptors in the activation of nuclear factor κB in human histiocytic lymphoma U-937 cells. *J Biol Chem* 1994; **269**: 31424.
40. Lin K-I, Lee S-H, Narayanan R, Baraban JM, Hardwick JM, Ratan RR. Thiol agents and Bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B. *J Cell Biol* 1995; **131**: 1149.
41. Chaturvedi M, Higuchi M, Aggarwal BB. Effect of tumor necrosis factors, interferons, interleukins and growth factors on the activation of NF-κB: evidence for lack of correlation with cell proliferation. *Lymphokine Cytokine Res* 1994; **13**: 309.
42. Zyad A, Benard J, Tursz T, Clarke R, Chouaib S. Resistance to TNF-α and adriamycin in the human breast cancer MCF-7 cell line: relationship to MDR1, MnSOD and TNF expression. *Cancer Res* **54**: 825.
43. Wong GH, Goeddel DV. Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism, *Science* 1988; **242**: 941.
44. Kharbanda S, Rubin E, Gunji H, *et al.* Camptothecin and its derivatives induce expression of the *c-jun* protooncogene in human myeloid leukemia cells. *Cancer Res* 1991; **51**: 6636.
45. Dixit V M, Marks RM, Sarma V, Prochownik EV. The antimitogenic action of tumor necrosis factor is associated with increased AP-1/*c-jun* proto-oncogene transcription. *J Biol Chem* 1989; **264**: 16905.
46. Mitchell PJ, Tjian R. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 1989; **245**: 371.
47. Bohmann D, Bos TJ, Admon A, Nishimura T, Bogt PK, Tjian R. Human protooncogene *c-jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* **238**: 1386.
48. Anyel P, Allegretto EA, Okino ST, *et al.* Oncogene *jun* encodes a sequence specific transactivator similar to AP-1. *Nature* 1988; **332**: 166.
49. Lee YJ, Galaoforo SS, Berns CM, *et al* Effect of ionizing radiation on AP-1 binding activity and basic fibroblast growth factor gene expression in drug-sensitive human breast carcinoma MCF-7 and multidrug-resistant MCF-7/ADR cells. *J Biol Chem* 1995; **270**: 28790.
50. Utsugi T, Mattern MR, Mirabelli CK, Hanna N. Potentiation of topoisomerase inhibitor-induced DNA strand breakage and cytotoxicity by tumor necrosis factor: enhancement of topoisomerase activity as a mechanism of potentiation. *Cancer Res* 1990; **50**: 2636.

(Received 26 June 1998; accepted 2 July 1998)