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Induction of oncogene addiction shift to NF-κB by camptothecin in solid tumor cells

Tomiteru Togano [a,1], Masataka Sasaki [a,1], Mariko Watanabe [a], Makoto Nakashima [a], Takashi Tsuruo [b], Kazuo Umezawa [c], Masaaki Higashihara [a], Toshiki Watanabe [d], Ryouichi Horie [a,*]

[a] Department of Hematology, School of Medicine, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan
[b] Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan
[c] Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-0061, Japan
[d] Laboratory of Tumor Cell Biology, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

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ABSTRACT

The biological basis of the resistance of solid tumor cells to chemotherapy is not well understood. While addressing this problem, we found that gastric cancer cell line St-4/CPT, lung cancer cell line A549/CPT, and colon cancer cell line HT-29/CPT, all of which are resistant to camptothecin (CPT), showed strong and constitutive nuclear factor (NF)-κB activity driven by IκB kinase compared with their parental cell lines St-4, A549, and HT-29. A new NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), reduced viability and induced apoptosis in St-4/CPT, A549/CPT, and HT-29/CPT cell lines, while their parental cell lines were resistant to DHMEQ. The results in this study present an example of the shift in signals that support the survival of solid tumor cells to NF-κB during the acquisition of resistance to CPT. The results also indicate that solid tumor cells that become resistant to chemotherapy may be more easily treated by NF-κB inhibitors.

Introduction

Multi-modal chemotherapy is widely used for the treatment of cancers. This strategy targets the basic mechanisms of cell survival involved in DNA replication and the division of cells [1]. However, it has become obvious that this strategy faces limitations caused by toxic effects on normal cells, especially for the treatment of solid tumors. Therefore, the establishment of new therapeutic strategies is needed.

Normal cells transform into cancer cells after multi-step tumorigenic events caused by the deregulation of oncogenes and tumor suppressor genes. Therefore, cancer cells are thought to bear multiple deregulations that support their survival. However, a recent study has indicated that the survival of cancer cells is dependent on a small number of deregulated signals [2]. This phenomenon, called "oncogene addiction," provides a rationale for molecular targeted therapy [3].

This strategy targets molecules involving oncogene addiction and intensifies the specificity of the treatment, thereby minimizing undesirable toxicity to normal cells. Recently, molecular targeted therapy has begun to be widely used in clinical practice and there are several concrete examples of outstanding success in the treatment of leukemia bearing particular cytogenetic abnormalities [4–6]. To develop a molecular targeted strategy, it appears important to identify the signals to which cancer cells are addicted for survival. Particular cytogenetic abnormalities or somatic initiating mutations are frequently reported in hematopoietic tumors, especially in leukemia and lymphoma [7,8]. On the other hand, recent cancer genome sequencing studies failed to find prevalently mutated genes in solid tumors including colorectal, breast, pancreatic and lung cancers [9–12], indicating that the status of oncogene addiction in solid tumors appears to be more complicated than that in hematopoietic tumors.

NF-κB represents a family of inducible transcription factors composed of a variety of homo- or heterodimers formed by five components: c-Rel, p65 (RelA), RelB, p50, and p52 subunits. NF-κB exists as an inactive complex with IκB regulatory proteins in the cytoplasm. Various signaling pathways converge into IκB kinase (IKK)-mediated degradation of IκB proteins and the subsequent release of uncomplexed NF-κB, which then migrates into the nucleus, binds to the κB enhancer motif in the promoter region of target genes, and finally induces transcription. c-Rel, p65, and p50 are involved in the classical (canonical) pathway activated by IKK complex consisting of IKKα, IKKβ, and their regulatory subunit IKKγ (NEMO), whereas RelB and p52 are involved in an alternative (non-canonical) pathway activated by IKKα [13].

* Corresponding author. Fax: +81 42 778 8441.
E-mail address: rhorie@med.kitasato-u.ac.jp (R. Horie).
[1] These authors contributed equally to this work.

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Activation of NF-κB has been connected with resistance against apoptosis and tumorigenesis [14]. NF-κB activation has also been connected with the resistance of malignant cells to chemotherapies [15,16].

In this study we focused on solid tumors that become resistant to chemotherapeutic agents and tried to find a prevalent addiction signal among them. We report the shift in survival basis of solid tumor cells to NF-κB after acquirement of resistance against a chemotherapeutic agent, topoisomerase inhibitor camptothecin (CPT) in lung, stomach, and colon cancer cell lines and discuss the significance of applying this finding to clinical medicine.

Materials and methods

Cells. Human solid tumor cell lines resistant to CPT (gastric cancer cell line St-4/CPT; lung cancer cell line A549/CPT; and colon cancer cell line HT-29/CPT) and their parental cell lines (St-4, A549, and HT-29) used in this study have been described in a previous paper [17]. Resistance of St-4/CPT, A549/CPT, and HT-29/CPT to CPT compared to their parental cell lines was confirmed prior to the study. These cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum with antibiotics.

Chemicals. Dehydroxymethylepoxyquinomicin (DHMEQ) is an NF-κB inhibitor that directly binds to NF-κB and blocks nuclear localization of NF-κB [18,19]. DHMEQ was dissolved with dimethylsulfoxide (DMSO). DHMEQ or DMSO was used for experiments at indicated concentrations as was used in a previous report [20]. Bisbenzimide H 33342 fluorochrome (Hoechst 33342) was purchased from Calbiochem (Bad Soden, Germany).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was carried out according to the methods described previously [20]. For detecting NF-κB binding, a double-stranded oligonucleotide containing the κB site of the promoter for the mouse H-2Kb class I major histocompatibility antigen gene was used as a probe. The nucleotide sequence is 5′-GAT CCG GCT GGG AAT CCC CGC TGG GAA TCC CCA TCT A-3′. In the control EMSA, a double-strand oligonucleotide containing the Oct-1 consensus sequence (Promega, Madison, WI, USA) was used as a probe. Antibodies used for super-shift assays were as follows: goat polyclonal antibody for NF-κB p50 (C-19), rabbit polyclonal antibody for NF-κB p65 (RelA) (C-20) and RelB (C-19), and mouse monoclonal antibody for both c-Rel (B-6) and NF-κB p52 (C-5) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Mouse IgG antibody (Sigma, St. Louis, MO, USA) served as a control.

Immunoblot analysis. Immunoblot analysis was performed as previously described [21]. Antibodies used were as follows: rabbit monoclonal antibody for ataxia telangiectasia mutated (ATM) and phospho (pS1981) ATM (both from Epitomics, Inc., Burlingame, CA, USA), anti-IKKα/β (H-470) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.), phospho-IKKα/β (Ser176/180)(16A6) rabbit monoclonal antibody (Cell Signaling Technology Inc., Danvers, MA, USA), and anti-α tubulin (TU-02) mouse monoclonal IgM antibody (Santa Cruz Biotechnology, Inc.). Alkaline phosphase-conjugated secondary antibodies used were donkey anti-rabbit IgG antibody (Chemicon International Inc., Temecula, CA, USA) and goat anti-mouse IgM antibody (Santa Cruz Biotechnology, Inc.).

Cell viability assay. The effects of DHMEQ on cell viability were assayed by color reaction with the tetrazolium salt WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-[4-nitrophenyl]-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt) (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). After incubation with DHMEQ or DMSO at the indicated concentrations and time points, cells were treated with the tetrazolium salt according to the manufacturer's recommendations and the results were measured by a microplate reader (Bio-Rad, Richmond, CA, USA) at a test wavelength of 450 nm and a reference wavelength of 630 nm.

Analysis of apoptosis and caspase activity. To quantify apoptosis, cells were labeled with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Biosciences, Palo Alto, CA, USA) and then subjected to flow cytometric analysis. Cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences) and fluorescence microscopy. Activity of caspase-3 was determined by using green fluorochrome-labeled inhibitors of caspases (FLICA)-3/7 (FLICA Apoptosis Detection Kit; Immunochemistry Technologies, LLC, Bloomington, MN, USA). For detection of nuclear DNA, cells were stained with Hoechst 33342 and photographed through a UV filter and an microscope (BX50F; Olympus, Tokyo, Japan).

Statistical analysis. Differences between mean values were assessed by two-tailed *t*-test. A *p*-value <0.05 was considered to be statistically significant.

Results and discussion

Induction of constitutive NF-κB activity in St-4/CPT and A549/CPT cell lines

NF-κB activation has been connected with the resistance of cancer cells to chemotherapy [15,16]. Treatments with topoisomerase inhibitors like CPT have been reported to induce transient NF-κB activation via IκB kinase (IKK), which confers solid tumor cells resistance to apoptosis [22]. Therefore, we first examined the status of NF-κB activity in St-4/CPT and A549/CPT cell lines, both of which are resistant to CPT, and their parental cell lines St-4 and A549 by EMSA analysis. The St-4/CPT and A549/CPT cell lines showed strong and constitutive NF-κB binding activity compared with the St-4 and A549 cell lines, although their migration patterns were different (Fig. 1A). This was also confirmed by immunostaining with antibody for activated NF-κB p65 (Chemicon International Inc.) and their staining pattern was generally homogenous (data not shown).

Electrophoretic mobility shift assay components of NF-κB that are constitutively activated in St-4/CPT and A549/CPT cell lines were analyzed by super-shift assays. The results revealed that the NF-κB constitutively activated in these cell lines consisted of p50, p65, and c-Rel, which indicates activation of the classical pathway (Fig. 1B). Our results show that NF-κB induction by CPT not only emerges as transient but also as a constitutive mechanism. NF-κB constitutively activated in CPT-resistant cells consisted of p50, p65, and c-Rel, which is in accordance with components previously reported in the transient induction of NF-κB by CPT [23].

The mechanism of constitutive NF-κB activity in St-4/CPT, A549/CPT, and HT-29/CPT cell lines

Because constitutive induction of NF-κB activity accompanied with CPT resistance in solid tumor cells has not yet been reported, we tried to elucidate the mechanism of this finding. Previous reports indicated that transient induction of NF-κB activity by CPT is mediated by ataxia terangiectasia mutated (ATM) and IKKγ. ATM, a member of phosphoinositide 3-kinase related protein kinases (PIKKs), is involved in genome stability, cellular responses to DNA damage, and cell cycle control [24]. In response to DNA damage, ATM phosphorylated at serine 1981 and its activated forms complex with IKKγ, resulting in the phosphorylation of IKKα and IKKβ, which causes activation of the classical NF-κB pathway [25–27].

Based on these backgrounds, we examined the status of activation in the ATM-IKK pathway in CPT-resistant cell lines and their parental cell lines. Because we found that colon cancer cell line HT-29/CPT also revealed strong and constitutive NF-κB activity

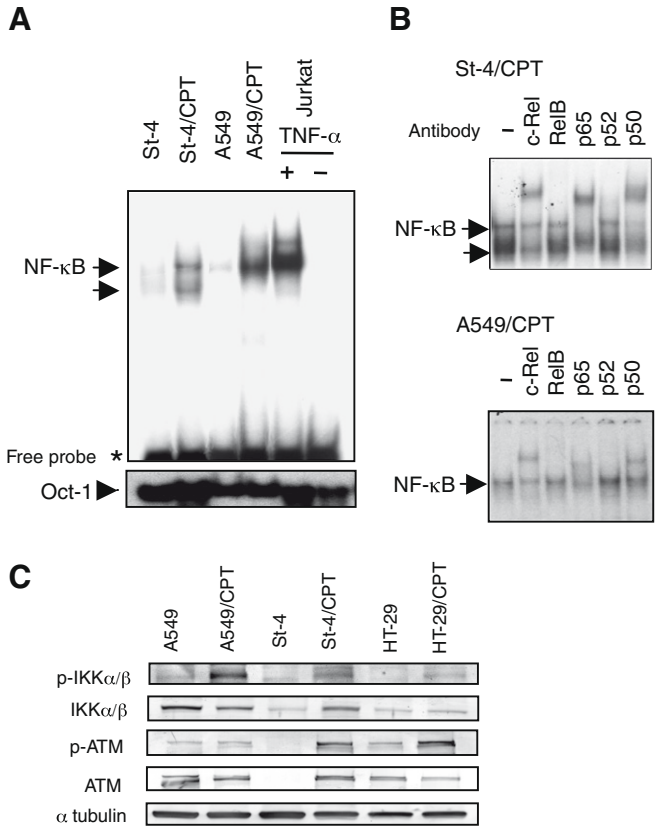

Fig. 1. Induction of constitutive NF-κB activity in camptothecin-resistant cell lines and its mechanism. (A) Strong and constitutive NF-κB activity in St-4/CPT and A549/CPT cell lines resistant to camptothecin. Nuclear extracts (2 μg) were examined for NF-κB binding activity by electrophoretic mobility shift assay with a radio-labeled NF-κB-specific probe. Jurkat cells treated with or without 10 ng/ml of tumor necrosis factor (TNF)-α served as a control. The arrows on the upper panel indicate bands corresponding to NF-κB binding activity. The arrowhead on the lower panel indicates binding activity for the control probe, Oct-1. The position of free probe is indicated by an asterisk. (B) NF-κB subcomponent analysis. Subcomponents of NF-κB constitutively activated in St-4/CPT and A549/CPT cell lines resistant to camptothecin were determined by super-shift analysis. Nuclear extracts (2 μg) were subjected to super-shift analysis with antibodies specific for c-Rel, p50, RelB, p52, and p65 or control antibody (–). Cell lines used are indicated on the top. (C) Expression and phosphorylation of IKKα/β and ATM. Immunoblot analysis was performed to detect the expression and phosphorylation in A549/CPT, St-4/CPT, and HT-29/CPT, and their parental cell lines. Whole cell lysates (25 μg) were subjected to the analysis. The expression of α tubulin served as an internal control. Antibodies used are shown on the left; cell lines used are shown on the top. p-IKKα/β, phosphorylated IκB kinase α/β; p-ATM, phosphorylated ataxia terangiectasia mutated.

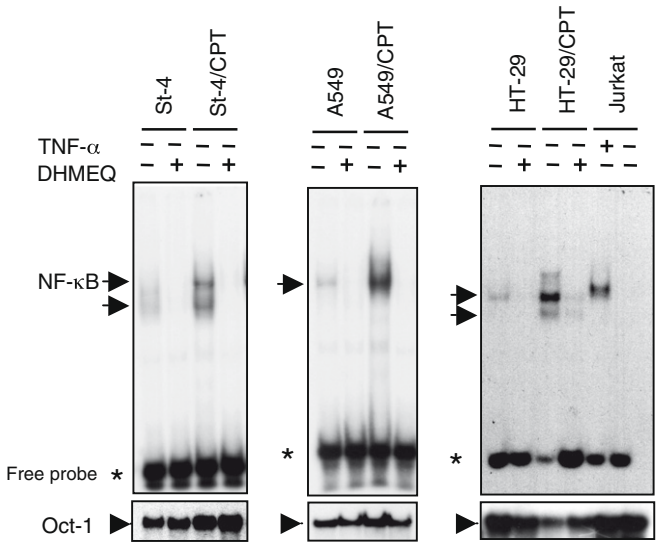

Fig. 2. Inhibition of constitutive NF-κB activity by DHMEQ in camptothecin-resistant cell lines. St-4/CPT, A549/CPT, and HT-29/CPT cell lines and their parental cell lines St-4, A549, and HT-29 were treated with 10 μg/ml of DHMEQ (+) or DMSO alone (–) for 7 h. Nuclear extracts (2 μg) were examined for NF-κB binding activity by electrophoretic mobility shift assay with a radio-labeled NF-κB-specific probe. The results of St-4/CPT, A549/CPT, and HT-29/CPT cell lines and their parental cell lines are presented. The Jurkat cell line treated with or without 10 ng/ml of TNF-α served as a control and is included in the results with HT-29 and HT-29/CPT cell lines. The arrows on the upper panel indicate bands corresponding to NF-κB binding activity. The arrowheads on the lower panel indicate binding activity for the control probe, Oct-1. The positions of free probe are indicated by asterisks.

consisting of p50, p65, and c-Rel compared with its parental cell line HT-29 (NF-κB activity in HT-29/CPT cells shown in Fig. 2 and other data not shown), we performed the experiments using St-4/CPT, A549/CPT, and HT-29/CPT and their parental cell lines. Analysis of the phosphorylation status of IKKα/β showed increased phosphorylation of IKKα/β in these three CPT-resistant cell lines compared with their parental cell lines, indicating that IKKα/β is responsible for the constitutive activation of NF-κB (Fig. 1C). Analysis of the expression and phosphorylation of ATM revealed the induction of ATM and its phosphorylation in St-4/CPT cells and increase of phosphorylation alone in HT-29/CPT cells, whereas no significant changes in the expression of ATM and its phosphorylation in A549/CPT cells were found (Fig. 1C).

These results indicate that the constitutive activation of IKK is a common mechanism triggering constitutive NF-κB activity in CPT-resistant cells. Because we could show activation of ATM in two cell lines, St-4/CPT and HT-29/CPT, other PIKKs, ATM- and Rad-3-related (ATR) kinase, DNA-dependent protein kinases (DNA-PKcs) [24] or other unknown factors might be involved in constitutive IKK activation in A549/CPT cells. Moreover, the parental cell lines were treated by CPT again and again during the acquisition of CPT resistance [17], indicating that repetitive activation of the PIK-Ks-IKK-NF-κB pathway caused its deregulation, and was therefore independent of CPT stimulation.

DHMEQ efficiently blocks constitutive NF-κB activity in St-4/CPT, A549/CPT, and HT-29/CPT cell lines

We next examined the effects of the NF-κB inhibitor DHMEQ against constitutive NF-κB activity in the three cell lines St-4/CPT, A549/CPT, and HT-29/CPT. Treatment of St-4/CPT, A549/CPT, and HT-29/CPT cells with DHMEQ almost completely abrogated strong and constitutive NF-κB activity in these cell lines (Fig. 2).

We recently reported that DHMEQ directly targets NF-κB and that its inhibitory effect is more potent on p65, c-Rel, and RelB than on p50 and p52. Almost complete abrogation of NF-κB in CPT-resistant cell lines by DHMEQ indicates that the strong and constitutive NF-κB activity consisting of p65, c-Rel, and p50 in these cell lines does not contain the p50 homodimer, which might interfere with the complex containing p65 and c-Rel as an inactive form of NF-κB [13].

DHMEQ induces apoptosis in St-4/CPT, A549/CPT, and HT-29/CPT cell lines, but not in their parental cell lines

To study the significance of the strong and constitutive NF-κB activity in the growth of CPT-resistant cell lines, we examined the effects of DHMEQ on cell viability. Results showed that DHMEQ treatment reduced the cell viability of St-4/CPT, A549/CPT, and HT-29/CPT cell lines in a dose-dependent manner, but did not reduce the cell viability of their parental cell lines St-4, A549, and HT-29 (Fig. 3, left panels). Fragmentation of the nuclei of CPT-resistant cell lines was clearly demonstrated 48 h after DHMEQ treatment, by Hoechst 33342 staining (Fig. 3, right panels).

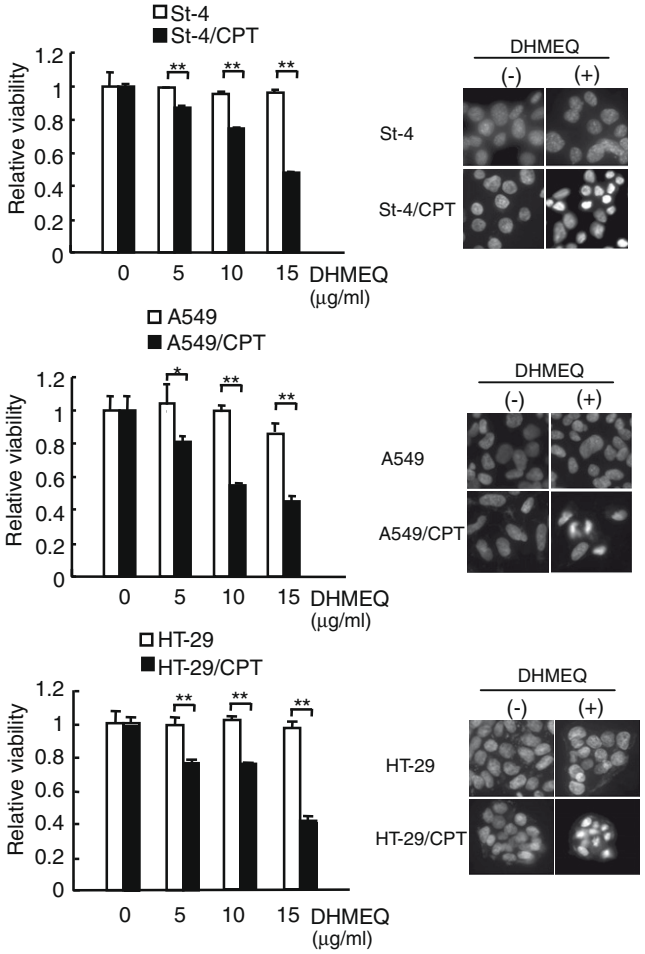

Fig. 3. Reduction of viability and induction of nuclear fragmentation by NF-κB inhibition in camptothecin-resistant cell lines. Dose-dependent reduction of cell viabilities of St-4/CPT, A549/CPT, and HT-29/CPT cell lines resistant to camptothecin and their parental cell lines St-4, A549, and HT-29 by DHMEQ. These cell lines were treated with the indicated concentrations of DHMEQ or DMSO alone for 48 h. The viability of the cells was determined by WST-8 assay and the relative levels compared with those of DMSO-treated cells are presented. Data represent the mean and standard deviation of triplicate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. Cell lines used are indicated on the top of each graph. Nuclear fragmentation in cell lines resistant for camptothecin treated with 20 μg/ml of DHMEQ (+) or DMSO alone (−) for 48 h was detected by staining with 10 μM Hoechst 33342, as described in the text, and is presented on the right.

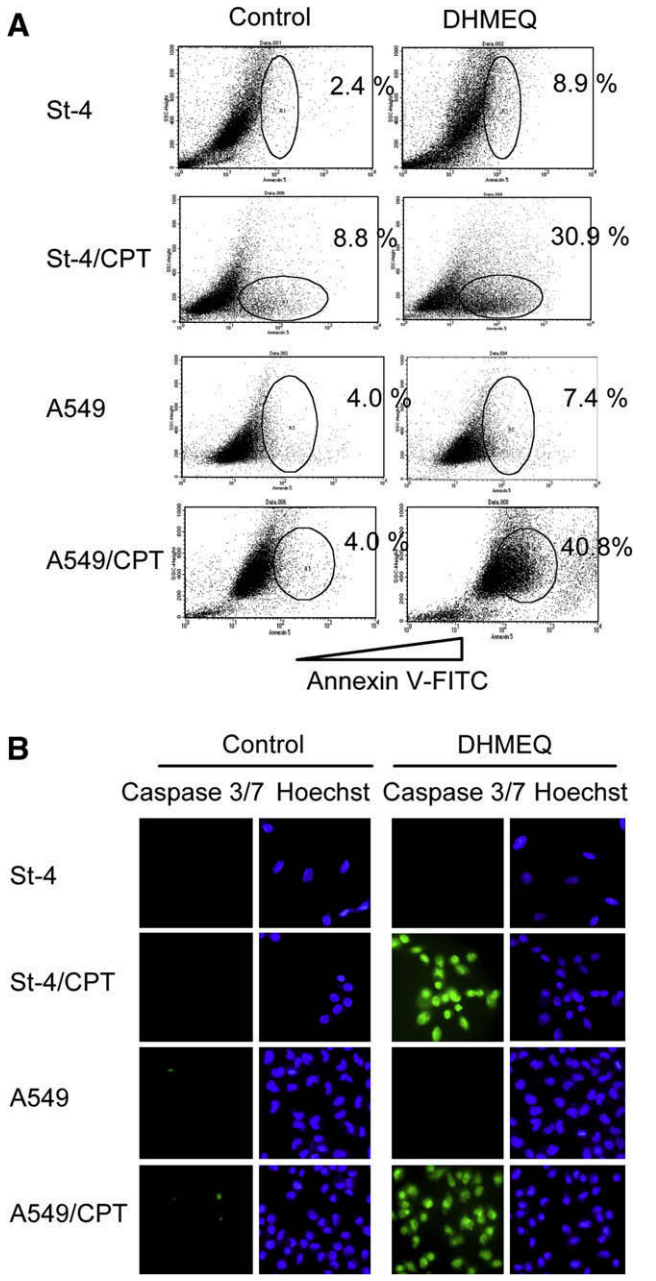

Fig. 4. Induction of Annexin V positivity and activation of caspase-3/7 by NF-κB inhibition in camptothecin-resistant cell lines. (A) Flow cytometric analysis of Annexin V-reactive cells. St-4/CPT, A549/CPT, and HT-29/CPT cell lines resistant to camptothecin and their parental cell lines St-4, A549, and HT-29 were treated with 20 μg/ml of DHMEQ or DMSO alone (control) for 48 h. After labeling with FITC-conjugated Annexin V, cells were analyzed by flow cytometry. Representative results with St-4/CPT and A549/CPT cell lines and their parental cell lines St-4 and A549 are presented. The percentage of cells positive for Annexin V is indicated in each graph. The horizontal axis indicates FITC, while the vertical axis represents side scatter characteristics. Data shown are representative of three independent experiments. (B) St-4/CPT, A549/CPT, and HT-29/CPT cell lines resistant to camptothecin and their parental cell lines St-4, A549, and HT-29 were treated with 10 μg/ml of DHMEQ or DMSO alone (control) for 8 h and fixed on slides. Caspase-3/7 activities in these cells after DHMEQ treatment were detected by green fluorochrome-labeled inhibitors of caspases (FLICA)-3/7 and nuclear DNA was stained with Hoechst 33342. The results were observed by microscopy as described in the Materials and Methods section. Representative results with St-4/CPT and A549/CPT cell lines and their parental cell lines St-4 and A549 are presented. Jurkat cells treated with or without 10 ng/ml of TNF-α served as a control (data not shown).

We next examined whether DHMEQ induces apoptosis in the St-4/CPT, A549/CPT, and HT-29/CPT cell lines by analyzing Annexin V reactivity and the caspase pathway. Flow cytometric analysis showed a significant increase in the number of Annexin V-positive cells after DHMEQ treatment compared with their parental cell lines. Representative results with St-4/CPT and A549/CPT along with their parental cell lines St-4 and A549 are shown (Fig. 4A). We also showed the cleavage of caspase-3/7, confirming that DHMEQ-induced apoptosis in CPT-resistant cells is associated with activation of the caspase pathway. Representative results with St-4/CPT and A549/CPT cell lines and their parental cell lines St-4 and A549 are shown (Fig. 4B).

Significance of oncogene addiction shift and its implication for clinical translation

The results presented in this study clearly indicate a change of major signaling pathways, in which solid tumor cells become "addicted" to NF-κB during the acquisition of resistance against CPT, although the addicted status of the parental cell lines might be complex and unknown. Our results, which indicate that induc-

tion of the constitutive NF-κB activity in cells resistant to CPT is a consequence of induction of the PIKKs-IKK pathway over and over by repetitive CPT treatment, support the above hypothesis.

Currently, molecular targeting strategy is widely used for the treatment of solid tumors. However, compared with hematopoietic tumors like chronic myelogenous leukemia or acute promyelocytic leukemia, most solid tumors are still resistant [4,5]. This difference might be caused by the complex nature of addiction status in solid tumors [9–12]. Most chemotherapeutic regimens currently used for solid tumors contain agents such as topoisomerase inhibitors like CPT used in this study or agents that cause DNA damage and transient NF-κB induction. Therefore, our results indicate that the shift of oncogene addiction to NF-κB may take place in clinical practice and that NF-κB is a molecular target of solid tumors resistant to chemotherapy.

Our results indicate that solid tumor cells that become resistant to chemotherapy may be more easily treated by NF-κB inhibitors. The results also suggest a potential treatment by "trapping" solid tumor cells to the "targetable" signal by an artificial addiction shift. A new NF-κB inhibitor DHMEQ, which selectively targets p65 and c-Rel, but not p50 may be one potential candidate to target "trapped" solid tumor cells.

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