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(-)-Menthol Inhibits DNA Topoisomerases I, II α and β and Promotes NF-Î B expression in Human Gastric Cancer SNU-5 Cells

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Abstract. *It has been reported that (-)-Menthol can inhibit the growth of rat liver epithelial tumor cells and is a potent chemopreventive agent. The purpose of the present experiment was to examine and identify cellular processes leading to cell death which are affected by (-)-Menthol in human gastric SNU-5 cancer cells. Cell death (cytotoxicity) was examined and analyzed by trypan blue stain and flow cytometric methods. It was shown that (-)-Menthol inhibited the proliferation of the cells in a dose- and time-dependent manner, inhibited topoisomerase I , IIα and IIβ, but promoted the levels of NF-ÎB gene expression based on the Western blot and polymerase chain reaction (PCR) and cDNA microarray methods. These data suggest that (-)-Menthol may induce cytotoxicity through inhibiting gene expression of topoisomerase I, IIα and IIβ and promoting the gene expression of NF-ÎB in SNU-5 cells.*

Most approaches to cancer chemotherapy have focused on the idea that cytotoxic drugs can be used to eradicate proliferating neoplastic cells. Cytotoxicity is associated with the presence of drug-induced damage to the genetic material, and DNA has served admirably as a primary focus for drug development. Other cellular targets should also be vulnerable, however, and, over the past decade, the plasma membrane in particular has received considerable attention as a therapeutic locus. In the past, there were only vague notions as to how membrane disruption could lead to cell death. Cell-surface signal transduction and growth control pathways represent new targets for the rational development of new cancer therapies.

(-)-Menthol ([1-α]-5-Methyl-2-[1-methylethyl]-cyclohexanol), is an aromatic compound, and is an important constituent of four essential oils, namely eucalyptus, lemongrass, palmarosa and peppermint. It comes from the *Mentha haplocalyx* Briq, *Mentha haplocalyx* Briq. Var. *piperascens* and *Mentha piperita* L. and has been reported to contain antibacterial and antifungal activity (1, 2). It has been demonstrated that (-)-Menthol can inhibit the growth of rat liver epithelial tumors cells (3), and can act as a potent chemopreventive agent during DMBA initiation of rat mammary tumors (4). Our laboratory has demonstrated that (-)-Menthol inhibits N-acetyltransferase activity in human hepatoma cells (5) and the distribution and metabolism of 2-aminofluorene in various tissues of Sprague-Dawley rats (6). However, there is no available information on the effect of (-)-Menthol on gastric cancer cells. The observed correlation between cytotoxicity and topoisomerase I inhibition strongly suggests that topoisomerase I-mediated DNA cleavage assays can be used as a guide to the development of superior analogues in this series.

In this study, SNU-5 human gastric cancer cells were treated with (-)-Menthol and analyzed for the correlation between cytotoxicity and topoisomerases. We also investigated the effect of (-)-Menthol on cell death associated with different levels of topoisomerases. Our results demonstrated that (-)-Menthol can induced cell death and affect topoisomerase I.

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Materials and Methods

Chemicals and reagents. (-)-Menthol, trypan blue, Tris-HCl, ribonuclease-A, triton X-100 and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); potassium phosphates, dimethyl sulfoxide (DMSO) and TE buffer were purchased from Merck Co. (Darmstadt, Germany); 80%

Iscove's modified Dulbecco's medium, glutamine, fetal bovine serum (FBS) and penicillin-streptomycin, trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Human gastric cancer cell line (SNU-5). The human gastric cancer SNU-5 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were immediately placed into 75-cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 and 95% air atmosphere in 80% Iscove's modified Dulbecco's medium supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% glutamine.

Cell viability determination by using trypan blue exclusion and flow cytometry. SNU-5 cells were plated in 12-well plates at a density of $1x10^5$ cells/well and grown for 24 h. (-)-Menthol, at final concentrations of 0.2, 0.4, 0.6, 1.6 and 2.4 mM, was then added, while only DMSO (solvent) was added to the control, and cells were grown for a different period of time. For determining cell viability, the trypan blue exclusion and flow cytometry protocols were used as previously described (7).

DNA damage electrophoresis analysis in SNU-5 cells followed (-)-Menthol treatment. SNU-5 cells were plated in 6-well plates at a density of $2x10^6$ cells/well and grown for 24 h. Various concentrations of (-)-Menthol were then added, as described above, and cells were grown for 24 h for DNA damage electrophoresis assay as described. DNA was prepared using the GENOME DNA isolation kit protocol (BIO 101, La Jolla, CA, USA), as described previously (7).

Western blotting for examining the effect of (-)-Menthol on topoisomerase I and II of SNU-5 cells. The total protein was collected from human gastric cancer SNU-5 cells cotreated with or without 1.6 mM (-)-Menthol before topoisomerase I and II were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as described previously (7).

Reverse transcriptase polymerase chain reaction (RT-PCR). The total RNA was extracted from SNU-5 cells by using the Qiagen RNeasy Mini Kit as described previously (17). Total RNA (1.5 μg), 0.5 μg of oligo-dT primer and DEPC (diethyl pyrocarbonate)-treated water were combined into a micro-centrifuge tube to a final volume of 12.5 μl. The entire mixture was heated at 70˚C for 10 min and chilled on ice for at least 1 min. The subsequent procedures for conducting reverse transcription were exactly the same as those in the instruction manual (First-strand cDNA synthesis kit, Invitrogen, Carsbad, CA, USA). The reverse transcription products from total RNA served as a template for PCR. When amplifying target cDNA, components in 50 μl of solution were as follows: 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 20 pmoles of each primer, cDNA template corresponding to the amount synthesized from 50 ng of total RNA and 2 units of DyNAzyme DNA polymerase. The sequence of primers was as follows: topoisomerase I: F TCTTC CCATGAAACTATAAATGGC and R CAAAGCTGAAAACCG CAGG topoisomerase II: F GGAGATTTGTACAAACATAC and R AAAATGTCTGCCTTACAA; topoisomerase IIα: F ACACTC AGCCTCTTATGTGC and R ATCAAATGTTGTCCCCG; topoisomerase IIβ F GCCCAAAGAGCACAAACATT and R CAGA CAGTAGTGACATTTCAATG topoisomerase IIIαF CCAGCT GAAGCCCACTGT and R CCTTGCATTACACCGTCCTT. Act b: GCTCGTCGTCGACAACGGCTC and CAAACATGATCT GGGTCATCTTCTC.

Microarray hybridization for examining the effect of (-)-Menthol on topoisomerase I, IIα, IIβ, IIIα, IIIβ and NF-Î B of SNU-5 cells. The total RNA was extracted from SNU-5 human gastric cancer cells treated with or without 1.6 mM (-)-Menthol by using the Qiagen RNeasy Mini Kit at the indicated time. The total RNA was used for cDNA synthesis and labelling, microarray hybridization, fluor-labelled cDNA hybridization to their complements on the chip, and the resulting localized concentrations of fluorescent molecules were detected and quantitated (Asia BioInnovations Corporation, Taipei, Taiwan).

Results

The effect of (-)-Menthol on the cell viability of SNU-5 cells. The effect of (-)-Menthol on cell viability was evaluated using flow cytometric assay. Various exposures (6, 12, 24, and 48 h) to (-)-Menthol dramatically decreased the cell number in a dose-dependent manner (Figure 1A, B, C and D). The concentration required for inhibition of growth by 50% was approximately 1.62 mg/mL for 24-h incubation. The control cells treated with vehicle alone showed no significant decrease in cell number.

The effect of (-)-Menthol on the DNA damage of SNU-5 cells. To determine whether the decrease of cell viability by (-)-Menthol was associated with DNA damage, we initially isolated DNA from SNU-5 cells treated with or without (-)-Menthol. Then we conducted gel electrophoresis to examine the DNA damage, as shown in Figure 2. The results demonstrated that (-)-Menthol induced DNA damage before leading to cell death.

The effecs of (-)-Menthol on the topoisomerase I, IIa, IIβ and NF-Î B of SNU-5 cells. In order to investigate the mechanism by which (-)-Menthol causes cell death, we also tested the effects of this compound on the proteins and gene expression levels of topoisomerase I, IIα, IIβ and NF-Î B, important regulators of cell death (DNA damage) pathway that lead to necrosis. As shown in Figure 3, Western blot analysis revealed that (-)-Menthol treatment decreased topoisomerase I, IIα, IIβ and NF-Î B protein levels. As shown in Figure 4A, B, C and D, PCR analysis revealed that (-)-Menthol treatment decreased topoisomerase I, IIα, IIβ and IIIβ levels of gene expression. However, the result showed that (-)-Menthol induced NF-Î B gene expression and did not affect topoisomerase IIIα. The effects of (-)-Menthol on the gene expression of topoisomerase I, IIα, IIβ, IIIα, IIIβ and NF-Î B from SNU-5 cells were also confirmed by cDNA microarray (Figure 5A, B, C and F) and are in agreement with the results from PCR.

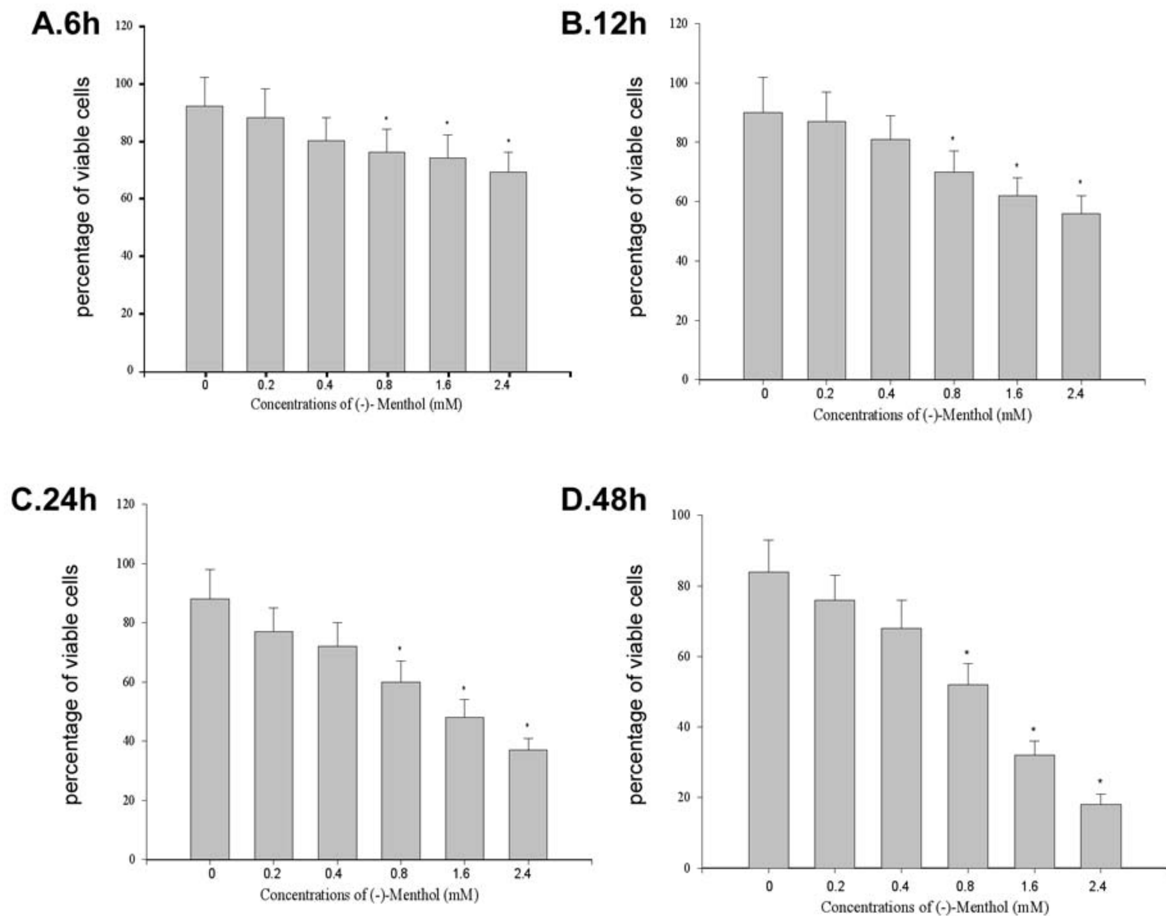

Figure 1. *Percentage of viable SNU-5 cells after (-)-Menthol treatment for 6, 12, 24 and 48 hours. SNU-5 cells ($5x10^5$ cells/well; 12-well plates) were plated in 80% Iscove's modified Dulbecco's medium + 10% FBS with various concentrations of (-)-Menthol for 24 hours. Then cells were collected by centrifugation and the viable cells were determined by the trypan blue exclusion test, as described in Materials and Methods. Data represents mean±S.D. of three experiments. *$p<0.05$.*

Discussion

(-)-Menthol-induced cytotoxicity in SNU-5 cells was dose- and time-course dependent. (-)-Menthol did not induce significant apoptosis but did induce necrosis based on the analysis by flow cytometry and DNA fragmentation protocols. Although (-)-Menthol has been reported to exert a potent chemopreventive action during DMBA initiation of rat mammary tumors (4), the exact molecular mechanism is still unclear. So far, the molecular mechanism of (-)-Menthol-induced necrosis and apoptosis in human cancer cells is still unclear.

The present results demonstrated that (-)-Menthol associated with topoisomerase-mediated DNA damage might be a possible mechanism by which (-)-Menthol exerts its cytotoxicity. It is known that activation of signaling pathways follows DNA damage induced by poisons of topoisomerase and can lead to cell death by apoptosis (8). Therefore, targeting the inhibition of topoisomerases may be a clinical approach for the development of more effective therapeutic strategies. Arimondo and Helene (9) pointed out that amsacrine, daunorubicin, etoposide (acting on type II topoisomerases), camptothecin and indolocarbazole derivatives of the antibiotic rebeccamycin (acting on type Iβ topoisomerases) have been shown to stimulate DNA cleavage by topoisomerases leading to cell death. We showed that (-)-Menthol reduced topoisomerases I and II levels, which may be the cause of induction of cell death. However, further investigation is needed regarding whether or not (-)-Menthol could be used as a potential anticancer chemotherapeutic agent. Bareet *et al.* (10), with *in vitro* studies, demonstrated a dual catalytic inhibitory activity of a new compound against topoisomerases I and

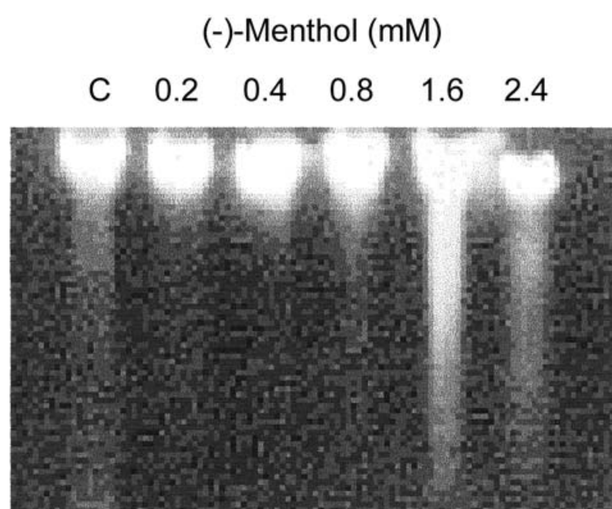

Figure 2. *DNA damage of SNU-5 cells after (-)-Menthol treatment for 24 hours. SNU-5 cells (5x10^6 cells/well; 6-well plates) were plated in 80% Iscove's modified Dulbecco's medium + 10% FBS with various concentrations of (-)-Menthol for 24 hours. Then cells were collected by centrifugation, DNA was isolated and gel electrophoresis was performed, as described in Materials and Methods.*

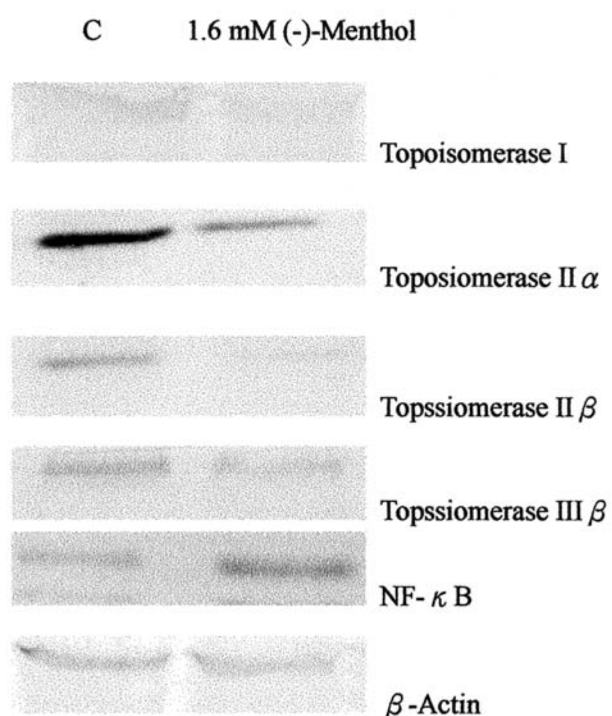

Figure 3. Down-regulation of topoisomerases I, IIα, IIβ and NF-ÎB by (-)-Menthol treatment. SNU-5 cells were treated with 1.6 mM (-)-Menthol for 24 hours. The cells were washed with PBS, lysed and the resulting cell lysates were subjected to immunoblotting with anti-topoisomerase I, IIα, IIβ and NF-ÎB antibodies, as described in Materials and Methods.

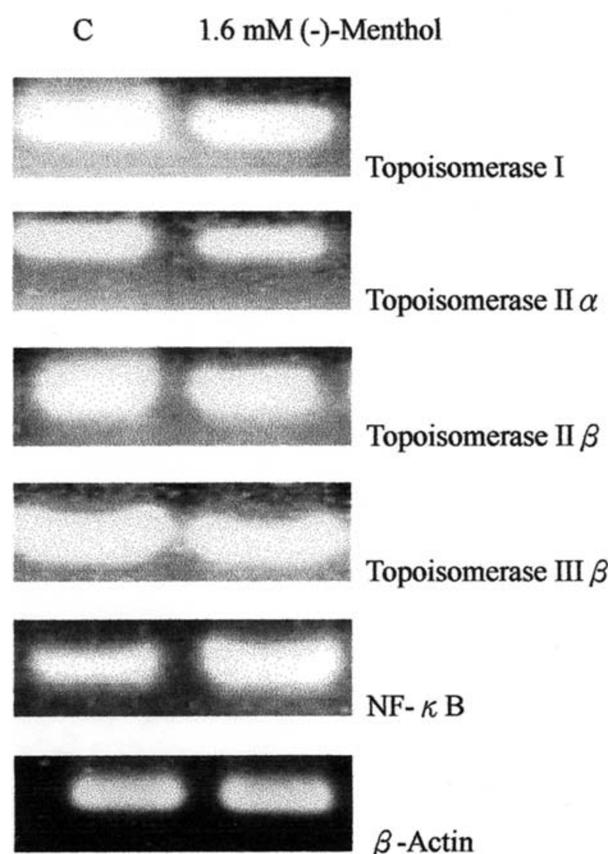

Figure 4. *Down-regulation of the mRNA levels of topoisomerases I, IIα, IIβ and NF-ÎB by (-)-Menthol treatment. SNU-5 cells were treated with 1.6 mM (-)-Menthol for 24 hours. The cells were washed with PBS, total RNA was isolated and subjected to PCR for topoisomerase I, IIα, IIβ and NF-ÎB primers, as described in Materials and Methods.*

Table I. *List of genes that were affected in (-)-Menthol-treated human gastric cancer cells (SNU-5).*

Name	Normalized expression ratio	Accession number
DNA topoisomerase I (relaxes supercoiled DNA)	0.425906	12458
DNA topoisomerase IIα (control cell proliferation)	0.931178	4829
DNA topoisomerase IIβ (nuclear regulatory subunit chromatin)	0.895885	5091
DNA topoisomerase IIIα (human DNA topoisomerase III mRNA)	0.969773	8817
DNA topoisomerase IIIβ (may relax DNA torsion up-replication)	0.638034	15903
NF-Î B	1.519896	16988

(Anti-pathogen response/Integral membrane /receptor and signaling)

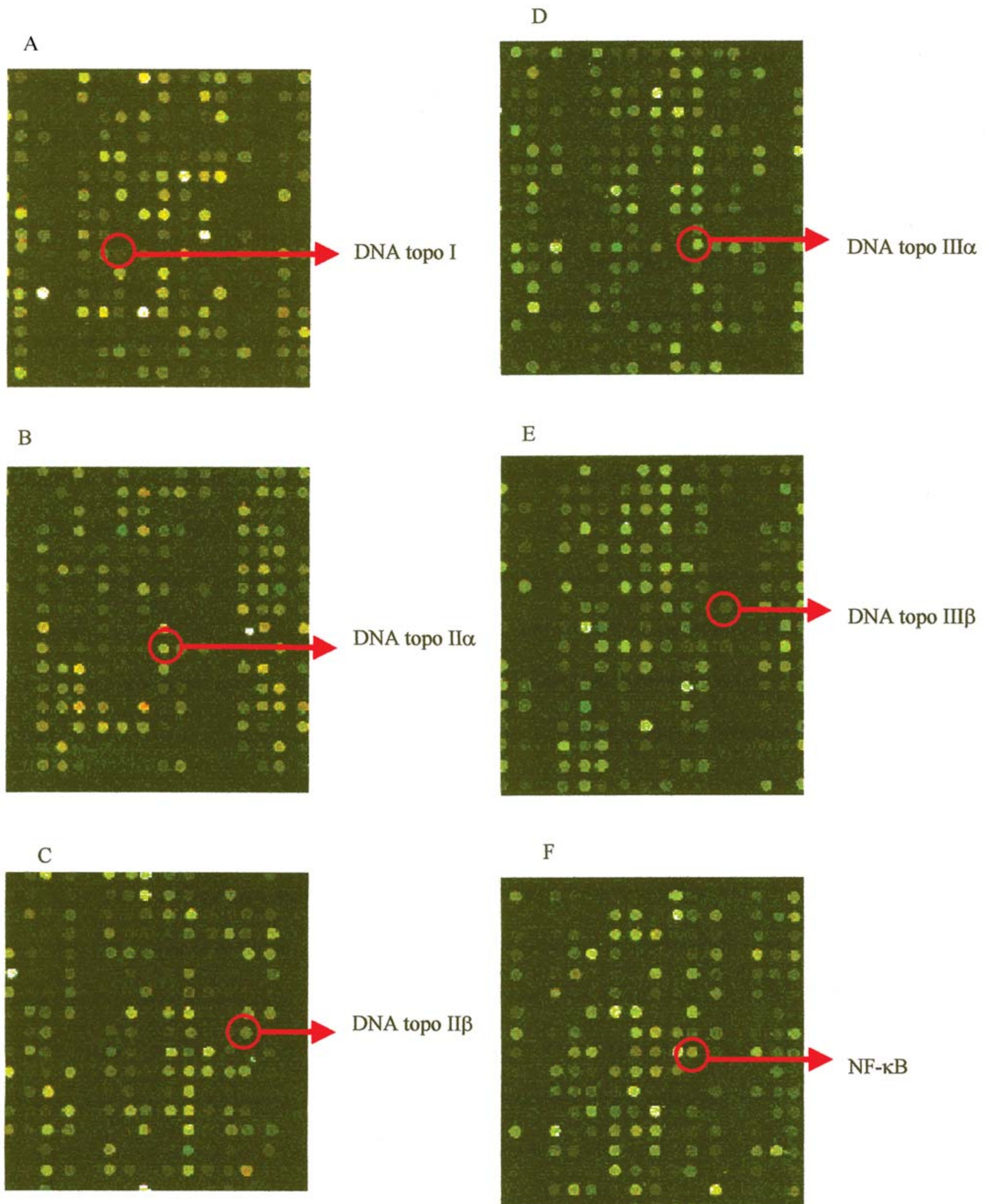

Figure 5. *cDNA microarrays of DNA topoisomerases I, IIα, IIβ, IIIα, IIIβ and NF-ÎB genes in human SNU-5 cells treated with 1.6 mM (-)-Menthol. SNU-5 cells (5x10^6 cells/well) in 6-well plates were treated with or without 1.6 mM (-)-Menthol for 24 hours. The total RNA was extracted and prepared for cDNA hybridization. Red color spot shows up-regulation and green color spot shows down-regulation. Circle mark (DNA topoisomerase I, IIα, IIβ, IIIα, IIIβ and NF-ÎB) show the genes that are down-regulated (panel A, B, C, D, E and F).*

II by a new mechanism involving interference with the DNA binding activity of these enzymes instead of DNA intercalation. It has been demonstrated that the enhacement of topoisomerase I cleavage most probably results from a combination of conformation and electrostatic effects, which then lead to enzyme poisoning and, finally, to cell death (11). It is well known that NF-Î B is an inducible transcription factor involved in the regulation of genes during inflammatory and immune responses (12, 13). Therefore, the inappropriate regulation of NF-Î B has been associated with cancer occurrence (14). It has also been reported that topoisomerase poisons can induce activation of NF-Î B (15). Our results also showed that (-)-Menthol increased the levels of NF-Î B, which may lead to cell death.

Acknowledgements

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