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Induction of Apoptosis in Leukemic Cells by Homovanillic Acid Derivative, Capsaicin, through Oxidative Stress: Implication of Phosphorylation of p53 at Ser-15 Residue by Reactive Oxygen Species

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ABSTRACT

Capsaicin (*N*-vanillyl-8-methyl-1-nonenamide) is a homovanillic acid derivative found in pungent fruits. Several investigators have reported the ability of capsaicin to inhibit events associated with the promotion of cancer. However, the effects of capsaicin on human leukemic cells have never been investigated. We investigated the effects of capsaicin on leukemic cells *in vitro* and *in vivo* and further examined the molecular mechanisms of capsaicin-induced apoptosis in myeloid leukemic cells. Capsaicin suppressed the growth of leukemic cells, but not normal bone marrow mononuclear cells, via induction of G_0-G_1 phase cell cycle arrest and apoptosis. Capsaicin-induced apoptosis was in association with the elevation of intracellular reactive oxygen species production. Interestingly, capsaicin-sensitive leukemic cells were possessed of wild-type p53, resulting in the phosphorylation of p53 at the Ser-15 residue by the treatment of capsaicin. Abrogation of p53 expression by the antisense oligonucleotides significantly attenuated capsaicin-induced cell cycle arrest and apoptosis. Pretreatment with the antioxidant *N*-acetyl-L-cystein and catalase, but not superoxide dismutase, completely inhibited capsaicin-induced apoptosis by inhibiting phosphorylation of Ser-15 residue of p53. Moreover, capsaicin effectively inhibited tumor growth and induced apoptosis *in vivo* using NOD/SCID mice with no toxic effects. We conclude that capsaicin has potential as a novel therapeutic agent for the treatment of leukemia.

INTRODUCTION

Capsaicin is the active principle ingredient of the hot chilli pepper Capsicum, which contains about 0.1–1.0% of capsaicin (1). Spicy foods may play some role in human carcinogenesis, and to date one single epidemiological study has demonstrated that there was a correlation between hot chilli pepper consumption and incidence of gastric cancer (2); however, other studies have failed to provide evidence for its genotoxic potential (3, 4). Capsaicin extracts have been extensively investigated for their effects on genotoxicity and mutagenicity *in vitro* as well as *in vivo*, but the study results are conflicting (5–7). In several studies, the tumor-initiating or -promoting potential of capsaicin was observed (8–11), whereas in other studies the chemoprotective effects of capsaicin were demonstrated (5, 12–14). Moreover, it has been reported that capsaicin inhibits cellular growth of neuroblastoma and hepatocarcinoma cells through the induction of apoptosis (15–17). However, the mechanism of capsaicin-induced apoptosis remains unclear, and the effects of capsaicin on human leukemic cells have never been studied.

The tumor suppressor protein p53 regulates the cellular response to DNA damage by mediating cell cycle arrest, DNA repair, and cell death (18, 19). The mechanisms involved in p53-mediated cell death remain controversial, and regulation of p53 function is complicated. Phosphorylation at the Ser-15 residue of p53 is critical for p53-dependent transactivation. In addition, accumulation of p53 protein by inhibiting the interaction between p53 and MDM2 stimulates p53-dependent transactivation (20). In response to stress signals, levels of p53 protein are rapidly increased, and its activity is enhanced after phosphorylation at the Ser-15 residue, resulting in the up-regulation of downstream genes, including the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and the proapoptotic gene *Bax*. In turn, increased levels of Bax induce mitochondrial depolarization, release of cytochrome *c*, and activation of a caspase cascade, leading to apoptosis (9, 21–25). Ataxia telangiectasia mutated kinase has been shown to phosphorylate the Ser-15 residue of p53, leading to apoptotic signal transduction (26, 27). Several studies have demonstrated that reactive oxygen species (ROS) generation phosphorylates and activates p53 in an ataxia telangiectasia mutated-dependent manner (28–32).

In the present study, we show that the homovanillic acid derivative capsaicin inhibits the proliferation of various leukemic cells. Capsaicin dramatically suppressed the growth of leukemic cells with wild-type p53, through the induction of G_0-G_1 cell cycle arrest and apoptosis. We further investigated the molecular mechanisms of capsaicin-induced apoptosis in leukemic cells *in vitro* as well as its antitumor effects *in vivo*.

MATERIALS AND METHODS

Cell Culture. NB4 promyelocytic leukemia and Kasumi-1 myeloid leukemia cell lines were generous gifts from Dr. M. Lanotte (Hôpital St. Louis, Paris, France; Ref. 33) and Dr. H. Asou (Hiroshima University, Hiroshima, Japan; Ref. 34), respectively. Retinoic acid-resistant acute promyelocytic leukemia cell line UF-1 was established in our laboratory (35). The human leukemic cell lines, including HL-60, K562, KU812, and U937, and NIH3T3 cells as a positive control for wild-type p53, were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). Bone marrow samples from eight patients with acute leukemia and three normal volunteers were obtained according to appropriate Human Protection Committee validation at the Keio University School of Medicine (Tokyo, Japan) with written informed consent. Mononuclear cells were separated by Lymphoprep (Nycomed Pharma As, Oslo, Norway). Cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2. UF-1 and primary cells from the patients were grown in RPMI 1640 with 15% fetal bovine serum (Hyclone Laboratories, Logan, MT) under standard culture conditions. The morphology was evaluated by cytospin slide preparation with Giemsa staining, and the viability was assessed by trypan blue dye exclusion.

Reagents. Capsaicin was purchased from Sigma (St. Louis, MO) and dissolved in 100% ethanol, and *N*-tert-butoxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Calbiochem, La Jolla, CA) was dissolved in DMSO (Sigma).

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NAC, buthionine sulfoximine, catalase, and superoxide dismutase were purchased from Sigma.

Cell Cycle Analysis. Cells (1×10^6) were suspended in hypotonic solution (0.1% Triton X-100, 1 mM Tris-HCl (pH 8.0), 3.4 mM sodium citrate, and 0.1 mM EDTA) and stained with 50 μg/ml of propidium iodide. DNA content was analyzed by FACSCalibur (Becton Dickinson, San Jose, CA). The population of cells in each cell cycle phase was determined using Cell ModiFIT software (Becton Dickinson).

Assays for Apoptosis. Apoptotic cells were quantified by Annexin V-FITC and propidium iodide double staining using a staining kit from PharMingen (San Diego, CA). The mitochondrial transmembrane potential ($\Delta\psi$m) was determined by flow cytometry. Briefly, cells were washed twice with PBS and incubated with 1 μg/ml Rhodamine 123 (Sigma) at 37°C for 30 min. Rhodamine 123 intensity was determined by flow cytometry.

Caspase Assays. Caspase-3-related protease activity was determined by using a commercially available kit (PharMingen) according to the manufacturer's instructions. Briefly, cells were fixed and permeabilized using the Cytofix/Cytoperm for 20 min at 4°C, pelleted, and washed with Perm/Wash buffer (PharMingen). Cells were then stained with polyclonal antibody against the active form of caspase-3 (PharMingen) for 20 min at room temperature, washed in Perm/Wash buffer, stained with goat antirabbit-FITC (Super Techs, Bethesda, MD), and analyzed by flow cytometry. In the caspase inhibitor assay, cells were pretreated with a synthetic pan-caspase inhibitor (20 μM; *N*-tert-butoxy-carbonyl-Val-Ala-Asp-fluoromethylketone) for an hour before addition of capsaicin.

Measurement of ROS Production. To assess the generation of ROS, we incubated control and capsaicin-treated cells with 5 μM dihydroethidium (Molecular Probes, Eugene, OR), which is rapidly oxidized to the fluorescent intercalator ethidium by cellular oxidants. Cells (1×10^5) were stained with 5 μM dihydroethidium for 15 min at 37°C and were then washed and resuspended in PBS. The oxidative conversion of dihydroethidium to ethidium was analyzed by flow cytometry.

Cell Lysate Preparation and Immunoblotting. Cells were collected by centrifugation at $700 \times g$ for 10 min, and then the pellets were resuspended in a lysis buffer [1% NP40, 1 mM phenyomethylsulfonyl fluoride, 40 mM Tris-HCl (pH 8.0), and 150 mM NaCl] at 4°C for 15 min. Mitochondrial and cytosolic fractions were prepared with digitonin-nagarse treatment. Protein concentrations were determined using a detergent-compatible protein assay system (Bio-Rad, Richmond, CA). Cell lysates (15 μg of protein/lane) were fractionated in 12.5% or 7.5% SDS-polyacrylamide gels before being transferred to the membrane (Immobilon-P membrane; Millipore, Bedford, MA) according to standard protocol. Antibody binding was detected by using the enhanced chemiluminescence kit with hyper-enhanced chemiluminescence film (Amersham, Buckinghamshire, United Kingdom). β-Actin was used as an indicator for equality of lane loading. Blots were also stained with Coomassie Brilliant Blue to confirm that equal amounts of protein extracts were present in each lane. The following antibodies were used in this study: anti-Rb, -cytochrome *c* (PharMingen), -MDM2 (Oncogene, Boston, MA), -p53, -phospho Rb (Ser-780; Cell Signaling, Beverly, MA), -cyclin D1, -Bax, -p21$^{WAF1/CIP1}$, and -β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). We used p53-phosphorylated kit (Cell Signaling) to detect phosphorylated p53. For detection of wild-type p53 protein expression, cell lysates were precipitated with anti-p53 wild-type monoclonal antibody (PAb1620; Oncogene) and then blotted with anti-p53 polyclonal antibody (Cell Signaling). Expression of wild-type p53 was also determined by flow cytometry.

Reverse Transcription-PCR, PCR, Gel Electrophoresis, and Sequencing. Total cellular RNAs were isolated from the cells by using RNA easy kit (Qiagen K.K., Tokyo, Japan). Random primed, first strand cDNAs were synthesized from 1 μg of total RNAs using Superscript II reverse transcriptase (Life Technologies, Inc. Gaithersburg, MD) according to the manufacturer's instructions. PCR was carried out for 30 s at 94°C, 30 s at 55°C, and 20 s at 72°C for 40 cycles. Primer sequences for p53 were sense (nucleotide 225-244) 5′-TGCACCAGCGACTCCTACAC-3′ and antisense (nucleotide 892-873) 5′-CTGGGTGAGGCTCCCCTTTC-3′. The PCR products were analyzed on 1% agarose gel. To normalize the amount of RNA, we used amplification of the human β-actin gene as a control. Exons 4-11 of the "hot spots" region of p53 were amplified by PCR using the following primers: 5′-GCCAAGTCTGTGACCTGCACG-3′ (exon 4) and 5′-TCAGTCTGAGTCAGGCCCTTC-3′ (exon 11). The amplified product was cloned into a pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) and sequenced with both M13 forward primer and M13 reverse primer included in the TOPO TA cloning kit (Invitrogen) according to the manufacturer's recommendation. DNA sequencing was performed on an ABI PRISM 310 genetic analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA). All of the oligonucleotides were obtained from Sawaday Technology (Tokyo, Japan).

Antisense Oligonucleotides for p53. The p53-antisense oligodeoxynucleotide targeted the following region of the initiation codon: 5′-CGGCTCCTCCATGGCAGT-3′. Its scrambled oligodeoxynucleotide (5′-ACTGCCATGGAGGAGCCG-3′) and the mismatch sequence (5′-CGGGTCCTCTACGCTAGT-3′) were designed as a negative control. These oligodeoxynucleotides were modified by phosphorothioate to enhance their stability, and they had no similarity to other mammalian genes as shown by Basic Local Alignment Search Tool search analysis. After 24 h of preincubation with these oligonucleotides, NB4 cells were treated with capsaicin and 1 μM each oligonucleotide for 24 h.

Animal Model and Experimental Design. We have established a system of human all-*trans* retinoic acid-sensitive acute promyelocytic leukemia model in NOD/SCID mice by using NB4 cells (36). Briefly, NOD/SCID mice (The Jackson Laboratory, Bar Harbor, ME) were pretreated with 3 Gy of total body irradiation, which is a sublethal dose that was expected to enhance the acceptance of xenografts. Subsequently, the mice were inoculated s.c. with NB4 cells (1×10^7 cells) in their logarithmic growth phase, and the inoculated NB4 cells rapidly formed s.c. tumors at the injection site. Fourteen days after implantation of the cells, mice with the transplanted cells were randomly assigned to be injected with 5% ethanol ($n = 15$; 50 μl) or capsaicin (50 mg/kg) in 5% ethanol ($n = 15$; 50 μl) as an emulsion fluid administered daily. After 6 days of the treatment, mice were sacrificed and dissected to measure tumor weights. The study was approved by the Animal Care and Use Committee at the Keio University School of Medicine, Tokyo, Japan. When the mice showed severe wasting or when observations were finished, mice were sacrificed according to the UKCCCR guidelines (37). Tumors were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and then stained with anti-single-strand DNA (Dako Japan Co., Ltd, Kyoto, Japan) for detection of apoptotic cells (38).

Statistical Analysis. Tumor weights and numbers of mitotic cells are expressed as the mean ± SD. Differences of both parameters were analyzed for significance by Student's *t* test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Effects of Capsaicin on Cellular Proliferation of Various Leukemic Cells. We first investigated the effects of capsaicin on cellular proliferation of seven leukemic cell lines that included NB4, UF-1, Kasumi-1, HL-60, K562, KU812, and U937 cells. Capsaicin inhibited cellular growth of all leukemic cells, but not normal bone marrow mononuclear cells, in a dose- and time-dependent manner (Fig. 1, *A* and *B*). Interestingly, capsaicin was particularly sensitive to NB4 and Kasumi-1 leukemic cells (Fig. 1, *A* and *B*), both of which expressed PAb1620-reactive wild-type p53 (Fig. 2, *A* and *B*). In addition, sequencing analysis of p53 (exons 4-11) was performed on both cell lines that have wild-type p53 (data not shown). But the other leukemic cell lines had defective p53 (Fig. 2, *A* and *B*). As previous investigations have reported, these cell lines contain mutated p53 alleles (39–42). Because capsaicin dramatically decreased cellular growth of NB4 and Kasumi-1 cells with the lowest IC_{50}, we used NB4 cells for our subsequent investigations. Cultivation with capsaicin increased the population of cells in the G_0-G_1 phase with a reduction of cells in the S-phase, which was followed by a marked increase of a sub-G_1 population at 24 h (Fig. 1*C*). Annexin V-positive apoptotic fractions were detected beginning 4 h after exposure to capsaicin, and these fractions dramatically increased in a time-dependent manner (Fig. 1*D*). Annexin V and propidium iodide double-positive cells were increased at 24 h after treatment, indicating that capsaicin induced

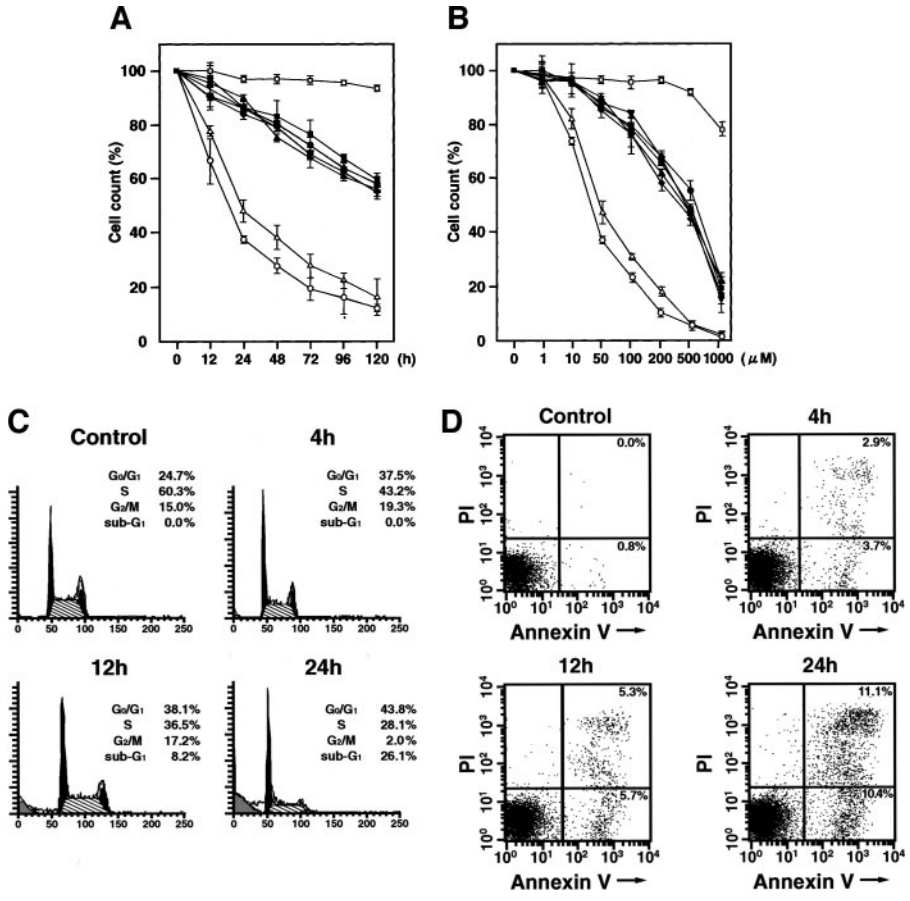

Fig. 1. Capsaicin inhibits growth of myeloid leukemic cells via G_0-G_1 phase cell cycle arrest followed by apoptosis. *A* and *B*, various myeloid leukemic cells [NB4 (○), Kasumi-1 (△), UF-1 (●), HL-60 (■), U937 (▲), KU812 (◆), K562 (▼)] and peripheral blood cells from a healthy donor (□) were treated with 50 μM capsaicin for various times (0–120 h; *A*) and concentrations (0–1000 μM) for 24 h (*B*). Cell viability was assessed by trypan blue dye exclusion. Results are expressed as the mean of three duplicate experiments, and the SD was within 5% of the mean. *C*, cell cycle analysis. Cells were cultured with 50 μM capsaicin for 0–24 h and stained with propidium iodide (PI) as described in "Materials and Methods." DNA content was analyzed by flow cytometry. G_0-G_1, G_2-M, and S indicate the cell phase, and sub-G_1 DNA content refers to the proportion of apoptotic cells. Each phase was calculated by using the cell ModiFIT program. Three duplicate experiments were performed with similar results. *D*, induction of apoptosis by the treatment of capsaicin in NB4 cells. Cells were cultured with 50 μM capsaicin for the indicated times, stained with Annexin V-FITC, and analyzed by flow cytometry. Three duplicate experiments were performed with similar results.

necrosis in addition to apoptosis in this fraction (Fig. 1*D*). These results showed that capsaicin-induced *in vitro* growth inhibition of leukemic cells was mediated by causing G_0-G_1 cell cycle arrest and apoptosis.

Capsaicin-Induced Death Signaling Is Mediated through the Mitochondrial Pathway. Treatment with capsaicin for 3 h significantly induced caspase-3 activity in NB4 cells (Fig. 3*A*). Capsaicin-induced apoptosis was completely blocked by the treatment with *N*-tert-butoxy-carbonyl-Val-Ala-Asp-fluoromethylketone (pan caspase inhibitor; Fig. 3*B*). After treatment with capsaicin for 3 h, low Rh123 staining in NB4 cells indicated an increase in the loss of mitochondrial $\Delta\psi$m (Fig. 3*C*). Capsaicin induced a substantial release of cytochrome *c* from the mitochondria into the cytosol within 3 h. In addition, capsaicin induced a translocation of Bax from cytosol to mitochondria (Fig. 3*D*). These results indicate that capsaicin-induced apoptosis in early phase NB4 cells is mediated through the mitochondrial-dependent pathway.

ROS Generation Triggers Capsaicin-Induced Apoptosis. Previous investigation has reported that capsaicin induces inhibition of growth and the NADH oxidase activity in HeLa cells (43). Other examination has shown that capsaicin-induced apoptosis in tumor cells is associated with the generation of ROS (44). Therefore, we analyzed the production of intracellular ROS in NB4 cells. Treatment with capsaicin in NB4 cells showed within 0.5 h a dramatic increase in intracellular ROS compared with control cells (Fig. 4*A*). Treatment with a thiol antioxidant, NAC, completely blocked the generation of ROS and attenuated capsaicin-induced apoptosis in NB4 cells (Fig. 4*B*). The addition of 1 mM buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase, induced glutathione (GSH) depletion and synergistically enhanced capsaicin-induced apoptosis (data not shown). Superoxide is a major component of ROS in the mitochondria and is converted rapidly to H_2O_2 by superoxide dismutase. Most H_2O_2 is degraded further to H_2O by the enzymes catalase and glutathione peroxidase. We thus examined the effect of more specific antioxidants on capsaicin-induced cell death. Interestingly, apoptosis induced by capsaicin could be blocked completely by pretreatment with catalase in both NB4 (Fig. 4*C*) and Kasumi-1 (Fig. 4*D*) cells. However, superoxide dismutase partially inhibited capsaicin-induced cell death (Fig. 4, *C* and *D*).

Expression of Cell Cycle- and Apoptosis-Associated Proteins in NB4 Cells. To characterize the molecular mechanism of capsaicin-induced cell cycle arrest followed by apoptosis in NB4 cells, we examined the expression of cell cycle- and apoptosis-associated proteins during the treatment with capsaicin. Expression of $p21^{WAF1/CIP1}$ proteins was dramatically increased with reduction of cyclin D1 protein expression, dephosphorylation of Rb, and up-regulation of p53 and Bax at 3 h after treatment (Fig. 5). Interestingly, the Ser-15 residue of p53 became significantly phosphorylated after an hour of exposure to capsaicin (Fig. 5). In contrast, the phosphorylation levels of p53 at other residues did not change in response to treatment with capsaicin (data not shown).

Phosphorylation of p53 at the Ser-15 Residue in NB4 Cells Treated with Capsaicin. p53 protein was accumulated during the treatment with capsaicin in NB4 cells expressing wild-type p53 (Fig. 5). In addition, Western blot analysis using the antibody specific to the phosphorylated Ser-15 of p53 revealed that the Ser-15 residue of p53 became phosphorylated immediately after the treatment with capsaicin (Figs. 5 and 6*A*). Interestingly, the inhibition of ROS generation by pretreatment of NAC inhibited capsaicin-induced phosphorylation of p53 at the Ser-15 residue (Fig. 6*A*). In addition, pretreatment of cells with catalase caused complete inhibition of capsaicin-induced p53

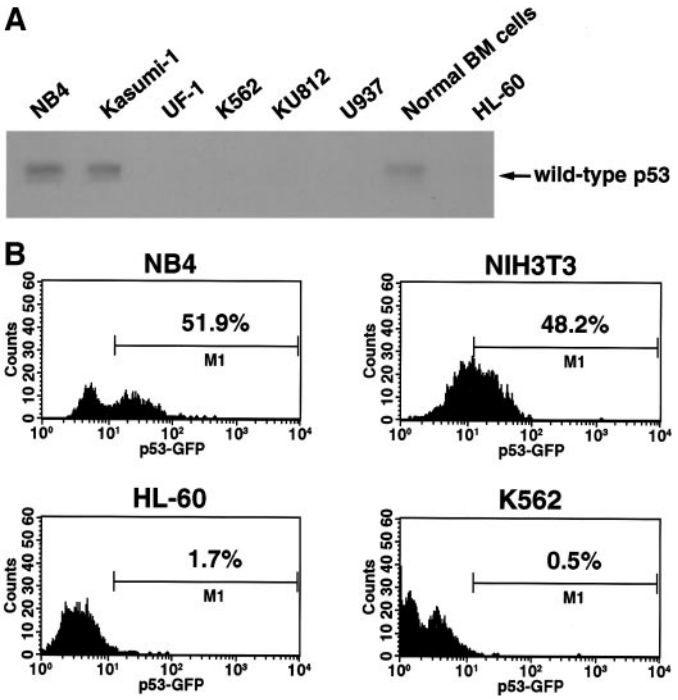

Fig. 2. Expression of wild-type p53 protein in various leukemic cells and normal bone marrow cells. *A*, wild-type p53 protein was immunoprecipitated with anti-p53 wild-type monoclonal antibody followed by blotting with the p53 polyclonal antibody. NB4 and Kasumi-1 cells are positive, but the other leukemic cells (HL-60, UF-1, K562, KU812, and U937) are negative for wild-type p53. Also, normal bone marrow mononuclear cells are positive. *B*, expression of wild-type p53 protein was analyzed by flow cytometry. NIH3T3 cells were used as a positive control. *GFP*, green fluorescent protein.

activation (Fig. 6*A*). These results suggest that H_2O_2 generation plays an essential role in p53 stabilization by phosphorylation at the Ser-15 residue (31, 32).

To examine whether inhibition of p53 expression can block capsaicin-induced cell cycle arrest and apoptosis in NB4 cells, we used antisense (AS) oligonucleotide for p53. Pretreatment with 1 μM p53 AS oligonucleotides prevented the capsaicin-induced increase in p53 protein levels in NB4 cells (Fig. 6*B*). In contrast, pretreatment with 1 μM scrambled and mismatch oligonucleotides did not significantly alter the expression of p53, and AS oligonucleotide did not modulate the expression of MDM2 and Bcl-2 proteins, indicating the specificity of the p53 AS oligonucleotide used in this study (Fig. 6*B*). Pretreatment with 1 μM p53 AS, but not scrambled or mismatched, oligonucleotides for 24 h significantly abrogated capsaicin-induced cell cycle arrest as well as apoptosis (Fig. 6*C*), suggesting that apoptotic cell death in capsaicin-treated NB4 cells is because of the ability of capsaicin to stimulate the accumulation of p53.

Effects of Capsaicin on Primary Cells from Patients with Leukemia. Among the leukemia cell lines, NB4 and Kasumi-1 cells expressing wild-type p53 were the most sensitive to capsaicin, whereas p53-defective cells including HL-60 and UF-1 cells were less sensitive to this agent. We therefore analyzed the association between the sensitivity to capsaicin and the status of p53 in freshly isolated cells from eight patients and found that the expression of wild-type p53 mRNA contributed to the sensitivity to capsaicin-induced apoptosis in the leukemic cells (Table 1). We also analyzed the levels of intracellular ROS generation during the treatment with capsaicin in capsaicin-sensitive and capsaicin-less sensitive cells and found that treatment with capsaicin caused a significant ROS generation with decreased intracellular GSH in capsaicin-sensitive cells (NB4 and leukemic cells from patient 1; Fig, 7, *A* and *B*). In contrast, in capsaicin-less sensitive cells (HL-60, UF-1, and leukemic cells from patient 6), intracellular ROS generation and GSH levels were less modulated by capsaicin (Fig. 7, *A* and *B*).

Capsaicin Induced Apoptosis *in Vivo*. Our *in vitro* data prompted us to examine whether the effects of capsaicin are equally demonstrable *in vivo*. Tumor weight significantly decreased in the mice that received an injection of capsaicin ($P < 0.001$, mean weight: 2.16 $\pm$ 0.53 g in the capsaicin-treated

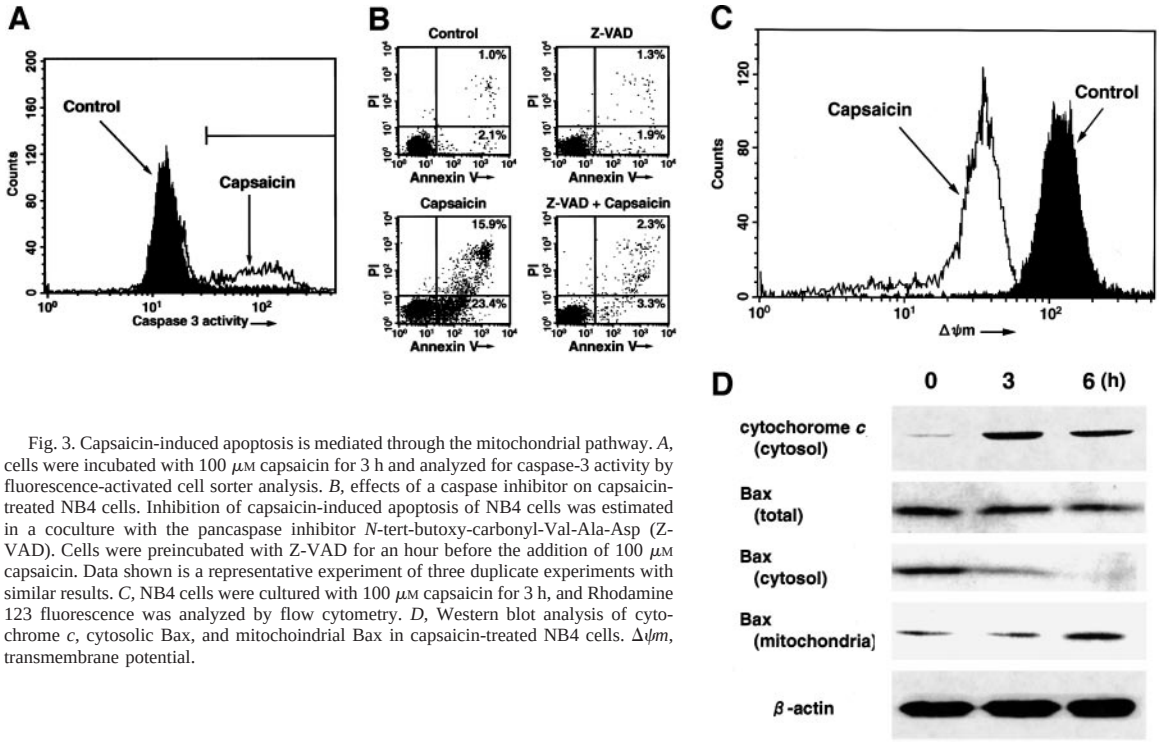

Fig. 3. Capsaicin-induced apoptosis is mediated through the mitochondrial pathway. *A*, cells were incubated with 100 μM capsaicin for 3 h and analyzed for caspase-3 activity by fluorescence-activated cell sorter analysis. *B*, effects of a caspase inhibitor on capsaicin-treated NB4 cells. Inhibition of capsaicin-induced apoptosis of NB4 cells was estimated in a coculture with the pancaspase inhibitor *N*-tert-butoxy-carbonyl-Val-Ala-Asp (Z-VAD). Cells were preincubated with Z-VAD for an hour before the addition of 100 μM capsaicin. Data shown is a representative experiment of three duplicate experiments with similar results. *C*, NB4 cells were cultured with 100 μM capsaicin for 3 h, and Rhodamine 123 fluorescence was analyzed by flow cytometry. *D*, Western blot analysis of cytochrome *c*, cytosolic Bax, and mitochoindrial Bax in capsaicin-treated NB4 cells. $\Delta\psi m$, transmembrane potential.

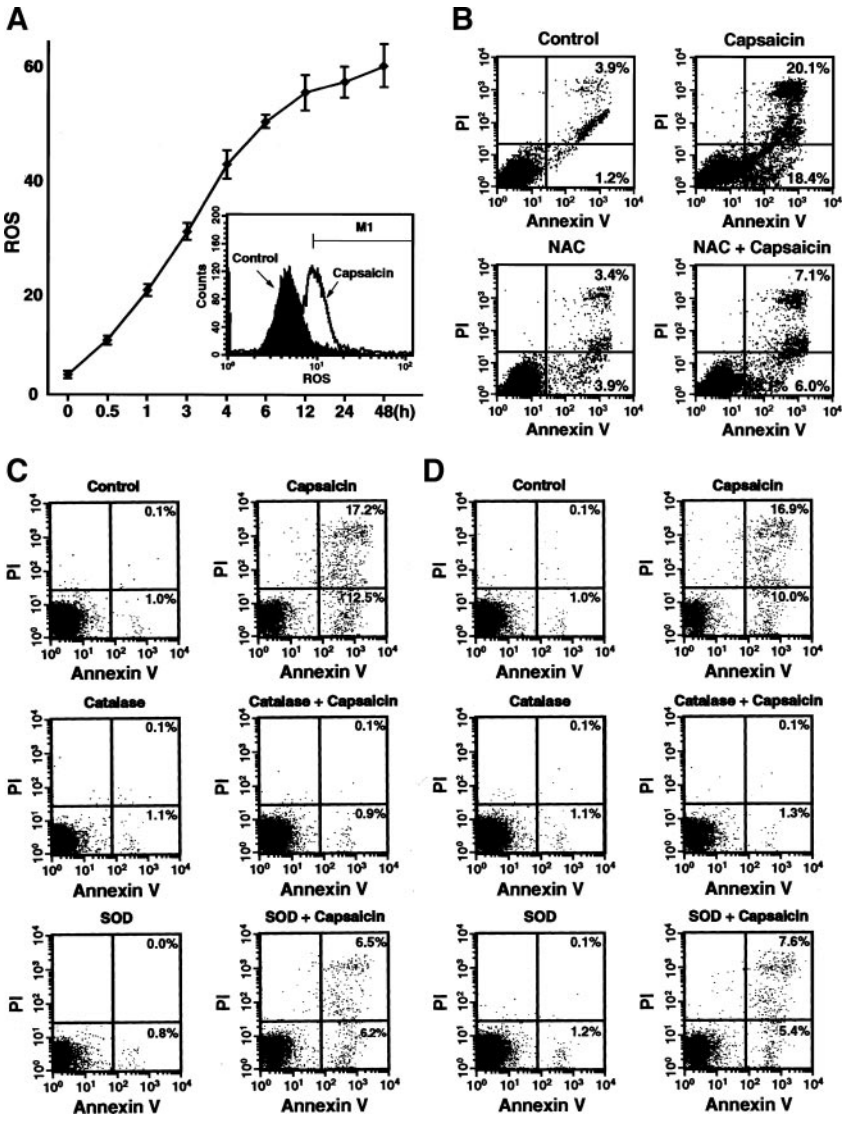

Fig. 4. Reactive oxygen species (ROS) generation by capsaicin in NB4 cells. *A*, to determine the intracellular concentration of ROS, we cultured NB4 cells treated with capsaicin for the indicated time with dihydroethidium (DHE), and the fluorescence was measured by flow cytometry. DHE-derived fluorescence in NB4 cells treated for 6 h with 100 μM of capsaicin is shown in the boxed panel. *B*, cells were pretreated with 100 μM *N*-acetyl-L-cystein (NAC) and then treated with 100 μM of capsaicin for 24 h, and induction of apoptosis was examined via Annexin V/propidium iodide (PI)-double staining. *C* and *D*, effect of specific antioxidants on capsaicin-induced apoptosis in NB4 (*C*) and Kasumi-1 (*D*) cells. Cells were treated with 400 units/ml catalase or 200 units/ml superoxide dismutase and then treated with 50 μM capsaicin for 24 h. Induction of apoptosis was examined by Annexin V/PI-double staining. Representative data of three independent experiments were shown. *SOD*, superoxide dismutase.

group *versus* 4.71 ± 1.49 g in the control group; Fig. 8*A*). Pathological analysis at autopsy revealed no capsaicin-induced tissue changes in any of the organs. These results suggest that capsaicin had no toxic effects on mice during this treatment. When we evaluated tumor cell proliferation by counting the number of apoptotic cells by single-strand DNA staining, we observed a significant increase in the capsaicin-treated group ($P < 0.001$, approximately 8-fold increase; Fig. 8*B*).

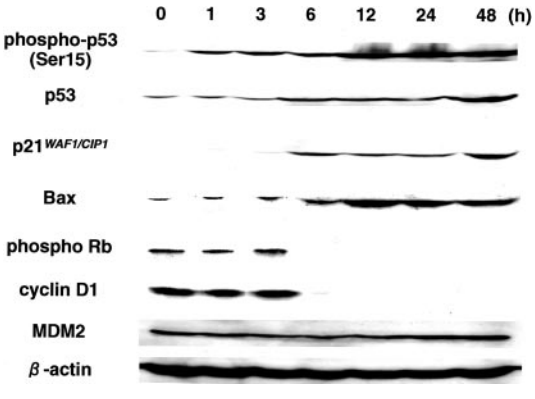

Fig. 5. Expression of the apoptosis- and cell cycle-associated proteins. NB4 cells were treated with 100 μM capsaicin for the indicated times. Cell lysates (15 μg/each lane) were fractionated on 12.5% SDS-polyacrylamide gels and analyzed by Western blotting with antibodies against apoptosis- and cell cycle-associated proteins. (pSer15-p53, p53, cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$, Bax, pSer-Rb, cyclin D_1, and MDM2) Reblotting with β-actin staining demonstrated that equal amounts of protein were present in each lane.

DISCUSSION

To date, several studies have revealed the ability of capsaicin to inhibit events associated with the initiation, promotion, and progression of cancer (5, 12–14). In contrast, other studies have suggested the tumor-initiating potential of capsaicin (8, 9), and several epidemiological studies have shown that chilli pepper consumers were at greater risk for gastric cancer than were nonconsumers (2). In this study, we have showed that capsaicin inhibits cellular growth of leukemic cells via inducing apoptosis-modulating ROS production. Interestingly, capsaicin dramatically induced apoptosis of myeloid leukemic cells expressing wild-type p53.

In our study, capsaicin-sensitive cells expressed wild-type p53 among the leukemic cell lines (NB4 and Kasumi-1 cells) and fresh samples. There was a report that NB4 promyelocytic leukemia cells have point mutations at codon 273 and 248 of the p53 gene (45), but we could not detect any mutations of the p53 gene in NB4

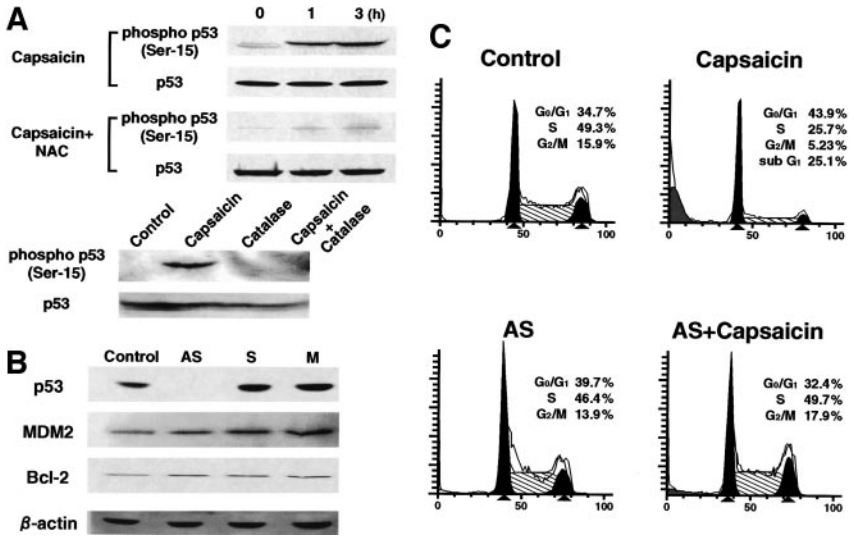

Fig. 6. Cell cycle arrest and apoptosis via a p53-dependent pathway in capsaicin-sensitive NB4 cells. *A*, phosphorylated Ser-15 residue of p53 was increased during capsaicin treatment of NB4 cells within an hour, and *N*-acetyl-L-cystein (NAC) inhibited phosphorylation of p53 by the treatment with capsaicin. In addition, specific antioxidant, catalase, completely blocked capsaicin-induced phosphorylation of the Ser-15 residue of p53. *B*, antisense (AS) oligonucleotide for p53 inhibits p53 expression in a sequence-specific manner. After 24 h of preincubation with 1 μM antisense, sense (S), or mismatch (M) oligonucleotides, NB4 cells were cultured in the presence of capsaicin and fresh oligonucleotides (1 μM) for 24 h. Cell lysates (30 μg/each lane) were fractionated on 12.5% SDS-polyacrylamide gels and analyzed by Western blotting with antibodies against p53, MDM2, and Bcl-2 proteins. Reblotting with β-actin staining demonstrated that equal amounts of protein were present in each lane. *C*, capsaicin-induced cell cycle arrest and apoptosis were abrogated by p53 AS oligonucleotide. Cells were preincubated with 1 μM oligonucleotide for 24 h before treatment with capsaicin. After preincubation, cells were treated with capsaicin for 24 h, and then cell cycle distribution was examined.

cells used in this investigation by sequencing analysis. In addition, we confirmed that our NB4 and Kasumi-1 cells expressed wild-type p53 protein using monoclonal antibody that recognizes conformational epitope of wild-type p53; therefore, we conclude that our NB4 and Kasumi-1 cells have wild-type p53. In contrast, we did not observe phosphorylation and accumulation of p53 during the treatment with capsaicin in less sensitive cells, which did not express wild-type p53. These cell lines have been reported to have mutated p53 gene as follows: HL-60 (major deletions), K562 (an early translational stop codon at 148), KU812 (a point mutation in codon 132 resulting in replacement of lysine with arginine), and U937 (46 base pairs deletion from codon 132) cells (39–42). Bax and $p21^{WAF1/CIP1}$ are well-known target genes of p53, and we demonstrated that capsaicin induced higher levels of these proteins in capsaicin-sensitive NB4 cells.

We showed that capsaicin induces phosphorylation of p53 at the Ser-15 residue after an hour of exposure, resulting in stabilization and protein accumulation. In addition, abrogation of p53 expression by the AS oligonucleotides could significantly inhibit induction of G_0-G_1 phase cell cycle arrest and apoptosis after treatment with capsaicin. However, normal bone marrow cells expressed wild-type p53, but capsaicin did not inhibit growth of the normal cells. Therefore, expression of wild-type p53 may be necessary but not sufficient for inducing apoptosis by capsaicin. Previous studies have reported that p53 gene mutations are infrequent and are found in only 5–10% of fresh acute myelogenous leukemia patients (46–48). In contrast, alterations of p53 gene are more frequent in myeloid leukemia cell lines, which might have the advantage in establishment of cell lines (42). Moreover, it has been reported that p53 mutations are associated with significantly poorer response to intensive chemotherapy and induce drug resistance by interfering with the normal apoptotic pathway in patients with acute myelogenous leukemia (49). Consistent with these reports, we showed that leukemic cells from seven of eight acute myelogenous leukemia patients express wild-type p53, and these cells are sensitive to capsaicin. Taken together, these results suggest that induction of p53 plays an essential role in G_0-G_1 cell cycle arrest and apoptosis in capsaicin-treated leukemic cells but not in normal cells.

Table 1 *Expression of wild-type p53 mRNA and sensitivity to capsaicin-induced apoptosis*[a]

Cells	Expression of wild-type p53 mRNA[d]	Annexin V single-positive cells		
		Control (%)	Capsaicin (%)	Fold increase[b]
Pt 1[e]	+	8.9 (12.6)[c]	63.4 (88.2)	7.1
Pt 2	−	4.5 (5.2)	5.2 (6.8)	1.2
Pt 3	+	3.7 (5.2)	40.3 (57.7)	10.9
Pt 4	+	6.8 (12.5)	50.9 (88.8)	7.5
Pt 5	+	6.2 (8.3)	64.3 (84.7)	10.4
Pt 6	+	4.2 (9.5)	53.4 (84.6)	12.7
Pt 7	+	3.8 (8.0)	43.5 (72.8)	11.4
Pt 8	+	3.3 (7.2)	46.2 (63.4)	14.0

[a] Cells were separated by Lymphoprep sedimentation procedure and subsequently cultured with 100 μM capsaicin for 24 h.

[b] Induction of apoptosis was measured by Annexin V single-positive cells and expressed as a fold increase of the percentage of control Annexin V single-positive cells.

[c] Percentage of Annexin V/P propidium iodide-double positive cells.

[d] Expression of wild-type p53 mRNA was examined by reverse transcription-PCR, and then PCR products were analyzed on 1% agarose gel. Result was expressed as positive (+) or negative (−).

[e] Pt 1, 2; acute lymphoblastic leukemia (ALL), Pt 3–8; acute myeloid leukemia (AML). *Pt*, patient.

Recent studies have demonstrated that mitochondria play an essential role in death signal transduction (50, 51). Bax in mitochondria is known to play an important role in the loss of $\Delta\psi$m (50, 51), and distribution of $\Delta\psi$m constitutes a critical step in a p53-dependent apoptotic pathway. In response to a capsaicin signal, Bax is induced and transported from the cytosol to mitochondria (data not shown), corresponding to a decline in $\Delta\psi$m followed by cytochrome *c* release and caspase activation. These results suggest that capsaicin-induced death signaling is mediated through the mitochondrial-dependent pathway.

Several studies have demonstrated that ROS generation phosphorylates p53 at the Ser-15 residue in an ataxia telangiectasia mutated-dependent manner (31, 32). Consistent with previous studies (52–54), we detected that capsaicin-induced apoptosis in NB4 cells and in fresh leukemic cells from patients expressing wild-type p53 was associated with a significant increase in the levels of intracellular ROS, after GSH depletion. Capsaicin-less sensitive cells have defective p53; however, capsaicin could generate intracellular ROS in these cells. Interestingly, pretreatment with NAC, an excellent supplier of GSH, inhibited phosphorylation of p53 at the Ser-15 residue in the presence of capsaicin, indicating that ROS acts upstream of p53 phosphorylation by capsaicin. Moreover, reduction of H_2O_2 by catalase inhibited phosphorylation of p53 at the Ser-15 residue and apoptosis. In contrast, capsaicin-less sensitive cells were p53 defective, and capsaicin induced lower levels of ROS generation with less modulation of GSH. In addition, we failed to observe phosphorylation or induction of p53 during the treatment with capsaicin in these cells. We

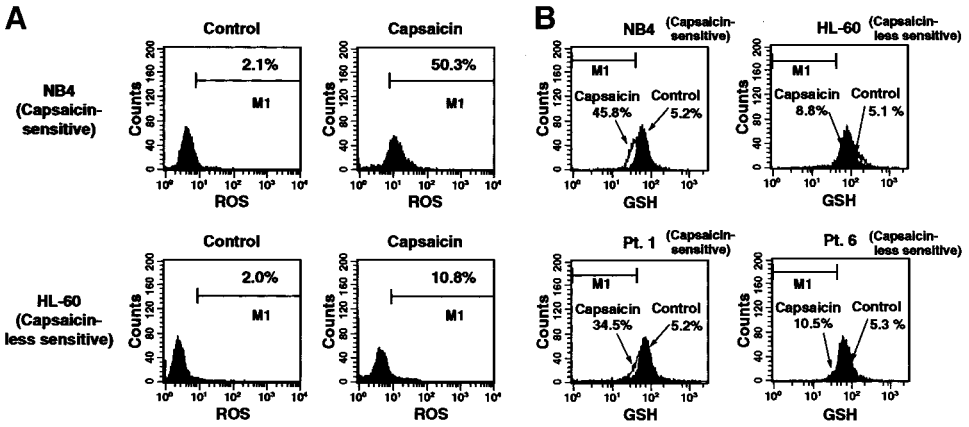

Fig. 7. Sensitivity to capsaicin in various leukemic cell lines and primary cells from patients. Intracellular levels of reactive oxygen species (ROS; *A*) and glutathione (GSH; *B*) were measured by flow cytometry in representative capsaicin-sensitive (NB4) and -less sensitive (HL-60) cell lines. *Pt.*, patient.

demonstrated that NAC also prevented capsaicin-less sensitive cells from apoptosis induced by a high dose of capsaicin (data not shown). Recent studies have reported that ROS is not only an upstream activator of the p53 pathway, but it is also a critical component of the downstream mediator of p53-dependent apoptosis, because overexpression of wild-type p53 produces ROS in association with apoptosis (55). The generation of ROS has been suggested to be a representative pathway of mitochondrial disruption in a p53-independent manner (56). It is likely that overgeneration of ROS plays some role in capsaicin-induced mitochondrial depolarization and apoptosis in p53-defective cells (57). Our data indicate that H_2O_2 can be a specific ROS species that plays mainly an essential role in capsaicin-induced p53 activation. Taken together, these data strongly indicate the existence of the following two downstream pathways that reduce $\Delta\psi m$ originating from H_2O_2 production by capsaicin: (*a*) one rapidly and with high sensitivity phosphorylatin p53 at the Ser-15 residue, leading to transportation of Bax to mitochondria, loss of $\Delta\psi m$, and early phase apoptosis in capsaicin-sensitive leukemic cells and (*b*) a second inducing a direct disruption of $\Delta\psi m$ by H_2O_2 generation independent of p53 in p53-defective cells but with less sensitivity to capsaicin. Additional studies are needed to clarify the exact mechanism of capsaicin-induced apoptosis in leukemic cells.

The therapeutic approach to acute leukemia is usually chemotherapy, but severe side effects and complications such as serious infection and bleeding because of anticancer drugs are major problems in the clinical setting. In particular, the side effects of drugs might be fatal in older patients or in immunocompromised patients, which highlights the urgent need for novel effective and less toxic therapeutic approaches. A component of the hot pepper Capsicum, capsaicin, is a natural compound and widely consumed as a food additive throughout the world, which indicates that it is less toxic to humans than current chemotherapeutic drugs. Because we could not observe any organ damage during *in vivo* experiments using a NOD/SCID mice leukemia model and because capsaicin did not affect cellular proliferation of normal bone marrow cells from healthy volunteers, we conclude that capsaicin might be developed as a new potent anticancer agent for the management of hematological malignancies.

In summary, we propose a model of cell cycle arrest and apoptosis induced by capsaicin through an oxidative stress in leukemic cells. Our data strongly indicate that capsaicin is particularly sensitive to leukemic cells expressing wild-type p53, which phosphorylated at Ser-15 residue by producing ROS during the treatment of capsaicin. Therefore, homovanillic acid derivative, capsaicin, has potential as a novel molecular targeted therapeutic agent against serine residue of the p53 gene for the treatment of leukemia, particularly in elderly and immunocompromised patients.

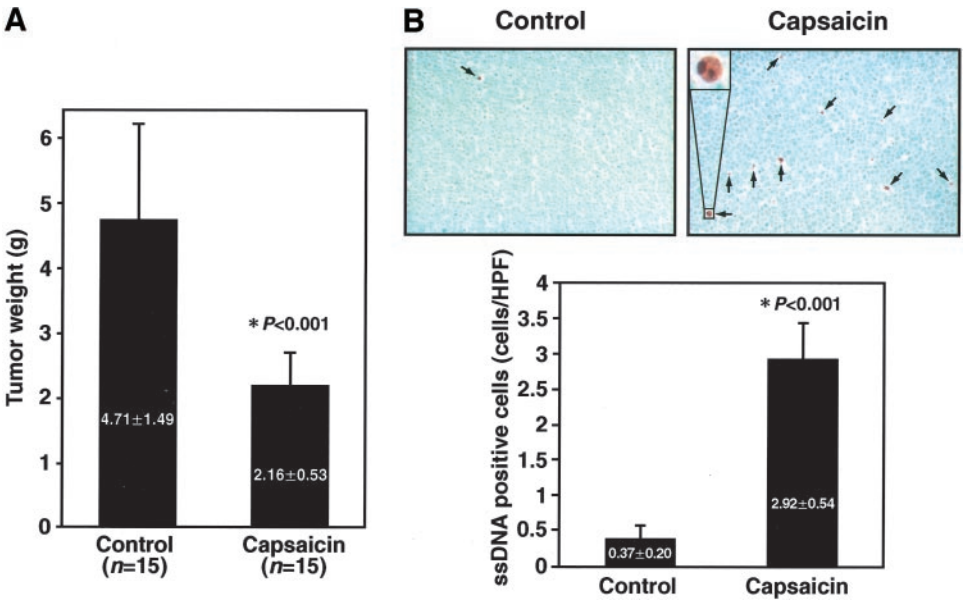

Fig. 8. Capsaicin-mediated apoptosis of leukemic cells *in vivo* using a NOD/SCID mice model. *A*, NB4 cells (1×10^7 cells) were inoculated s.c. into NOD/SCID mice. Fourteen days after transplantation, 5% ethanol (control; $n = 15$) or capsaicin (15 mg/kg; $n = 15$) was given daily for 6 days, after which mice were sacrificed and tumor weights were measured. *B*, the tumor sections were fixed and stained with anti-single-strand (ss)DNA antibody. We counted the apoptotic cells in the corresponding fields of control and capsaicin-treated tumor sections (10 sections/mouse, $n = 15$). *Arrows* indicate single-strand DNA (ssDNA)-positive cells. Original magnification, ×400. *HPF*, high power field.

REFERENCES

1. Monsereenusorn, Y., Kongsamut, S., and Pezalla, P. D. Capsaicin–a literature survey. Crit. Rev. Toxicol., *10:* 321–339, 1982.
2. Lopez-Carrillo, L., Hernandez Avila, M., and Dubrow, R. Chilli pepper consumption and gastric cancer in Mexico: a case-control study. Am. J. Epidemiol., *139:* 263–271, 1994.
3. Venkat, J. A., Shami, S., Davis, K., Nayak, M., Plimmer, J. R., Pfeil, R., and Nair, P. P. Relative genotoxic activities of pesticides evaluated by a modified SOS microplate assay. Environ. Mol. Mutagen., *25:* 67–76, 1995.
4. Richeux, F., Cascante, M., Ennamany, R., Sabourеau, D., and Creppy, E. E. Implication of oxidative stress and inflammatory process in the cytotoxicity of capsaicin in human endothelial cells: lack of DNA strand breakage. Toxicology, *147:* 41–49, 2000.
5. Surh, Y. J., and Lee, S. S. Capsaicin, a double-edged sword: toxicity, metabolism, and chemopreventive potential. Life Sci., *22:* 1845–1855, 1995.
6. Park, K. K., and Surh, Y. J. Effects of capsaicin on chemically-induced two-stage mouse skin carcinogenesis. Cancer Lett., *114:* 183–184, 1997.
7. Yun, T. K. Update from Asia: Asian studies on cancer chemoprevention. Ann. N. Y. Acad. Sci., *889:* 157–192, 1999.
8. Zhang, Z., Hamilton, S. M., Stewart, C., Strother, A., and Teel, R. W. Inhibition of liver microsomal cytochrome P-450 activity and metabolism of the tobaccospecific nitrosamine NNK by capsaicin and ellagic acid. Anticancer Res., *13:* 2341–2346, 1993.
9. Miyashita, T., and Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. Cell, *80:* 293–299, 1995.
10. Toth, B., Rogan, E., and Walker, B. Tumorigenicity and mutagenicity studies with capsaicin of hot peppers. Anticancer Res., *4:* 117–119, 1984.
11. Agrawal, R. C., and Bhide, S. V. Biological studies on carcinogenicity of chillies in Balb/c mice. Indian J. Med. Res., *86:* 391–396, 1987.
12. Surh, Y. J., Lee, B. C., Park, K. K., Mayne, S. T., Liem, A., and Miller, J. A. Chemoprotective effects of capsaicin and diallyl sulfide against mutagenesis or tumorigenesis by vinyl carbamate and *N*-nitrosodimethylamine. Carcinogenesis (Lond.), *16:* 2467–2471, 1995.
13. Modly, C. E., Das, M., Don, P. S. C., Marcelo, C. L., Mukhtar, H., and Bickers, D. R. Capsaicin as an *in vitro* inhibitor of benzo(a)pyrene metabolism and its DNA binding in human and murine keratinocytes. Drug Metab. Dispos., *14:* 413–416, 1986.
14. Teel, R. W. Effects of capsaicin on rat liver S9-mediated metabolism and DNA binding of aflatoxin. Nutr. Cancer, *15:* 27–32, 1991.
15. Richeux, F., Cascante, M., Ennamany, R., Sabourеau, D., and Creppy, E. E. Cytotoxicity and genotoxicity of capsaicin in human neuroblastoma cells SHSY-5Y. Arch. Toxicol., *73:* 403–409, 1999.
16. Lee, S. Y., Nam, D. H., and Kim, J. A. Induction of apoptosis by capsaicin in A172 human glioblastoma cell. Cancer Lett., *161:* 121–130, 2001.
17. Jung, M. Y., Kang, H. J., and Moon, A. Caspase-induced apoptosis in SK-Hep-1 hepatocarcinoma cells involves bcl-2 downregulation and caspase-3 activation. Cancer Lett., *165:* 139–145, 2001.
18. Ko, L. J., and Prives, C. p53: puzzle and paradigm. Genes Dev., *10:* 1054–1072, 1996.
19. Shieh, S. Y., Taya, Y., and Prives, C. DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser 20, requires tetramerization. EMBO J., *18:* 1815–1823, 1999.
20. Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell, *91:* 325–334, 1997.
21. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. The *mdm-2* oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell, *69:* 1237–1245, 1992.
22. Levine, A. J. p53, the cellular gatekeeper for growth and division. Cell, *88:* 323–331, 1997.
23. Jimenez, G. S., Khan, S. H., Stommel, J. M., and Wahl, G. M. p53 regulation by post-translational modification and nuclear retention in response to diverse stresses. Oncogene, *18:* 7656–7665, 1999.
24. Ashcroft, M., and Vousden, K. H. Regulation of p53 stability. Oncogene, *18:* 7637–7643, 1999.
25. Lakin, N. D., and Jackson, S. P. Regulation of p53 in response to DNA damage. Oncogene, *18:* 7644–7655, 1999.
26. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science (Wash. DC), *281:* 1677–1679, 1998.
27. Nakagawa, K., Taya, Y., Tamai, K., and Yamaizumi, M. Requirement of ATM in phosphorylation of the human p53 protein at serine 15 following DNA double-strand breaks. Mol. Cell. Biol., *19:* 2828–2834, 1999.
28. Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L., and Pelicci, P. G. The $p66^{shc}$ adaptor protein controls oxidative stress response and life span in mammals. Nature (Lond.), *402:* 309–313, 1999.
29. Li, P. F., Dietz, R., and von Harsdorf, R. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. EMBO J., *18:* 6027–6036, 1999.
30. Xie, S., Wu, H., Cogswell, J., Lu, L., Jhanwar-Uniyal, M., and Dai, W. Reactive oxygen species-induced phosphorylation of p53 on serine 20 is mediated in part by polo-like kinase-3. J. Biol. Chem., *276:* 36194–36199, 2001.
31. Banin, S., Moyal, L., Shieh, S. Y., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science (Wash. DC), *281:* 1674–1677, 1998.
32. Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev., *13:* 152–157, 1999.
33. Lanotte, M., Martin-Thouvenin, V., Njiman, S., Balerini, P., Valensi, F., and Berger, R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood, *77:* 1080–1086, 1991.
34. Asou, H., Tashiro, S., Hamamoto, S., Otsuji, A., Kita, K., and Kamada, N. Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. Blood, *77:* 2031–2036, 1991.
35. Kizaki, M., Matsushita, H., Takayama, N., Muto, A., Ueno, H., Awaya, N., Kawai, Y., Asou, H., Kamada, N., and Ikeda, Y. Establishment and characterization of a novel acute promyelocytic leukemia cell line (UF-1) with retinoic acid-resistant features. Blood, *88:* 1824–1833, 1996.
36. Fukuchi, Y., Kizaki, M., Kinjo, K., Awaya, N., Ito, M., Kawai, Y., Umezawa, A., Hata, J., Ueyama, Y., and Ikeda, Y. Establishment of a retinoic acid resistant acute promyelocytic leukemia model in hGM-CSF transgenic SCID mice. Br. J. Cancer, *78:* 878–884, 1998.
37. Workman, P., Balman, A., Hickman, J. A., McNally, N. J., Mitchison, N. A., Pierepoint, C. G., Raymond, R., Rowlatt, C., Stephens, T. C., and Wallace, J. UKCCCR guidelines for the welfare of animals in experimental neoplasia. Br. J. Cancer, *58:* 109–113, 1998.
38. Naruse, I., Keino, H., and Kawarada, Y. Antibody against single-strand DNA detects both programmed cell death and drug-induced apoptosis. Histochemistry, *101:* 73–78, 1994.
39. Wolf, D., and Rotter, V. Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. Proc. Natl. Acad. Sci. USA, *82:* 790–794, 1985.
40. Neubauer, A., He, M., Schmidt, C. A., Huhn, D., and Liu, E. T. Genetic alterations in the p53 gene in the blast crisis of chronic myelogenous leukemia: analysis by polymerase chain reaction based techniques. Leukemia (Baltimore), *7:* 593–600, 1993.
41. Feinstein, E., Cimino, G., Gale, R. P., Alimena, G., Berthier, R., Kishi, K., Goldman, J., Zaccaria, A., Berrebi, A., and Canaani, E. p53 in chronic myelogenous leukemia in acute phase. Proc. Natl. Acad. Sci. USA, *82:* 6293–6297, 1991.
42. Sugimoto, K., Toyoshima, H., Sakai, R., Miyagawa, K., Hagiwara, K., Ishikawa, F., Takaku, F., Yazaki, Y., and Hirai, H. Frequent mutations in the p53 gene in human myeloid leukemia cell lines. Blood, *79:* 2378–2383, 1992.
43. Morre, D. J., Chueh, P-J., and Morre, D. M. Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. Proc. Natl. Acad. Sci. USA, *92:* 1831–1835, 1995.
44. Lee, Y. S., Nam, D. H., and Kim, J-A. Induction of apoptosis by capsaicin in A172 human glioblastoma cells. Cancer Lett., *161:* 121–130, 2000.
45. Fleckenstein, D. S., Uphoff, C. C., Drexler, H. G., and Quentmeier, H. Detection of p53 gene mutations by single strand conformational polymorphism in human acute myeloid leukemia-derived cell lines. Leuk. Res., *26:* 207–214, 2002.
46. Fenaux, P., Jonveaux, P., Quiquandon, I., Lai, J. L., Pignon, J. M., Loucheux-Lefebvre, M. H., Bauters, F., Berger, R., and Kerckaert, J. P. p53 gene mutations in acute myeloid leukemia with 17p monosomy. Blood, *78:* 1652–1657, 1991.
47. Fenaux, P., Preudhomme, C., Quiquandon, I., Jonveaux, P., Lai, J. L., Vanrumbeke, M., Loucheux-Lefebvre, M. H., Bauters, F., Berger, R., and Kerckaert, J. P. Mutations of the p53 gene in acute myeloid leukaemia. Br. J. Haematol., *80:* 178–183, 1992.
48. Hu, G., Zang, W., and Deisseroth, A. B. p53 gene mutations in acute myelogenous leukaemia. Br. J. Haematol., *81:* 489–194, 1992.
49. Wattel, E., Preudhomme, C., Hecquet, B., Vanrumbeke, M., Quesnel, B., Dervite, I., Morel, P., and Fenaux, P. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. Blood, *84:* 3148–3157, 1994.
50. Sarkaria, J. N., Bushby, E. C., Tibbetts, R. S., Roos, P., Taya, Y., Karnitz, L. M., and Abraham, R. T. Inhibition of phosphoinositide 3-kinase related kinase by the radiosensitizing agent wortmannin. Cancer Res., *58:* 4375–4382, 1998.
51. Green, D. R., and Reed, J. C. Mitochondria and apoptosis. Science (Wash. DC), *281:* 1309–1312, 1998.
52. Troyano, A., Fernandez, C., Sancho, P., de Blas, E., and Aller, P. Effect of glutathione depletion on antitumor drug toxicity (apoptosis and necrosis) in U937 human promonocytic cells. The role of intracellular oxidation. J. Biol. Chem., *276:* 47107–47115, 2001.
53. Palozza, P., Serini, S., Torsello, A., Boninsegna, A., Covacci, V., Maggiano, N., Ranelletti, F. O., Wolf, F. I., and Calviello, G. Regulation of cell cycle progression and apoptosis by β-carotene in undifferentiated and differentiated HL-60 leukemia cells: possible involvement of redox mechanism. Int. J. Cancer, *97:* 593–600, 2000.
54. Huang, P., Feng, P., Oldham, E. A., Keating, M. J., and Plunkett, W. Superoxide dismutases as a target for the sensitive killing of cancer cells. Nature (Lond.), *407:* 390–395, 2000.
55. Johnson, T. M., Yu, Z. X., Ferrans, V. J., Lowenstein, R. A., and Finkel, T. Reactive oxygen species are downstream mediators of p53-dependent apoptosis. Proc. Natl. Acad. Sci. USA, *93:* 11848–11852, 1996.
56. Ye, J., Wang, S., Leonard, S. S., Sun, Y., Butterworth, L., Antonini, J., Ding, M., Rojanasakul, Y., Vallyathan, V., Castranova, V., and Shi, X. Role of reactive oxygen species and p53 in chromium (VI)-induced apoptosis. J. Biol. Chem., *274:* 34974–34980, 1999.
57. Vrablic, A. S., Albright, C. D., Craciunescu, C. N., Salganik, R. I., and Zeisel, S. H. Altered mitochondrial function and overgeneration of reactive oxygen species precede the induction of apoptosis by 1-*O*-octadecyl-2-methyl-rac-glycero-3-phosphocholine in p53-defective hepatocytes. FASEB J., *15:* 1739–1744, 2001.