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Inhibition by Differentiation-inducing Agents of Wild-type p53-dependent Apoptosis in HL-60 Cells

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The product of the p53 tumor-suppressor gene has been shown to function in apoptosis and cell cycle regulation. However, there is little information regarding the regulation of apoptosis in cell differentiation. We investigated the relationship between p53-dependent apoptosis and differentiation induction using human promyelocytic leukemia HL-60 cells transfected with pMAMneo expression vectors containing dexamethasone-inducible wild-type p53 (wt-p53) cDNA inserts. Continuous exposure of the pMAMneo/wt-p53 transfectants to 1 μM dexamethasone for more than 24 h caused overexpression of wt-p53 followed by cell death with morphological changes typical of apoptosis. Using the wt-p53-inducible HL-60 cells, we examined the effects of differentiation inducers on the wt-p53-dependent apoptosis. All-*trans* retinoic acid (all-*trans* RA) at 1 nM or granulocyte macrophage colony-stimulating factor (GM-CSF) at 35 pM inhibited the wt-p53-induced apoptosis over a 42-h treatment. The apoptosis inhibition by GM-CSF, but not all-*trans* RA, was abolished by specific inhibitors of protein kinase C. These results suggest that extracellular signals involved in the differentiation induction could modulate the wt-p53-dependent apoptosis through protein kinase C-dependent and independent pathways.

Key words: Apoptosis — p53 — GM-CSF — Retinoic acid — Protein kinase C inhibitor

Apoptosis in cancer cells can be induced by a variety of external or internal signals, such as glucocorticoid treatment,[1] withdrawal of growth factors,[2] treatment with antibodies against Fas antigen,[3] overexpression of c-*myc*,[4] p53,[5–7] or c-*rel*,[8] and conversely can be inhibited by *bcl*-2 overexpression.[9] Of the apoptosis activators, the product of the p53 tumor-suppressor gene has been clearly shown to be responsible for directing apoptosis, which suggests that apoptosis is a mechanism of the cellular defense against cancer development.[10] On the other hands, normal cells are usually under the control of differentiation. Therefore, both cell differentiation and apoptosis are thought to be mechanisms for homeostasis.[11]

However, there has been little information concerning relationships between apoptosis and cell differentiation. It therefore seemed interesting to study the relationship between p53 expression involved in apoptosis induction and the external signals that affect cell differentiation. In the present study, we established wt-p53-inducible HL-60 cell clones and investigated the effects of differentiation-inducing agents, such as all-*trans* retinoic acid (all-*trans* RA) and granulocyte macrophage colony-stimulating factor (GM-CSF), on p53-induced apoptosis.

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MATERIALS AND METHODS

Reagents Dexamethasone, all-*trans* RA and geneticine were purchased from Sigma Chemical Company (St. Louis, MO). Plasmid vector pMAMneo was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Recombinant human GM-CSF was a generous gift from Kirin Brewery Co., Ltd. (Tokyo). Calphostin C was kindly provided by Dr. T. Tamaoki, Kyowa Hakko Co., Ltd. (Tokyo). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) was purchased from Seikagaku Corp. (Tokyo). Anti-p53 monoclonal antibodies were purchased from Oncogene Science, Inc. (Seattle, WA).

Cells and DNA transfection HL-60 cells and wt-p53 transfectants were cultured in RPMI 1640 medium with 15% heat-inactivated fetal bovine serum. Wt-p53 cDNA was obtained from WI-38/VA13 cells by polymerase chain reaction cloning,[12,13] and this cDNA was inserted into pMAMneo, a dexamethasone-inducible expression vector. pMAMneo and pMAMneo/wt-p53 were linearized by *Nde*I digestion and were transfected into HL-60 cells by electroporation. Among the geneticine-resistant cells obtained, wt-p53 cell clones inducible by dexamethasone treatment were screened and more stable subclones, named 6A4, 11B2 and B517S2, were selected. In the standard p53-induction experiment, cells were seeded at $1–5\times10^5$ cells/ml and dexamethasone was

added at a final concentration of 0.1–1 μM. Cell viability was determined by trypan blue dye exclusion assay.

Identification of apoptotic cells After an appropriate treatment with dexamethasone, cells were cytocentrifuged, fixed with methanol, stained with Wright-Giemsa according to the standard method, analyzed for apoptotic morphology on a light microscope Nikon Optiphoto (Nikon, Tokyo), and photographed with a Nikon FX-35A camera.

DNA fragmentation assay Equivalent amounts of DNA were electrophoresed in 2% agarose gel containing ethidium bromide (0.25 μg/ml) in 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8) at 50 V. DNA was visualized by an ultraviolet transilluminator (Atto Corp., Tokyo) and photographed with a Polaroid camera equipped with Polapan type 667 films (Polaroid, UK).

Quantitation of fragmented DNA Fragmented and intact DNAs were quantitated according to the method of Colotta *et al.*[14] with some modifications. Briefly, 10^6 cells were harvested and lysed in a lysis buffer (0.2% Triton X-100, 10 mM EDTA, 10 mM Tris-HCl, pH 7.5). Fragmented DNA and intact DNA were separated by brief centrifugation, and were subjected to trichloroacetic acid (TCA) precipitation. DNA in TCA-insoluble fractions was extracted with NaOH/perchloric acid, mixed with diphenylamine reagent[15] and then incubated at 37°C overnight. Reaction solutions were transferred to flat-bottomed 96-well plates, and DNA was quantitated by measuring absorbance at 595 nm on a microplate reader (Bio-Rad, Model 450). The DNA fragmentation ratio was calculated by means of the following formula: DNA fragmentation ratio = recovered low-molecular-weight DNA/total DNA (low-molecular-weight DNA + intact DNA). Quantitation experiments were performed in triplicate.

Southern blot analysis Aliquots of 10 μg of DNA were digested with *Bam*HI restriction enzyme followed by ethanol precipitation. Digested DNA was electrophoresed in 0.8% agarose gel and blotted on a nylon membrane. The blotted DNA was hybridized with ^{32}P-labeled, random-primed p53 cDNA probes and analyzed by autoradiography using X-Omat film (Kodak, Rochester, NY).

Western blot analysis of p53 Sample proteins were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted on a nitrocellulose membrane. The blotted membrane was hybridized with an anti-p53 antibody, PAb1801 (final 0.1 μg/ml). After washing, the membrane was hybridized with horseradish peroxidase-conjugated anti-mouse IgG polyclonal antibodies. Antibody-hybridized antigens were detected with an ECL detection system (Amersham International plc, Little Chalfont, UK) and recorded by 1- to 10-min exposures on Kodak X-Omat film.

Inhibition study with protein kinase C inhibitors B517S2 cells were pretreated for 20 h with or without 35 pM GM-CSF or 1 nM all-*trans* RA in the presence of 1 μM dexamethasone, and then 10 nM calphostin C or 15 μM H-7 was added. Cells were further incubated for 24 h, and cell viability was determined by trypan blue dye exclusion assay.

RESULTS

Isolation and characterization of wt-p53 inducible HL-60 cells The human promyelocytic leukemia cell line HL-60 lacks the endogenous p53 gene and hence expresses no p53 protein.[16] We constructed an expression vector, pMAMneo, containing the wt-p53 cDNA under the dexamethasone-inducible promoter and transfected HL-60 cells with the vector. We obtained more than 300 clones of geneticine-resistant transfectants and selected three p53-inducible cell clones with stable phenotypes, namely 6A4, 11B2 and B517S2.

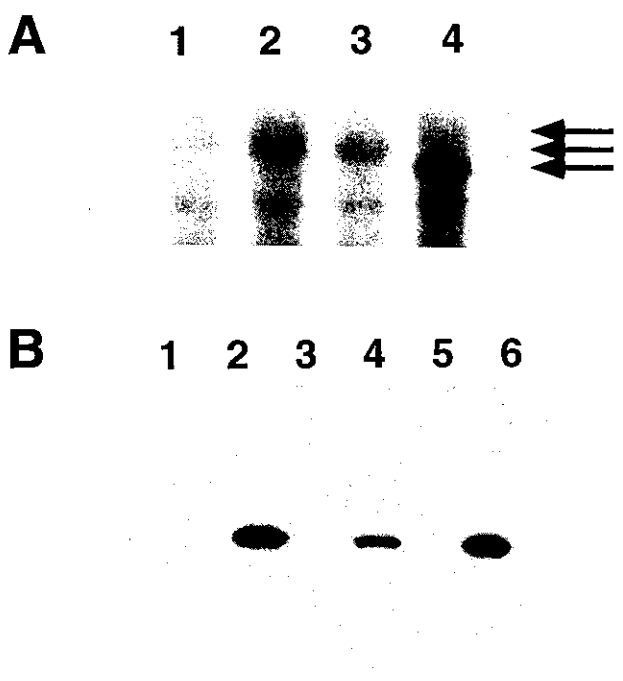

Fig. 1. (A) Southern blot analysis of wt-p53 transfectants. Lane 1, HL-60; lane 2, wt-p53 transfectant clone 6A4; lane 3, clone 11B2; lane 4, clone B517S2. Arrows indicate the exogenous p53 gene specifically detected in the transfectants. (B) Western blot analysis of wt-p53 protein. Whole cell lysates were prepared from wt-p53-induced cells after treatment with 1 μM dexamethasone for 20 h (lanes 2, 4 and 6) or uninduced (lanes 1, 3 and 5). Lanes 1 and 2, clone 6A4; lanes 3 and 4, clone 11B2; lanes 5 and 6, clone B517S2.

Southern blot analysis showed that integrated exogenous p53 cDNAs were detected at different sizes in each of the three clones (6A4 at 18.5 kilobase pair (kbp), 11B2 at 15.5 kbp and B517S2 at 9 kbp) (Fig. 1A), indicating that each clone was independent and contained a single copy of the exogenous p53 cDNA. The B517S2 clone produced the highest level of the wt-p53 protein among the three (Fig. 1B) and exhibited the highest stability for wt-p53 induction, as well as good viability after freezing and thawing. We, therefore, focused on the B517S2 clone for differentiation induction experiments.

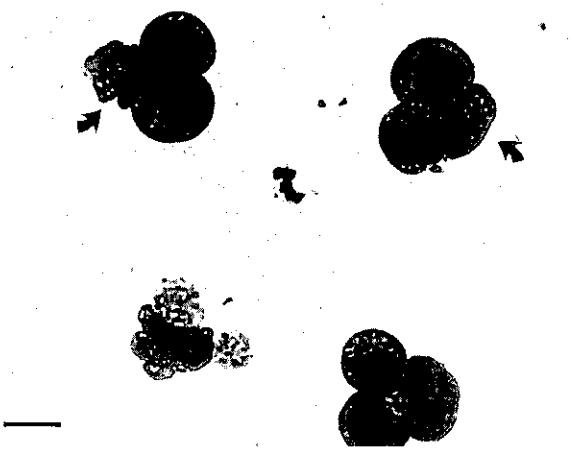

Fig. 2. Morphology of apoptotic cell death after wt-p53 induction. Clone B517S2 cells (pMAMneo/wt-p53 transfected) were cultured for 68 h with 1 μM dexamethasone, subjected to Wright-Giemsa staining and photographed. Arrows indicate apoptotic cells. Bar, 33 μm.

The continuous expression of wt-p53 reduced cell viability but did not induce differentiation toward granulocytes or macrophages. However, a portion of the B517S2 cells that expressed high levels of the wt-p53 protein showed typical apoptotic morphology, characterized by marked reduction in cell size, prominent chromatin condensation and fragmented nuclei (Fig 2). DNA from the dexamethasone-treated wt-p53 transfectants demonstrated a typical apoptotic DNA fragment pattern corresponding to multimers of nucleosome-size DNA (Fig. 3, lanes 2, 4 and 6). These morphological and biochemical examinations confirmed that the wt-p53-induced cell death in HL-60 cells was, indeed, apoptosis.

We further examined in detail the relationship between wt-p53 expression level and cell death under the fine control of dexamethasone concentration (Fig. 4). When clone B517S2 cells were treated with 0.01 μM dexamethasone, the expression level of wt-p53 was very low (Fig. 4B, lane 1), and cell death rarely occurred (Fig. 4A). On the other hand, at 0.1 μM dexamethasone, the expression of wt-p53 reached the maximum level after a 20-h treatment (Fig. 4B, lanes 5 and 6) and apoptosis was induced as frequently as by the treatment with 1 μM dexamethasone (Fig. 4A). Only transient overexpression of the wt-p53 for less than 20 h was insufficient to induce apoptosis (data not shown). The results indicated a good correlation between the level of continuous wt-p53 expression and the frequency of cell death. Further overexpression of wt-p53 may be needed to induce complete apoptosis in HL-60 cells.

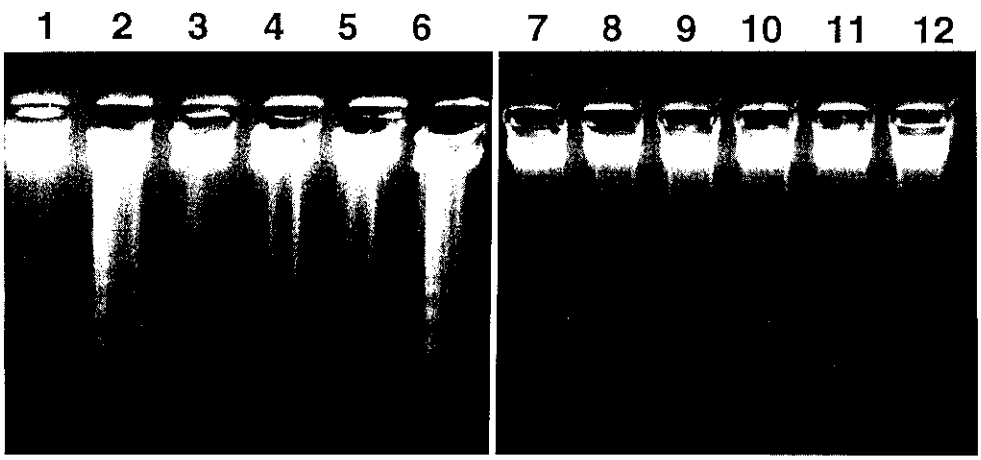

Fig. 3. Induction of DNA fragmentation by continuous expression of wt-p53. Three wt-p53 inducible clones (6A4, 11B2 and B517S2), two mock-transfected clones (H6 and E2), and parental HL-60 cells were treated with 1 μM dexamethasone (lanes 2, 4, 6, 8, 10, and 12) or without (lanes 1, 3, 5, 7, 9, and 11) for 68 h. Lanes 1 and 2, clone 6A4; lanes 3 and 4, clone 11B2; lanes 5 and 6, clone B517S2; lanes 7 and 8, HL-60; lanes 9 and 10, clone H6; lanes 11 and 12, clone E2.

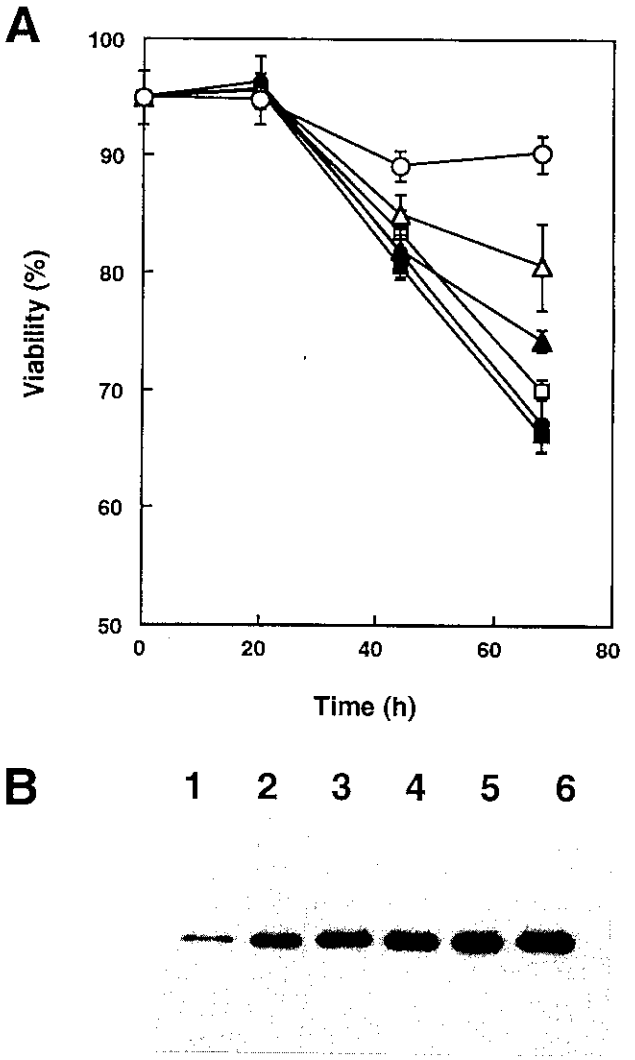

Fig. 4. Correlation of wt-p53 expression level and apoptosis. (A) Cell viability change on treatment with dexamethasone at various concentrations. Clone B517S2 cells were cultured in the presence of dexamethasone, and at intervals cell viability was determined by trypan blue dye exclusion assay. Concentrations of dexamethasone: 1 μM (closed circles), 0.1 μM (closed squares), 0.06 μM (open squares), 0.04 μM (closed triangles), 0.02 μM (open triangles), 0.01μM (open circles). (B) Expression levels of wt-p53 proteins induced by dexamethasone at various concentrations. After a 20-h incubation with various concentrations of dexamethasone, wt-p53 was detected by western blot analysis. Concentrations of dexamethasone; lane 1, 0.01 μM; lane 2, 0.02 μM; lane 3, 0.04 μM; lane 4, 0.06 μM; lane 5, 0.1 μM; lane 6, 1 μM.

Influence of all-*trans* RA and GM-CSF on wt-p53-dependent apoptosis It is known that the exposure of HL-60 cells to all-*trans* RA or GM-CSF induces differentiation toward granulocytes.[17,18] Furthermore, all-*trans*

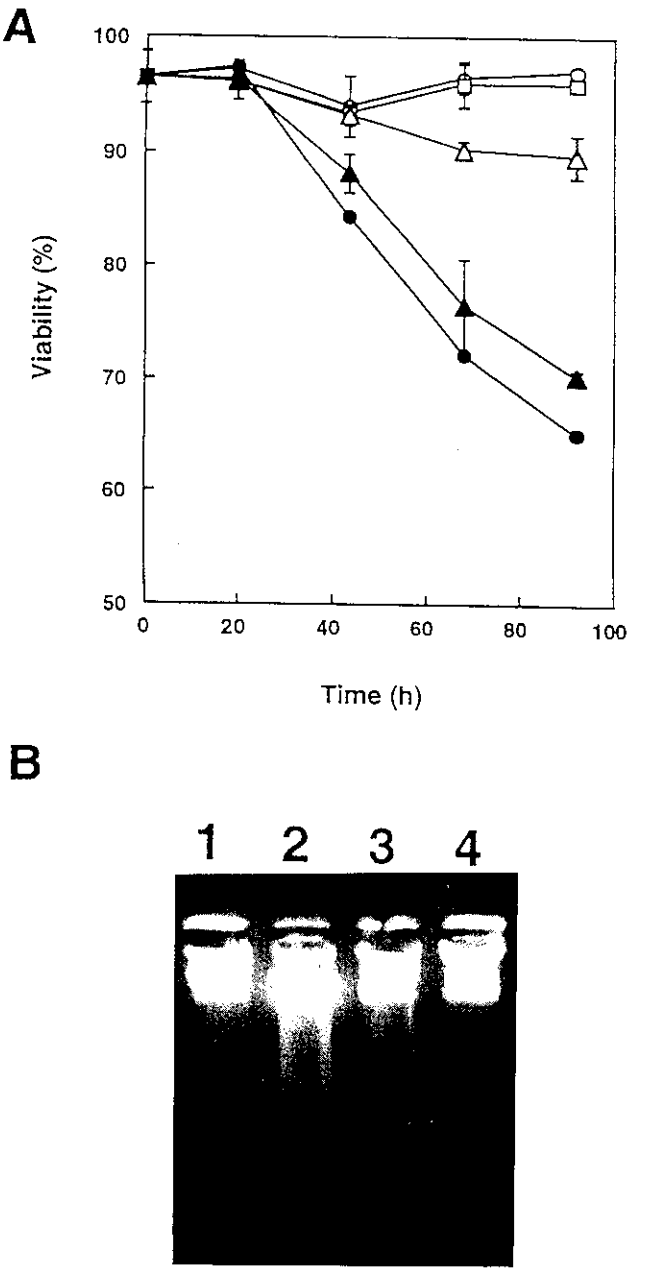

Fig. 5. Effects of all-*trans* RA and GM-CSF on wt-p53-induced apoptosis. (A) B517S2 cells were incubated with all-*trans* RA at various concentrations in the presence of 1 μM dexamethasone. Cell viability was determined at each time period by trypan blue dye exclusion assay. Concentration of all-*trans* RA: none (open circles), 1 μM dexamethasone (closed circles), 1 μM dexamethasone and 10 nM all-*trans* RA (open squares), 1 μM dexamethasone and 1 nM all-*trans* RA (open triangles), 1 μM dexamethasone and 0.1 nM all-*trans* RA (closed triangles). (B) After a 42-h incubation, B517S2 cells were harvested and 6 μg of each DNA was analyzed for fragmentation. Lane 1, none; lane 2, 1 μM dexamethasone; lane 3, 1 μM dexamethasone and 35 pM GM-CSF; lane 4, 1 μM dexamethasone and 1 nM all-*trans* RA.

RA induces not only differentiation but also apoptosis at terminal differentiation.[19] Therefore, the effects of all-*trans* RA and GM-CSF on wt-p53-dependent apoptosis were investigated using wt-p53-inducible cells. Treatment with all-*trans* RA prevented apoptosis induction by wt-p53 overexpression. The effect of all-*trans* RA was dose-dependent (Fig. 5A) and 1 n*M* all-*trans* RA was sufficient for inhibition of the DNA fragmentation caused by wt-p53 overexpression (Fig. 5B, lane 4). All-*trans* RA and GM-CSF induced differentiation toward granulocytes equally in the control cells and the wt-p53 transfectants in the absence or presence of dexamethasone (data not shown). GM-CSF treatment at 35 p*M* for 42 h also inhibited DNA fragmentation caused by wt-p53 overexpression in the presence of dexamethasone (Fig. 5B, lane 3). However, unlike in the all-*trans* RA treatment, the inhibitory effect of GM-CSF on apoptosis extended only up to 48 h and then apoptosis by overexpression of wt-p53 gradually occurred (data not shown). These inhibitory effects on the induction of DNA fragmentation were quantified by diphenylamine reaction.[15] DNA was extracted from each group of cells subjected to an appropriate treatment for 42 h, and the amount of fragmented DNA was determined by measuring the absorbance at 595 nm (Table I). This experiment confirmed that 14% of DNA was fragmented in the cells with only wt-p53 overexpressed, but in the cells treated further with all-*trans* RA or GM-CSF, DNA fragmentation decreased to 6%.

Table I. Quantitation of DNA Fragmentation

Cell clones and treatment	Released DNA fragments (% of total DNA)
B517S2 (pMAMneo/p53+)	
Control	4.0±0.3
0.1 μM Dex	14.7±0.4
0.1 μM Dex+35 p*M* GM-CSF	6.7±0.7
0.1 μM Dex+1 n*M* all-*trans*RA	6.4±1.4
H6 (pMAMneo)	
0.1 μM Dex	4.0±0.2

Cells were cultured for 44 h with appropriate treatment, and then examined for the percentage of DNA fragmentation, as described in "Materials and Methods." The data are mean± SD of triplicate experiments.

Effects of protein kinase C inhibitors on suppression of apoptosis by RA or GM-CSF It has been suggested that a signal transduction pathway through protein kinase C could be involved in an apoptosis mechanism.[20] As shown in Fig. 6, inhibitors specific for the protein kinase C regulatory domain, such as calphostin C and H-7, did not significantly influence apoptosis induction by wt-p53 overexpression. However, both calphostin C and H-7 were able to abrogate apoptosis inhibition by GM-CSF. But all-*trans* RA-mediated inhibition of apoptosis was unaffected by protein kinase C inhibitors (Fig. 6).

DISCUSSION

Our data indicated that sustained overexpression was required for apoptosis to be induced in HL-60 cells, and low levels of wt-p53 expression did not affect cell growth. Previous studies showed that wt-p53 expression alone or followed by irradiation caused growth suppression.[21-23] Because of the known functions of the wt-p53 protein,[24] we assume that specific conditions, such as cell type, wt-p53 expression level, and deficiency of mutant p53, may

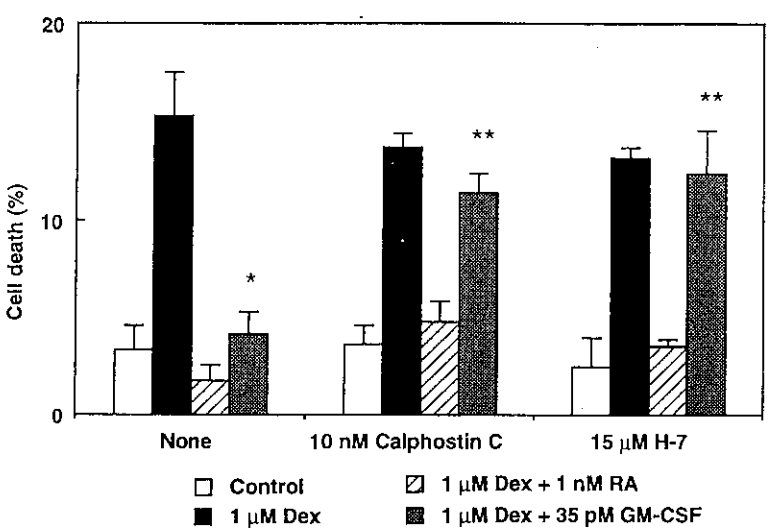

Fig. 6. Effect of protein kinase C inhibitors on apoptosis inhibition by GM-CSF or all-*trans* RA. B517S2 cells were pretreated for 20 h with dexamethasone (Dex) in the presence or absence of GM-CSF or all-*trans* RA and then treated for the following 24 h with 10 n*M* calphostin C or 15 μM H-7. Cell viability was determined by trypan blue dye exclusion assay. The levels of cell death in the presence of protein kinase C inhibitors (★★) were significantly higher than that in the absence of protein kinase C inhibitor (★) ($P<0.01$).

be essential for apoptosis induction in cancer cells. However, the functions of the wt-p53 protein have not been fully established yet, and neither have the effectors necessary for apoptosis induction been identified.

We have presented here data suggesting that differentiation inducers, such as all-*trans* RA and GM-CSF, inhibit wt-p53-induced apoptosis. GM-CSF has been reported to inhibit apoptosis induced by growth factor removal, and this apoptosis inhibition was abrogated by protein kinase C inhibitors.[20] We observed that protein kinase C inhibitors, such as H-7 and calphostin C at nontoxic concentrations[25] also suppressed the apoptosis-inhibitory effect of GM-CSF, but not that of all-*trans* RA. An activator of protein kinase C, 12-O-tetradecanoylphorbol-13-acetate (TPA) also inhibited the wt-p53-induced apoptosis (data not shown). Therefore, intracellular signals induced by GM-CSF may activate a protein kinase C pathway to inhibit apoptosis, but all-*trans* RA-mediated signals do not. Neither all-*trans* RA nor GM-CSF inhibited apoptosis induced in HL-60 cells by etoposide, an anti-cancer drug, or by serum deprivation (data not shown). Thus, the inhibitory effects of all-*trans* RA and GM-CSF on apoptosis may be specific to the wt-p53-dependent pathway. Yonish-Rouach *et al.*[5] have previously reported that the p53-induced apoptosis was inhibited by interleukin-6, which induces differentiation of M1 cells. Alternatively, apoptosis-inducing signals by wt-p53 overexpression could be regulated by extracellular signals involved in differentiation induction.

c-Myc is highly expressed in HL-60 cells by gene amplification,[26] and further overexpression of c-Myc could induce apoptosis.[4] However, there was no significant difference in the expression level of c-Myc proteins between control and wt-p53-overexpressing cells, even after a 42-h treatment (data not shown). Although Bcl-2 overexpression is known to block apoptosis,[9] all-*trans* RA or GM-CSF treatment did not enhance the Bcl-2 expression. All-*trans* RA rather reduced Bcl-2 expression, at least on 24-h treatment (data not shown). Thus, overexpression of c-Myc or Bcl-2 by itself may not be enough to explain the wt-p53 activity for apoptosis induction or the inhibitory effects of all-*trans* RA and GM-CSF in HL-60 cells.

The observations described here appear to contradict the previous report by Martin *et al.*[19] that all-*trans* RA caused terminal differentiation and apoptosis in HL-60 cells. In our experiments, apoptosis inhibition was caused by extremely low concentrations of all-*trans* RA. This inhibition occurred at an earlier stage than complete induction of terminal differentiation in spite of the decrement of Bcl-2 expression. Therefore, earlier events in differentiation induction may have a role in inhibiting apoptosis by overexpression of wt-p53.

The wt-p53 protein reportedly has transcriptional factor activity,[27] and p53 binding elements have been identified. The p53-binding sequence is not unique, and a loose consensus sequence (RRRCATGYYYRRRCATGYYY) exists.[28] This may explain why wt-p53 can induce several streams of genes, including WAF1[29] and GADD45,[24] involved in growth arrest. It is likely that other genes involved in apoptosis and differentiation are also induced by wt-p53. Alternatively, adequate positive signals for cell survival and differentiation may be required to compensate for wt-p53-induced negative signals, since the overexpression of wt-p53 is abnormal during cell growth. It is important to identify the molecules involved in such signal transductions. Further investigations using the wt-p53-inducible cells should help us to reach a better understanding of the role of the p53 protein in apoptosis and differentiation.

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