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Induction of Apoptosis by Quercetin: Involvement of Heat Shock Protein

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ABSTRACT

Quercetin, a widely distributed bioflavonoid, inhibits the growth of tumor cells. The present study was designed to investigate the possible involvement of apoptosis and heat shock protein in the antitumor activity of quercetin. Treatment with quercetin of K562, Molt-4, Raji, and MCAS tumor cell lines resulted in morphological changes, including propidium iodide-stained condensed nuclei (intact or fragmented), condensation of nuclear chromatin, and nuclear fragmentation. Agarose gel electrophoresis of quercetin-treated tumor cells demonstrated a typical ladder-like pattern of DNA fragments. In addition, the hypodiploid DNA peak of propidium iodide-stained nuclei was revealed by flow cytometry. Quercetin induced apoptosis in cells at G_1 and S in a dose- and time-dependent manner. The apoptosis-inducing activity of quercetin was enhanced by cycloheximide and actinomycin D. A nuclease inhibitor, aurintricarboxylic acid, inhibited quercetin-induced apoptosis, whereas deprivation of intracellular calcium by EGTA had no effect. 12-*O*-Tetradecanoylphorbol-13-acetate and H-7 did not affect the induction of apoptosis by quercetin. The synthesis of HSP70 was inhibited by quercetin when determined by immunocytochemistry, Western blot analysis, and Northern blot analysis. Quercetin-treated tumor cells were not induced to show aggregation of HSP70 in the nuclei and nucleolus in response to heat shock, resulting in apoptosis. By contrast, when tumor cells were first exposed to heat shock, no apoptosis was induced by quercetin. In addition, pretreatment of tumor cells with HSP70 antisense oligomer that specifically inhibited the synthesis of HSP70 enhanced the subsequent induction of apoptosis by quercetin. These results suggest that quercetin displays antitumor activity by triggering apoptosis and that HSP70 may affect quercetin-induced apoptosis.

INTRODUCTION

Cell death in a multicellular organism can occur by two distinct mechanisms, apoptosis or necrosis (1–3). The apoptosis plays an important role in embryonic development, metamorphosis, hormone-dependent atrophy, and tumor growth as a physiological event regulating the cell number or eliminating damaged cells (1–11). Cells undergoing apoptosis are characterized by reduced cell volume, condensed chromatin in the nucleus, formation of internucleosomal DNA fragmentation, and loss of membrane integrity, as well as generation of apoptotic bodies (1, 4, 7). The mechanism underlying this type of cell death is, however, not thoroughly understood (1, 3, 5–7, 9). Previous studies have shown that this cell death involves an active participation of the affected cell in its self-destruction via activation of specific genes and synthesis of new proteins (3, 5).

Recent studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents including cisplatin, cytarabine, camptothecin, amsacrine, etoposide, and teniposide (1, 2, 6, 10–13). There is accumulating evidence that the efficacy of antitumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis (1–3, 5, 6, 13, 14).

Quercetin, 3,3′,4′,5,7-pentahydroxy flavone, is one of most widely distributed bioflavonoids in the plant kingdom (15–20) and is a component of most edible fruits and vegetables. While humans consume approximately 1 g of flavonoid daily in the diet, quercetin is hardly absorbed and passes through the gastrointestinal tract. Quercetin inhibits the growth of malignant cells through various mechanisms: inhibition of glycolysis, macromolecule synthesis and enzymes; freezing cell cycle; and interaction with estrogen type II binding sites (15–20). In addition, the flavone inhibited the induction of heat shock proteins and thermotolerance without affecting other protein synthesis (15, 16, 18). The exact mechanisms responsible for the antitumor effect of quercetin, however, is not thoroughly understood yet. The present study was designed to investigate whether quercetin exerts cytotoxic activity against tumor cells by inducing apoptosis and to examine the possible role of heat shock proteins in the phenomenon.

MATERIALS AND METHODS

Agents. Quercetin was purchased from Nacalai Tesque (Kyoto, Japan). An inhibitor of the catalytic site of protein kinase H-7, [1-(5-isoquinolinesulfonyl)-2-methyl-piperazine dihydrochloride]; a protein kinase C activator, TPA;[2] a protein synthesis inhibitor, cycloheximide; an RNA synthesis inhibitor, actinomycin D; and a calcium chelator, EGTA; were obtained from Sigma Chemical Co. An endonuclease inhibitor, ATA, was from Kanto Chemical (Tokyo, Japan). Final concentrations of solvent (dimethyl sulfoxide) used were less than 0.2% and were not found to affect apoptosis.

Cell Culture and Quercetin Treatment. The K562 human chronic myeloid leukemia, Molt-4 acute T-lymphocytic leukemia, Raji Burkitt lymphoma, and MACS mucinous cystoadenocarcinoma of ovary cell lines were used in the present study. Exponentially growing cells were exposed to varying concentrations of quercetin for varying time intervals.

Treatment with Agents. Tumor cells were treated with 50 μg/ml cycloheximide, 10 μg/ml actinomycin D, 100 μM ATA, 0.1 mM TPA, 20 μM H-7, or 1 mM EGTA for 2 h prior to quercetin treatment. The above doses were effective in mediating their activity in preliminary experiments.

Assessment of Apoptosis. Cell viability was determined by a trypan blue dye exclusion test. Morphological analysis of apoptosis was performed after Wright-Giemsa staining under a light microscope and after staining with PI under fluorescence microscopy (4).

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described (11, 13). Briefly, cells (3×10^6) were lysed with 0.5 ml lysis buffer (5 mM Tris-HCL (pH 8), 0.25% Nonidet P-40, and 1 mM EDTA), followed by the addition of RNase A (Sigma) at a final concentration of 200 μg/ml, and incubated for 1 h at 37°C. Cells were then treated with 300 μg proteinase K/ml for an additional h at 37°C. After addition of 4 μl loading buffer, 20-μl samples in each lane were subjected to electrophoresis on a 1.5% agarose at 50 V for 3 h. DNA was stained with ethidium bromide.

Flow cytometric analysis was also performed to identify hypodiploid/apoptotic cells and to measure the percentage of hypodiploid cells after PI staining in hypotonic buffer as described (4, 6, 7, 13). Briefly, cell pellets were suspended in 1 ml hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma), and the cells were analyzed by the use of FACScan (Becton and Dickinson, Mountain View, CA) with Cell Fit software. Hypodiploid cells appeared in the cell cycle distribution as cells with DNA content less than G_1. In addition, cell cycle analysis was performed simultaneously.

Heat Shock and HSPs. Nuclease-resistant phosphorothioate oligodeoxynucleotides (antisense, sense, and nonsense) were synthesized automatically with 381 DNA synthesizer and purified by high-pressure liquid chromatography and reverse-phase chromatography as described (21). HSP70

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[2] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ATA, aurintricarboxylic acid; HSP, heat shock protein; ABC, avidin biotin complex; PI, propidium iodide.

antisense oligomer (5'-CGCGGCTTTGGCCAT-3') was complementary to the initiation codon and four downstream codons of human HSP70 mRNA (22). The corresponding sense oligomer (5'-ATGGCCAAAGCCGCG-3') and nonsense oligomer (5'-CGGGTATGCTTCGCC-3') were used as controls. The specific inhibition of HSP70 expression in tumor cells by this antisense oligomer was analyzed by Western blot, quantitative immunofluorescence, and Northern blot methods.

Intracellular distribution of HSP was analyzed by indirect immunoperoxidase staining of either single cell suspensions fixed on slides or on tissue culture chamber slides (23). Briefly, cells were fixed with paraformaldehyde, permeabilized with Triton X-100, washed, and incubated with glycine-containing phosphate-buffered saline. Monoclonal antibodies used as the first antibody in an ABC method were anti-HSP25 (clone IAP-9; Sigma), anti-HSP70 (clone BRM-22; Sigma), and anti-HSP90 (Funakoshi, Tokyo).

Western Blot Analysis. Western blot analysis was performed as described previously (24). Briefly, 5×10^6 cells were lysed in 1 ml lysis buffer, and the protein concentration was determined by the bicinchoninic acid protein assay reagent. The samples were denatured in sample buffer, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were electroblotted with Sartoblot onto a polyvinylidene difluoride membrane. The membrane blots were rinsed with TTBS [20 mM Tris, 500 mM NaCl, 0.05% Tween-20, (pH 7.5)] and blocked by 3% gelatin. The blots were incubated first with anti-HSP70 monoclonal antibody and then with a biotinylated second antibody, followed by a transfer to VECTASTAIN ABC. 3,3'-diaminobenzidine substrate kits for horseradish peroxidase (Vector Laboratories) were used for development of color.

Northern Blot Analysis. Total cellular RNA was isolated from tumor cells by guanidinium thiocyanate-calcium chloride method with minor modification, as described previously (15, 16). Equal amounts (20 μg) of total RNA were electrophoresed in formaldehyde-containing agarose gels, transferred to a nitrocellulose membrane, and hybridized with γ-^{32}P-labeled probes for HSP70 (Oncogene Science) or β-actin as an internal control.

RESULTS

Induction of Apoptosis by Quercetin. Treatment with quercetin of tumor cells resulted in morphological changes characteristic for apoptosis: a brightly red-fluorescent condensed nuclei (intact or fragmented) by fluorescence microscopy of PI-stained nuclei, blebbing, reduction of cell volume, condensation of nuclear chromatin, nuclear fragmentation, and apoptotic bodies (Fig. 1). Agarose gel electrophoresis of quercetin-treated cells demonstrated a ladder-like pattern of DNA fragments consisting of multiples of approximately 180–200 base pairs, consistent with internucleosomal DNA fragmentation (Fig. 2). The apoptosis-inducing effect of quercetin was dose- and time-dependent, being observed at 1 μM and reaching a maximum at 200 μM.

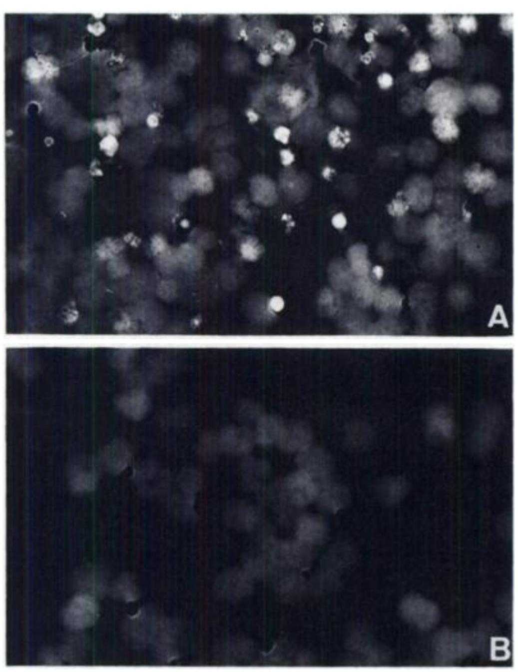

Fig. 1. Fluorescence microscopic appearance of PI-stained nuclei of quercetin-treated tumor cells. Raji cells were treated (*A*) or untreated (*B*) with 50 μM quercetin for 24 h, stained with PI, and examined under a fluorescence microscope (× 400).

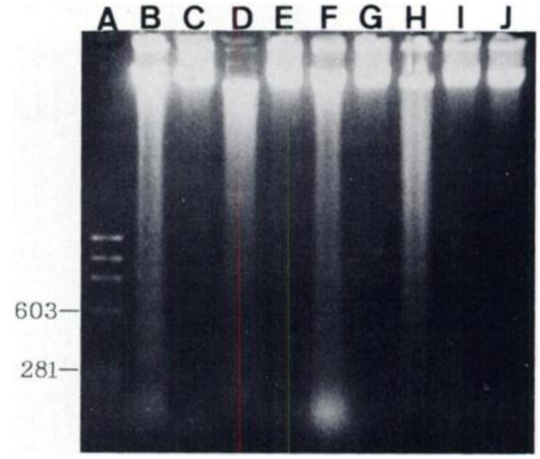

Fig. 2. Agarose gel electrophoretic patterns of DNA isolated from quercetin (50 μM; 48 h)-treated and untreated tumor cells. *Lane A*, marker. *Lane B*, quercetin-treated K562. *Lane C*, untreated K562. *Lane D*, quercetin-treated Molt-4. *Lane E*, Untreated Molt-4. *Lane F*, quercetin-treated MCAS. *Lane G*, untreated MCAS. *Lane H*, quercetin-treated Raji. *Lane I*, untreated Raji. *Lane J*, Raji treated with quercetin and ATA (100 μM).

By the use of flow cytometry, we could unequivocally assess the number of hypodiploid cells (apoptotic cells) and cells with diploid DNA content (nonapoptotic cells). Results obtained in flow cytometry strongly correlated with those in classical DNA fragmentation assays, DNA fragmentation in agarose gel electrophoresis, and cell counting with PI-staining fluorescence microscopy. A relative apoptotic index in flow cytometry varied from 0.91 to 0.96. Therefore, the quantitative assessment of hypodiploid cells by flow cytometry was used to estimate the number of apoptotic cells (Fig. 3).

Cell Cycle Specificity of Quercetin-induced Apoptosis. Cell cycle specificity of apoptosis induced by quercetin was analyzed by DNA fluorescence histogram. The number of G_1 and S cells among total cells (including apoptotic and nonapoptotic cells) decreased when hypodiploid cells increased in number by elevation of quercetin doses and prolongation of incubation time (Fig. 4). Similar results were obtained in all cell lines tested.

Effects of Various Agents on Quercetin-induced Apoptosis. Possible roles of protein and RNA synthesis in quercetin-induced apoptosis were considered. When tumor cells were first treated with cycloheximide or actinomycin D and then treated with quercetin for an additional 24 h in the presence of these agents, the number of hypodiploid cells increased in number (Table 1). The enhancement was dependent on doses of cycloheximide and actinomycin D (data not shown). Cycloheximide or actinomycin D alone also induced apoptosis. By contrast, an endonuclease inhibitor, ATA, inhibited the quercetin-induced apoptosis in a dose-dependent manner, as determined by flow cytometry and ethidium bromide-stained agarose gel analysis (Fig. 2). Deprivation of intracellular calcium by EGTA did not inhibit quercetin-induced apoptosis. Apoptosis induced by quercetin was not affected by an activator (TPA) or an inhibitor (H-7) of protein kinase C.

Roles of HSP70 in Quercetin-induced Apoptosis. In an attempt to explore the role of heat shock proteins in quercetin-induced apoptosis, we tested the effects of quercetin on the synthesis and intracellular distribution of heat shock proteins. When tumor cells were

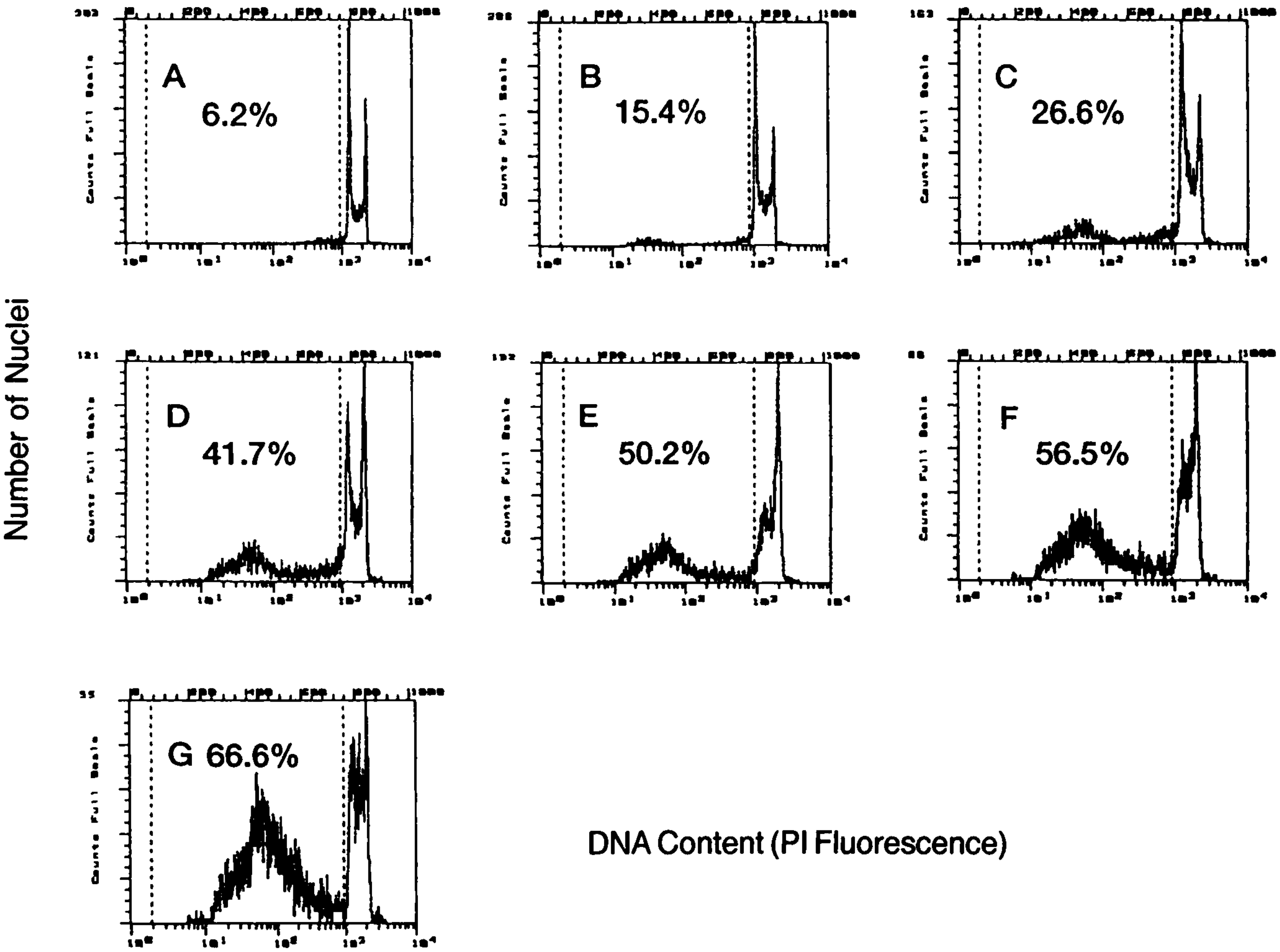

Fig. 3. DNA fluorescence histograms of PI-stained K562 in FL2-H. Cells were treated with varying doses of quercetin for 48 h. *A*, control. *B*, 1 μM. *C*, 25 μM. *D*, 50 μM. *E*, 100 μM. *F*, 150 μM. *G*, 200 μM.

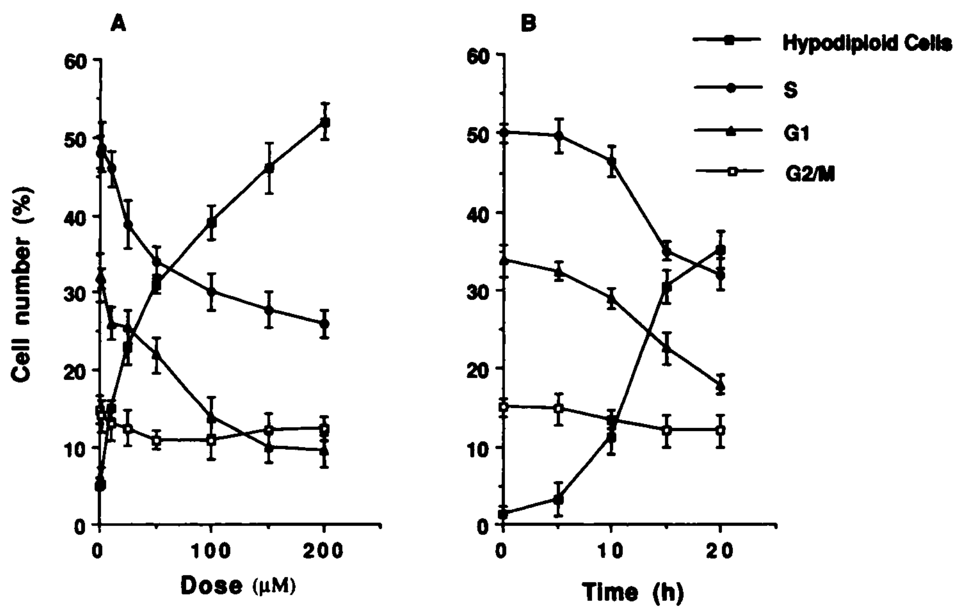

Fig. 4. Cell cycle specificity of quercetin-induced hypodiploid cells in DNA fluorescence histogram of FL2-A. K562 cells were treated with varying doses of quercetin for 20 h (*A*) or with 100 μM quercetin for varying time intervals (*B*). Results are expressed as means of triplicate samples; *bars*, SD.

treated with quercetin for at least 5 h and subsequently exposed to heat shock at 42°C, hypodiploid cells increased in number (Fig. 5). By contrast, the number of hypodiploid cells decreased when tumor cells were first heated and then treated with quercetin.

In the next set of experiments, intracellular localization of HSP70 was determined by immunohistochemical staining. HSP70 was localized in the cytoplasm and nuclei but not in nucleolus of untreated tumor cells (Fig. 6). Exposure to heat shock at 42°C resulted in

Table 1 *Effects of various agents on the induction of hypodiploid cells by quercetin*

K562 cells were pretreated with various agents for 2 h and then treated with 100 μM quercetin for an additional 24 h. Numbers of hypodiploid cells were calculated by DNA fluorescence histograms. Results are expressed as means ± SD of triplicate samples.

	% hypodiploid cells	
Treatment	Control	Quercetin
Medium	2.3 ± 1.1	40.1 ± 2.4
Cycloheximide	14.3 ± 2.3	75.5 ± 2.1[a]
Actinomycin D	19.3 ± 3.2	91.2 ± 5.4[a]
ATA	4.3 ± 3.4	8.6 ± 4.3
TPA	5.3 ± 4.3	37.3 ± 3.2
H7	6.5 ± 2.4	39.5 ± 4.2
EGTA	5.4 ± 4.5	42.3 ± 3.6

[a] Values are significantly different from those of controls according to *t* test at $P < 0.01$.

immediate aggregation of HSP70 in the nuclei, nucleolus, and cytoplasm. Considerable amounts of HSP70 accumulated by 2 h, which persisted for 4 h and returned to pretreatment values by 10 h. When tumor cells was first treated with quercetin for 5 h and then exposed to heat shock, HSP70 did not aggregate. Treatment with quercetin alone for 5 h did not induce apoptosis or HSP70 accumulation in the nuclei and cytoplasm. No HSP70 was found in apoptotic tumor cells. By contrast, no changes in intracellular distribution were observed with HSP90 and HSP25.

Continuous treatment of tumor cells with quercetin induced a time-dependent decrease in HSP70 when analyzed by Western blot analysis. In addition, pretreatment with quercetin inhibited the subsequent HSP70 response to heat shock (Fig. 7). This was confirmed at the mRNA level by the use of Northern blot analysis.

In an attempt to further confirm the role of HSP70 in quercetin-induced apoptosis, tumor cells were first treated with HSP70 antisense oligomer and then with quercetin. Treatment with HSP70 antisense oligomer resulted in an increase in the number of hypodiploid cells in response to quercetin (Fig. 8). By contrast, HSP70 sense oligomer had no effect.

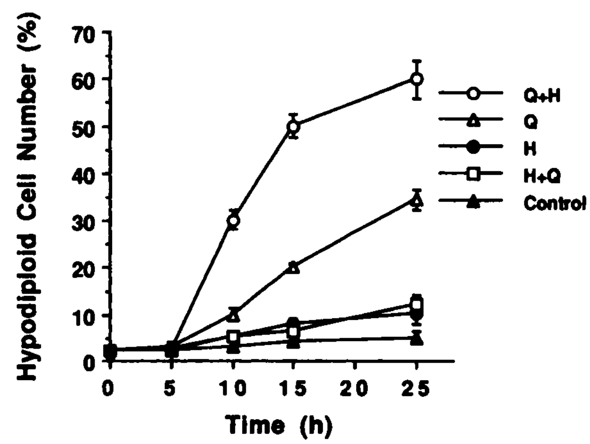

Fig. 5. Effects of heat shock on quercetin-induced hypodiploid cells. *Q+H*, K562 cells were first treated with 100 μM quercetin for 5 h, washed, heated at 42°C for 1 h, and finally treated with quercetin for 20 h. *Q*, 25-h treatment with quercetin. *H*, cells were exposed to heat shock and incubated at 37°C for 25 h. *H+Q*, cells were first exposed to heat shock and then treated with quercetin for 25 h. *Control*, untreated. Hypodiploid cells were determined by DNA fluorescence histogram. Results are expressed as means of triplicate samples; *bars*, SD.

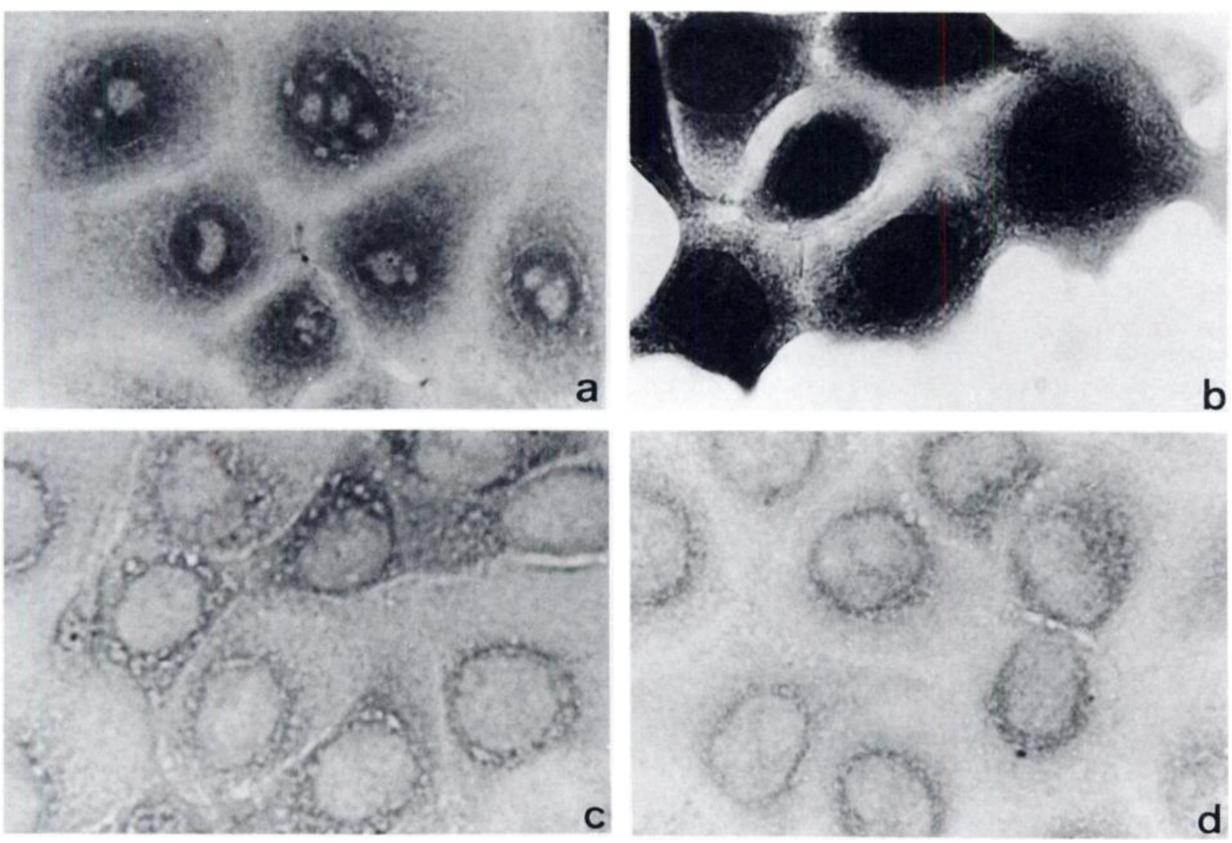

Fig. 6. Effects of heat shock and quercetin on intracellular HSP70. HSP70 in MACS was determined by immunocytochemical staining (ABC) on cell culture chamber slides (not counterstained by hemotoxylin; × 800). *a*, untreated. *b*, 2 h after heat shock. *c*, 5-h treatment with 100 μM quercetin. *d*, 5-h treatment with quercetin, followed by 1-h exposure to heat shock and 2-h recovery.

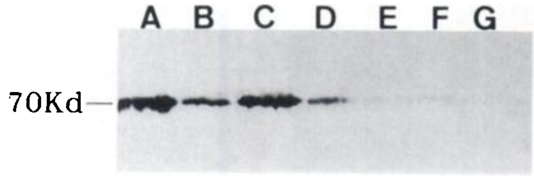

Fig. 7. Effects of quercetin on HSP70 determined by Western blot analysis. *Lane A,* MACS cells were exposed to heat shock at 42°C for 1 h and incubated at 37°C for 2 h. *Lane B,* cells were pretreated with 100 μM quercetin for 5 h, then exposed to heat shock and incubated at 37°C for 2 h. *Lane C,* control cells. *Lanes D, E, F,* and *G,* cells were treated with 100 μM quercetin alone for 5, 10, 15, and 20 h, respectively. Total cellular protein from 1 × 10^5 viable cells was added in each lane.

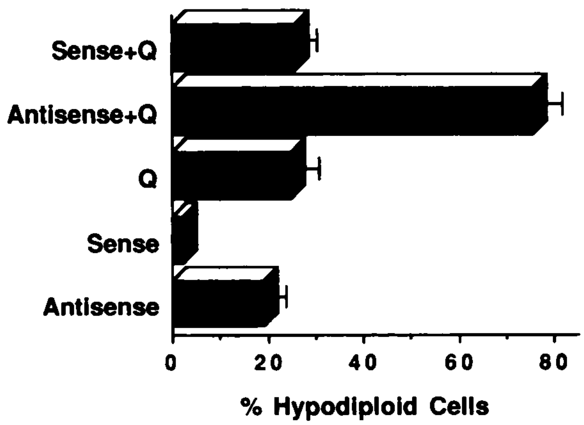

Fig. 8. Enhancement by HSP70 antisense of induction of hypodiploid cells by quercetin. K562 cells were first treated with 8 μM HSP70 antisense or sense oligomers for 24 h and then with 50 μM quercetin (*Q*) for an additional 24 h. Hypodiploid cells were calculated by DNA fluorescence histograms. Results are expressed as means of duplicate determination; *bars,* SD.

DISCUSSION

In the present study, several observations have been made concerning the apoptosis-inducing effect of quercetin and the role of heat shock proteins in the apoptosis. The present study has, to our knowledge, first demonstrated that quercetin triggers apoptosis in various tumor cells including K562, Molt-4, Raji, and MCAS. The effect was also observed with other tumor cell lines from gastric carcinomas, colon carcinoma, and lung carcinomas (data not shown). Tumor cells treated with quercetin displayed the characteristics of apoptosis induced by other agents (1–10). Quercetin was previously reported to have antitumor activity (15–20), although the mechanism responsible for the activity was not clarified in these studies. Our findings may indicate that quercetin displays antitumor activity by triggering apoptosis. Furthermore, the agent is found to elevate the number of hypodiploid cells (apoptotic cells) and reduced the number of cells at G_1 and S without affecting total cell numbers. Thus, quercetin may cause apoptosis mainly in cells at G_1 and S.

Recent studies have shown that quercetin inhibits specifically the synthesis of heat shock proteins at the level of mRNA accumulation and transcription without affecting the other protein synthesis (15, 16, 18). Heat shock proteins are known to play an important role in the protein metabolism and survival of cells (24–28). It seems thus likely that heat shock proteins may be involved in the apoptosis induced by quercetin. In fact, our findings presented in this communication indicate that quercetin inhibits the synthesis of HSP70 and changes the intracellular distribution of HSP70, which may be involved in the induction of apoptosis by quercetin. This conclusion is drawn from the following experimental data: (*a*) quercetin reduced or abrogated HSP70 in tumor cells before apoptosis occurred, and apoptotic cells failed to display these proteins; (*b*) untreated tumor cells responded to heat shock by HSP70 aggregation in the nuclei and nucleolus, with no or low apoptosis; (*c*) quercetin-treated cells showed no accumulation of HSP70 in the nuclei and nucleolus in response to heat shock; (*d*) exposure to heat shock induced considerable amounts of HSP70 in tumor cells, which in turn became unresponsive to quercetin; (*e*) treatment with HSP70 antisense oligomer of tumor cells inhibited HSP70 expression, which in turn resulted in an enhanced induction of apoptosis by quercetin.

It has been reported that the inhibition of RNA and/or protein synthesis abrogates the induction of apoptosis (1, 4, 7, 11). In other systems, however, the induction of apoptosis was not prevented by actinomycin D and cycloheximide (5). In the present study, the apoptosis-inducing effect of quercetin was augmented by actinomycin D and cycloheximide, the mechanism responsible for which is not understood yet. Cycloheximide was shown to inhibit the acquisition of thermotolerance, in which heat shock proteins are mainly involved (16, 18, 26). We are currently investigating the synergistic apoptosis-inducing effect of quercetin and cycloheximide or actinomycin D at the level of mRNA or protein synthesis.

In conclusion, the data presented in this report strongly indicate that quercetin induces apoptosis in tumor cells through inhibition of HSP70 synthesis and expression. These findings may be of importance to explore further the role of heat shock proteins in the growth and metabolism of tumor cells, to search for new antitumor agents by inhibiting heat shock protein synthesis, and to enhance the efficacy of hyperthermia by blocking HSP synthesis.

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