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Growth Suppression of Human Cancer Cells by Polyphenolics from Sweetpotato (*Ipomoea batatas* L.) Leaves

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Sweetpotato leaves (*Ipomoea batatas* L.) contain a high content of polyphenolics that consist of caffeic acid, chlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, and 3,4,5-tri-*O*-caffeoylquinic acid. We investigated the suppression of the proliferation of selected human cancer cells by phenolic compounds isolated from sweetpotato leaf. The human cancer cells used in this research included a stomach cancer (Kato III), a colon cancer (DLD-1), and a promyelocytic leukemia cell (HL-60). Caffeic acid and di- and tricaffeoylquinic acids dose-dependently depressed cancer cell proliferation, and the difference in sensitivity between caffeoylquinic acid derivatives and each kind of cancer cell was observed. Specifically, 3,4,5-tri-*O*-caffeoylquinic acid effectively depressed the growth of three kinds of cancer cells, and caffeic acid had an exceptionally higher effect against HL-60 cells than other di- and tricaffeoylquinic acids. In attempting to clarify the mechanism of growth suppression with the addition of the apoptotic inhibitor *N*-ethylmaleimide, we observed that the nuclear granulation in 3,4,5-tri-*O*-caffeoylquinic acid-treated HL-60 cells suggested apoptosis induction. This effect was confirmed by DNA fragmentation, an increase of caspase-3 activity, and expression of *c-Jun*. Growth suppression of HL-60 cells by 3,4,5-tri-*O*-caffeoylquinic acid was determined to be the result of apoptotic death of the cells. These results indicate that 3,4,5-tri-*O*-caffeoylquinic acid may have potential for cancer prevention.

KEYWORDS: Growth suppression; cancer cell; polyphenolics; sweetpotato leaf; apoptosis

INTRODUCTION

The sweetpotato leaf is consumed as a fresh vegetable in many parts of the world (*1*). Sweetpotato leaves have high nutritive value (*2*) and are an excellent source of antioxidative compounds, namely, polyphenolics (*3*). They also contain a high content of polyphenolics that consist of caffeic acid (CA) and caffeoylquinic acid derivatives (CQA derivatives) with plural caffeoyl groups bound to quinic acid (*3*). "Suioh", a new variety of sweetpotato for use of the leaf and petiole, was recently released from the National Agricultural Research Center for Kyushu Okinawa Region. Suioh is a bushy variety that produces many harvests in the summer. Moreover, the taste of the leaf and petiole is better than that of other cultivars. In addition, Suioh leaves have a higher content of polyphenol and DPPH radical scavenging activity than garland chrythanthemum, spinach, broccoli, cabbage, and lettuce (*4*). Therefore, Suioh is expected to be more widely accepted as a leafy vegetable in the near future.

CQA derivatives in sweetpotato leaves may exhibit many kinds of physiological functions other than radical scavenging activities. For example, 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA) inhibits the histamine secretion induced by concanavalin A plus phosphatidylserine from rat peritoneal mast cells (*5*). Chlorogenic acid (ChA), CA, and quinic acid (QA) suppress hepatoma cell invasion without altering cell proliferation (*6*). Recently, ChA, 3,5-diCQA, and 4,5-di-*O*-caffeoylquinic acid (4,5-diCQA) were identified as the primary antioxidants in edible chrysanthemums (*7*). Additionally, 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-triCQA) exhibits a selective inhibition of HIV replication (*8*), antimutagenicity (*9*), radical scavenging activity (*10*), and antidiabetes (*11*). However, no reports discuss the relationship between CQA derivatives and the effects on the growth of several kinds of cancer cells.

Several reports indicate that a variety of naturally occurring compounds may play a partial role in the prevention of human cancer (*12*). The studies demonstrate that the powerful antioxidation property of catechin in green tea and resveratrol in grape seed leads to cancer cell apoptosis, the suicide of the cell (*13*−*15*). The route to apoptosis is reported to be different in catechin and resveratrol; that is, polyphenols with antioxidation properties pass through various routes to arrive at apoptosis. These studies

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acknowledge the possibility that diet may play an important role in cancer and disease control. The compounds in food consumed in a daily diet are thought to be especially important for cancer prevention. Current investigation reveals that the sweetpotato stem and leaf in particular contain a high level of polyphenols, with a content that equals green tea. While the resveratrol content in the grape seed is not quite as high, it still has a high functionality. The objective of the present study is to demonstrate and explain how the CQA derivatives of the sweetpotato leaf depress the growth of cancer cells.

MATERIALS AND METHODS

Materials. QA and CA were purchased from Wako Pure Chemical Industries (Osaka, Japan). ChA was obtained from Sigma Chemical (St. Louis, MO). 3,4-Di-*O*-caffeoylquinic acid (3,4-diCQA), 3,5-diCQA, 4,5-diCQA, and 3,4,5-triCQA were purified (>97%) from sweetpotato leaves (*3*).

Cell Culture. Three kinds of cells were obtained as follows: stomach cancer cell line (Kato-III) from HSRRB (Osaka, Japan); colon cancer cell line (DLD-1) from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan); and promyelocytic leukemia cell line (HL-60) from the Riken Cell Bank (Tsukuba, Japan). The cells were suspended in an RPMI 1640 medium (Nissui, Tokyo, Japan) with 10% heat-inactivated fetal calf serum (FCS), 2 mM l-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (penicillin−streptomycin solution; Sigma Chemical) in a 5% CO_2 humidified incubator at 37 °C.

Cell Proliferation Assay. Kato-III, DLD-1 cells (1.0×10^5 cells/mL), and HL-60 cells (2.0×10^5 cells/mL) were cultured in the presence of each CQA derivative, to which was added 1% volume of culture medium. Each CQA derivative was then dissolved in 20% dimethyl sulfoxide (DMSO). After a 72 h incubation, the cell survival rate was measured by the WST-1 method [2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; Dojindo, Kumamoto, Japan] (*16*). Because the WST-1 assay is based on water soluble formazan reaction only by metabolic active cells, it detects both apoptotic and necrotic cell death. The cell proliferation percentage was defined as (absorbance in each well)/(absorbance in a blank well) × 100 (%). The absorbance in the microplate wells was measured at 450 nm with a dual-wavelength flying-spot scanning densitometer with a microplate system (Shimadzu Co., Kyoto, Japan).

Stability of CQA Derivatives in Medium. Each CQA derivative was dissolved in the test medium to a final concentration of 100 μM from a 10 mM stock solution in 20% DMSO. Test media were RPMI 1640 medium with 10% FCS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. They were incubated for 72 h at 37 °C and 5% CO_2 in six well plates not containing cells. The amount of remaining CQA derivatives was analyzed with high-performance liquid chromatography (HPLC) (*3*) every 24 h.

Chromatin Condensation. HL-60 cells were exposed to 500 μM 3,4,5-triCQA with and without 1 mM *N*-ethylmaleimide (NEM) of apoptosis inhibitor (Kisida, Osaka, Japan) (*17*) for 24 h. The nuclei of the dying cells were stained with 4 μM 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Dojindo), and living cells were stained with 4 μM 3′,6′-di(*O*-acetyl)-2′,7′-bis[N,N-bis(carboxymethyl)aminomethyl] fluorescein, tetra-acetoxymethyl ester (Calcein-AM; Dojindo) at 37 °C for 15 min. Stained nuclei were observed and photographed with an Olympus fluorescence microscope.

DNA Fragmentation. HL-60 cells (2.0×10^5 cells/mL) were treated with 500 μM 3,4,5-triCQA, to which was added 1% volume of culture medium, and were incubated at 37 °C for 0, 1, 3, 6, 12, and 24 h. For the fragmentation of nuclear DNA, 1.0×10^5 cells were collected by centrifugation and were lysed in 1 mL of the lysis buffer (50 mM Tris-HCl, pH 8.0) containing 100 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfide, and RNase A (0.1 mg/mL; Wako Pure Chemical Industries) at 37 °C for 30 min. After treatment with 0.1 mg/mL proteinase K (Wako Pure Chemical Industries), it was incubated at 37 °C for 30 min. DNA was prepared by using phenol/chloroform extraction and precipitated by ethanol. The DNA sample was separated by electrophoresis in 2% agarose gel and stained with ethidium bromide.

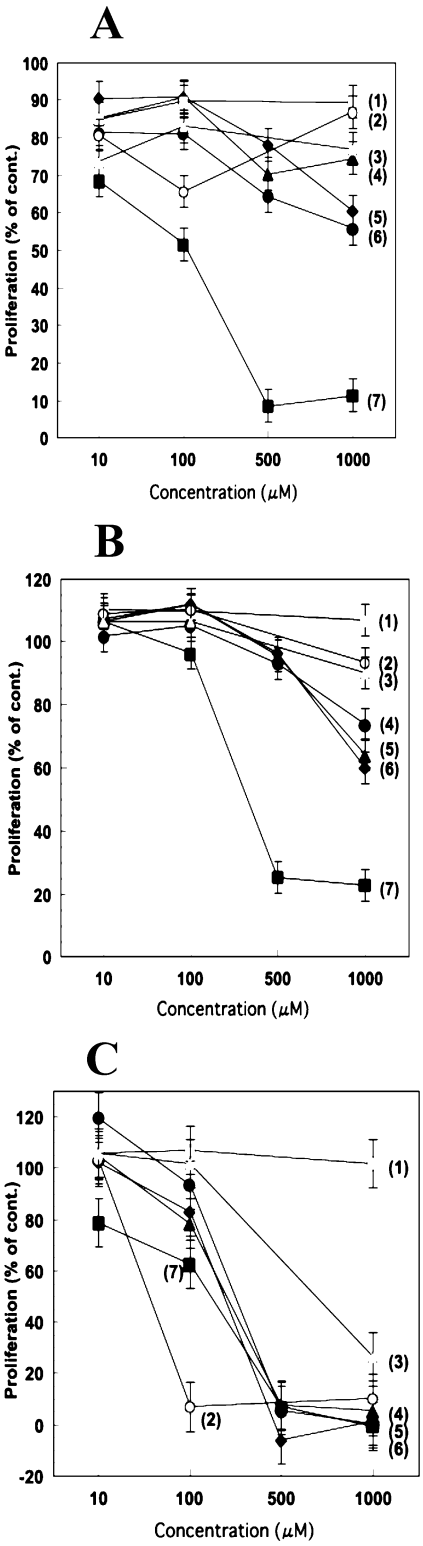

Figure 1. Effect of each polyphenol component on proliferation of Kato-III (**A**), DLD-1 (**B**), and HL-60 (**C**) cells. Data were represented as means ± SE for three determinations. Key: 1, quinic acid; 2, CA; 3, ChA; 4, 3,4-diCQA; 5, 3,5-diCQA; 6, 4,5-diCQA; and 7, 3,4,5-triCQA.

Activity of Caspase 3. HL-60 cells were exposed to 500 μM 3,4,5-triCQA for 0, 1, 2, and 3 h and collected, washed with phosphate-buffered saline, and suspended in 50 mM HEPES (pH 7.4) containing 5 mM CHAPS and 5 mM DTT. Cell lysates were clarified by centrifugation at 12000*g* for 15 min at 4 °C. Caspase 3 activity in the resultant supernatant was determined using the colorimetric activity

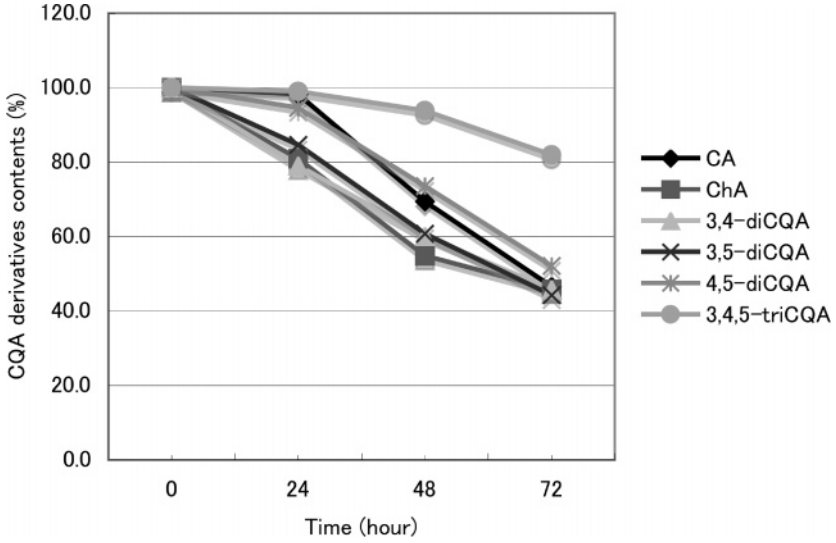

Figure 2. Stability in time of each 100 μM CQA derivative solutions in RPMI 1640 medium. Quantification of each 100 μM CQA derivative is based on HPLC peak area expressed as a percentage of 0 time samples.

assay kit (Sigma Chemical). Absorbance was measured at 405 nm with a dual-wavelength flying-spot scanning densitometer with a microplate system (Shimadzu Co., Kyoto, Japan).

Revere-Transcribed Polymerase Chain Reaction (RT-PCR) Analysis. HL-60 cells were exposed to 500 μM 3,4,5-triCQA for 0, 3, 6, 12, and 24 h, and RNA was extracted by Isogene (Nippon Gene, Tokyo, Japan). The total RNA (0.5 μg) was RT by a high-fidelity RNA PCR Kit (TaKaRa, Shiga, Japan). Expression of apoptosis-related genes was detected by the standard PCR method according to Hou et al. (*18*) using TaKaRa Ex taq (TaKaRa). One microliter for the cDNA synthesis was subjected to the PCR reaction for 30 and 25 cycles by using human *c-Jun* (apoptosis-related gene) and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as control because it is stably expressed at high levels in almost all tissues and cells. The *c-Jun* cDNA was amplified by using the sense primers (5′-CAACATGCTCAGGGAACAGG) at positions 261−280 and antisense primers (5′-GGTCCATGCAGTTCTTGGTC) at positions 531−550. The *GAPDH* was amplified by using the sense primers (5′-GACCCCTTCATTGACCTCAAC) at positions 143−162 and antisense primers (5′-CATACCAGGAAATGAGCTTG) at positions 965−984. The PCR products were separated on 1.2% agarose gel and stained with ethidium bromide.

RESULTS

Growth Suppression of Cancer Cells by Polyphenolics. **Figure 1** depicts growth suppression of three kinds of cancer cells by QA, CA, and CQA derivatives: a stomach cancer (Kato-III), a colon cancer (DLD-1), and a promyelocytic leukemia cell (HL-60). QA had no effect on the growth of each cancer cell at concentrations between 10 and 1000 μM. In Kato-III cells, 3,5- and 4,5-diCQAs suppressed 20−40% of the proliferation at concentrations of 100−500 μM. Additionally, 3,4,5-triCQA dose dependently inhibited 30−90% of the proliferation at concentrations of 10−500 μM. In DLD-1 cells, CA and ChA suppressed 10% of the proliferation at a concentration of 1000 μM, while 3,4-, 3,5-, and 4,5-diCQAs dose dependently suppressed 20−40% of the proliferation. Also, 3,4,5-triCQA inhibited 80% of the proliferation at over 500 μM. In HL-60 cells, CA inhibited 90% of the proliferation at a concentration over 10 μM, and ChA inhibited 80% of the proliferation at a concentration of 1000 μM. Also, 3,4-, 3,5-, and 4,5-diCQAs and 3,4,5-triCQA dose dependently suppressed 40−90% of the proliferation at concentrations between 100 and 1000 μM. One report discussed resolving the polyphenol in the cell culture medium at once (*19*). Therefore, the content of the

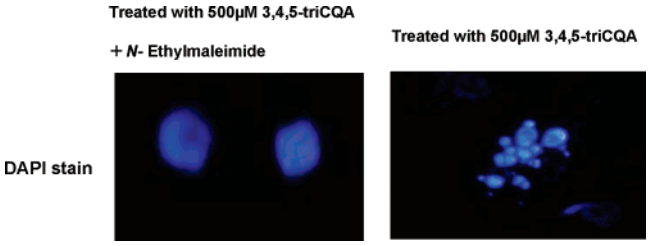

Figure 3. Morphological changes by 500 μM 3,4,5-triCQA in HL-60 cells stained with DAPI. NEM was added on the left side as an apoptosis inhibitor.

CQA derivatives in the cell culture medium changed over the time course (**Figure 2**). After 72 h, CA was 46.2% and ChA remained 45.8%. Additionally, 3,4-, 3,5-, and 4,5-diCQAs remained between 44 and 52%, while 3,4,5-triCQA remained at 82% and was proven to be stable in the culture solution. It is considered that 3,4,5-triCQA does not have strong hydrophilicity. Moreover, because it is resolved in the culture solution and decreasing is limited, the effect on the cancer cell can be expected to be long term.

Morphological Changes of 3,4,5-TriCQA-Treated HL-60 Cells. In order to explain the mechanism of cell growth suppression by polyphenols, morphological changes were examined in HL-60 cells, which are well-known in apoptosis research (*15*, *18*, *20*, *21*). HL-60 cells were treated with 3,4,5-triCQA in the presence or absence of NEM, an inhibitor of caspase, and used regularly as an apoptosis inhibitor (*17*) at a concentration of 500 μM. The 3,4,5-triCQA-treated cells were stained with Calcein-AM and DAPI in the presence of NEM but were stained with DAPI only in its absence (**Figure 3**). Granulation of the nucleus appeared as fluorescent blue in the detail of the DAPI-only stained cells (those in which NEM was absent). Nucleus granulation is a feature of the morphological change in apoptosis, and these results suggested that 3,4,5-triCQA may induce apoptosis in HL-60 cells. In addition, fluorescent yellow appeared by the calcein-AM stain when NEM was added to the 3,4,5-triCQA-treated cells. It was suggested that the death of 3,4,5-triCQA-treated cells could be suppressed by controlling caspase, which was the enzyme related to apoptosis.

DNA Fragmentation, Caspase 3 Activity, and *c-Jun* Expression of 3,4,5-TriCQA-Treated HL-60 Cells. The

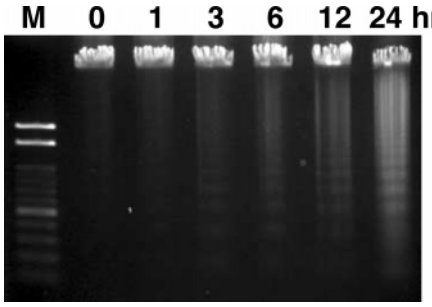

Figure 4. Time course of DNA fragmentation by 500 μM 3,4,5-triCQA in HL-60 cells. The cells were cultivated with 500 μM 3,4,5-triCQA for 0, 1, 3, 6, 12, and 24 h. The isolated DNA was loaded into a well of 2% agarose gel and electrophoresis. M is the 100 bp DNA marker.

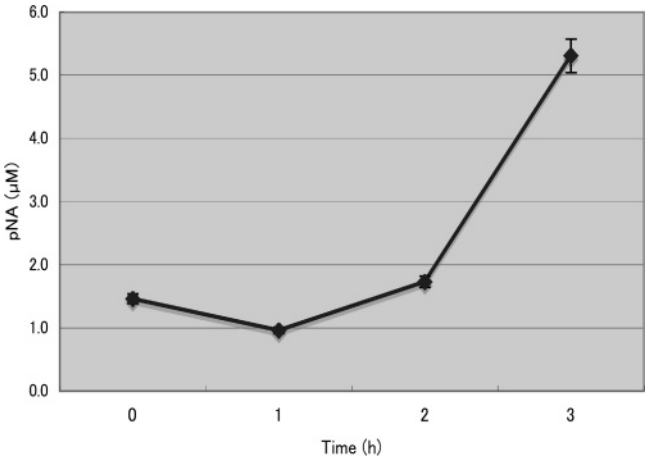

Figure 5. Time courses of caspase 3 activity in 500 μM 3,4,5-triCQA-treated HL-60 cells. The cells were prepared. Data were represented as means ± SE for three determinations.

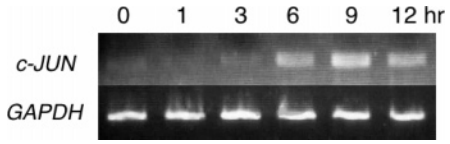

Figure 6. RT-PCR analysis of *c-JUN* and *GAPDH*. The cells were cultivated with 500 μM 3,4,5-triCQA for 0, 1, 3, 6, 9, and 12 h, and total RNA was isolated.

significant features of apoptosis are granulation of the nucleus, DNA fragmentation, and increased caspase activity. The relationship between DNA fragmentation and incubation time was examined in 3,4,5-triCQA-treated HL-60 cells. After 6 h of the treatment, the DNA ladder was clearly detected (**Figure 4**). One of the earliest and most consistently observed features of apoptosis is the induction of a series of cytosolic proteases, the caspases, which cleave protein substrates and give rise to characteristic apoptotic morphology. Caspase 3 has been implicated as a key protease that is activated during the early stages of apoptosis (*22*). Caspase 3 activity began to rise after 2 h of incubation (**Figure 5**) and increased more than three times after 3 h of incubation. Furthermore, *c-Jun* is one of the genes concerned with apoptosis induction (*15*, *20*). **Figure 6** illustrates the relationship between the expression of *GAPDH* and *c-Jun* for control and incubation times in 3,4,5-triCQA-treated HL-60 cells. The *c-Jun* was expressed 3 h after the treatment and reached its maximum expression in 9 h. Although it decreased a little, that level was maintained even as long as 12 h afterward. Within the same period, *GAPDH*, which is a housekeeping gene, was expressed during all incubation times. These results indicate that growth suppression of 3,4,5-triCQA-treated HL-60 cells is due to apoptosis induction.

DISCUSSION

Human cancer cell lines, especially the gastric and colorectal adenocarcinoma, were selected for this study because the cells of the digestive tract come into direct contact with food components. Identifying compounds that can prevent the development of cancer cells in these organs and including them in foods may serve as a preventative measure. CA, diCQAs, and triCQA dose dependently suppressed the growth of each cancer cell, but the intensities of growth suppression were dependent on the cancer cell variety used in this experiment (**Figure 1**). The CQA derivatives exhibited higher sensitivity toward the HL-60 cell than toward the Kato III and DLD-1 cells, and all of the CQA derivatives exhibited the typical feature of depressing the cell proliferation in the order of HL-60 > Kato-III > DLD-1. The HL-60 cell is more sensitive to apoptosis induction by various kinds of chemicals (*23*). Mishima et al. (*21*) also reported that 3,4,5-triCQA purified from Brazilian propolis inhibited the growth of HL-60. It is believed that the sensitivity is high because only the HL-60 cell is a floating cell; Kato-III is a half-adhesion cell, and DLD-1 is an adhesion cell. As for the same CQA derivatives, this research has demonstrated that sensitivity differs greatly by cell type, especially in relation to cell and tissue morphology. Therefore, future research will require that the choice of cell be matched to the development purpose. Cancer growth was only slightly affected by QA without caffeoyl groups in the structure, suggesting the necessity that the caffeoyl group be bound to QA. In contrast, CA suppressed HL-60 cell proliferation as effectively as the same level of 3,4,5-triCQA. No data are currently available to explain this variance. However, Kaul and Khanduja (*24*) indicated that CA was the most effective inhibitor of cancer promotion in mice skin. Recently, the vitamin K-free Simon extracts containing CA, ChA, and diCQAs markedly suppressed severe bone destruction mediated by abundant osteoclasts associated with adjuvant-induced arthritis in rats (*25*). Among these substances, CA exhibits the most powerful inhibitory effects on osteoclastogenesis.

The stability of CQA, which would influence cell proliferation control, was confirmed (**Figure 2**). However, a sharp decline was not detected by 24 h. Because CA, ChA, and diCQAs decreased linearly from 24 to 72 h, 50% remained in 72 h. However, if they act as a material that strongly causes apoptosis, a decrease after 24 h might not be problematic because after the cell is treated, caspase activity is sure to continue for at least 3 h (**Figure 5**). Moreover, 500 μM and 1 μM had the same results on the stability of CQA derivatives (data not shown).

The increase of living cells by NEM addition and nuclear granulation in 3,4,5-triCQA-treated HL-60 cells suggests that growth suppression by this compound is due to apoptosis induction (**Figure 3**). Apoptotic signal transduction and execution require the coordinated action of the cascade of caspases (*22*). This effect was confirmed by DNA fragmentation, increase of caspase 3 activity, and expression of *c-Jun* (**Figures 4**−**6**).

In recent years, advanced research of apoptosis pathways was conducted with the caspase family. It was known that caspase 9 is activated when the oxidation signal is received from mitochondria in the cell, although several types of signals received in the cell activate caspase 3. Apoptosis by caspase 9 seemed to be induced by the antioxidant activity of 3,4,5-triCQA. However, the expected caspase 9 activation did not

occur during the investigations (data not shown). The results confirmed that 3,4,5-triCQA does not induce a caspase 9 apoptosis pathway.

Cancer researchers generally accept the "two-step carcinogenesis" hypothesis that a cancer cell is generated from a normal cell through several steps involving mutation and promotion (*26*). Further cancer cell generation progresses in several steps, and suppression of these steps may result in cancer prevention. It was previously reported that the polyphenolics in isolated sweetpotato leaves effectively inhibited the mutagenicity induced in *Salmonella typhimurium* TA98 by the carcinogen Trp-P-1 (*9*). Several anticancer drugs are now known to induce apoptosis in cancerous cells, and apoptosis is considered to be their primary mechanism (*27*, *28*). The present results and antimutagenic data suggest that sweetpotato leaf polyphenolics, especially 3,4,5-triCQA, may suppress not only the mutation of normal cells but also the growth of cancer cells by apoptosis induction. The present study suggests that sweetpotato leaves are potential anticancer materials, but further in vivo investigation is necessary.

ABBREVIATIONS USED

CA, caffeic acid; CQA, caffeoylquinic acid; ChA, chlorogenic acid (3-*O*-caffeoylquinic acid); 3,4-diCQA, 3,4-di-*O*-caffeoylquinic acid; 3,5-diCQA, 3,5-di-*O*-caffeoylquinic acid; 4,5-diCQA, 4,5-di-*O*-caffeoylquinic acid; 3,4,5-triCQA, 3,4,5-tri-*O*-caffeoylquinic acid.

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