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Inhibition of Proteasome Activity by Various Fruits and Vegetables is Associated with Cancer Cell Death

MARINA S. CHEN, DI CHEN and Q. PING DOU

The Prevention Program, Barbara Ann Karmanos Cancer Institute and Department of Pathology, Wayne State University, School of Medicine, Detroit, Michigan, 48201, U.S.A.

Abstract. *There is a large amount of scientific evidence showing that fruits and vegetables lower the risk of cancer. However, the responsible molecular mechanisms remain poorly understood. Our previous studies have demonstrated that inhibition of proteasomal chymotrypsin-like activity is associated with cancer cell apoptosis, which may also be the major mechanism responsible for the anticancer effects of green tea polyphenols. In the current study, we tested the hypothesis that some fruits and vegetables inhibit tumor cell proteasome activity and that this inhibition contributes to their cancer-preventative activities. We report that the extracts of apple and grape are more potent than onion, tomato and celery in: (i) inhibiting the proteasomal chymotrypsin-like activity in leukemia Jurkat T cell extract; (ii) accumulating the polyubiquitinated proteins in intact Jurkat T cells; (iii) inducing activation of caspase-3/-7 and cleavage of poly(ADP-ribose) polymerase in intact Jurkat T cells; and (iv) inducing the appearance of spherical cells preferentially in prostate cancer PC-3 over the normal NIH 3T3 cell line. We also found that strawberry extract had some effect on Jurkat T cell extract and the prostate PC-3 cell line but not on intact Jurkat T cells. Our findings suggest that the proteasome is a cancer-related molecular target for, at least, the extracts of apple, grape and onion, and that the inhibition of proteasome activity by these fruits or vegetable may contribute to their cancer-preventative effects, although other molecular mechanisms may also be involved.*

Abbreviations: (-)-EGCG, (-)-epigallocatechin-3-gallate; PARP, poly(ADP-ribose) polymerase; AMC, 7-amido-4-methyl-coumarin.

Correspondence to: Q. Ping Dou, Ph.D., The Prevention Program, Barbara Ann Karmanos Cancer Institute and Department of Pathology, School of Medicine, Wayne State University, 516 HWCRC, 4100 John R, Detroit, Michigan, 48201, U.S.A. Tel: 313-966-0641, Fax: 313-966-7368, e-mail: doup@karmanos.org

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Fruits and vegetables are very important components of a healthy diet. Scientific evidence has shown that fruits and vegetables lower the risk of cancer (1-5). In 1989, a National Academy of Sciences report on diet and health recommended consuming 5 or more servings (80 grams per serving) of fruits and vegetables daily to reduce the risk of both cancer and heart disease (6). In 1997, an international review panel (World Cancer Research Fund–American Institute for Cancer Research) concluded that there was convincing evidence that a high intake of vegetables and fruits decreases the risk of many cancers (7). In 1998, the expert group commissioned by the Chief Medical Officer's Committee on Medical Aspects of Food and Nutrition Policy of the United Kingdom (COMA) reached similar conclusions (8). It is estimated that one third of all cancer deaths in the United States could be avoided through appropriate dietary modification such as increased consumption of fruits, vegetables and grain (9, 10). Diets rich in fruits and vegetables have been strongly recommended to prevent cancers. The evidence supporting this recommendation is mostly based on observational studies including case-control, cohort and prospective studies (11-13). However, the responsible molecular mechanisms for the anticancer effects associated with fruits and vegetables are still poorly understood.

Previously, we reported that ester bond-containing tea polyphenols, such as (-)-epigallocatechin-3-gallate [(-)-EGCG], potently and specifically inhibited the chymotrypsin-like activity of the proteasome *in vitro* and *in vivo* at the concentrations found in the serum of green tea drinkers. Inhibition of the proteasome by (-)-EGCG in several tumor and transformed cell lines results in G1 arrest or apoptosis (14-16). These results suggest that inhibition of the proteasome activity by green tea polyphenols may contribute to the cancer preventative effect of tea (17, 18).

The 26S proteasome is a large multi-subunit protease complex present in both the nucleus and cytoplasm of eukaryotic cells. It has been shown that the ubiquitin-proteasome system plays an important role in regulating cell growth and death and is the central non-lysosomal pathway

for protein degradation (19-21). Under normal conditions the lysosomal pathway degrades extracellular proteins imported into the cell by endocytosis or pinocytosis, whereas the proteasome controls degradation of intracellular proteins (19-21). There are at least three major proteasomal activities involved in target protein degradations: chymotrypsin-like, trypsin-like and caspase-like activities (19-21). It has been found that inhibition of the chymotrypsin-like, but not trypsin-like, activity is associated with tumor cell apoptosis (22, 23).

Apoptosis, also called programmed cell death or physiological cell death, is an essential and evolutionarily conserved property involved in the maintenance of multicellular organisms and found in all species from worms to man. Apoptosis is required in many fundamental biological processes including embryonic development, metamorphosis, hormone-dependent atrophy and chemically-induced cell death (24, 25). It is known that a group of proteases named caspases is central to the death machinery present in all eukaryotic cells. Caspases act in a self-amplification cascade. An activated upstream caspase, named initiator caspase and including caspase-2, -8, -9 and -10, will cleave and then activate one or more downstream caspases, named effector caspases including caspase-3, -6 and -7 (26). The activated effector caspases then cleave various cellular enzyme proteins such as poly(ADP-ribose) polymerase (PARP) (27), retinoblastoma protein (RB) (28) and Bcl-2 or cytoskeleton proteins (26), leading to apoptotic cell death.

In the current study, we hypothesized that some fruits and vegetables inhibit the cell proteasome activity and that this inhibition contributes to their cancer risk-reducing abilities (1-5). We report, for the first time, that extracts from apple and grape are more potent than those from onion, tomato and celery in inhibiting the proteasomal chymotrypsin-like activity *in vitro* and *in vivo*, inducing Jurkat T cell apoptosis and the appearance of spherical cells preferentially in the prostate cancer PC-3 cell over the normal NIH 3T3 cell line. These results suggest that, although various fruits and vegetables have cancer risk-lowering abilities, apple, grape and perhaps onion target the cancer cellular proteasome, while others may be involved in different molecular mechanisms.

Materials and Methods

Materials. Highly purified tea polyphenol (-)-EGCG (>95%) was purchased from Sigma Inc. (Saint Louis, MO, USA). Green tea extract (3% w/v) was prepared in water and further diluted as indicated in the text. Extracts of apple, grape, strawberry, onion, tomato and celery were prepared by using a Juiceman™ Juice Extractor, followed by high-speed centrifugation at 45,000 g for 30 min and sterile filtering. Fluorogenic peptide substrates Suc-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) and Ac-Asp-Glu-Val-Asp-AMC (for the caspase-3/-7 activities) were obtained from Calbiochem Inc. (San Diego, CA, USA). Polyclonal antibodies to human PARP and ubiquitin were purchased from Boehringer Mannheim (Indianapolis, IN, USA) and Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA), respectively.

Cell culture and whole cell extract preparation. Human leukemia Jurkat T and prostate cancer PC-3 cell lines were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum, 100 units/ml of penicillin and 100 μg/ml streptomycin. Normal murine NIH 3T3 fibroblasts were grown in DMEM medium containing 10% fetal calf serum, penicillin and streptomycin. All cells were maintained in 5% CO_2 atmosphere at 37˚C. Whole cell extract was prepared as described previously (15). Briefly, the cells were harvested and washed twice with phosphate-buffered saline (PBS) and homogenized in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40) for 30 min at 4˚C. Lysates were centrifuged at 12,000 g for 30 min at 4˚C and the supernatants were collected as whole cell extracts.

Cell-free proteasomal chymotrypsin-like activity assay. Protein extract (10 μg) of Jurkat T cells was incubated for 60 min at 37˚C with 40 μM of the fluorogenic peptide substrates (Suc-Leu-Val-Try-AMC) in 100 μl of an assay buffer (50 mM Tris-HCl, pH 7.5), without or with either purified (-)-EGCG (at 1, 5, 10 or 20 μM) or an extract (at 1, 2.5, 5 or 10% v/v). After incubation, hydrolyzed 7-amido-4-methyl-coumarin (AMC) fluorogenic group was measured by using a multi-well plate Wallac™ Victor^2 1420 Multilabel Counter with an excitation filter of 380 nm and an emission filter of 460 nm. Changes in fluorescence were calculated against non-treated control and plotted with statistical analysis using Microsoft Excel™ software.

Cell-free caspase-3/-7 activity assay. Cell-free caspase-3/-7 activities were determined by measuring the release of the AMC group from a caspase-3/-7 specific substrate Ac-Asp-Glu-Val-Asp-AMC. Briefly, Jurkat T cells were treated with 5% of an extract or 25 μM of (-)-EGCG for 3, 6, 12 or 24 h, followed by preparation of whole cell extracts. The cell extract (20 μg) was then incubated in 100 μl of the assay buffer (50 mM Tris-HCl, pH 7.5) along with 40 μM of caspase-3/-7 substrate in a 96-well plate. The reaction mixture was incubated at 37˚C for 2 h and the hydrolyzed fluorescent AMC groups were quantified as described above.

Western blot analysis. Jurkat T cells were incubated with either 25 μM of (-)-EGCG or 5% of an extract for up to 48 h. This was followed by preparation of whole cell extracts as described above. Equal amounts of protein extract (30 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) using a Trans-Blot^R Semi-Dry Transfer System (Bio-Rad, Hercules, CA, USA). The enhanced chemiluminescence (ECL) Western blot analysis was then performed using specific antibodies to PARP or ubiquitin.

Measuring cellular morphological changes. Human prostate cancer PC-3 cells or normal murine NIH 3T3 fibroblast cells were incubated with either (-)-EGCG (at 1, 10, 25 and 50 μM) or an extract (at 1%, 2.5%, 5% or 10%) for 12 h. Photographs of the treated cells were taken using phase-contrast inverted microscope (Leica; Wetzlar, Germany) (amplification 100x).

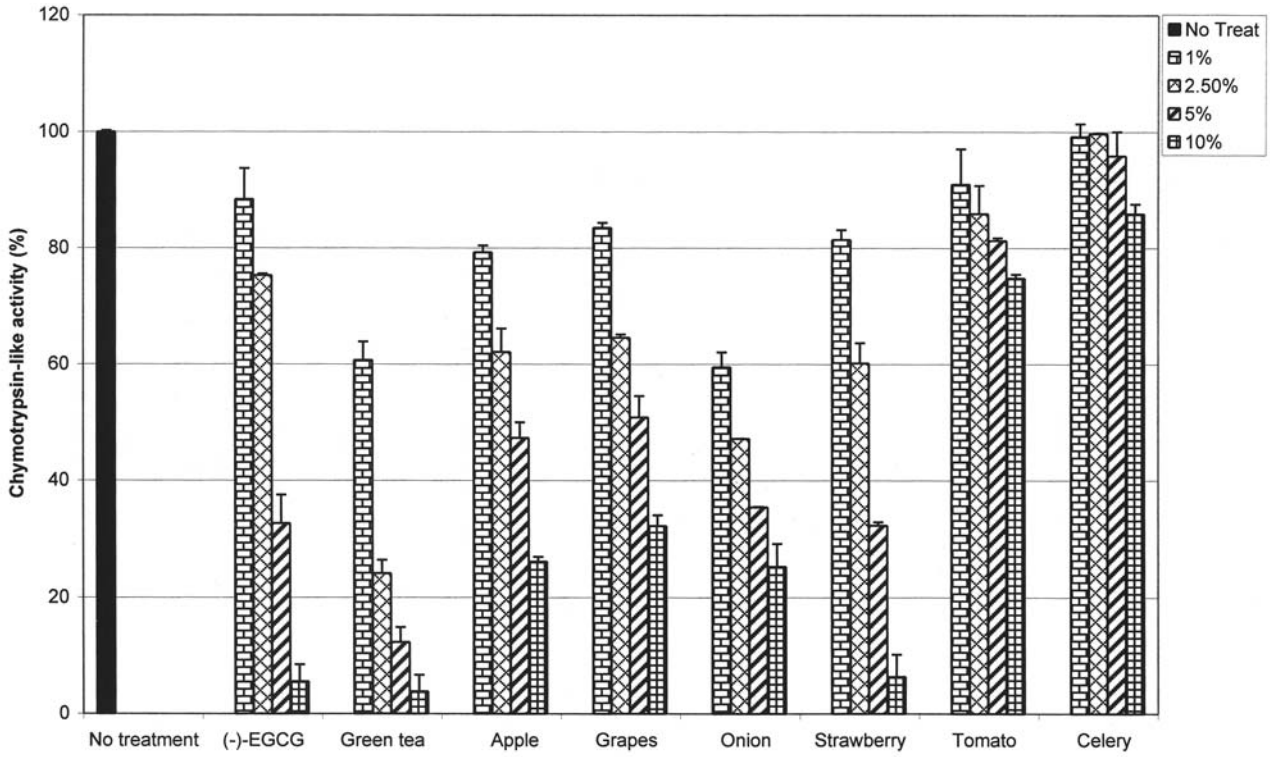

Figure 1. *Inhibition of the proteasome chymotrypsin-like activity in vitro by extracts of fruits and vegetables. A Jurkat T cell extract was incubated for 1 h with indicated concentrations of an extract or purified (-)-EGCG and fluorogenic peptide substrate, followed by measurement of free AMC groups as described in Materials and Methods. PBS was used as a negative control (no treatment). Values are mean triplicates and error bars denote standard deviations.*

Results

Effects of extracts of various fruits and vegetables on the proteasomal chymotrypsin-like activity in Jurkat T cell extract. We hypothesized that fruits and vegetables might inhibit the cellular proteasome activity and that this inhibition contributes to the reduction of cancer risk (1-5). Because inhibition of the proteasomal chymotrypsin-like activity is associated with cancer cell apoptosis (22, 23), we measured the effects of extracts from various fruits and vegetables on this activity in human leukemia Jurkat T cell extract. As expected, two positive controls, purified green tea polyphenol (-)-EGCG and green tea extract, inhibited the proteasomal chymotrypsin-like activity in a concentration-dependent manner (Figure 1). We found that extracts of apple, grapes, onion and strawberry also potently inhibited the proteasomal chymotrypsin-like activity (Figure 1). The inhibitions were concentration-dependent. At concentrations of 1, 2.5, 5 or 10% (v/v), apple, grape, onion and strawberry extracts exhibited inhibition of the proteasomal chymotrypsin-like activity by 20-40%, 40-50%, 50-70% and 70-90%, respectively (Figure 1). However, they were less potent when compared to green tea extract in inhibiting the proteasomal chymotrypsin-like activity under the same conditions (Figure 1). Tomato and celery, in contrast to apple, grape, onion and strawberry, showed little inhibitory activity. Even at 10% concentration, tomato and celery extracts only inhibited ~25 and ~15% of such activity, respectively (Figure 1).

Proteasome-inhibitory and apoptosis-inducing abilities of fruit and vegetable extracts in intact Jurkat T cells. Next we investigated whether the proteasome-inhibitory activities of the tested fruit and vegetable extracts *in vitro* correlated with their potencies in intact cells. We treated human leukemia Jurkat T cells for up to 48 h with 5% of each of the tested fruit or vegetable extracts, again using 5% of green tea extract or 25 μM of (-)-EGCG as positive controls. Following treatment, the cells were collected and assayed for increased levels of ubiquitinated proteins, which accumulate following the inhibition of cellular proteasome activity (19-21). As expected, green tea extract significantly increased the levels of ubiquitinated proteins, peaking at 6 and 12 h (Figure 2A); (-)-EGCG was also able to induce accumulation of ubiquitinated proteins although to a less extent, with levels peaking at 24 and 48 h (Figure 2A). The order of the abilities of various fruit and vegetable extracts to accumulate ubiquitinated proteins in Jurkat T cells was

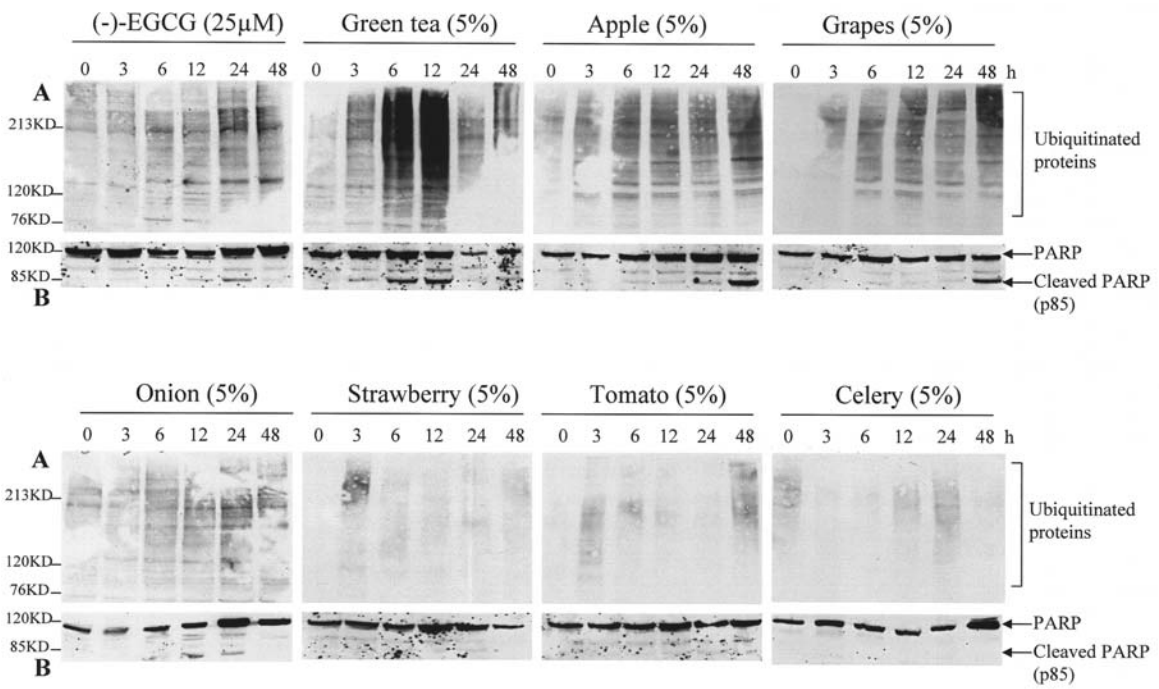

Figure 2. *Accumulation of ubiquitinated proteins (inhibition of the proteasome activity in vivo) and induction of PARP cleavage by fruit and vegetable extracts in intact Jurkat T cells. Jurkat T cells were treated with 5% of each extract or 25 μM of purified (-)-EGCG for indicated hours, followed by Western blot analysis using specific antibodies to ubiquitin (A) or PARP (B). The lanes of successive bands represent ubiquitinated proteins due to the inhibition of proteasome activity by tested extracts (A). The positions of full-length PARP and cleaved p85 fragment are indicated by arrows (B).*

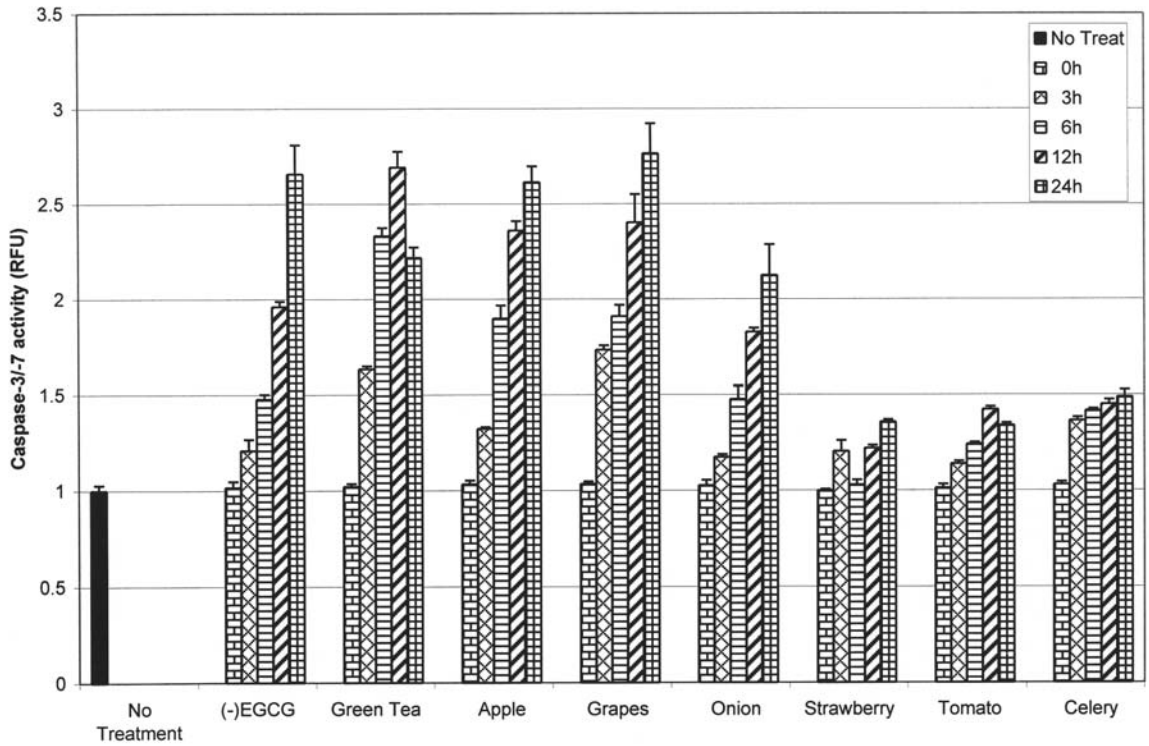

Figure 3. *Cell-free caspase-3/-7 activity assay. Jurkat T cells were treated with 5% of each extract and 25 μM of purified (-)-EGCG for different time points and then harvested. The prepared cell extracts (20 μg per reaction) were incubated with caspase-3/-7 substrate for 2 h. The measurement of free AMC groups was described in Materials and Methods. The graph is normalized. Values are means of triplicates and error bars denote standard deviations.*

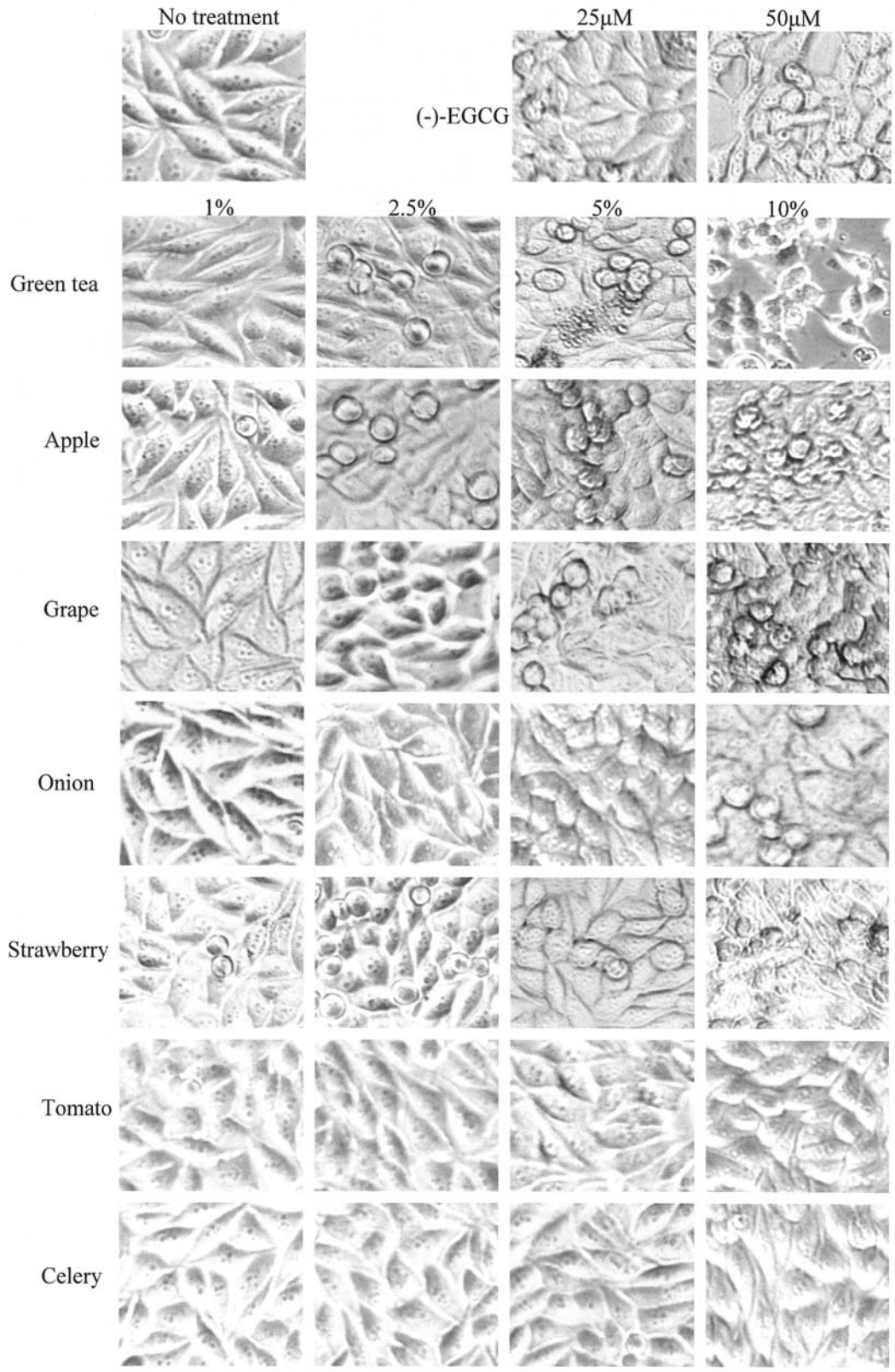

Figure 4. *Morphological changes of prostate cancer PC-3 cells induced by fruit and vegetable extracts. Human prostate cancer PC-3 cells were incubated with indicated concentrations of each extract or (-)-EGCG for 12 h. The photographs of morphological changes were taken using phase-contrast inverted microscopy (Magnification 100x).*

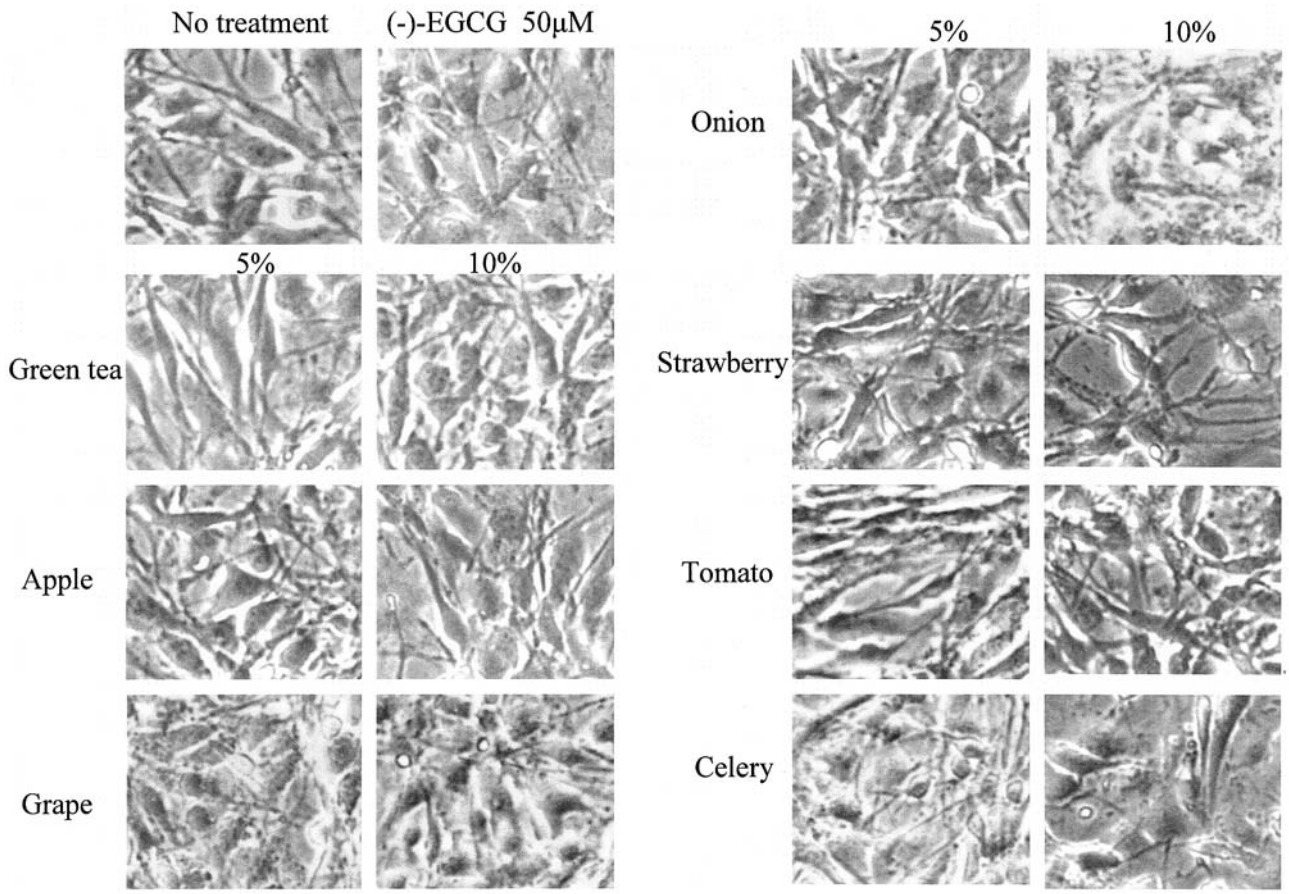

Figure 5. *Normal murine NIH 3T3 fibroblast cells are resistant to treatment by fruit and vegetable extracts. NIH 3T3 cells were incubated with indicated concentrations of each extract or (-)-EGCG for 12 h and morphological changes were measured by phase-contrast inverted microscopy (Magnification 100x).*

found to be: apple, grape > onion > strawberry, tomato, celery. Consistent with the *in vitro* data (Figure 1), apple and grape were more potent than tomato and celery but less potent than green tea (Figure 2A). However, different from the *in vitro* results, onion was found to be less potent than apple and grape in intact Jurkat T cells (Figure 2A) and, specifically, strawberry was inactive *in vivo* although it was quite potent *in vitro* (Figures 2 *vs*. 1).

It has been shown that inhibition of the proteasomal activity is associated with induction of tumor cell apoptosis (14-16, 22, 23). We compared the apoptosis-inducing activities among the fruit and vegetable extracts as measured by PARP cleavage and *in vitro* caspase-3/-7 activity assay. As positive controls, both green tea extract and (-)-EGCG induced cleavage of PARP (Figure 2B) and activation of caspase-3/-7 (Figure 3). The rank of the apoptosis-inducing abilities of the tested fruit and vegetable extracts was apple, grape > onion > strawberry, tomato, celery (Figures 2B and 3), consistent with the order of their potencies in inducing accumulation of ubiquitinated proteins (Figure 2A). Therefore, inhibition of the cell proteasome activity by these fruits and vegetables correlated well with induction of leukemia cell apoptosis.

Fruit and vegetable extracts induce morphological changes preferentially in prostate cancer over normal cells. After we determined the apoptosis-inducing potencies of various fruit and vegetable extracts in leukemia Jurkat T cells (Figures 2, 3), we studied their morphological effects on the human prostate cancer PC-3 cell line. Exponentially growing PC-3 cells were treated for 12 h with purified (-)-EGCG (as control) or an extract of green tea (as control), apple, grape, onion, strawberry, tomato or celery extract at various concentrations, followed by observing cellular morphological changes (Figure 4). The untreated PC-3 cells were elongated, indicative of healthy cells (no treatment), but became spherical after treatment with green tea extract at 2.5-10% or (-)-EGCG at 25-50 μM (Figure 4), which was followed subsequently by detachment of the cell and death. Similar morphological changes were also observed in PC-3

cells treated with apple or grape extracts at 2.5-10% and onion extract at 5-10% (Figure 4). These morphological changes and the number of cells displaying such changes were concentration-dependent (Figure 4). However, tomato and celery extracts showed no such effects even at 10% concentration (Figure 4). The morphological change-inducing potencies of apple, grape, onion, tomato and celery extracts matched with their abilities to inhibit the proteasome activity and induce apoptosis in Jurkat T cells (Figures 2, 3). These results further support our conclusion that proteasome inhibition is a common mechanism for anti-cancer activity. Although the strawberry extract had little effect on intact Jurkat T cells in accumulation of ubiquitinated proteins (Figures 2, 3), it was able to induce PC-3 cellular morphological changes (Figure 4), indicating the involvement of different molecular targets.

We then determined the effect of these fruit and vegetable extracts on normal murine NIH 3T3 fibroblasts. Impressively, under the same treatment (12 h), neither purified (-)-EGCG nor any extract of green tea or fruit and vegetable at 1, 2.5 and 5% was able to induce the appearance of any spherical cells as above (Figure 5) (data at 1, 2.5% not shown). At the highest concentration used (50 μM or 10%), only onion and celery extracts had some effect on cellular morphological changes (Figure 5). These data suggest that fruit and vegetable extracts are able to preferentially target cancer *over* normal cells.

Discussion

In this study, we tested the hypothesis that some fruits and vegetables inhibit tumor cell proteasome activity and that this inhibition is responsible for their reported cancer risk-lowering abilities (1-5). We tested the potencies of apple, grape, strawberry, onion, tomato and celery in inhibiting the proteasomal activity *in vitro* and *in vivo*, and correlated the results with their apoptosis-inducing activities. We found that apple and grape, like green tea extract and purified (-)-EGCG, were more potent than onion, tomato and celery in inhibiting cell-free proteasomal chymotrypsin-like activity and proteasomal activity in intact cells. Interestingly, the order of the potencies of these fruit and vegetable extracts in inhibiting the proteasome activity was found to be consistent with that of their abilities to induce Jurkat T cell apoptosis and the appearance of spherical cells preferentially in prostate cancer PC-3 *over* the normal NIH 3T3 cell line (Figures 1-4).

It has been demonstrated that the chymotrypsin-like activity of the proteasome is associated with cancer cell survival (14, 22, 23). Our previous studies have shown that (-)-EGCG is a potent and specific inhibitor of proteasomal chymotrypsin-like activity and that such inhibition is associated with cancer cell growth arrest and apoptosis (14-16). Studies from other groups using different proteasome inhibitors resulted in the same conclusions (29, 30). The results of this study demonstrate, for the first time, that the proteasomal chymotrypsin-like activity is also significantly inhibited *in vitro* by apple, grape, strawberrys, and onion extracts, but to a much lesser extent by tomato and celery extracts (Figure 1).

To determine whether the fruit or vegetable extracts are able to inhibit the proteasome activity *in vivo*, we treated Jurkat T cells with each extract, followed by Western blot analysis of whole cell lysates using an anti-ubiquitin antibody. Our data indicated that all examined extracts that could inhibit proteasomal activity *in vitro* could also inhibit proteasomal activity *in vivo*, except strawberry. It has been observed that strawberry extract inhibits the proteasome activity mainly *in vitro* but with little effect *in vivo*, and has little effect on activating caspase-3/-7 either (Figures 2, 3). The reasons remain to be further investigated.

Previous studies have shown that proteasome inhibitors induce tumor cell apoptosis (14-16). In this study, we found that apple and grape extracts are potent proteasome inhibitors. Consistently, these extracts are also able to induce apoptosis, as indicated by PARP cleavage and caspse-3/-7 activity. Our data also demonstrated that only the extracts that inhibit proteasomal chymotrypsin-like activity *in vivo* can induce activation of caspase-3/-7 and cleavage of PARP (Figures 1-3).

An ideal prerequisite for any anticancer agent would be to induce apoptosis selectively in cancer but not in normal cells. We tested the effect of extracts on the viability of cancer and normal cells. We found that normal fibroblasts were generally more resistant than prostate cancer cells to treatment by green tea, apple and grape extracts (Figure 5 *vs.* Figure 4). The order of induction of cellular morphological changes in cancer cells was again that of apple, grape > onion > tomato, celery (Figure 4).

In conclusion, our results have indicated that apple, grape and onion extracts are inhibitors of proteasomal activity *in vitro* and *in vivo*. Furthermore, such inhibition *in vivo* by these fruit and vegetable extracts is associated with induction of cancer cell apoptosis. This mechanistic study further supports other scientific evidence from epidemiological studies and strongly suggests that daily and regular consumption of fruits and vegetables may play an important role in prevention and/or suppression of cancer. Future studies should focus on uncovering detailed mechanisms responsible for the cancer-preventive properties of fruits and vegetables.

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