Routledge
Taylor & Francis Group

Nutrition and Cancer

ISSN: 0163-5581 (Print) 1532-7914 (Online) Journal homepage: www.tandfonline.com/journals/hnuc20

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To cite this article: Peter J. Ferguson, Elzbieta M. Kurowska, David J. Freeman, Ann F. Chambers & James Koropatnick (2006) In Vivo Inhibition of Growth of Human Tumor Lines by Flavonoid Fractions From Cranberry Extract, Nutrition and Cancer, 56:1, 86-94, DOI: 10.1207/s15327914nc5601_12

To link to this article: https://doi.org/10.1207/s15327914nc5601_12

Published online: 18 Nov 2009.

NUTRITION AND CANCER, *56*(1), 86–94

In Vivo Inhibition of Growth of Human Tumor Lines by Flavonoid Fractions From Cranberry Extract

Peter J. Ferguson, Elzbieta M. Kurowska, David J. Freeman, Ann F. Chambers, and James Koropatnick

Abstract: *Edible fruits and berries may serve as sources for novel anticancer agents, given that extracts of these foods have demonstrated cytotoxic activity against tumor cell lines. Semipurified, flavonoid-rich extracts of cranberry (*Vaccinia macrocarpa*) were shown previously to arrest proliferation of tumor cells and induce apoptosis. However, the ability of cranberry flavonoids to inhibit tumor growth in vivo has not been reported other than in a preliminary report. As model systems for testing this activity, human tumor cell lines representative of three malignancies were chosen: glioblastoma multiforme (U87), colon carcinoma (HT-29), and androgen-independent prostate carcinoma (DU145). A flavonoid-rich fraction 6 (Fr6) and a more purified proanthocyanidin (PAC)-rich fraction were isolated from cranberry presscake and whole cranberry, respectively, by column chromatography. Fr6 and PAC each significantly slowed the growth of explant tumors of U87 in vivo, and PAC inhibited growth of HT-29 and DU145 explants ($P < 0.05$), inducing complete regression of two DU145 tumor explants. Flow cytometric analyses of in vitro-treated U87 cells indicated that Fr6 and PAC could arrest cells in G1 phase of the cell cycle ($P < 0.05$) and also induce cell death within 24 to 48 h of exposure ($P < 0.05$). These results indicate the presence of a potential anticancer constituent in the flavonoid-containing fractions from cranberry extracts.*

Introduction

Although major progress has been made in understanding the biology of human cancers, with concomitant design and discovery of novel chemotherapy agents, there remains a critical need for clinically useful agents that can control the growth of solid tumors. Based on the association between diets high in fruits and vegetables and decreased incidence of some cancers, edible plants are increasingly being studied as potential sources of new anticancer agents (1–7). Cranberries (*Vaccinia macrocarpa*) are a rich source of phytochemicals. Extracts of this berry display antiproliferative activity against human tumor cell lines (8–11). Flavonoid-containing fractions of cranberry extracts induced cell cycle arrest and apoptosis in a human cancer cell line in vitro (10). In preliminary studies, cranberry extract fractions inhibited the growth of the human glioma cell line U87 in vivo when administered by injection to tumor-bearing mice (11), and growth of the breast carcinoma cell line MDA-MB-435 in mice was inhibited by cranberry when included as part of the animals' diet (12).

There are several tumor types for which it is of particular importance to find new treatments, considering their frequency in the population and/or poor response to standard treatment. Glioblastoma multiforme is the most common primary brain malignancy in adults, and has a median survival of less than 1 year (13). Colorectal cancer remains one of the top three leading causes of cancer death in North America, as does prostate cancer in men (13). Effective chemotherapy regimens for these tumors are very limited, leaving few second-line treatment choices when a tumor becomes refractory to the primary treatment. Proliferation of cell lines derived from each of these tumor types was shown to be inhibited by fractions of cranberry extracts in vitro (10), but it had not been demonstrated that these fractions could inhibit tumor growth in vivo. In the current study, fractions of cranberry extracts inhibited growth of all three of these tumor types (glioblastoma, and colorectal and prostate carcinomas) in vivo, even to the point of inducing complete regression of two xenografts of the human prostate tumor cell line DU145. To increase our understanding of the mechanism by which inhibition was induced, the glioblastoma cell line was assayed for cell cycle distribution and viability following treatment with the cranberry extracts in vitro. The in vitro assays suggest that tumor growth inhibition may result from cell cycle arrest and induction of cell death.

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Materials and Methods

Preparation of Cranberry Extracts

Cranberry extract and fractions were generously provided by Ocean Spray, Inc. (Lakeville-Middleboro, MA). Flavonoid-rich fraction 6 (Fr6) was isolated by standard chromatographic techniques from cranberry presscake as described previously (10). (Presscake is the remaining wet hulls after the juice is squeezed out.) Briefly, a 25°C water extract of blended cranberry presscake was eluted through a reverse-phase C18 Flash 40M cartridge column (Biotage, Inc., Charlottesville, VA). After elutions of inactive material using methanol and water, the Fr6 fraction was eluted using 1% acetic acid/methanol and was then freeze-dried. The proanthocyanidins (PAC) fraction was isolated from a whole cranberry extract by a similar process, except that the flavonoid-rich fraction (equivalent of Fr6) was eluted subsequently through a column of LH-20 Sephadex™ (Sigma-Aldrich, Inc., St. Louis, MO) using 50% ethanol followed by 80% acetone. The concentrations used hereafter refer to those of the freeze-dried powder on a wt/vol basis, upon solubilizing in the appropriate solvent [dimethylsulfoxide (DMSO) for cell culture, 10% DMSO in water for mouse injections].

In Vitro Assays of Antiproliferative Activity

Cell culture medium and fetal bovine serum were purchased from Invitrogen, Inc. (Burlington, Ontario, Canada). Cell culture plasticware was obtained from Invitrogen, Fisher Scientific (Unionville, Ontario, Canada), and VWR International (Mississauga, Ontario, Canada). The cell lines U87 glioma, HT-29 colon carcinoma, and DU145 prostate carcinoma were obtained from American Type Culture Collection (Rockville, MD). Cultured cell lines were maintained and cytotoxicity assays conducted as described previously (10). Briefly, proliferation was assayed in 96-well plates by staining of cells using the vital stain Alamar Blue™ (BioSource International, Inc., Camarillo, CA), and quantitated by fluorescence on a Wallac Victor plate reader (Perkin-Elmer, Boston, MA). Concentrations of extract that inhibited proliferation of cultured cells by 50% (IC_{50} values) were determined by interpolation of plotted data.

In Vivo Experiments

Female Balb/c mice and immunocompromised NCR-nu/nu, female mice, 4–6 weeks old (Taconic, Germantown, NY), were housed in a temperature- and humidity-controlled environment, with a 12-h light–dark cycle, in a pathogen-free isolation facility regulated and controlled by the University of Western Ontario. Female mice were used for all cell line xenografts to maintain consistency of any influences on drug disposition and metabolism. (Even though the tumors were grown in female mice, it would not be expected to influence the growth or treatment-response of the androgen-independent, prostate tumor cell line DU145.) The mice were maintained according to the *Guide for the Care and Use of Laboratory Animals*, set by the Canadian Council on Animal Care and adopted by The University of Western Ontario. Mice were fed a standard laboratory diet ad libitum.

Tumors were established by harvesting rapidly proliferating, cultured cells, and injecting subcutaneously into the right flank. Numbers of cells injected, in 75 µl saline, for each cell line were U87, 1×10^6; HT-29, 5×10^6; DU145, 4×10^6. When primary tumors became palpable, their maximum length and width were measured every 2 days with calipers. Volume was calculated assuming an ellipsoidal tumor shape (length × width^2 × π/6). When tumors became larger than approximately 1 cm^3, mice were euthanized by CO_2 asphyxiation.

Control groups comprised eight mice, and groups that received cranberry extracts contained six mice. Doses of 100 mg/kg PAC and 250 mg/kg Fr6 were administered intraperitoneally (ip) in a volume of not more than 80 µl of 10% DMSO in water. This amount of vehicle (control injections) had no detrimental effects on the animals. Injections were every 2 or 3 days from Day 2 (preliminary study, Ref. 11) or Day 3 (remainder of study) to 21 or 24 (total of 10 injections). Mice were weighed daily during the treatment period, and subsequently at twice-weekly intervals.

Flow Cytometric Assays

DNA (cell cycle) distribution: Extract treatment of U87 cells in 75-cm^2 flasks was initiated by introduction of 0.2 volume of a sixfold concentration of Fr6 or PAC in growth medium (final concentrations as given in legend for Fig. 7). Following the indicated incubation period, cells were harvested by trypsinization, and nuclei were prepared, treated with RNase A, and stained using propidium iodide (PI) (14). The nuclei were analyzed by flow cytometry (XL-MCL Flow Cytometer; Beckman Coulter, Hialeah, FL). DNA (cell cycle) distributions were analyzed using Multicycle for Windows Advanced DNA Cell Cycle Analysis software (Phoenix Flow Systems, San Diego, CA).

Apoptosis: Cultures were prepared and exposed to Fr6 or PAC as described above. Cells were harvested and, without fixing, were stained with fluorescein-conjugated annexin-V (BD Biosciences, Mississauga, Ontario, Canada) and PI. Cells that stained positively only for annexin-V were early apoptotic. Cells that were doubly positive for annexin V and PI were considered to be dead or dying, and for purposes of calculating the total population of dead and dying cells, were grouped with early apoptotic cells.

Statistical Analysis

Differences in the following measurements, between treated and control groups, were compared using a Student's *t*-test: the IC_{50} values for Fr6 and PAC against the three cell lines, average weights of mice, average tumor sizes in mice, length of time to reach specified tumor volumes, cell cycle

Table 1. Sensitivity of Cell Lines to Cranberry Extract Fractions, as Determined by In Vitro Proliferation Assay[a,b]

	IC_{50} (mg/L)	
Cell Line	Fr6	PAC
U87	128 ± 25 (7)	48 ± 12 (7)[c]
HT-29	168 ± 69 (4)	79 ± 5 (2)[c,d]
DU145	234 ± 75 (4)	96 ± 20 (5)[c,d]

a: Abbreviations are as follows: IC_{50}, concentrations of extract that inhibited proliferation of cultured cells by 50%; Fr6, flavonoid-rich fraction 6; PAC, proanthocyanidin fraction.
b: Mean ± SD (*n*).
c: IC_{50} value for PAC was significantly different from that of Fr6; $P < 0.05$.
d: Significantly different from U87; $P < 0.05$.

distribution, and size of populations of dead and dying cells. In all analyses, differences were considered to be statistically significant if the $P < 0.05$.

Results

In Vitro Inhibition of Proliferation of Human Tumor Cell Lines by Cranberry Extract Fractions

A series of human tumor cell lines was screened for sensitivity to cranberry preparations Fr6 and PAC. The cell lines reported in Table 1 are representative of tumors from three different tissues, selected for subsequent testing for in vivo sensitivity. The glioma cell line U87 was the most sensitive to PAC by approximately twofold. U87 tended to be more sensitive to Fr6 than the other two cell lines, but the apparent difference was not significant ($P = 0.16$ compared with HT-29; $P = 0.065$ compared with DU145).

Inhibition of Growth of Human Tumor Cell Lines in Nude Mice by Cranberry Extract Fractions

Before testing Fr6 and PAC against tumors in nude mice, it was necessary to identify doses at which these preparations would be toxic to the fewest animals. Balb/c mice were administered three ip injections of various doses of cranberry preparations, 2 days apart, while the animals were monitored for dose-dependent indicators of health (weight change, motility, skin changes). Tolerable doses of Fr6 and PAC in Balb/c mice were determined to be 250 mg/kg for each agent. Upon initiation of treatment of nude, tumor-bearing mice, it was apparent that these mice were much less tolerant of PAC. Doses of 100 mg/kg PAC and 250 mg/kg Fr6 were chosen to be used for injections every 2 days for 3 wk after a range of doses was tested in the nude mice. These doses resulted in minor toxicities that were manageable and from which the mice recovered: skin petechiae that resolved spontaneously after 3 or 4 days and weight loss (less than 20%) during treatment, from which the animals recovered even during the treatment period or else immediately after the treatment ended. During subsequent experiments, given that some animals died unexpectedly of unknown causes, treatment schedules were modified slightly to maintain better health of the animals. The schedules for specific experiments are detailed in the legends for Figs. 1, 2, 4, and 6, respectively.

In the initial, preliminary experiment, PAC (a dose of 100 mg/kg on Day 1 post-tumor injection and every second day up to Day 17) significantly slowed growth of U87 tumors ($P < 0.05$), when tumor sizes on each day were compared (11). These differences were significant on each day from the day that tumors were initially observed (Day 8) until the first day that control animals had to be euthanized due to large tumor size (Day 16). In addition, PAC significantly increased the time required for the U87 tumors to reach milestone sizes of 400, 500, and 600 mm^3 by approximately 48–59% (5–7.5 days; $P < 0.05$; data not shown).

Figure 1 is indicative of the effect of PAC (and also Fr6; data not shown) on weights of the mice during treatment. For the purposes of statistical analysis, it was apparent that there were three phases of treatment effects, and so the body weights within each phase were grouped and subjected to analysis for possible differences (by Student's *t*-test). The first phase (I) revealed a loss of weight in treated mice ($P < 0.05$) between the initiation of treatment and Day 6. During the recovery phase (II, Days 7–16), body weights remained significantly lower than controls ($P < 0.05$). In the final phase (III), from Days 17–25 (note that treatment ended on Day 17), the weights of treated mice were not significantly different from those of controls.

In the study that followed, the ability of PAC to inhibit U87 tumor growth in vivo was confirmed subsequently; the same action was demonstrated for Fr6 (Fig. 2). The injection schedule was modified slightly to begin on Day 3 in view of the potential for established tumors actively undergoing pro-

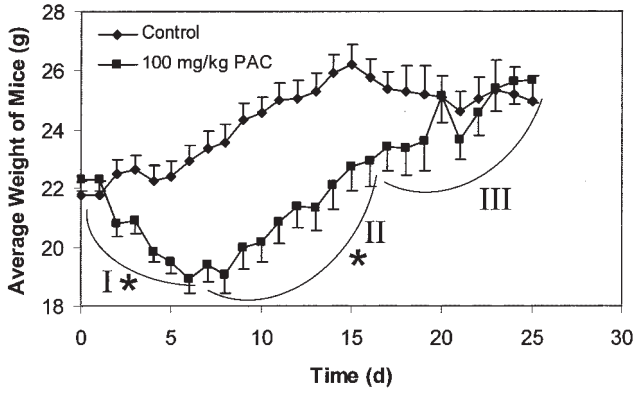

Figure 1. Effect of proanthocyanidins (PAC) treatment on body weights of nude, tumor-bearing mice. Mice were injected with PAC, 100 mg/kg intraperitoneally, on 1, 3, 5, 7, 9, 11, 13, 15, and 17 days after tumors were implanted (Day 0). For this experiment, data are presented as means ± SEM ($n = 12$ mice per group). *$P < 0.05$: measurements were grouped according to phase of initial weight loss (I, Days 0–6), recovery period (II, Days 7–16), and recovered (III, Days 17–25) for purposes of analysis by *t*-test.

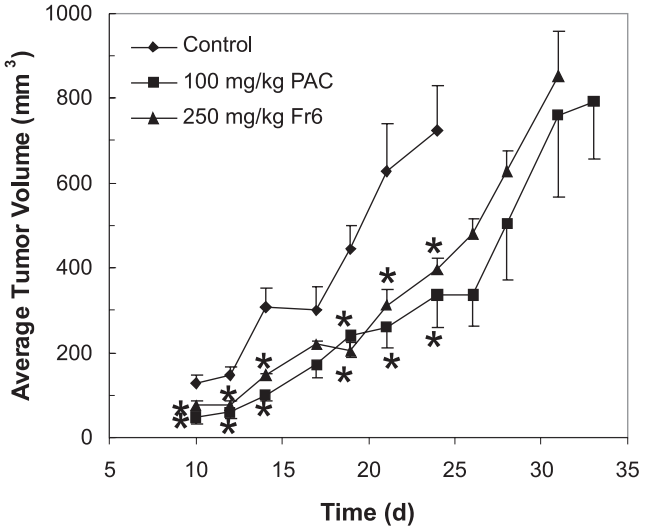

Figure 2. Effect of proanthocyanidins (PAC) and flavonoid-rich fraction 6 (Fr6) treatments on growth of U87 tumors in mice. One × 10^6 U87 glioma cells were implanted subcutaneously into the flank of female, nude mice. Mice were injected subsequently intraperitoneally with PAC at 100 mg/kg, Fr6 at 250 mg/kg, or vehicle on the following days after tumor implant: 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21. Tumor sizes were measured by caliper and volume was calculated assuming an ellipsoid (control, $n = 8$; treated, $n = 6$; means ± SEM). *$P < 0.05$.

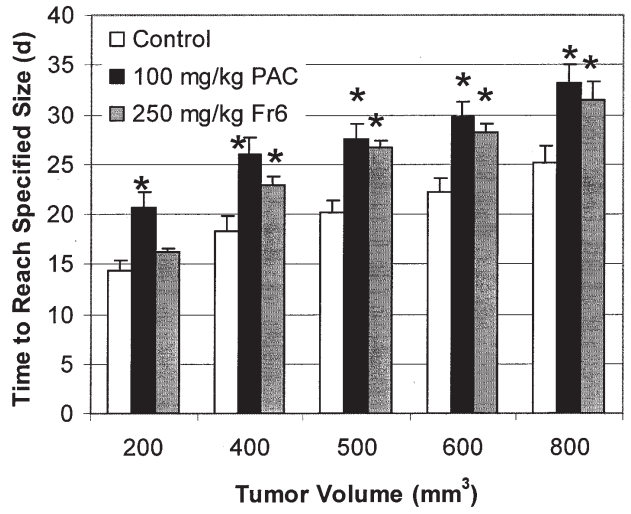

Figure 3. Effect of proanthocyanidins (PAC) and flavonoid-rich fraction 6 (Fr6) treatments on time required for U87 tumors to reach milestone sizes in mice. The length of time for tumors to reach the volumes indicated was determined by interpolation of plotted sizes for tumors in each individual animal. *$P < 0.05$. Data were derived from the experiment presented in Fig. 2.

liferation to be more sensitive to cytotoxic treatment than quiescent tumor cells immediately after injection. Mice received a total of 10 injections. On days when significant differences in tumor size between treated and control were evident, tumors in treated mice were 40–68% smaller than in controls. PAC and Fr6 significantly increased the time required for the tumors to reach milestone sizes (Fig. 3). Growth was delayed by up to 40%.

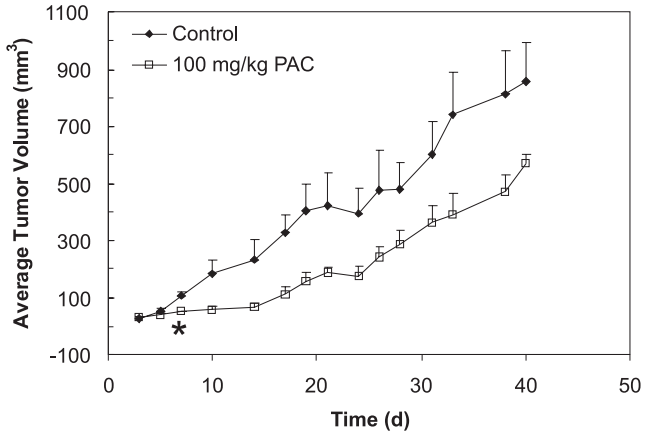

Figure 4. Effect of proanthocyanidins (PAC) treatment on growth of HT-29 tumors in mice. Five × 10^6 HT-29 colon carcinoma cells were implanted subcutaneously into the flank of female, nude mice. Mice were injected subsequently intraperitoneally with PAC at 100 mg/kg or vehicle on the following days after tumor implant: 3, 5, 7, 10, 12, 14, 17, 19, 21, and 24. Tumor sizes were measured by caliper and volume was calculated assuming an ellipsoid (control, $n = 8$; treated, $n = 6$; means ± SEM). *$P < 0.05$.

The treatments for which data are shown in Figs. 1–3 generally were well tolerated. Weight loss (less than 20% total body weight) returned to normal during and following the end of treatment. One Fr6-treated animal, in apparently good health, died of unknown causes following the sixth injection (Day 13). This may have resulted from an embolism or inadvertent injection of extract directly into a blood vessel. A second PAC-treated mouse died of apparent abdominal infection, potentially introduced as a result of the frequency of injection.

The ability of PAC to inhibit tumor growth in vivo was then investigated using tumor cell lines derived from two different tissues: colon and prostate. For these experiments, mice were given an extra day after the third, sixth, and ninth injections to recover from potential treatment-associated toxicities, particularly the previously observed weight loss and idiopathic deaths. PAC significantly inhibited the growth of both HT-29 colon tumor and DU145 prostate tumor in nude mice. HT-29 tumor growth was delayed significantly (up to 8 days), after which time heterogeneity in control tumor growth rate reduced significance (Fig. 4). In each group (control and treated) there was one animal in which the HT-29 tumors failed to establish; therefore, these were omitted from the calculation of averages to plot tumor growth (Fig. 4) and for the purpose of calculating the time required to reach milestone sizes (Fig. 5). PAC delayed growth by approximately 10 to 14 days, equivalent to a 75% (the time to reach a volume of 200 mm^3) to 34% (time to reach 1,000 mm^3) delay.

The average size of PAC-treated DU145 prostate tumors was significantly smaller at 71 days (Fig. 6). Two slow-growing tumors in the control group and heterogeneity in the length of time tumors took to become established contributed to a lack of significance in differences in the length of time tumors took to grow to the milestone sizes of 200 and 400

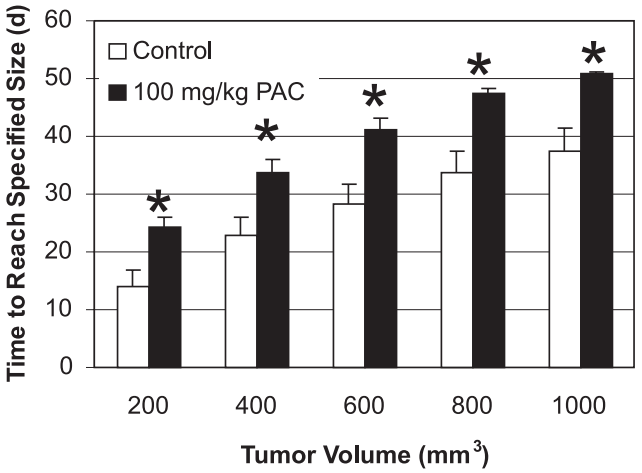

Figure 5. Effect of proanthocyanidins (PAC) treatment on time required for HT-29 tumors to reach milestone sizes in mice. The length of time for tumors to reach the volumes indicated was determined by interpolation of plotted sizes for tumors in each individual animal. *$P < 0.05$. Data were derived from the experiment presented in Fig. 4.

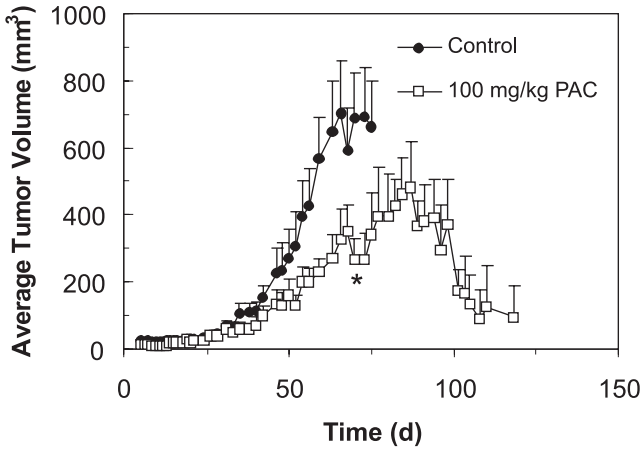

Figure 6. Effect of proanthocyanidins (PAC) treatment on growth of DU145 tumors in mice. Four × 10^6 DU145 prostate carcinoma cells were implanted subcutaneously into the flank of female nude mice. Mice were injected subsequently intraperitoneally with PAC at 100 mg/kg or vehicle on the following days after tumor implant: 3, 5, 7, 10, 12, 14, 17, 19, and 21. Tumor sizes were measured by caliper and volume was calculated assuming an ellipsoid (control, $n = 8$; treated, $n = 6$; means ± SEM). *$P < 0.05$.

mm3. All control tumors reached a size of 1,000 mm3 by 52 to 108 days (when animals were euthanized). However, of the group of four PAC-treated tumors, only one reached a size of 600 mm3 (this animal died of an unknown cause on Day 100). (Of six mice in the PAC-treated group, two were lost to toxic events of unknown cause early in the treatment protocol.) Two of these PAC-treated tumors reached sizes of 400 and 500 mm3, respectively, but completely regressed by 108 days. A third PAC-treated tumor (585 mm3 at maximum size) had shrunk to 285 mm3 when the experiment was ended at 120 days, 100 days after treatment had ended. Figure 6 demonstrates this regression between Days 87 and 118. The plot for control tumors was discontinued at Day 75, given that the average tumor size was affected greatly by the loss of large tumors due to euthanization of animals.

Alteration of Cell Cycle Kinetics in Human Glioma Cell Line by Cranberry Extract Fractions

Fr6 and PAC were tested for their ability to alter cell cycle kinetics of U87 cells by analyzing DNA distribution by flow cytometry at 24 and 48 h of exposure (Fig. 7). At concentrations that caused significant inhibition of proliferation (i.e., decreased cell numbers at 4 days compared with untreated control cells), both Fr6 and PAC induced a significant increase in the proportion of U87 cells in G1 by 24 h, with a concomitant decrease in the number of cells in S phase. By 48 h, the cell cycle distribution resembled that of control cells (drug concentration = 0 on *x*-axis).

Induction of Cell Death in Human Glioma Cell Line by Cranberry Extract Fractions

Concentrations of Fr6 and PAC similar to those used to generate the data shown in Fig. 7 were tested for their ability to induce cell death, using a flow cytometric, double-staining method for cells actively undergoing apoptosis and dead cells. Annexin V staining alone distinguished early apoptotic cells, and cells that were positive for both annexin V and PI were considered late apoptotic or dead, including those killed by nonapoptotic mechanisms. Grouping these populations collectively as "dead and dying cells" (Fig. 8), both cranberry extract preparations induced significant cell death at concentrations that caused inhibition of proliferation by 4 days. Paclitaxel was used as a positive control. For the lower concentrations (in particular, 100 and 200 mg/L Fr6 and 25, 50, and 100 mg/L PAC), the percentage of dead and dying cells increased over time. Cells that had detached from the growth surface of the flasks, and were included in all analyses, contributed less than 10% to the total fraction of dead and dying cells. This floating fraction comprised mainly dead cells: for treatments with the two highest concentrations of each preparation, at increased time of exposure, up to 75% of these detached cells were dead, as determined by positive annexin V and PI staining (data not shown).

Discussion

The extracts used in these studies were obtained from cranberry presscake (the material remaining following the extraction of juice) (Fr6) or from homogenized whole berry (PAC). Both extracts are believed to contain the majority of flavonoids in cranberry, based on the process by which they were isolated. Both fractions significantly inhibited proliferation of tumor cell lines in vitro (10,11) and PAC inhibited the growth of three cell lines in vivo (this study); Fr6 inhibited growth of U87 in vivo. Other fractions consisting of sugars, proteins, acids, and waxy materials, respectively, were

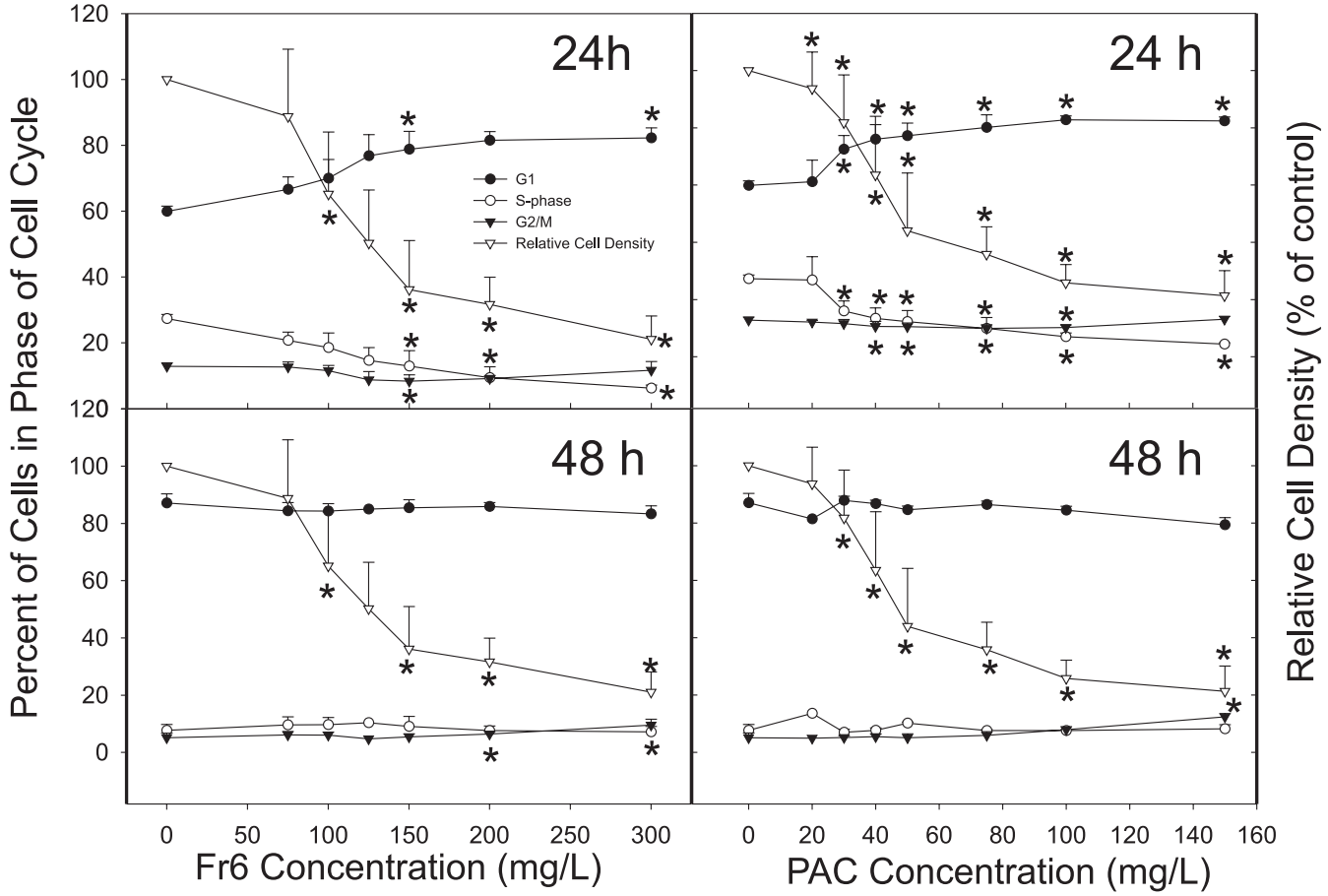

Figure 7. Cell cycle distribution analysis of flavonoid-rich fraction 6 (Fr6) -treated and proanthocyanidins (PAC) -treated U87 cells. Rapidly proliferating U87 cells were exposed to 0 to 300 mg/L Fr6, or 0 to 150 mg/L PAC, and at 24 and 48 h were tested flow cytometrically for DNA content (cell cycle distribution). Data points represent summarized data from three to five separate determinations (means ± SD). The percentages of cells in G0/G1 phase, S phase, and G2/M phases were determined at 24 and 48 h. Relative cell density (96-well plate, Alamar Blue assay) was determined at 4 days for each exposure for each experiment. Concentrations of extract that inhibited proliferation of cultured cells by 50% for Fr6 and PAC for this set of experiments were 128.4 ± 25.1 and 47.8 ± 12.2 (means ± SD; $n = 5$), respectively. *$P < 0.05$ compared with dimethylsulfoxide control.

demonstrated previously to contain no antiproliferative activity (data not shown).

A previous in vitro assessment of sensitivity of cell lines to the cranberry extracts suggested that the three cell lines used in the present study were of sufficient sensitivity to respond to in vivo treatment if they were implanted into immunocompromised mice. An earlier in vivo screen of the prostate cancer cell line LNCaP and the breast carcinoma cell line MDA-MB-435 failed to show in vivo suppression of growth or tumor regression induced by Fr6 or PAC (data not shown). However, the initial in vivo screen of the U87 cell line indicated that this line would serve as a model system for subsequent studies. The weights of most of the treated mice began to recover even during the treatment period, and had recovered totally by the time treatment was completed (Fig. 1). However, several mice developed irreversible weight loss requiring euthanasia or died of unknown causes. Therefore, the dosing schedule was modified to allow mice an extra day of recovery after every third injection, which helped to minimize weight loss and treatment-related deaths. Any loss of appetite caused by treatment was transient, which suggests that treatment could be continued for longer than described here. Longer treatments will be attempted in future studies to assess capacity to effect complete regression of tumors.

It is clear from the growth curves of the control and treated U87 tumors (Fig. 2), and based on the time required to reach milestone sizes (Fig. 3), that both the Fr6 and PAC preparations slowed the growth of these explants. Of course, the dose of extract that can be used against the mice is limited by toxicity to the animals, as monitored by weight loss. On a weight-per-weight basis, the allowable dose of PAC was 40% of that of Fr6, which is indicative of the higher purity of active but also toxic components in the mixture obtained at the second chromatography step in the preparation of this product. It remains to be established whether the ingredient or ingredients causing toxicity to the animals is or are the same as or different from those responsible for antitumor activity. If toxic constituents do not contribute to the anticancer potential of the extract, future steps to purify the antitumor component may allow for higher dosing of the active ingredient that yields an even more effective arrest of U87 tumor growth.

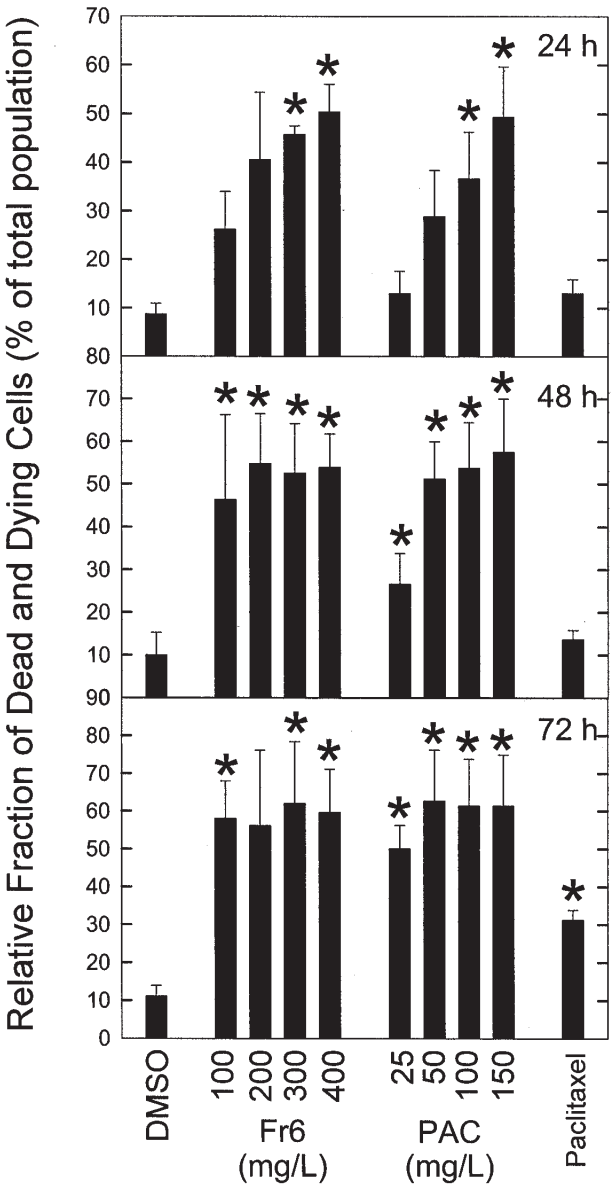

Figure 8. Induction of cell death in flavonoid-rich fraction 6 (Fr6) -treated and proanthocyanidins (PAC) -treated U87 cells. Rapidly proliferating U87 cells were exposed to 0–400 mg/L Fr6 or 25–150 mg/L PAC and after 24, 48, and 72 h were tested flow cytometrically for incidence of dead and dying cells. Bars indicate the percent of the entire population of cells that was dead and dying, summarized from three to four experiments (means ± SD). *$P < 0.05$ compared with dimethylsulfoxide (DMSO) control. Relative proliferation of treated cells, compared with DMSO-treated controls after 4 days for 100, 200, 300, and 400 mg/L Fr6 was, respectively, 74.1 ± 12.7%, 28.7 ± 7.2%, 21.4 ± 3.9%, and 14.1 ± 3.0% (n = 3–4) (means ± SD). Relative proliferation in 25, 50, 100, and 150 mg/L PAC was, respectively, 95.8 ± 10.7%, 61.4 ± 12.9%, 30.5 ± 5.8%, and 17.9 ± 0.7% (n = 3) (means ± SD). Relative proliferation in 4 nM paclitaxel was 27.2 ± 6.2% (n = 4).

PAC was also able to inhibit in vivo growth of two other cell lines derived from tumors that arose in two different tissues. The appearance of palpable HT-29 tumors in mice was delayed significantly by administration of PAC (Fig. 4). In addition, the delay in reaching each of the milestone sizes caused by PAC treatment was significant (Fig. 5).

The inhibition of growth of prostate carcinoma DU145 tumors in vivo was only significant at one time point, but remarkably, the PAC-treated tumors began to regress after they had reached approximately 400 to 600 mm^3 (Fig. 6). None of the control tumors regressed in size prior to reaching 1,000 mm^3 (at which point the animals must be euthanized), although it is not known if regression would have occurred if the mice had been kept alive. Additional studies are required to determine if a lengthier treatment, possibly with a lower dose of PAC, would be able to induce earlier and complete regressions of DU145 tumors.

Flavonoids have been demonstrated to induce cell death or cell cycle arrest depending on the flavonoid and on the concentration (5,15–19). In addition, arrest may occur in G1 or G2, depending on the cell type (5,15). Fr6 was shown previously to arrest MDA-MB-435 breast tumor cells in G2/M phase of the cell cycle after 24 h of treatment, with a concomitant decrease in the fraction of cells in S phase, and no change in the G1 fraction (10). In contrast, both Fr6 and PAC arrested glioma U87 cells by 24 h in G1, and less so in G2/M, with a resulting decrease in the S-phase fraction (Fig. 7). After another 24 h, however, the DNA profile resembled that of the control cells. This may have been due to the death of the arrested cells, which occurred in a concentration- and time-dependent manner (Fig. 8). The changes in cell cycle distribution were all observed at concentrations of Fr6 or PAC at which proliferation during 4 days was significantly decreased by up to 60–70%. These semipurified cranberry extracts contain mixtures of unidentified compounds (Freeman and Ferguson, HPLC data not shown), which may contribute different effects on cellular growth regulation, possibly in different proportions depending on the cell type that is being tested.

Physiological events that occur only in whole animals (innate immune functions in nude mice, angiogenesis, interactions with stromal cells, and others) may be affected by cranberry extract in a manner that facilitates tumor regression. Effects of this type are not known or addressed by the present study, but are under investigation. This study confirmed a preliminary finding that Fr6 and PAC slowed the growth of a glioma cell line explant in vivo. Larger studies are planned to confirm and potentially improve the in vivo inhibitory effect of the cranberry extracts on growth of all three tumor types tested herein, possibly by extending the treatment schedule. The complete regression of two of the DU145 tumors is highly suggestive of potential success against this tumor type.

The component or components of cranberry extract that are responsible for the antitumor activity observed in this study are believed to belong to the flavonoid family of

phytochemicals. Flavonoids comprise four major groups: proanthocyanidins, anthocyanins, flavan-3-ols (catechins), and flavonols. Cranberries contain compounds from each group (20), but it is not known which constituent is responsible for their anticancer activity. Flavan-3-ol monomers (which include catechins) constitute approximately 7% of total PAC (21). Catechin and epicatechin inhibit proliferation of tumor cell lines (22). In cranberry, the flavan-3-ols generally exist in the form of polymers of two to at least 12 units (PAC) (21,23,24). Studies of their antiproliferative activity have not been reported.

Studies were undertaken to determine whether four flavonoids, identified as potential cranberry constituents, might be responsible for the antiproliferative activity of Fr6 and PAC. Fractionation of Fr6 and PAC was performed by high-performance liquid chromatography (HPLC) and all fractions were tested for antiproliferative activity. The retention times of resveratrol, quercetin, myricetin, and epigallocatechin gallate were then determined and superimposed on the fraction chromatograms. Only epigallocatechin gallate coeluted with the cytotoxic activity (data not shown). In addition, against three cell lines that differed in relative sensitivity to Fr6 and PAC, the four candidate compounds did not display the same relative antiproliferative activity (data not shown). Further purification will be required to identify the active constituent in the cranberry extracts.

Two laboratories have isolated cranberry components that have antiproliferative activity. One group of components is a trio of triterpene hydroxycinnamates (8), which behave physicochemically differently from Fr6 or PAC with respect to solubilities and HPLC elutions. A second laboratory identified epicatechin polymers and anthocyanin glycosides in antiproliferative crude fractions of cranberry, but individual active ingredients were not identified (9). There remains a need to identify the active components of Fr6 and PAC, with the intent of obtaining a clinically useful preparation.

PAC demonstrated promising, significant antitumor activity against three tumor types that are notoriously refractory to traditional chemotherapy agents (13,25–27). Growth of U87 glioblastoma was also inhibited in vivo by Fr6. Various flavonoids are reported to inhibit proliferation, induce apoptosis, and/or inhibit malignancy-related functions (e.g., metalloprotease) in glioma cell lines (28–32), the colon carcinoma cell line HT-29 (33), and the androgen-independent prostate carcinoma cell line DU145 (22,33,34). Otherwise, information on the cytotoxic activity of cranberry flavonoids against human cancer cell lines is limited.

The very high mortality rates of glioblastoma, metastatic colon cancer, and androgen-refractory prostate cancer indicate the dire need of new agents that can eradicate or at least control these malignancies. The demonstrated ability of cranberry extracts to inhibit the growth of human tumor explants representative of these three tumor types in vivo indicates that cranberry is potentially an important source of a novel anticancer agent.

Acknowledgments and Notes

This study was undertaken at the London Regional Cancer Program, London Health Sciences Centre, London, Ontario, Canada. The authors would like to acknowledge the expert technical assistance of Ling He and Jennifer MacDonald, KGK Synergize, Inc., with the animal studies, and Wendy Brown and Michael Keeney, London Health Sciences Centre, with the flow cytometry studies. Advice with flow cytometry from Karen Morley, University of Western Ontario, and with statistical analysis from Larry Stitt, University of Western Ontario, was also appreciated. Editorial advice was provided by Dr. Rinee Mukherjee, KGK Synergize, Inc.

This work was presented in part at the World Cancer Research Fund International/American Institute for Cancer Research International Research Conference on Food, Nutrition and Cancer, July, 2004 (11). The research project was supported by a grant from the American Institute for Cancer Research. Dr. Chambers is a Canada Research Chair in Oncology, supported by the Canada Research Chairs Program.

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Submitted 7 November 2005; accepted in final form 18 July 2006.

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