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Cranberry Proanthocyanidins Induce Apoptosis and Inhibit Acid-Induced Proliferation of Human Esophageal Adenocarcinoma Cells

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The occurrence of esophageal adenocarcinoma and its only recognized precursor lesion, Barrett's esophagus, has rapidly increased during the past three decades. The precise reason for the rise remains to be elucidated, but increasing rates have been linked to multiple nutritional factors. Plant-based diets have generally been associated with a reduction of risk for esophageal adenocarcinoma and those of animal origin with risk escalation. Moreover, a number of recent in vitro and limited in vivo investigations have reported that cranberry extracts affect multiple cancer-associated processes in breast, colon, prostate, and other cancer cell lines of epithelial origin. Thus, this study sought to investigate the chemopreventive potential of a cranberry proanthocyanidin rich extract (PAC) in SEG-1 human esophageal adenocarcinoma (EAC) cells. PAC pretreatment significantly inhibited the viability and proliferation of EAC cells in a time- and dose-dependent manner. Moreover, PAC (50 μg/mL) significantly inhibited acid-induced cell proliferation of SEG-1 cells. PAC treatment induced cell cycle arrest at the G1 checkpoint and significantly reduced the percentage of SEG-1 cells in S-phase following 24 and 48 h of exposure. PAC treatment also resulted in significant induction of apoptosis. Thus, PAC modulates cell cycle regulation, aberrant proliferation, and apoptosis, all key biological processes altered during progression to esophageal adenocarcinoma. These findings support that further mechanistic studies are warranted to more fully elucidate the inhibitory potential of PAC against esophageal cancer.

KEYWORDS: Cranberry proanthocyanidins; cell cycle; antiproliferative; esophageal cancer; SEG-1

INTRODUCTION

The occurrence of esophageal adenocarcinoma and its only recognized precursor lesion, Barrett's esophagus, has increased at an alarming rate during the past three decades throughout most of the Western world (*1–3*). Esophageal cancer is now the seventh leading cause of cancer-related deaths among U.S. males (*4*). In 2007 an estimated 15560 new incident cases of esophageal cancer are expected and 13940 deaths (*4*). Furthermore, the five-year survival rate for those diagnosed with esophageal cancer remains poor, only 15.6% for all stages, reflecting the insidious nature of this malignancy and the need to develop improved preventive and treatment strategies. The precise reason for the rapid increase remains to be elucidated, but increasing rates have consistently been linked to chronic reflux (*5–7*) and obesity (*8–10*). Other risk factors linked to the development of esophageal adenocarcinoma include dietary patterns and specific nutritional factors, the presence of hiatal hernia, and potential medication use (*11–17*). Plant-based diets have generally been associated with a reduction of risk for esophageal adenocarcinoma and those of animal origin with risk escalation (*15–17*).

Research supports that cranberry proanthocyanidins have a role in maintaining urinary tract health (*18–21*); however, the anticancer potential of cranberry or cranberry constituents is not well characterized. A number of in vitro investigations utilizing breast, colon, prostate, lung, oral cavity, and epithelial cells have found that cranberry extracts affect the cancer-associated processes of proliferation and apoptosis (reviewed in refs *22–27*). In addition, one recent in vivo investigation reported that cranberry extracts inhibited the growth of glioma, colon, and prostate tumors in nude mice (*28*). However, to our knowledge the cancer inhibitory potential of cranberries or cranberry constituents has not been evaluated against esophageal cancer utilizing in vivo models or in vitro systems. We have previously reported on the esophageal cancer inhibitory potential of other berries (black raspberries) with bioactive constituents following a preclinical assessment (*29*) and in a recent pilot study conducted in patients with Barrett's esophagus (*30*). Thus, given our positive results with black raspberries and building

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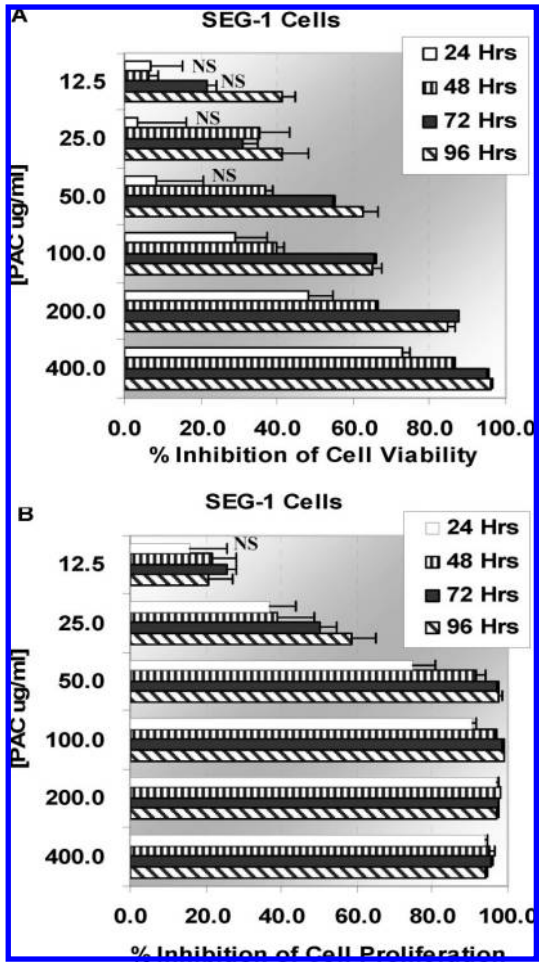

Figure 1. PAC treatment inhibits SEG-1 cell viability and cell proliferation in a time- and dose-dependent manner. (**A**) Effects of PAC treatment (12.5, 25, 50, 100, 200, and 400 μg/mL) on cell viability 24, 48, 72, and 96 h post PAC treatment. Cell viability was measured using the WST-1 assay. (**B**) Effects of PAC treatment (12.5, 25, 50, 100, 200, and 400 μg/mL) on cell proliferation 24, 48, 72, and 96 h post PAC treatment. Cell proliferation was measured using the BrdU assay. Data are expressed as a percentage of vehicle-treated cells, mean $\pm$ SE, and a Students *t* test was performed to determine significant differences due to PAC treatment. NS indicates PAC-treated cells were not significantly different from vehicle-treated cells; all other groups were significantly different.

evidence that cranberry extracts inhibit the growth of a number of epithelial-derived tumor cell lines, we embarked on the screening of a proanthocyanidin-rich cranberry extract (PAC) for potential antiesophageal cancer effects. We utilized two human esophageal adenocarcinoma cell lines: SEG-1, a cell line that can be stimulated to proliferate through acid exposure; and a non-acid-responsive line, BIC-1. Thus, in this initial evaluation we sought to investigate the chemopreventive potential of PAC to inhibit cell viability, cell proliferation (acid-induced and noninduced), and the effects of PAC on cell cycle phase, cellular morphology, and apoptosis.

MATERIALS AND METHODS

Cranberry Extract. Cranberry fruit (*Vaccinium macrocarpon* Ait.) was collected at the Marcucci Center for Blueberry and Cranberry Research, Chatsworth, NJ. The cranberry PAC-rich powder was obtained by our study collaborator, Dr. Amy Howell of Rutgers University, Chatsworth, NJ, and prepared as previously reported (*18*). Briefly, purified cranberry proanthocyanidin extract was isolated using solid-phase chromatography according to well-established methods for

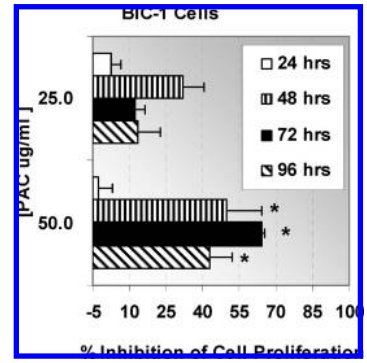

Figure 2. PAC (25 or 50 μg/mL) inhibits cell proliferation of BIC-1 esophageal cells. Data are expressed as a percentage of vehicle-treated cells, mean $\pm$ SE, and asterisks indicate a statistically significant difference compared to vehicle-treated cells ($P \leq 0.05$, two-tailed *t* test).

proanthocyanidin isolation (*18, 20, 21*). Cranberry fruit was homogenized with 70% aqueous acetone and filtered, and the pulp was discarded. The collected cranberry extract was concentrated under reduced pressure to remove the acetone. The cranberry extract was suspended in water, applied to a preconditioned C-18 solid phase chromatography column, and washed with water to remove sugars, followed by acidified aqueous methanol to remove acids. The fats and waxes were retained on the C-18 sorbent. The total polyphenolic fraction containing anthocyanins and flavonol glycosides as well as the proanthocyanidins (confirmed using reverse phase HPLC with diode array detection) was eluted with 100% methanol and dried under reduced pressure. The total polyphenolic fraction was suspended in 50% EtOH and applied to a preconditioned Sephadex LH-20 column, which was washed with 50% EtOH to remove low molecular weight phenolics (anthocyanins and flavonol glycosides). Proanthocyanidins that remained adsorbed to the LH-20 were eluted from the column with 70% aqueous acetone. Elution of the proanthocyanidins was monitored using diode array detection at 280 nm. The absence of absorption at 360 and 450 nm confirmed that anthocyanins and flavonol glycosides were removed. Acetone was removed under reduced pressure, and the resulting purified proanthocyanidin extract was freeze-dried. The presence of proanthocyanidins with A-type linkages was confirmed using matrix-assisted laser desorption ionization (MALDI-TOF MS) or electrospray ionization (DI/ESI MS) as previously described (*18*). In summary, current technologies including ^{13}C NMR, electrospray mass spectrometry, MALDI-TOF MS, and acid-catalyzed degradation with phloroglucinol have all been utilized to characterize the profile and concentration of proanthocyanidins present in the extract under evaluation (*18, 20, 21*). As previously reported, the proanthocyanidin molecules consisted of epicatechin units with mainly DP of 4 and 5 containing at least one A-type linkage (*20*).

Cell Culture. The SEG-1 and BIC-1 human esophageal cancer cell lines were utilized in this series of experiments. These cell lines were each derived from a Barrett's-associated adenocarcinoma of the distal esophagus and were kindly provided by Dr. David Beer of the University of Michigan, Ann Arbor, MI. Cells were grown in Dulbecco's modification of Eagle's medium (DMEM) containing L-glutamine (2.0 mM), penicillin (10^4 units/mL), streptomycin (10^4 μg/mL), sodium pyruvate (1 mM), and 5–10% fetal bovine serum (FBS) depending on the experiment. Cells were maintained as monolayers at 37 °C with 95% air and 5% CO_2 throughout all studies.

Assessment of Cell Viability and Cell Proliferation. Cells were plated in sterile 96-well plates (1×10^4 cells/well) and incubated for 32 h prior to treatment with PAC at concentrations of 12.5, 25.0, 50.0, 100.0, 200.0, and 400.0 μg/mL. Each drug concentration was tested in replicates of six per experimental time point. PAC was dissolved in a very small amount of 100% alcohol, which was then diluted with DMEM phenol-free complete media with 5% FBS to reach the desired concentration of PAC (resulting in a final concentration of alcohol of less than or equal to 0.001% in the cell culture). Simultaneous measurements of cell viability and cell proliferation were assessed at 24, 48, 72, and 96 h following PAC treatment. Cell viability and cell

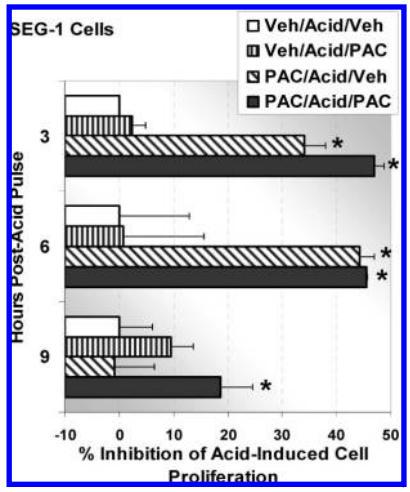

Figure 3. PAC (50 μg/mL) inhibits acid-induced cell proliferation of SEG-1 esophageal cells. Asterisks indicate a significant difference compared to controls treated with vehicle, acid-pulsed, and replenished with medium and vehicle ($P \leq 0.05$, two-tailed t test). Data are shown as the mean percent proliferation compared to the controls.

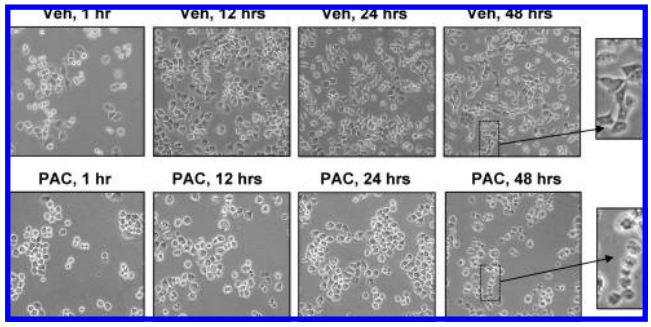

Figure 4. Changes in SEG-1 cell morphology and density following treatment with PAC (50 μg/mL) for 1, 12, 24, and 48 h (magnification 100× and 200× for enlarged subpopulation of cells at 48 h). The effects of PAC treatment are evident as early as 12 h, and by 48 h cell density is decreased and numerous cells shrink and exhibit a rounded morphology characteristic of apoptosis.

proliferation were measured in the same plate utilizing the WST-1 (catalog no. 1644807) and BrdU (catalog no. 1647229) colorimetric ELISA kits as described by the manufacturer (Roche Applied Science, Indianapolis, IN). Briefly, the viability assay is based on the ability of metabolically active cells to cleave the tetrazolium salt, WST-1, into a formazen dye. The BrdU cell proliferation assay is based on the incorporation of the pyrimidine nucleoside analogue BrdU instead of thymidine during DNA synthesis.

Effects of PAC on Acid-Induced Cell Proliferation. Acid reflux is considered to be an important risk factor for esophageal adenocarcinoma progression and is known to increase cellular proliferation and modulate other key cancer-related pathways. Thus, we assessed the inhibitory potential of PAC against acid-induced cell proliferation in acid-responsive SEG-1 cells. Briefly, cells were pretreated with PAC (50 μg/mL) or vehicle for 24 h, pulsed with acidified medium (pH 3.5) or medium alone for 20 min in a humidified chamber at 37 °C, then rinsed with PBS and replenished with fresh medium and vehicle or PAC (50 μg/ml) as indicated by the experimental groups shown in **Figure 2**. Cell proliferation was measured by the BrdU colorimetric ELISA as described above at 3, 6, and 9 h post acid exposure. Acid-induced proliferation maximizes approximately 6 h post acid exposure in SEG-1 EAC cells (data not shown), which is how the time points of 3, 6, and 9 h were chosen for evaluation.

Determination of Cell Cycle Phase and Apoptosis by Flow Cytometry. SEG-1 cells were plated in T25 flasks at 1.5×10^6 cells/flask and incubated for 24 h prior to treatment with PAC at 50.0 μg/mL or the vehicle (<0.001% ETOH). PAC or vehicle was added in

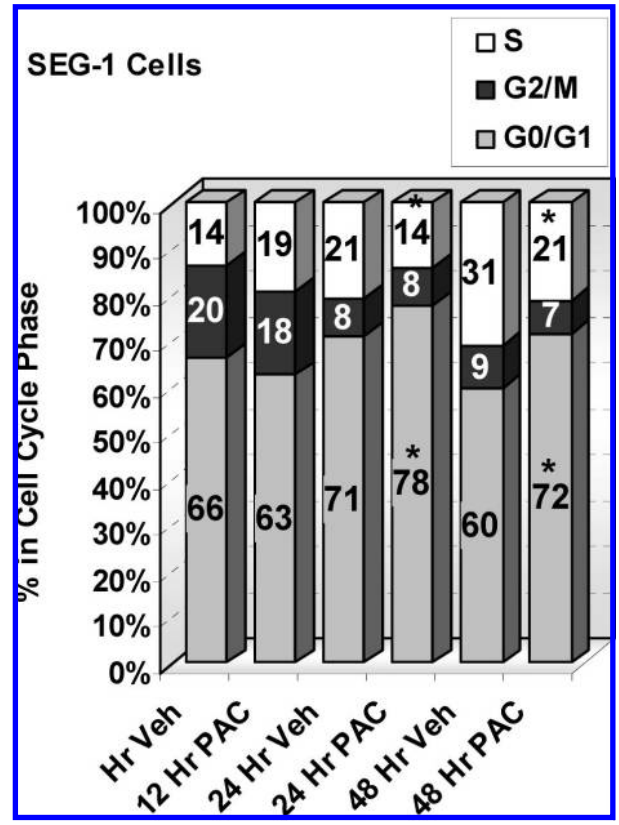

Figure 5. Effects of PAC on SEG-1 cell cycle distribution following 12, 24, and 48 h of treatment. Mean percent of cells in specific cell cycle phases is displayed on the bar graphs per treatment and time point. Asterisks indicate a significant difference between PAC- (50 μg/mL) and vehicle-treated cells at the same time point ($P < 0.05$, two-tailed t test). Pretreatment with PAC for 24 and 48 h significantly decreases the percentage of cells in S-phase and significantly increases the percentage of cells in the G1 checkpoint.

DMEM phenol-free complete medium with 5% FBS. Each evaluation was performed in at least triplicate per experimental time point. The propidium iodide (PI) detection method was employed (BD Sciences, Palo Alto, CA) to access cell cycle distribution at 12, 24, and 48 h post-treatment with PAC or vehicle in SEG-1 cells. In brief, cells were collected at each time point and fixed in ice-cold 70% ETOH, washed in PBS, and stained with PI. Annexin V-FITC staining methods were employed to detect apoptotic events following 24 and 48 h of treatment with PAC or vehicle, as previously described (*31*). Analysis of the cell cycle data was made using ModFit LT software (Verity, Topsham, ME) to determine the percentage of cells in each phase of the cell cycle (G0/G1, S, G2/M) on the basis of DNA content histograms, and for apoptosis data WinMDI software was utilized (Joseph Trotter; http://pingu.salk.edu.software.html). A FACSCalibur flow cytometer was employed for cell cycle and apoptosis analysis utilizing a minimum of 20000 and 10000 cells per treatment, respectively.

Statistical Analysis. Values are expressed as the mean ± SE or SD, as indicated in the displayed results. The significance of the difference between the control or vehicle-treated and each experimental test condition was analyzed using Student's t test. All tests were two-sided, and a value of $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Inhibition of SEG-1 Cell Viability and Proliferation by PAC. Anticancer activity has been reported for whole cranberry extracts as well as specific cranberry fractions in numerous in vitro studies utilizing cancer cell lines and in limited in vivo investigations (*22–28*). However, to our

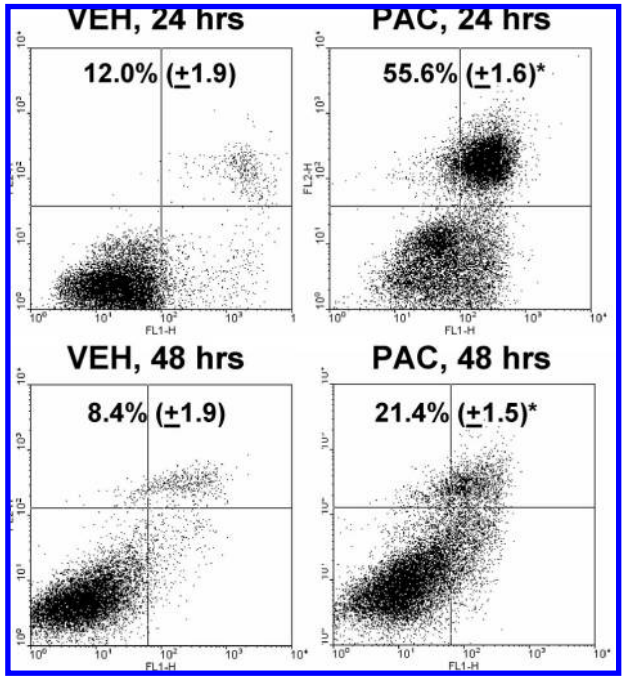

Figure 6. PAC (50 μg/mL) induces apoptosis of SEG-1 esophageal cells. Asterisks indicate a significant difference compared to vehicle-treated cells at the same time point ($P < 0.05$, two-tailed t test). Data are shown as the mean percent total apoptosis (±SD).

knowledge the cancer inhibitory potential of cranberries or cranberry constituents has not been evaluated against esophageal cancer utilizing in vivo models or in vitro systems. Thus, to initially screen PAC for potential antiesophageal cancer effects, we utilized an acid-responsive human esophageal adenocarcinoma cell line, SEG-1, and evaluated the ability of PAC to inhibit cell viability and cell proliferation over time and at multiple concentration levels. As indicated in **Figure 1A**, pretreating SEG-1 cells with PAC for 48 h or more at concentrations of 25 μg/mL or greater significantly inhibited cell viability. The inhibition appeared to be both dose- and time-dependent with the greatest declines evident at 72 h of treatment and beyond. However, when PAC was administered at concentrations of 100 μg/mL or greater, significant declines in viability occurred as early as 24 h. Similarly, PAC significantly inhibited SEG-1 cell proliferation when administered at concentrations of 25 μg/mL or greater; however, there was essentially no additional inhibition of SEG-1 cell proliferation by PAC at concentrations above 50 μg/mL. In addition, we evaluated the antiproliferative effects of PAC in a second esophageal adenocarcinoma cell line, BIC-1. As displayed in **Figure 2**, pretreatment of BIC-1 cells with 25 μg/mL PAC for 48 h inhibited cell proliferation by 32.1% ($P = 0.07$), whereas 50 μg/mL PAC significantly inhibited BIC-1 cell proliferation by 49.8, 64.5, and 43.1% at 48, 72, and 96 h, respectively. Consequently, additional studies with PAC were carried out utilizing the concentration of 50 μg/mL, which moderately affected cell viability but strongly inhibited proliferation of two different esophageal adenocarcinoma cell lines, SEG-1 and BIC-1.

Esophageal adenocarcinoma cell lines have been employed to gain insight into the mechanisms by which reflux of acid and bile salts promotes progression to Barrett's esophagus and ultimately esophageal adenocarcinoma. Reflux of bile and stomach acid has been linked to esophageal adenocarcinoma in human cohorts, and recent in vitro studies have documented specific cellular responses to acid or bile exposure, including alterations in genes associated with increased cell proliferation and survival, with a corresponding decrease in apoptosis via increases in ERK-, p38-, and JNK-mediated MAP kinase signaling pathways (*32–36*). Morgan et al. reported that exposing SEG-1 EAC cells to acid resulted in immediate down-regulation of genes associated with apoptosis, early alteration in genes up-regulating cell proliferation, and consistent up-regulation of genes associated with cell cycle progression across the time course (*36*). Thus, next we evaluated the ability of PAC (50 μg/mL) to inhibit acid-induced cell proliferation in SEG-1 cells over time. As displayed in **Figure 3**, PAC significantly reduced acid-induced cell proliferation, with maximum reductions occurring 3 and 6 h post acid pulse. Treating SEG-1 cells with PAC before and after the acid pulse (PAC/Acid/PAC) significantly inhibited cell proliferation, by 47, 46, and 19% at 3, 6, and 9 h, respectively. In addition, pretreatment alone with PAC (PAC/Acid/Veh) significantly reduced acid-induced SEG-1 cell proliferation at 3 and 6 h by 34 and 44%, respectively. However, by 9 h post acid pulse the protective effect imparted by PAC pretreatment alone was no longer evident. In addition, acid pulsing SEG-1 cells and treating with PAC post acid pulse (Veh/Acid/PAC) only did not significantly reduce cell proliferation, suggesting that the inhibitor must be present prior to acid exposure to offer maximum protection. This phenomenon is an important consideration for future in vivo and clinical chemopreventive evaluations of PAC given that episodic reflux is common in patients with Barrett's esophagus. Thus, PAC may impart the greatest benefit when consumed multiple times a day or at minimum prior to reflux.

The PAC-induced inhibition of cell viability and cell proliferation were paralleled by reductions in cell density and cellular morphology as illustrated in **Figure 4**. Pretreatment of SEG-1 cells with PAC (50.0 μg/mL) resulted in reduced cell density as early as 12 h compared to vehicle-treated SEG-1 cells, and the difference was maintained through 48 h without any PAC replenishment. These data parallel the significant PAC-associated reductions in cell viability and cell proliferation. Loss of cell cycle checkpoint control and subsequent uncontrolled cell proliferation are characteristic of numerous cancers, including esophageal adenocarcinoma. Given our knowledge that PAC inhibited cell viability and proliferation, including acid-induced proliferation of esophageal adenocarcinoma cells, next we utilized flow cytometry to assess the effects of PAC on inducing cell cycle arrest. Pretreatment of SEG-1 cells with PAC (50 μg/mL) resulted in a significant increase in the percentage of cells at the G1 checkpoint and significant declines in the percentage of cells in S-phase at 24 h and to a greater extent following 48 h of treatment with PAC (**Figure 5**).

In addition, following 48 h of PAC treatment, SEG-1 cells exhibit morphological changes, including cell shrinkage and rounding, consistent with apoptosis induction (**Figure 4**), leading us to evaluate the ability of PAC (50 μg/mL) to induce apoptosis. As shown in **Figure 6** PAC treatment of SEG-1 cells resulted in significant induction of apoptosis 24 and 48 h after treatment, with the greatest magnitude of induction following 24 h. Vehicle-treated SEG-1 cells experienced 12% apoptosis at 24 h compared with 55.6% induction in PAC-treated SEG-1 EAC cells. In summary, our results show that a cranberry proanthocyanidin rich extract has potent effects on cell cycle regulation, cell viability, cell proliferation, and apoptosis of esophageal adenocarcinoma cells. The specific molecules mediating the pro-apoptotic, antiproliferative, and positive cell cycle regulatory effects of PAC are currently under investigation.

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