Antitumor Activities of Apple Extracts

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This work was supported by the Overseas Chinese Scholars Fund of Educational Ministry, China (No. [2003] 406).

Received October 24, 2005; accepted December 22, 2005.

OBJECTIVE To examine the antitumor activities of fresh apple extracts.
METHODS Fuji apple extracts were tested for their anti-LS-174T-proliferative activities, for their effect on expression of PCNA, and ability to induce apoptosis in a LS-174T cell line.
RESULTS Apple extracts inhibited LS-174T cellular proliferation in a concentration and time dependent manner. The apple extracts equivalent to a concentration of 50 mg/ml inhibited the proliferation of the LS-174T cells by 34.5±1.2% after 48 h and 47.5±1.8% after 72 h respectively. Apple extracts inhibited PCNA expression and induced apoptosis of the LS-174T cells at concentrations above 12.5mg/ml.
CONCLUSION Apple extracts can inhibit PCNA expression and induction of apoptosis in LS-174T cells which may contribute to their inhibitiory effect on cellular proliferation.

KEYWORDS: apple, LS-174T cell lines, phytochemicals, PCNA, apoptosis.

Numerous epidemiological studies have shown that individuals who regularly consume fruits and vegetables have a decreased risk of cancer.[1] Phytochemicals in fruits and vegetables are at the frontier of a new perspective of nutrition which concerns the role of nutrients in the prevention and retardation of chronic diseases such as cancer and cardiovascular diseases. Therefore, modifications in the diet may be a plausible strategy for the prevention of cancer. Apples contain an array of phytochemicals including vitamins and polyphenols. Several of these phytochemicals have independently been shown to have anticancer effects. Quercetin has been shown to inhibit the proliferation of azoxymethanol-induced colonic epithelial tumor cells in mice.[2] Phytochemicals in fruits and vegetables exert their protective effects by a variety of different mechanisms and may function as blocking agents which prevent carcinogenesis. Other phytochemicals are suppressing agents that inhibit the expression of neoplastic cells which have already been exposed to a carcinogen.[3] Some phytochemicals are antioxidants that scavenge free radicals and thus reduce oxidative stress which has been implicated in cancer formation. In this study, apple extracts were tested in a colon cancer cell line to determine their antitumor activities. We also sought to investigate the effect on expression of PCNA and apoptosis by an immunohistochemical staining method in order to identify the role of apple extracts in inhibition of cancer cellular proliferation.

MATERIALS AND METHODS

Apple preparation and storage

Fresh Fuji apples (100 *g*) were harvested at a ripe stage and extracted

using 200 ml 80% acetone as reported (weight divided by volume is 0.5).[4] The extracts were stored at -70℃ until used.

Measurement of cell proliferation

Human colonic adenocarcinoma LS-174T cells (purchased from the Cell Institute of Chinese Medical Academy of Science, Shanghai, China) were maintained in DMEM medium supplemented with 10% FBS (Gibco, Life Technologies, Grand Island, NY) at 37℃ in a 5% CO_2 incubator. Cell concentrations of 2.5×10^4/ml in the growth media were placed in each well of a 96-well flat-bottom plate. The cell number was determined from a linear response curve during 96 h of cell growth. After 4 h of incubation at 37℃ in 5% CO_2, the growth medium was removed and media containing various concentrations (0, 0.5, 5, 12.5, 25, 50 mg/ml) of apple extracts were added to the cells. Control cultures received the solvent minus the apple extracts and blank wells contained 100 μl of growth medium with no cells. After 48 and 72 h of incubation, cell proliferation was determined using the colorimetric MTS assay (MTS-based cell titer 96 nonradioactivity cell proliferation assay) (Promega, Madison, WI), a colorimetric method utilizing a tetrazolium reagent. Cell proliferation (%) was determined at 48 and 72 h from the MTS absorbance (490 nm) reading for each concentration compared to the control. At least three replications for each sample were used to determine the cell proliferation (%) value.

Immunohistochemistry

Immmunohistochemistry detection of PCNA expressed in LS-174T cells

LS-174T cells were grown on gelatin-coated Thermanox coverslips (Nunc, Roskilde, Denmark) and various concentrations (0, 5, 12.5, 25 mg/ml) of apple extracts added. After 48 h when the cells were near 80% confluentcy they were rinsed in PBS and fixed in cold acetone for 2 minutes, followed by incubation in 3% hydrogen peroxide. Slides were then incubated with primary antibody against PCNA at 4 ℃ overnight (Boster company, Wuhan, China), followed by incubation with biotinylated secondary antibody for 30 min at room temprature. After a 15-min wash in PBS, the slides were treated with streptavidin-horseradish peroxidase complex at room temperature for 30 min, then incubated with 0.05% of 3,3-diaminobenzidine tetrahydrochloride dihydrate. Finally, the slides were counterstained with hematoxylin. A negative control was performed by PBS substitution for the PCNA antibody staining.

Apoptosis studies

For programmed cell death (apoptosis) studies, LS-174T cells were treated with various concentrations (0, 5, 12.5, 25 mg/ml) of apple extracts for 48 h, rinsed in PBS and fixed in cold acetone for 2 min. DNA fragmentation was assayed by the TUNEL reaction, according to the manufacturer's instructions (Roche Co, Switzerland). The nuclei were counterstained with hematoxylin and apoptotic cells were counted as a percentage of the total nuclei in 7~10 independent fields. The mean value of three independent experiments was considered as the percentage of cells undergoing apoptosis.

RESULTS

Cell proliferation

Cell proliferation was analyzed at 48 and 72 h after the LS-174T cells had been cultured with an equivalent of 0, 0.5, 5, 12.5, 25, 50 mg/ml of apple extracts in the media using the MTS assay. LS-174T cell proliferation was inhibited in a dose and time-dependent manner after exposure to the apple extracts. At 48 h, the inhibited rate was 9.1±1.0%, 14.08±1.60%, 22.2±1.2%, and 35.0±1.2% respectively at the concentrations from 5 to 50 mg/ml of apple extracts. At 72 h, the inhibited rate was 12.5±0.9%, 32.9±0.6%, 42.5±1.4%, and 47.5±1.8% respectively at the concentrations from 5 to 50 mg/ml of apple extracts (Fig.1).

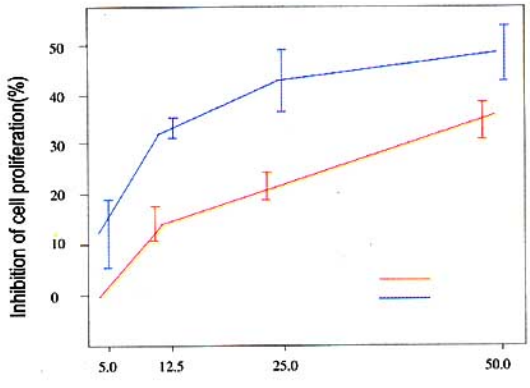

Fig.1. Antiproliferative activity of apple extracts.

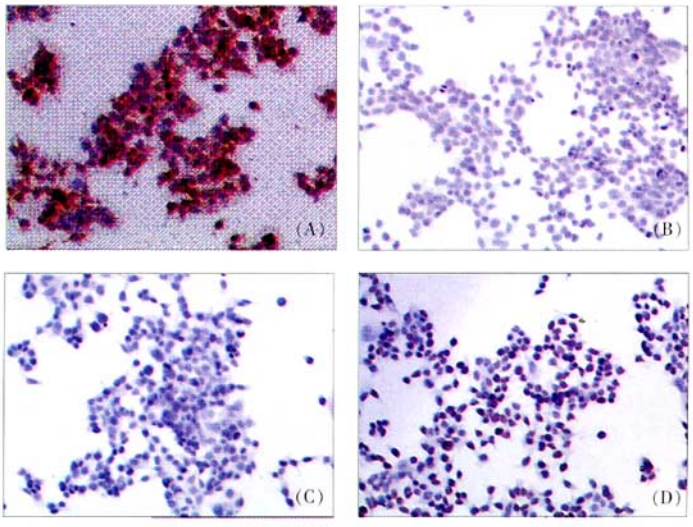

Fig.2A. PCNA, control. **Fig.2B.** PCNA, 25 mg/ml of apple extracts. **Fig.2C.** TUNEL, control. **Fig.2D.** TUNEL, 25 mg/ml of apple extracts.

Immunohistochemistry

Expression of PCNA was observed in most nuclei of LS-174T cells in the control group (90.0±3.0%, Fig. 2A). The positive rate of PCNA decreased with the concentration of apple extracts increasing from 5 to 25 mg/ml (84.2±2.2%, 70.6±1.9%, 52.7±0.9% respectively, Fig.2B). Apple extracts induced apoptosis of LS-174T cell lines obviously above the dose of 12.5 mg/ml. The positive rate of apoptosis increased with the concentrations of apple extracts increasing from 5 to 25 mg/ml (12.3±0.2%, 30.7±0.7%, 51.7±1.3% respectively, Figs.2C,D).

DISCUSSION

Many studies have highlighted the importance of diet in the maintenance of good health and the prevention of cancer. In recent comprehensive reviews, which include approximately 200 studies each, higher intake of fruits and vegetables were significantly associated with a reduced risk of cancer at most sites.[5,6] For people who consume low amounts of fruits and vegetables, the risk of cancer is twice that of people who consume higher quantities of fruits and vegetables.[5] The mechanism of action of many phytochemical inhibitors of carcinogenesis are poorly understood, making it difficult to organize them into a precise pattern. Researchers classify inhibitors into three categories according to the time in the carcinogenic process at which they are effective. The first consists of compounds that prevent the formation of carcinogens from precursor substances. In the second are compounds that inhibit carcinogenesis by preventing carcinogenic agents from reaching or reacting with critical target sites in the tissues. These inhibitors are called "blocking agents" which is descriptive of their mechanism of action. They exert a barrier function. The third category of inhibitors acts subsequent to exposure to carcinogenic agents. These inhibitors are termed "suppressing agents" since they act by suppressing the expression of neoplasia in cells previously exposed to doses of carcinogens that otherwise would cause cancer.[7]

Here we used apple extracts to determine their inhibitory effects on LS-174T cellular proliferation and expression of PCNA and apoptosis. The results showed that apple extracts tested at a concentration of ≥ 12.5 mg/ml had inhibitory activity ($P<0.01$). Cellular proliferation was inhibited in a dose-dependent manner when exposed to apple extracts at a concentration >5 mg/ml. It is well known that PCNA expression has a positive relationship with cancer cellular proliferation , since there was an inverse relationship between apoptosis and cell proliferation, the results showed that the apple's combination of phytochemicals (phenolic acids and flavonoids) decreased the expression of PCNA within the LS-174T cells and also induced apoptosis. Therefore, we suggest that this strong inhibition of tumor cell proliferation in vitro could be due to decreased PCNA expression and induction of apoptosis by the apple extracts.

In summary, this study suggests that the natural combination of phytochemicals in fruits is critical to their antiproliferative activity. Our results will provide a wide prospect for developing more natural anti-tumor drugs with little or without serious side effects.

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