Human colon cancer HT-29 cell death responses to doxorubicin and Morus Alba leaves flavonoid extract

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Abstract: The mechanistic basis for the biological properties of Morus alba flavonoid extract (MFE) and chemotherapy drug of doxorubicin on human colon cancer HT-29 cell line death are unknown. The effect of doxorubicin and flavonoid extract on colon cancer HT-29 cell line death and identification of APC gene expression and PARP concentration of HT-29 cell line were investigated. The results showed that flavonoid extract and doxorubicin induce a dose dependent cell death in HT-29 cell line. MFE and doxorubicin exert a cytotoxic effect on human colon cancer HT-29 cell line by probably promoting or induction of apoptosis.

Key words: Cell death, Doxorubicin, Flavonoid, Viability.

Introduction

Colorectal cancer (CRC) is the third most frequent cancer worldwide and includes about 9.4% of global cancer cases. An increased CRC incidence is observed in developed countries as comparison with less developed ones (1-4). Many evidences from epidemiological studies support the association between cancer risk and dietary food intake (5). Epidemiological studies suggest that fruits and vegetables rich of phytochemical compounds may contribute to a reduced risk of certain cancers particularly colon cancer (1). Oriental medicinal herbs are rich of Flavonoids. Polyphenols are introduced as major component of mulberry fruit with chemopreventive activity and chemotherapeutic effects (6). Flavonoids have antioxidant, antiviral, anti-allergic, anti-inflammatory, antitumor and free radical scavenging properties. Based on flavonoids chemical structures, they are classified to many derivatives such as flavonols, flavones, flavanones, flavanols, isoflavones and anthocyanidins. According to epidemiological studies, a significant decrease of stomach cancer risk was found with total dietary flavonols and dietary quercetin consumption. These results might be due to anti-inflammatory and antitumor effects of these nutrients (5). The findings of a study conducted by Sang et al. suggested that mulberry fruit induces human glioma cells death through ROS-dependent mitochondrial pathway (6). The adenomatous polyposis coli (APC) gene is a key tumor suppressor gene and its inactivation is a major initiating event in colorectal tumorigenesis (7). APC promoter hypermethylation contributes to the loss of APC expression in CRCs with allelic loss on 5q (8). Apoptosis is a cellular process and defined as programmed cell death pathway morphologically, which is focused of clinicians who study and treat cancer. CRC pathogenesis and its resistance to chemotherapeutic drugs and radiotherapy could be due to apoptotic function disorders, both of which act, at least in part, by killing cancer cells (9).

Some food constituents such as flavonoids contribute to prevention of CRC and are able to induce apoptosis following DNA damage, which is an important mechanism of cancer prevention (9). Poly (ADP-ribose) polymerase (PARP) is an enzyme that catalyzes post-translational modification of protein. Poly(ADP-ribose)lation of protein is involved in a variety of biological processes including chromatin structural regulation, transcription, DNA repair, DNA replication, telomere homeostasis, cell division, cell proliferation, cell death and other physiological and pathological functions (10, 11). PARP inhibitors represent a new class of promising drugs in anticancer therapy. In a pilot study, PARP expression in testicular germ cell tumors (GCTs) was evaluated and the correlation between expression patterns and clinic pathological variables was identified by Michal Mego et al (12). They showed that PARP is overexpressed in testicular germ cell tumors compared to normal testis.

The aim of our study was to assess and compare the effect of flavonoids extract of MFE, doxorubicin and MFE plus doxorubicin combination on cell death or growth suppression of human colon cancer HT-29 cell line. In order to obtain direct biological evidence that whether APC gene expression and PARP protein level alteration are responsible for death cells of colon cancer HT-29 cell line after treatment with MFE and doxorubicin drug, the APC gene expression and PARP protein level were investigated.
Materials and Methods

Extraction and purification of flavonoids from mulberry leaves

Mulberry leaves were collected from gardens in North east of Tehran and confirmed by an herbalist and a herbarium code was considered for it. A modified method of Liu et al. was used for extraction and determination of flavonoids (13). Dried Morus alba leaves were used for extraction of total flavonoids. Identification of plant was verified by an herbalist. Powdered material was extracted twice with 70% ethanol solution (v/v) at 90 ℃ for 2h. After filtration and centrifugation (3000 rpm, 15 min), the solvent was evaporated and the aqueous extract was condensed under reduced pressure. One mL of a diluted solution containing flavonoids, 0.7 mL of 5% (w/w) NaNO2, and 10 mL of 30% (v/v) ethanol were combined and stirred for 5 min. Then 0.7 mL of 10% AlCl3 (w/w) was added and the mixture was stirred for 6 min. Then, 5 mL of 1 mol/L NaOH was added. Finally, the NKA-9 macroporous resins were chosen for purification of the separated MTF from the crude extract. Subsequently, the solution was diluted to 25 mL with 30% (v/v) ethanol prior to the measurement. After 10 min standing, the absorbance of the solution was measured at 500 nm with a Carry-100 Spectrophotometer (Bio-Varian Model, Australia). The MTF solution was diluted to 1, 1.5, 2 mg/mL and stored at 4°C before using.

Determination of flavonoids amount

The contents of flavonoids were expressed in mg/g of dry weight by comparing with standard curve of rutin. The yield of flavonoids was calculated using the following formula: \( Y = (6.404A + 0.2806) BV/W \) (mg/g) where: \( A = \) absorbance (500 nm), \( B = \) dilution factor, \( W = \) dry weight of cactus skin precisely measured (g), \( V = \) volume of the extracting agent (mL) (13).

Cell Culture

The HT-29 cell line was obtained from National Center of Cell Sciences, Pastor Institute of Iran (Tehran-Iran). The HT-29 cell line was cultured at 37°C with 5% CO2 in RPMI Medium containing 10% heat inactivated fetal bovine serum (FBS), 1% streptomycin. Cultures at ~80% confluence were routinely split 1:5 in 10 cm culture dishes as follows. The cells were washed twice in pre-warmed PBS. Trypsin-EDTA 0.25% was added to the dishes and incubated at 37°C for 1-5 minutes. The cells were detached from the dishes, 1 mL pre-warmed culture medium was added and the cells transferred to a 50 mL Falcon tube. Cells were spun down at 1500 rpm and plated in new dishes with fresh culture medium.

Cell count

The HT-29 cell line was harvested and seeded into 96-well plates at a density of 15000 cells per well for further experiments. Cells were counted before and after being seed in the 96-well plates. Cells were stained with trypsin blue and counted using neobar lam.

Cell viability: MTT assay

Alive cells were quantified by MTT assay, which is based on the cleavage of the tetrazolium salt (MTT) by metabolically active cells to form a purple formazan dye that is water-insoluble. MTT was cleaved by all living, metabolically active cells such as cancer cells (HT-29). The generated formazan amount is directly proportional to the homogenous cell population. More formazan was produced by active cells than resting cells. These properties are due to cleavage of MTT only by active mitochondria. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) has been used to develop quantitative colorimetric assay for mammalian cell survival and proliferation. MTT was dissolved in PBS at 5 mg/mL concentration and filtered to sterileize and remove the small insoluble residue present in some batches of MTT. Cells were seeded in 96-well culture plates (15000 cell/well in 100 μL culture medium RPMI for HT-29 cell line) and incubated overnight at 37 °C and 5% CO2. Then cells morphology was observed in invert microscope before exposure to flavonoids extract. After overnight growth, supernatants in the culture plates were aspirated out and then 10μL of flavonoid extract solution in concentrations of 0.1, 0.5, 1, 1.5, 5, 10, 50, 100, 500, 1000 μg/mL and 10μL of doxorubicin in concentration of 0.01, 0.1, 0.5, 1, 10, 20, 50, 100, 1000 μg/mL were added to HT-29 cell in each well of 96-well culture plates separately. Then 90 μL of culture medium was added to each well. Treated cells were incubated overnight at 37 °C and 5% CO2 separately. Morphology of cells was observed by invert microscope after exposure to different concentrations of flavonoid extract. After overnight incubation, supernatants were aspirated out and 10 μL MTT solution (50 mg/10 mL PBS) was added to each well then 90 μL of culture medium was added to each well and the plates were incubated for 4 hours in the case of HT-29 cell line. Supernatants were replaced by 100 μL DMSO and plates were shaken at 37 °C in shaker incubator for 15 minutes. Then absorbance at 570 nm wavelength was recorded using ELISA reader. All absorbance values were corrected against blank wells which contained growth media alone.

PARP assay

PARP concentration of the control and treated HT-29 cells with MFE and doxorubicin and combination of MFE + doxorubicin at a concentration of 500 μg/mL was identified according to the manufacturer’s instruction of PARP ELISA kit (Biocompare Company, USA). HT-29 cells were seeded overnight (50,000 cells/well) and treated for 24 hours with 500 μg/mL MFE, 500 μg/mL doxorubicin and (500 μg/mL MFE + 500 μg/mL doxorubicin) combination for 24 hours fixed for detecting adherent cells and analyzed. Medium cell culture and blank were used as positive and negative controls respectively.

Real time PCR assay of APC expression

RNA isolation, cDNA synthesis, and Quantitative Real-time PCR of APC

Total RNA of the control and treated HT-29 cells with MFE, doxorubicin and combination of MFE-doxorubicin at a concentration of 500 μg/mL were isolated according to the manufacturer’s instruction of TRIZOL reagent (Invitrogen). Real-time PCR was performed using an ABI apparatus (Applied Biosystems, USA) according to the manufacturer’s instructions of TRIZOL.
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Concentrations of doxorubicin was also studied. As shown in Figure 2, changes in cell death by doxorubicin were similar to those estimated for MFE by MTT assay, suggesting that the reduction in cell viability by MFE was mainly due to induction of cell death. The IC50 100 and 500 µg/mL were identified for doxorubicin and MFE respectively. To ensure whether MFE-induced cell death was due to increase of APC (adenomatous polyposis coli) gene expression, controls and three groups of HT-29 cells were exposed to 500 µg/mL MFE, 500 µg/mL doxorubicin and (500 µg/mL MFE + 500 µg/mL doxorubicin) combination for 24 hours. APC expression was determined in treated cells and controls by quantitative real-time PCR. According to Figure 3, APC mRNA level of treated HT-29 cells with MFE for 24 hours was significantly increased by 76% relative to controls (4.31 times, p<0.024). 33% and

Statistical analysis

All statistical calculations were performed with the SPSS16 statistical software package (SPSS for Windows, Chicago, IL). Data were expressed as means ± SD and were statistically analyzed by using independent sample t-test. Each experiment was tested in triplicate. p<0.05 was considered statistically significant.

Results

Cell viability and cell death were identified in HT-29 cell line, which exposed to 0.1-1000 µg/mL of total MFE for 24 hours by MTT assay. As it shown in Figure 1, MFE treatment resulted in a reduction of cell viability in a dose dependent manner. The cell viability diminished to 50% of controls after treatment with 500 µg/mL of MFE for 24 hours. To compare the cytotoxic effect of MFE on HT-29 cell viability with current chemotherapy drugs, HT-29 cells treatment with different concentrations of doxorubicin was also studied. As shown in Figure 2, changes in cell death by doxorubicin were similar to those estimated for MFE by MTT assay, suggesting that the reduction in cell viability by MFE was mainly due to induction of cell death. The IC50 100 and 500 µg/mL were identified for doxorubicin and MFE respectively. To ensure whether MFE-induced cell death was due to increase of APC (adenomatous polyposis coli) gene expression, controls and three groups of HT-29 cells were exposed to 500 µg/mL MFE, 500 µg/mL doxorubicin and (500 µg/mL MFE + 500 µg/mL doxorubicin) combination for 24 hours. APC expression was determined in treated cells and controls by quantitative real-time PCR. According to Figure 3, APC mRNA level of treated HT-29 cells with MFE for 24 hours was significantly increased by 76% relative to controls (4.31 times, p<0.024). 33% and

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Table 1. PARP concentration of three treated HT-29 cells groups with 500 µg /mL of MFE, 500 µg /mL of doxorubicin, (500 µg/mL MFE + 500 µg/mL doxorubicin) combination for 24 hours in relation to controls.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>PARP Concentration (ng/ml)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mulberry leaves extract</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.41</td>
<td>0.01</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.94</td>
<td>0.03</td>
</tr>
<tr>
<td>Control</td>
<td>1.41</td>
<td></td>
</tr>
<tr>
<td>Mulberry leaf extract and Doxorubicin</td>
<td>0.99</td>
<td>0.01</td>
</tr>
<tr>
<td>Control</td>
<td>1.41</td>
<td></td>
</tr>
</tbody>
</table>

38% raise were observed for APC expression of treated HT-29 cells with doxorubicin (1.51 times, P=0.03) and doxorubicin-MFE combination (1.63 times, P=0.023) relative to controls, respectively. These results indicate that MFE and doxorubicin induce a significant loss of cell viability through APC expression increase.

To determine whether alteration of PARP level is involved in MFE-induced loss of cell viability, the PARP level of exposed HT-29 cells to MFE, doxorubicin and MFE-doxorubicin combination was examined by ELISA assay. As shown in figure 4 and table 1, the PARP level of HT-29 cells was significantly reduced in the presence of 500 µg/mL MFE (P=0.01), 500 µg/mL doxorubicin (P=0.03) and (500 µg/mL MFE + 500 µg/mL doxorubicin) combination (P=0.01) as comparison to the control cells. It is interesting to note that PARP reduction of exposed HT-29 cells to MFE was higher than those treated with doxorubicin and combination of MFE-doxorubicin.

Discussion

Our results showed that the viability reduction of treated cells with MFE was higher than those were exposed to doxorubicin. In agreement with our result, the strong and inverse linear association of flavonoids and CRC risk was also identified by Evropi and et al. (14). They showed a significant dose dependent reduction of CRC risk that was associated with increased consumption of flavonols, quercetin, catechin and epicatechin. According to the results of a study by Bobe et al. (15), high consumption of flavonols, which are at higher levels in beans, onions, apples and tea, are associated with a decreased risk of advanced adenoma recurrence. Consistent with our results, a significant decreased risk of CRC was reported by Italian hospital-based case-control study (36%) and Scottish population-based case-control studies (27%) for those who are in the highest quartile of flavonol intake (15). An inverse association was also identified between flavones and breast cancer risk by Greek et al (16).

In the present study we showed that a reduction of PARP concentration occurs in vitro in colon cancer HT-29 cell line in response to treatment with MFE and doxorubicin. The findings from our study confirm that PARP concentration decrease could be a useful marker to assess cell death in HT-29 cell line in cell culture condition. It remains to be determined how MFE and doxorubicin causes the release of cytochrome c or activate caspase-3 which are responsible for apoptosis. As the cytochrome c/caspase-3 pathway is essential part for understanding the mechanism of apoptosis, thus, currently we are investigating to uncover the possibility by which the apoptosis occurs. However, our primary results showed that flavonoid-induced apoptosis is stimulated by the release of cytochrome c to the cytosol, by procaspase-9 processing, and through a caspase-3-dependent mechanism. Our results suggested that PARP is a possible negative regulator of cell growth in vitro and as well as probably in vivo, which should be demonstrated. Our analyses indicated that PARP reduction level in HT-29 cell culture condition was associated with a cells death progression.

In a variety of tumor cell lines, overexpression of PAPR-1 has been identified, which was associated with malignant progression. High concentration of PAPR-1 has also been detected in malignant lymphoma cells compared to normal lymph nodes, adjacent non-tumor tissues, or hyperplastic polyps (17). A high correlation was also found between high levels of PAPR-1 and poor prognosis in early breast cancer. In this type of cancer, PAPR-1 was indicated to be the major component of tumor cells in response to DNA damage. PAPR-1 has a critical role for maintaining tumor cells genetic stability. High expression of PAPR-1 was also found in moderate differentiate hepatocellular carcinomas (HCC). Consistently, poly-ADP-ribosylation was increased in HCC, colon carcinomas, cervical cancer, melanoma and basal cell carcinoma. The results of previous studies have shown that PAPR-1 overexpression is related with progression of prostate cancer, and also
is as a potential independent predictor of aggressiveness among the pathological patterns, which is related to this type of tumor (17–21).

It has been shown that PARP inhibitors can affect death of tumor cells. Inhibition of PARP up to 90% could suppress DNA repair. It has been reported that PARP 1 inhibiting alone might be sufficient to induce tumor cell death and avoid the toxic effects of chemotherapy and radiation. This type of therapy is proposed to target only tumor tissues and protect the normal tissues (22). Recently, a variety of agents such as iniparib (BSI 201) and olaparib (AZ 2281) have been introduced as PARP inhibitors to elevate cytotoxic therapy without increasing side effects and to kill cancer cells with DNA repair defect as a single agent in breast and ovarian cancer. PARP inhibitors have selectivity for the tumor cells over normal cells due to genomic instability of some tumor cells (22). PARP inhibitors vary in their route of administration, toxicity profile, and efficacy and resistance mechanism. It should be noted that resistance against PARP inhibitors has been observed like many other therapies. Accordingly, an increased PARP expression has been shown in tumor cells (22, 23). Single strand DNA breaks are repaired by base excision repair (BER). Cell death seems to be occurred by BER inhibition and this makes PARP proteins to be ideal targets for anticancer therapy. It has been revealed that BER function and DNA repair are disturbed by PARP inhibitors. Therefore, PARP inhibitors could affect tumor cells death (22, 23). We assumed that decrease of PARP concentration and continuous APC expression have inhibitory effects on HT-29 cancer cells growth and, therefore, attempted to examine the MFE and anticancer drug doxorubicin effects on APC expression and PARP level of HT-29 cancer cells. As shown in Figure 3 treatment of HT-29 cells with MFE, doxorubicin and their combination induced the ACP level expression elevation in relation to the control cells. Accordingly, increased ACP expression level exposed HT-29 cell to MFE was more than those were exposed to doxorubicin and combination of MFE- doxorubicin. Therefore, the elevated ACP expression in treated HT-29 cells could be due to probably anti-proliferative activity of MFE, doxorubicin and their combination. APC inactivation was reported to be the earliest genetic event that happens in adenomas development into colorectal cancer (24). Inactivation of APC due to mutations was found in 80% of all human colon tumors. It was known that HT-29 cells contains two carboxyl-terminal- truncated APC proteins including 100 kDa and 200 kDa instead of intact APC protein. Stable expression of full-length APC was not found in this and other colorectal cancer lines containing mutant APC (25). Morin et al. (26), by using a inducible APC expression system in CRC H-29 cells containing endogenous inactive APC alleles showed a significant decrease of cell growth. They also showed that reduction of cell growth was due to the induction of cell death through apoptosis. They demonstrated that apoptosis plays an important role in reduction of size and number of cells not only in advanced tumors but also at the very earliest stages of neoplasia. It has been also identified that nonsteroidal anti-inflammatory drugs (NSAIDs) as cancer chemotherapeutic agents can decrease the size and number of colorectal tumors. NSAIDs were reported to replace the physiologic function of APC that was destroyed by mutation (26).

As it shown in Figure 3, the effect of combination of MFE and doxorubicin on cell viability is lower than that of just MFE. It seems that some exposed functional groups of MFE that are critical for reduction of PARP level, induction of APC expression level and HT-29 cells death might be buried after exposure to doxorubicin in cell culture. Therefore, it is proposed that only MFE cytotoxic effect is more effective than its combination with doxorubicin for HT-29 cells death and apoptosis.

Taking together, our findings suggest that MFE and doxorubicin exert a cytotoxic effect by probably promoting or induction apoptosis. These data could be potentially useful for identification of other chemo preventive agents.

Also our study revealed that cytotoxic effect of MFE on HT-29 cells was higher than those observed for doxorubicin. Thus, HT-29 cell line treatment with combination of MFE and doxorubicin does not show a synergistic effect on death and apoptosis of HT-29 cells. Clearly, additional pre-clinical studies using appropriate in vivo animal models as well as carefully designed pharmacokinetics studies are needed before clinical testing of MFE as cancer preventive or therapeutic agents.

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