Full Length Research Paper

Effects of recombinant MAP30 on cell proliferation and apoptosis of human esophageal carcinoma EC-1.71 cells

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Momordica anti-HIV protein of 30 kDa (MAP30) was found to have anti-tumor activities. Therefore, in the present study the effects of MAP30 on the proliferation and apoptosis of human esophageal carcinoma EC-1.71 cells were investigated. The antiproliferative activities were determined using methyl thiazolyl tetrazolium (MTT) assay, colony formation and tumor xenograft model of nude mice. Morphological changes were observed by microscopy. The normal, apoptotic and necrotic cells were identified by fluorescence staining. The percentage of apoptosis and the proportion of the periodic cells were displayed by flow cytometry analysis. The level of mitochondria membrane potential (ΔΨm) was determined by 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) fluorescent probe assay and the content of cytochrome C (Cyt C) was investigated by enzyme-linked immunosorbent assay (ELISA). The activity of Caspase-12 was investigated by Caspase-12 Fluorometric Assay. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein expression was investigated by immunocytochemical staining. Our results indicated that MAP30 could suppress the cells proliferation, induce the cells apoptosis, decrease the proportion of cells in S phase, and increase the proportion of cells in G₀/G₁ phases. Treated with MAP30, the ΔΨm decreased, the Cyt C content and the Caspase-12 activity increased, and the NF-κB protein translocated. In conclusion, the recombinant MAP30 could suppress the esophagus carcinoma 1.71 (EC-1.71) cells proliferation and induce their apoptosis.

Key words: Momordica charantia, MAP30, EC-1.71 cells, apoptosis, mitochondria membrane potential, Cyt C, Caspase-12, NF-κB.

INTRODUCTION

Momordica anti-human immunodeficiency virus (HIV) protein of 30 kDa (MAP30) is a type of ribosome inhibiting protein (RIP) widely used in traditional Chinese medicine (Lee-Huang et al., 1990). It possesses multiple properties: N-glycosidase activity (Barbieri et al., 1994), anti-protein synthesize, inhibition activity of HIV-1 integrase (Au et al., 2000), inducing deoxyribonucleic acid (DNA) inactivation (Wang et al., 2000) and so on. Moreover, MAP30 has no toxic effect on normal cells because it cannot ingress into these cells (Lee-Huang et al., 1995; Schreiber et al., 1999). Recent interest in MAP30 has been stimulated by the reports of MAP30

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potent anti-tumor activity against human cancer cell lines. It had been demonstrated that MAP30 had obvious anti-tumor effects on brain tumor, mammary tumor and melanoma (Lee-Huang et al., 2000). Esophageal carcinoma is one of the most common malignant tumors worldwide. However, little is known about the effect of MAP30 on esophageal carcinoma in vitro and in vivo. Therefore, it is an attractive topic whether MAP30 is capable of preventing esophageal carcinoma. This study was to evaluate the ability of MAP30 to inhibit human esophageal carcinoma cell line EC-1.71 proliferation in vitro and in vivo, and to induce the apoptosis of EC-1.71 cells.

MATERIALS AND METHODS

Cell culture and MAP30

EC-1.71 cell line (alternatively named as EC/CUHK1) was established from a well-differentiated human esophageal carcinoma. Cells were grown in culture at 37°C and 5% CO2 in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Beyotime Institute of Biotechnology, Shanghai, China). Recombinant MAP30 was cloned from the Momordica charantia leaves and expressed in our Lab.

Cell viability assay

Measurement of cell growth inhibition with methyl thiazolyl tetrazolium (MTT) (Sigma, USA) assay. Cells were plated in 96-well plates and 10, 20, 30, 40, 50 and 60 μg/ml MAP30 were added, respectively. The control group was given only culture medium. After 24, 48, 72 and 96 h, 20 μl MTT (5 g/L) was added to each well and the plates were incubated at 37°C for 4 h. Then all culture medium supernatant were replaced with 100 μl dimethyl sulfoxide (DMSO) (Sigma, USA). The plates were shaken for 15 min and the absorbance was measured by standard enzyme-linked immunosorbent assay (ELISA). The cells inhibition rate was calculated according to the following formula:

\[
\text{Cells} \ (%) = \left[1 - \frac{A_{\text{treated}}}{A_{\text{control}}}\right] \times 100.
\]

Results were measured in triplicate. Based on the growth inhibitory rates of each group of drugs on cells, SPSS software was used to calculate 50% inhibitory concentrations (IC50).

Colony formation assay

Cells were plated in 100 mm tissue culture dishes (1000/dish) and MAP30 (20, 30 and 40 μg/ml, respectively) were added after 24 h. Fifteen (15) days later, colonies with a diameter of more than 0.5 mm were counted and photographed. Colony forming efficiency was calculated according to the following formula:

\[
\text{Colony forming efficiency} \ (%) = \frac{\text{colony number}}{\text{cell number inoculated}} \times 100.
\]

Morphological changes examined by light and electron microscopy

For studies with light microscopy, cells were treated with 20 μg/ml MAP30 for 24, 48 and 72 h, respectively. Morphological changes were observed. For studies with electron microscopy, cells were cultured with 20 μg/ml MAP30 for 48 h, then fixed with 2% paraformaldehyde/2% glutaraldehyde for 0.1 M phosphate buffer (pH 7.4); followed by 1% osmium tetroxide. After dehydration, the sections were stained with uranyl acetate and lead citrate for observation under a JEM 2000 EX electronmicroscope (JEC, USA).

Identification of normal, apoptotic and necrotic cell by fluorescence staining

The identification of normal, apoptotic and necrotic cell was carried out using a normal /apoptotic/necrotic cell detection kit (NANJING KEYGEN BIOTECH. CO., LTD) according to the manufacturer’s instruction. The cells were treated with MAP30 (20 and 30 μg/ml, respectively) for 48 h. The control group was given only culture medium containing no drugs. Each treatment was tested in tetrad flasks. Cells were observed under a fluorescence microscope equipped with a 510 nm excitation filter and photos were taken by a Nikon eclipse TE300 and Nikon Digital Sight (Nikon, JAPAN). The cells apoptotic/necrotic efficiency was calculated according to the following formula:

\[
\text{Cells apoptotic efficiency} \ (%) = \left[ \frac{\text{nonage apoptotic cells} + \text{advanced stage apoptotic cells}}{\text{cell number} \times 100} \right.
\]

\[
\text{Cells necrotic efficiency} \ (%) = \left[ \frac{\text{necrotic cells}}{\text{cell number} \times 100} \right.\]

Apopotosis and cell cycle distribution

Cellular DNA content and cell distribution were quantified by flow cytometer (FCM) using propidium iodide (PI) (Sigma, USA). The cells were treated with MAP30 (20 and 30 μg/ml, respectively) for 48 h and the control group was given only culture medium containing no drugs. Each treatment was tested in tetrad flasks. The cells (1 ×106) were collected and fixed in 70% alcohol at 4°C overnight, the cells were then centrifuged for 5 min at 1000 rpm. The supernatant was removed and the cells were washed twice with phosphate buffer saline (PBS). Then the cells were re-suspended in 3 ml PBS, then 3 ml phosphate-citrate buffer (0.2M Na2HPO4 and 0.1M citric acid) was added into the cells. After 30 min incubation, cells were collected and re-suspended in 150 μl PI and 200 μl RNaseA (Sigma, USA). The samples were incubated at room temperature in dark for 30 min and analyzed by a Fluorescence Activated Cell SorterOrtho (FACS) 420 FCM (Becton Dickinson Co., USA) in triplicate. Cell populations in the sub-G1 area were quantified from a standard count of 105 cells using MULTICYCLE software (PHEONIX Co., USA).

Evaluation of mitochondrial membrane potential (ΔΨm)

The ΔΨm of the cells was investigated by a mitochondrial membrane potential assay kit with JC-1 (BioVision, Inc., USA) according to the manufacturer’s instruction. Cells were seeded into 24-well plates (7×104/well) and 20 μg/ml MAP30 was added into treated wells 24 h later.

The negative control wells were added only culture medium containing no drugs. The positive control wells were added to carbonylcyanide-m-chlorophenylhydrazone (CCCP). Each treatment was tested in tetrad wells. All the above plates were incubated for 6, 12, 24 and 48 h, respectively. Expressions of JC-1 J-aggregates and JC-1 monomers were observed under a fluorescence microscope and photos were taken by a Nikon eclipse TE300 and Nikon Digital Sight (Nikon, JAPAN).
The cells were treated with 20 μg/ml MAP30 for 48 h and the control group was untreated with MAP30. The cells were collected, washed twice with PBS, re-suspended in 0.5 ml PBS, mixed with egg white, fixed in 10% buffered formalin and embedded in paraffin. The paraffin blocks were cut into 4 μm thick sections. Expressions of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Sigma, USA) were detected using streptavidin-peroxidase (SP) method.

In vivo anti-tumor activity

Ten 7-week old male nude mice (Balb/c, Experimental Animal Center, Yangzhou University, China) were randomized into 2 groups (treated and control group, n, 5). They were inoculated subcutaneously with transplanted EC-1.71 cells (5×10⁶/mouse). Mice in treated group were inoculated with the cells treated with 20 μg/ml MAP30 for 48 h beforehand, while mice in control group were inoculated with the cells untreated. Thirty (30) days later, the mice were sacrificed, and their tumors were removed and weighed immediately. Tumor inhibitory rate was calculated according to the following formula:

Tumor inhibitory rate (%) = 1-(Wtreated/Wcontrol) ×100

RESULTS

Effect of Momordica anti-HIV protein of 30 kDa (MAP30) on EC-1.71 cells in vitro

The results of MTT assay suggested that MAP30 could inhibit the growth of cells in a dose-dependent manner (Table 1). Cells growth were suppressed by 10.0, 22.9, 27.2, 33.0, 40.8 and 46.8%, respectively after treated with 10, 20, 30, 40, 50 and 60 μg/ml MAP30 for 96 h. The IC₅₀ was 82.86 μg/ml. Figure 1 showed the cell colony-forming, and the colony forming efficiency of control group, 20, 30 and 40 μg/ml MAP30-treated group was 39.3±1.032%, 15.1±0.958%, 13.3±0.832%, and 12.3±0.798%, respectively (P<0.01).

The results of light microscope revealed that when treated with MAP30 for 48 and 72 h some cells became round, blunt and shrinkage, smaller in size, membrane blebbing, holes, cytoplasmic extrusions and formation of apoptotic bodies, and became detached and suspended in the medium, especially for 72 h. Otherwise, the cells were regular and grew fully in patches and confluent, rarely sloughing off in the control and 24 h group (Figure 2). The ultrastructural and morphological changes were significantly observed under electron microscope. As shown in Figure 3, nuclear fragmentation, chromosome condensation, and cell
Figure 1. Clony-forming of EC-1.71 cells. 200X (A→D: Control group, 20, 30, 40 µg/ml MAP30 group). Under microscope: in the control group, the shapes of the colonies were in order, the morphous of the cells were good and the disposition between the cells was tight; in the MAP30 group, the shapes of the colonies were in disorder, the cells number of the colony was few, the cells adherence to the wall of the flasks were unlight, the morphous of the cells were bad and the disposition between the cells was very loose.

Figure 2. Morphological changes observed under light microscope. 400X (A: Controls; B→D: EC-1.71 cells treated with 20 µg/ml MAP30 for 24, 48 and 72 h). Compared with control group, the cells treated with MAP30 for 48 and 72 h became round, blunt and shrinkage, smaller in size, membrane blebbing, holes, cytoplasmic extrusions and formation of apoptotic bodies under light microscope.
Figure 3. Morphological changes observed under electronic microscope 10000X. After incubated with 20 μg/ml MAP30 for 48 h, nuclear fragmentation, chromosome condensation, cell shrinkage and ER expansion and broaden were visible.

Figure 4. Morphological changes of EC-1.71 cells treated by MAP30 for 48 h under fluorescence microscope 200X (A: control group, B: 20 μg/ml MAP30 group, C: 30 μg/ml MAP30 group). Nonage apoptotic cells: green nucleoid, irregular cells, e.g. crescent-shaped; advanced stage apoptotic cells: orange nucleoid, chromosome condensation, nuclear fragmentation, vary size; necrotic cells: oval cells, uniform orange yellow nucleoid, regular cells; normal cells: round, and nucleoid were uniform green. When treated with MAP30 for 48 h, apoptotic cells could be seen, but necrotic cells were not found. In control group, the cells were normal and no apoptotic and necrotic cells.

cell shrinkage were visible and endoplasmic reticulum (ER) were expansion and broaden.

As shown in Figure 4, the results of fluorescence staining revealed that when treated with MAP30 for 48 h, some apoptotic cells could be seen under fluorescent microscope. The nonage and advanced stage apoptotic cells were visible, while necrotic cells were not found. Otherwise, in control group, the cells were regular in morphology, which were round, and the nucleoid was uniform green. But apoptotic and necrotic cells were not found.

The cells apoptotic efficiency of control group, 20 and 30 μg/ml MAP30 group was 0.513±0.925%, 27.1±1.503% and 26.9±1.832%, respectively (MAP30 group P<0.01 versus control group, 20 μg/ml P>0.05 versus 30 μg/ml MAP30 group).

FCM with only PI staining showed that cells treated with 20 and 30μg/ml MAP30 for 48h resulted in a higher number of cells in the G<sub>0</sub>/G<sub>1</sub> phase (50.18 and 52.40%, respectively) compared with that in the control group (43.52%). This increase was coupled with the decreased
Table 2. Effects of MAP30 on apoptosis and cell cycle progression of EC-1.71 in vitro (n=3, means ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (µg/ml)</th>
<th>G₀/G₁</th>
<th>S</th>
<th>G₂+M</th>
<th>Apoptosis index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>43.52±1.514Δ</td>
<td>51.55±0.709*</td>
<td>4.92±1.067</td>
<td>2.87±0.137*</td>
</tr>
<tr>
<td>Experiment</td>
<td>20</td>
<td>50.18±1.050ΔΔ1</td>
<td>47.51±0.306*</td>
<td>2.31±0.551</td>
<td>4.67±0.636**</td>
</tr>
<tr>
<td>-</td>
<td>30</td>
<td>52.40±0.431 ΔΔ2</td>
<td>45.68±0.917**</td>
<td>1.93±0.351</td>
<td>7.29±0.300**</td>
</tr>
</tbody>
</table>

**1, **2, *P < 0.01 pairwise comparison illustrated the effect of MAP30 on the induction of apoptosis was in a dose-dependent manner. **1, **2, *P < 0.01 pairwise comparison and ΔΔ1, ΔΔ2, ΔP < 0.01 pairwise comparison illustrated that cells treated with MAP30 resulted in a higher number of cells in the G₀/G₁ phase and decreased percentage of cells in S phase.

Figure 5. Effect of MAP30 on ΔΨm in EC-1.71 cells under fluorescent microscope, 200X. MAP30 treated cells showing a majority of cells stained green due to low ΔΨm. After treated with MAP30 for 6, 12, 24 and 48 h, the red color decreased gradually and at the same time the green color increased.

percentage of cells in S phase (47.51 and 45.68%, respectively) whereas 51.55% in the control cells. As shown in Table 2, FCM analysis also revealed the effect of MAP30 on the induction of apoptosis was in a dose-dependent manner.

In the current study, the effect of MAP30 on ΔΨm was examined by evaluating the changes in fluorescence intensity of cells stained with JC-1. In healthy cells with high ΔΨm, JC-1 formed J-aggregates with intense red fluorescence. In apoptotic or unhealthy cells with low ΔΨm, JC-1 remained in the monomeric form (green fluorescence). As shown in Figure 5 after treated with MAP30 for 6, 12, 24 and 48 h, the red color decreased gradually while the green color increased gradually. This indicated that a majority of MAP30 treated cells were stained green due to low ΔΨm. The result of ELISA showed
Figure 6. Effect of MAP30 on Cyt C of EC-1.71 cells in vitro. The concentration of Cyt C reached the peak at 24 h and then fell-off after 24 h. MAP30 group P < 0.01 versus control group at 24 h.

Table 3. Effects of MAP30 on Caspase-12 activity of EC-1.71 in vitro. (n=3, means ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (µg/ml)</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>5248.412 ± 180.3968*</td>
<td>8288.555 ± 556.2641*</td>
</tr>
<tr>
<td>Experiment</td>
<td>20</td>
<td>6687.137 ± 216.230**1</td>
<td>13717.7927 ± 608.6507**1</td>
</tr>
<tr>
<td>-</td>
<td>30</td>
<td>7796.375 ± 383.1511**2</td>
<td>16702.2127 ± 433.4613**2</td>
</tr>
</tbody>
</table>

The caspase-12 activity was determined by Caspase-12 Fluorometric Assay Kit. EC-1.71 cells were treated with 20 and 30 µg/ml MAP30 for 48 and 72 h. **P < 0.01 versus * and ***P < 0.01 versus * illustrated that MAP30 could increase the cells Caspase-12 activity. ** P < 0.01 versus *** illustrated it was in a time-dependent manners, ***1 P < 0.01 versus ***2 and **1 P < 0.05 versus **2 illustrated it was in a dose-dependent manners.

Figure 7. NF-κB expression in human EC-1.71. 200X (A: control group; B: MAP30 group). The masculine sites were stained yellow or yellowish brown. When treated with 20 µg/ml MAP30 for 48 h, the NF-κB translocated from nuclei to cytoplasm.

showed that the releasing of Cyt C was increased in the cells's supernate that had been cultured with MAP30. The concentration reached the peak at 24 h and then fell-off after 24 h (Figure 6).

The results of caspase-12 activity assay demonstrated that the viable cells number were increased in the cells that had been cultured with 20 and 30 µg/ml MAP30 for 48 and 72 h in a time- and dose-dependent manners (P<0.05) (Table 3).

But Immunohistochemical analysis results showed that the NF-κB translocated from nuclei to cytoplasm in the MAP30-treated cells (Figure 7).
Effect of Momordica anti-HIV protein of 30 kDa (MAP30) on EC-1.71 cells in vivo

To determine whether MAP30 could inhibit tumor growth in vivo, an equal number of EC-1.71 cells were injected into the right flanks of nude mice. Tumor growth was entirely inhibited in treated group while the average tumors weight of the control group was 0.532±0.102g when the mice were killed on the 30th day. The tumor inhibitory rate was 100%.

DISCUSSION

The most attractive bioactive substances isolated from *M. charantia* are RIPs. RIPs have been classified into Types 1 and 2. Type 1 RIPs possess anti-tumor activities. The most significant mechanism for Type 1 RIPs anti-tumor is inducing tumor cells apoptosis (Chiu et al., 2001; Xia et al., 2003; Narayanan et al., 2004; Xiong et al., 2007). RIPs have an N-glycosidase activity and can make nucleosome fragmentation (Bagga et al., 2003) and so that the protein ratio increased between pro-apoptotic genes and anti-apoptosis gene on DNA and protein level directly or indirectly and then induced the cells apoptosis. MAP30 belongs to Type 1 RIPs and can induce the tumor cells apoptosis and has no toxic effect on normal cells (Lee-Huang et al., 1995; Schreiber et al., 1999). In this study, the results of MTT, colony formation assay and tumor xenograft model of nude mice showed that MAP30 could suppress the EC-1.71 cells proliferation both in vitro and in vivo. Treated with 20 μg/ml MAP30 for 48 and 72 h, microscope showed apoptotic morphological changes. Further fluorescence staining, not only confirmed that MAP30 could induce the EC-1.71 cells apoptosis, but also the normal, apoptotic and necrotic cells were identified. The results of FCM showed that MAP30 could induce cells apoptosis by blocking cell cycle in G0/G1 phases (a higher number of cells in G0/G1 phase and a decreased percentage of cells in S phase) and the effect of MAP30 on the induction of apoptosis was in a dose-dependent manner (P<0.05). All these results suggested that MAP30 could inhibit the EC-1.71 cells proliferation and xenografts by induction cells apoptosis. Lee-huang et al. (2000) also found that MAP30 could inhibit the MDA-MB-231 human breast cancer cells human breast tumor cells proliferation and xenografts by inducting cells apoptosis.

There are many signal transduction pathways to regulate cells apoptosis: including mitochondrial pathway, death receptor pathway, ER pathway (Choi et al., 2009; Chae et al., 2004) and some other pathways. Disruption in Ca²⁺ homeostasis, inhibition of protein glycosylation and accumulation of misfolding proteins may induce ER stress which is associated with a range of diseases. Normally, the unfolded protein response (UPR) is activated to protect cells that are experiencing ER stress. However, sustained or intense ER stress can lead to apoptosis (Szegedzki et al., 2003). The mechanisms of ER stress-pathway are probably associated with (1) mitochondrial/Apaf-1 depend pathways. The up-regulation of UPR can enhance the sensitivity of mitochondrial to the proapoptosis factor (Milhavet et al., 2002). This can lead to mitochondrial outer membrane permeability (MOMP) increasing (Kirkegaard and Jäättelä, 2009) and then MOMP increasing results in ΔΨm decreasing, Cyt C and some apoptosis inducing factors (AIF) releasing from mitochondria (De Bruin and Medema, 2008). The release of Cyt C will start the cells apoptosis. (2) The caspase-12 activation pathways. The caspase-12 pathways mechanism of apoptosis induced by ER-pathway demonstrated that ER stress-induced apoptosis can be mediated through the activation of caspase-12 which is localized to the outer membrane of the ER. The activation of caspase-12 eventually leads to activation of apoptosis (Rao et al., 2001). In this study, we demonstrated that MAP30 could induce a time-dependent decrease in ΔΨm and the releasing of Cyt C were increased in the cells’s supernate when treated with MAP30. Using caspase-12 fluorometric assay, we found that viable cell number was increased in the cells that had been cultured with MAP30 in a time- and dose-dependent manners (P<0.05). Furthermore, we found that ER were expanded and broaden under electron microscope when the cells expressed apoptosis. These results implied that MAP30 induced apoptosis in EC-1.71 cells via ER stress-pathway [mitochondrial/Apaf-1 depend pathways (including a reduction of ΔΨm and releasing of Cyt C) or the caspase-12 activation pathways] or via ER stress-pathway associated mitochondria-dependent apoptosis mechanism.

NF-κB is a nuclear transcription factor, which controls different biological processes including cell growth, apoptosis and so on (Li et al., 2007; Gilmore, 2006). Classical NF-κB is usually kept in an inactive form in the cytoplasm, but the liberation and nuclear translocation of classical NF-κB will affect the cells apoptosis. Some reports indicated that NF-κB has a dual effect on apoptosis. However, the commonly accepted view is that the activation of NF-κB leads to apoptosis resistance (Dolcet et al., 2005; Karin and Ben-Neriah, 2000; Inoue et al., 2007). In the current study, the expressions of NF-κB were observed to be translocated from the nuclei to the cytoplasm in the cells treated with MAP30, which suggested that MAP30-induced apoptosis was associated with NF-κB pathways. This finding supported the idea that the inactivation of NF-κB could promote the cells apoptosis.

Taken together, our study had demonstrated that MAP30 could inhibit human esophageal carcinoma EC-1.71 cells proliferation in vitro and in vivo, and could induce the cells apoptosis. MAP30 induced apoptosis in EC-1.71 cells maybe via ER stress-pathway or via ER stress-pathway associated mitochondria-dependent
apoptosis mechanism, inducing NF-κB protein translocated from nucleus to endochylem. As we know, the different apoptosis pathways were not isolated, but intercommunication, which developed into a complex network. (Basu et al., 2009). So next step, we will aim to study the apoptosis mechanisms of MAP30 and the relationship between different apoptosis pathways.

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