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mda-7/IL-24 induces apoptosis in human HepG2 hepatoma cells by endoplasmic reticulum stress

XIAOFENG ZHANG*, XIAOYAN KANG*, LEHUA SHI, JIN LI, WEN XU, HAIHUA QIAN, MENGCHAO WU and ZHENGFENG YIN

Department of Molecular Oncology, Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University, Shanghai 200438, P.R. China

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Abstract. mda-7/IL-24 shows tumor-suppressor activity in a broad spectrum of human cancer cells. However, the molecular mechanism by which mda-7/IL-24 induces apoptosis is not well understood and most likely involves different pathways depending on the tumor. We examined the apoptotic effect of the adenovirus-mediated mda-7/IL-24 (Ad.mda-7) on human HepG2 hepatoma cells. We found that blocking the endoplasmic reticulum (ER) stress inhibited apoptosis induced by Ad.mda-7 and down-regulated the expression of caspase-12, Bax and caspase-3. The treatment of subcutaneous tumor xenografts of HepG2 cells with Ad.mda-7 inhibited tumor growth and angiogenesis. As in the *in vitro* studies, we found that blocking ER stress prevented Ad.mda-7 from inducing apoptosis in liver cancer cells *in vivo*. Our studies suggest that Ad.mda-7 induces apoptosis of HepG2 cells mainly through activation of the ER stress pathway.

Introduction

The *mda-7* gene has been widely studied as a tumor inhibitor ever since it was first identified by Jiang *et al* (1) during subtraction hybridization using a cDNA library of actively proliferating and terminally differentiated human HO-1 melanocytes. They provided preliminary evidence that mda-7 can inhibit the proliferation of melanoma cells and promote their terminal differentiation. In 2001, Huang *et al* (2) described the chromosome location and gene structure of *mda-7*. Later, Caudell *et al* (3) defined the cytokine property of the mda-7 protein in a series of experiments, naming it interleukin-24 (mda-7/IL-24) and placing it in the IL-l0 family. A series of studies have demonstrated that adenovirus-mediated mda-7/IL-24 (Ad.mda-7) selectively induces apoptosis of various types of tumor cells without inducing harmful effects in normal cells.

The molecular mechanism by which mda-7/IL-24 selectively induces apoptosis of tumor cells is complex. Moreover, the mechanism varies depending on the tumor cell line (4). It induces apoptosis of ovarian cancer cells mainly through the fas/fasL pathway (5), melanoma cells through the p38 MAP kinase (MAPK) pathway (6), lung cancer cells through the PKR pathway (7), malignant cerebral glioma through the JNK pathway (8) and pancreatic cancer cells through the inhibition of the Wnt/PI3K pathway (9). Other studies have shown that intracellular mda-7/IL-24 is located in the endoplasmic reticulum (ER) and Golgi complex and that it may be involved in the apoptosis signal transduction pathway that is mediated by ER stress (10).

Hepatocellular carcinoma (HCC) is among the five most deadly cancers and its incidence has been on the rise in recent years (11). Some studies have shown that mda-7/IL-24 induces apoptosis in HCC (12,13), although the mechanisms are unclear. Since the ER chaperone protein BiP/GRP78 has been identified as an intracellular target of the antitumor activity of mda-7/IL-24 (14), we hypothesized that the apoptotic effect of Ad.mda-7 in liver cancer cells may involve the activation of the ER stress pathway. In the present study, we investigated the mechanism of apoptosis induced by Ad.mda-7 in human HepG2 hepatoma cells *in vitro*, as well as *in vivo* in a nude mouse model.

Materials and methods

Construction of recombinant adenovirus. Replication-defective adenovirus-5 (Ad5) carrying the gene for mda-7/IL-24 was constructed using the AdEasy™ Adenoviral Vector System (Quantum Biotech, Montreal, Canada). Briefly, recombinant adenovirus plasmid pAd.mda-7 carrying human mda-7/IL-24 cDNA was constructed and transfected into 293 cells to generate the recombinant adenovirus vector Ad.mda-7. The virus containing this construct was isolated using plaque

Correspondence to: Dr Zhengfeng Yin, Department of Molecular Oncology, Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University, 225 Changhai Road, Shanghai 200438, P.R. China
E-mail: yinzfk@yahoo.com.cn

*Contributed equally

Abbreviations: ER, endoplasmic reticulum; HCC, hepatocellular carcinoma; MOI, multiplicities of infection; MVD, microvascular density

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screening, purification and amplification. At the same time, recombinant adenovirus Ad.GFP carrying green fluorescent protein (GFP) was constructed as a control.

Cell culture and treatment. Human HepG2 hepatoma cells and normal liver L02 cells were purchased from the American Type Culture Collection. HepG2 and L02 cells were incubated in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C, 5% CO_2. Trypan-blue staining confirmed that cell activity was >95%. At 24 h before transfection with the recombinant adenovirus vector at different multiplicities of infection (MOI), cells were transferred to a 6-well plate at $2x10^5$ cells/well. At 2 h after transfection, the medium was changed and cells were cultured for another 72 h. Thapsigargin (TG, 2 μM; Calbiochem, San Diego, CA, USA) was used as the positive control to induce the ER stress pathway; cells were treated for 72 h. Before the induction of cell apoptosis, cells were treated for 30 min with the ER inhibitor calpastatin I (ALLN, N-Ac-L-L-norleucinal, 25 μM; Calbiochem).

Cell proliferation assay. Cells were collected and transferred to a 96-well plate at $5x10^3$ cells/well. After 24 h, Ad.mda-7, Ad.GFP, ALLN+Ad.mda-7 or ALLN+Ad.GFP viruses were added at MOI 10 and cells were cultured for another 3 days, when cell survival and growth were examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), as described in the literature (15). The samples included a zeroing well (medium) and control well (untreated cells). All data were standardized to the readings for the untreated cells in the corresponding row. All experiments were repeated in triplicate.

Western blot analysis. Cells were harvested in a lysis buffer and subjected to Western blotting, as described previously (16). The detecting antibody protein included rabbit anti-human BiP/GRP78, rabbit anti-human caspase-12 (Biovision, Mountain View, CA, USA), rabbit anti-human caspase-3, rabbit anti-human P-p38 MAPK and rabbit anti-human ß-actin, rat anti-human Bax, and rat anti-human caspase-8 (Cell Signaling, Beverly, CA, USA).

Flow cytometry (FACS). Cells ($2x10^6$) were collected, fixed in 75% ethanol at 4˚C overnight and stained with propidium iodide (PI) for 30 min. Cell populations at different stages of the cell cycle were analyzed with a FACSaria flow cytometer (Becton Dickinson, Mountain, CA, USA). The data obtained were treated with modifit3.0 software.

Establishment of HepG2 tumor models. BALB/c nude mice (4-5 weeks old, 20-25 g) were provided by the Shanghai Experiment Animal Center of the Chinese Academy of Sciences. The experiments were conducted according to the regulations for experimental animal management at the Second Military Medical University (Shanghai, China). HepG2 cells ($5x10^6$/mouse) were seeded under the costal back skin subcutaneously. Starting on day 5, nodules began to appear at the seeding site. When the tumor grew to 4-5 mm in diameter, experimental treatment was initiated. Twenty nude mice whose tumors were similar in size (4-5 mm in diameter) were chosen and equal numbers were assigned to two groups: The Ad.mda-7 treatment group and the Ad.GFP control group. Ad.mda-7 or Ad.GFP was administered by means of intra-tumor single point injection at a dose of $2x10^8$ pfu/100 μl for each tumor on three occasions separated by intervals of 3 days. The animals were sacrificed by vertebral dislocation 3 weeks after the first injection. The tumor was weighed and measured for a) the maximum diameter and b) the minimum diameter. Tumor volume was calculated according to the formula: tumor volume=$axb^2/2$. The tumor was then fixed with 10% para-formaldehyde. Another 12 tumor-bearing nude mice (4-5 mm in diameter of tumor) were chosen and split equally between an ALLN+Ad.mda-7 treatment group and an Ad.mda-7 control group. On three occasions separated by 3-day intervals, ALLN (100 mg/kg) was administered intraperitoneally, followed 1 h later by an intra-tumor single point injection of Ad.mda-7 ($2x10^6$ pfu/100 μl). The animals were sacrificed 24 h after the last injection. A portion of tumor tissue was fixed with 10% paraformaldehyde and the remaining portion was used to detect caspase-12, Bax and caspase-3 by Western blotting.

Evaluation of the tumor tissue by immunohistochemistry, microvascular density (MVD) and TUNEL. The tumor tissue was sectioned using basic pathology techniques. Immuno-histochemical staining was conducted with the EliVision™ plus kit (Zymed, San Francisco, CA, USA) according to the manufacturer's instructions. The primary antibodies were rabbit anti-mouse ki-67 and rabbit anti-human CD31 (Santa Cruz, CA, USA). The apoptosis index of tumor tissue cells was determined using the Fluorescein-FragEL™ DNA fragmentation detection kit (Oncogene, Cambridge, MA, USA) according to the manufacturer's instructions. For each condition, five fields were observed at high power (original magnification, x400) and the number of positive cells per 200 cells in each field was determined. The number of micro-vessels in five fields (original magnification, x400) was recorded and the mean value was used as the MVD of the tumor according to Weidner's artificial counting method (17).

Statistical analysis. The data are expressed as means ± SD. Tests for significance of differences were performed by ANOVA or Student's t-test as appropriate. $P<0.05$ was considered statistically significant.

Results

Ad.mda-7 induces apoptosis of HepG2 cells by activating the ER stress pathway and up-regulating Bax, caspase-3 and p38 MAPK. Previous studies have shown that Ad.mda-7 induces growth inhibition and apoptosis in SMMC-7721 hepatoma cells (13). Our results using MTT and FACS were consistent with this conclusion in HepG2 cells, whereas we saw no negative effects in normal liver LO2 cells (Fig. 1A and B). Intracellular mda-7/IL-24 has been shown to localize in the ER and Golgi and to interact with BiP/GRP78 protein via its C and F ring (14). In the present experiments, HepG2 cells transfected with Ad.mda-7 expressed high levels of BiP/GRP78 based on Western blotting results (Fig. 1C). Caspase-12 is another ER stress-related protein. These cells also

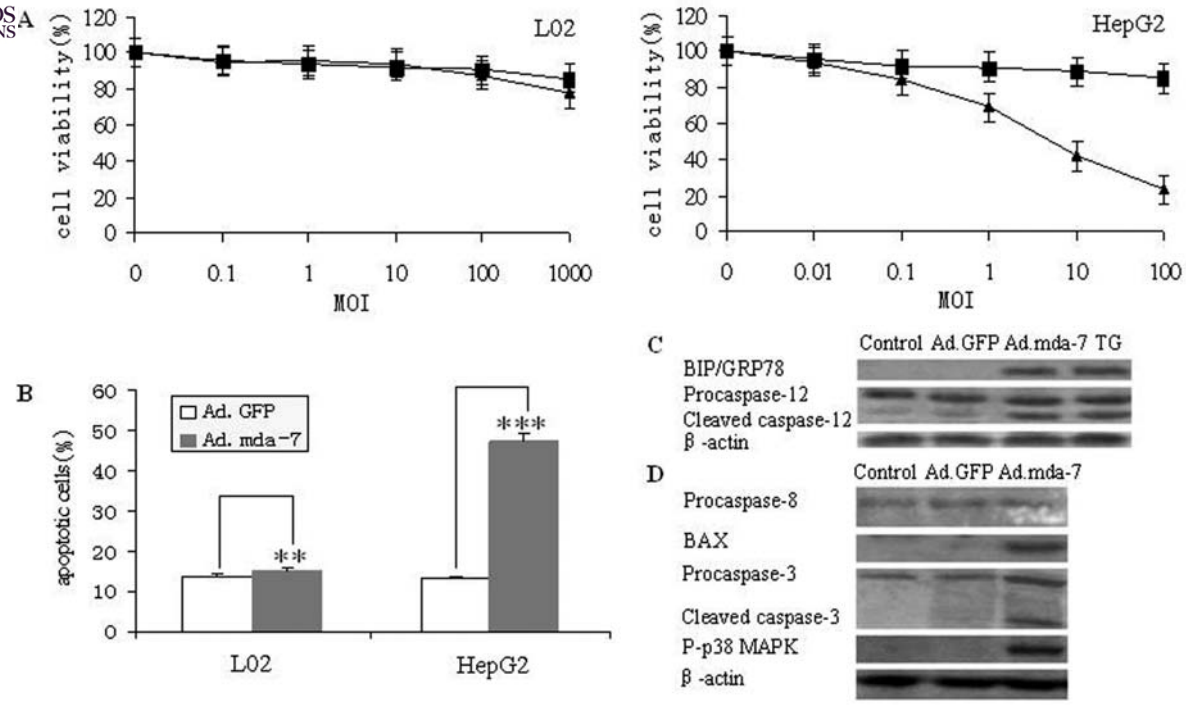

Figure 1. Ad.mda-7 induced growth inhibition and apoptosis in HepG2 cells. (A) LO2 and HepG2 cells were cultured for 24 h after plating and then infected with Ad.mda-7 (▲) or Ad.GFP (■) at different MOIs. MTT assays were performed 3 days after infection. (B) Percent of apoptotic cells determined by FACS 3 days after infection with Ad.mda-7 or Ad.GFP. Results of (A) and (B) are expressed as the average of triplicate samples ± SD. The values were normalized to untreated cells. $^{**}P>0.05$ and $^{***}P<0.01$. (C) HepG2 cells were treated for 48 h with PBS (control), Ad.GFP, Ad.mda-7, or TG (2 μM). BiP/GRP78 and caspase-12 were detected by Western blot. ß-actin was used as the internal standard. (D) HepG2 cells were treated with PBS (control), Ad.GFP, or Ad.mda-7 for 48 h. Caspase-8, Bax, caspase-3 and p38 MAPK were detected by Western blot.

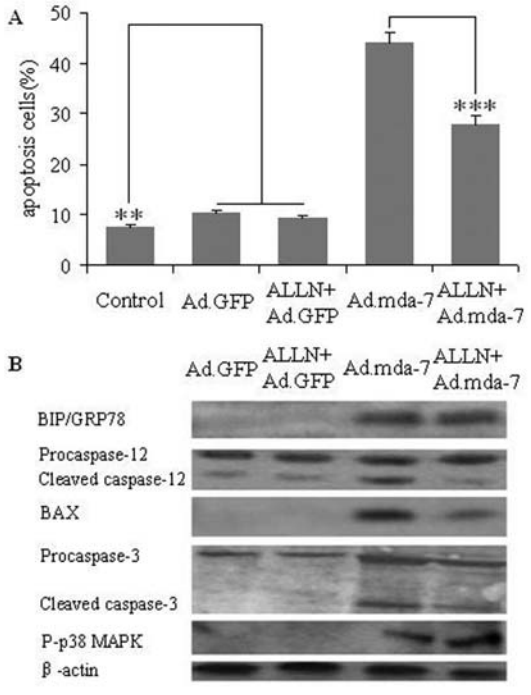

Figure 2. Blocking ER stress inhibited Ad.mda-7-induced apoptosis of HepG2 cells. HepG2 cells were treated with ALLN (25 μM) for 30 min, then infected with Ad.mda-7 or Ad.GFP. (A) After 72 h, cells were harvested for FACS analysis to determine the percentage of apoptotic cells. Values were normalized to the untreated cells (control). Results are expressed as the average of triplicate samples ± SD. $^{**}P>0.05$ and $^{***}P<0.01$. (B) After 48 h BiP/GRP78, caspase-12, Bax, caspase-3 and p38 MAPK were detected by Western blot. ß-actin was used as the internal standard.

showed the presence of an activated degraded caspase-12 fragment (48 kDa) by Western blotting (Fig. 1C). Likewise, caspase-3, normally present as a zymogen (32 kDa), was detected in its activated 17 kDa form, while caspase-8 was detected only as the zymogen. Synthesis of Bax protein was activated 48 h after transfection. In addition, we found that p38 MAPK was phosphorylated (Fig. 1D). These results indicate that Ad.mda-7 induces apoptosis of HepG2 cells likely by activating the ER stress pathway and by up-regulating the expression of Bax and caspase-3, as well as the phosphorylation of p38 MAPK.

Blocking ER stress inhibits apoptosis of HepG2 cells induced by Ad.mda-7 and down-regulates caspase-12, Bax and caspase-3. We used calpastatin I (ALLN) to investigate whether blocking the ER stress pathway would affect the growth of HepG2 cells transfected with Ad.mda-7. Experiments using treatments at different doses showed that 25 μM of ALLN did not produce toxicity to HepG2 cells (date not shown), although it did markedly inhibit apoptosis induced by Ad.mda-7, as measured by FACS (Fig. 2A). Western blotting of HepG2 cells transfected with Ad.mda-7 showed that ALLN treatment markedly inhibited the activation of caspase-12, Bax and caspase-3 (Fig. 2B). The levels of BiP/GRP78 were unaffected by ALLN treatment, as was the level of phosphorylated p38 MAPK. These results show that Ad.mda-7 induces apoptosis of HepG2 cells through the ER stress pathway and that this effect is closely related to the mitochondrial pathway and that the apoptotic effect can be blocked by ALLN.

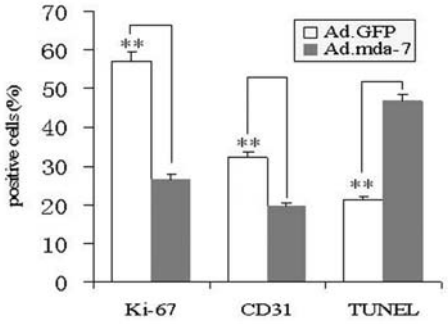

Figure 3. Ad.mda-7 inhibited growth, proliferation and angiogenesis of HepG2 tumors, and induced HepG2 cell apoptosis *in vivo*. The expression of ki-67 and CD31 in HepG2 tumors was analyzed by immunohistochemistry 3 weeks after the initial injection of a transgenic virus and apoptotic HepG2 cells were detected by TUNEL. The number of positive cells was counted as described in Materials and methods. The results are expressed as means ± SD of three randomly selected mice. $^{**}P<0.01$.

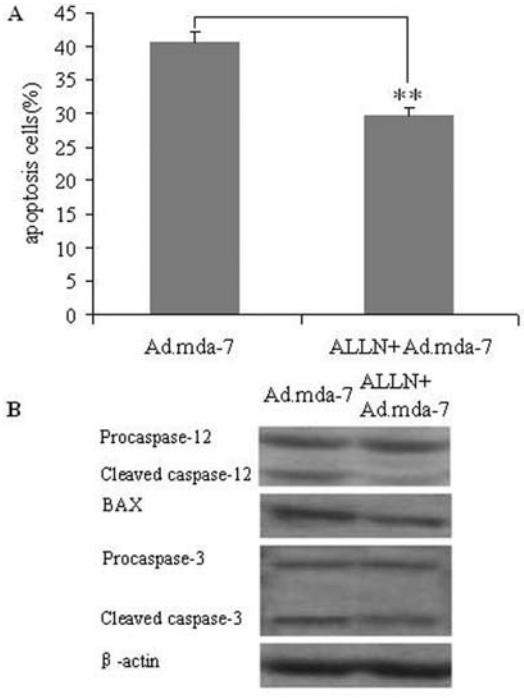

Figure 4. Blocking ER stress protected HepG2 cells from Ad.mda-7-induced apoptosis *in vivo*. At 24 h after the last administration of Ad.mda-7 or ALLN+Ad.mda-7 *in vivo*, (A) the percentage of apoptotic cells was analyzed by TUNEL; the percentage was calculated from the number of positive cells out of 200 cells per field under high power (original magnification, x400). $^{**}P<0.01$. (B) Caspase-12, Bax and caspase-3 were detected by Western blot. ß-actin was used as the internal standard.

Ad.mda-7 inhibits growth, proliferation and angiogenesis of HepG2 tumors in vivo. The cell proliferation-associated antigen, Ki-67, is one of the most widely used cell proliferation-associated markers (18). CD31 is one of the markers of neo-vascularization and its antibody is often used for the detection of MVD (19). In established HepG2 tumors, the intratumoral administration of Ad.mda-7 significantly inhibited tumor growth. The tumor volume in the Ad.mda-7 treatment group and the Ad.GFP control group was 312.6±30.24 mm^3 and 520.6±30.00 mm^3 (P=0.001) and the tumor mass was 0.321±0.031 g and 0.534±0.030 g (P=0.001). More importantly, we demonstrated that Ad.mda-7 treatment significantly down-regulated the expression of Ki-67 and CD31, compared with Ad.GFP-treated tumors, as evidenced by immunohistochemistry and assessment of the proliferation index (PI) and MVD (Fig. 3). These results indicate that *in vivo*, Ad.mda-7 inhibits the growth and proliferation of HepG2 tumors and prevents tumor neo-vascularization.

Ad.mda-7 induces HepG2 cell apoptosis and this effect can be inhibited in vivo by blocking ER stress. Using TUNEL, we found that the number of apoptotic HepG2 cells increased markedly with Ad.mda-7 treatment compared to treatment with Ad.GFP (Fig. 3). We then injected ALLN into HepG2 tumors intraperitoneally. No drug toxicity was observed with the dose of ALLN used (100 mg/kg). However, the apoptotic rate of HepG2 tumors in the ALLN+Ad.mda-7 group was significantly lower than in the Ad.mda-7 group, as detected by TUNEL (Fig. 4A). This indicates that ALLN prevents Ad.mda-7 from inducing apoptosis of HepG2 cells *in vivo*. Furthermore, Western blotting showed that the expression of caspase-12, Bax and caspase-3 in the ALLN+Ad.mda-7 group was down-regulated (Fig. 4B). These results show that the ER stress pathway may be involved in apoptosis induced by Ad.mda-7 in HepG2 cells *in vivo*.

Discussion

Although continuous progress has been made in the diagnosis and treatment of HCC over the past decades, a significant number of patients are already in the advanced stages of the disease when they seek medical help. In addition, a high recurrence of HCC makes it difficult for available therapies to improve the prognosis significantly (11). Progress in gene therapy may provide new possibilities for the treatment of HCC (20). One such agent that has shown promise in preclinical studies is known as mda-7/IL-24, which has passed a phase I clinical trial and is in phase II/III of a clinical trial in melanoma patients (21). We confirm in the present study that Ad.mda-7 inhibits the growth and proliferation of HepG2 cells, without causing significant adverse effects in normal liver L02 cells. Additionally, we carried out an *in vivo* experiment that showed that Ad.mda-7 inhibits growth and proliferation of HepG2 cells and inhibits angiogenesis in tumors. Furthermore, we found that mda-7/IL-24 induces apoptosis of HepG2 cells by activating the ER stress pathway *in vitro* and *in vivo*.

The ER is an important place for protein processing and the maintenance of intracellular homeostasis. Prolonged stress of the ER, for example, due to hypoxia or various physiochemical conditions may trigger the unfolded protein response (UPR) and a Ca^{2+} imbalance. These responses, collectively known as 'ER stress', result in cell apoptosis (22). For example, thapsigargin (TG) can cause an intracellular Ca^{2+} imbalance and activate caspase-12, thereby promoting ER stress (23). Some studies have found that calpain in the ER regulates caspase-12 without participating directly in ER stress. The activation of caspase-12 causes cell apoptosis, whereas calpastatin I (ALLN), an ER inhibitor, can inhibit caspase-12 activation (24). Immunofluorescent staining has shown that intracellular mda-7/IL-24 is located in the ER and Golgi

SPANDIDOS PUBLICATIONS and interacts with the ER-resident protein 78 through its C and F ring (14). Our results show that Ad.mda-7 up-regulates the expression of BiP/GRP78. We also found that caspase-12 is activated after Ad.mda-7 activates BiP/GRP78. We found that treating HepG2 cells with ALLN significantly reduces apoptosis and growth inhibition induced by Ad.mda-7 *in vitro* and *in vivo*, indicating that the ER stress pathway plays a role in apoptosis of HepG2 cells induced by Ad.mda-7.

Studies have shown that caspase activation through the death receptor pathway, the mitochondrial pathway, and the ER stress pathway cause cell apoptosis. Caspase-8 is the primary initiator of the death receptor pathway, while caspase-3 is the main caspase effector in the mitochondrial pathway (25). Bax plays an important role in the induction by Ad.mda-7 of the mitochondrial apoptosis pathway (26). We found that infecting HepG2 cells with Ad.mda-7 increases the expression of Bax and caspase-3, as well as the phosphorylation of p38 MAPK, without affecting the levels of caspase-8. When ALLN is used to block the activation of caspase-12 and the ER stress pathway, however, expression of Bax and caspase-3 is significantly down-regulated *in vitro* and *in vivo*. We therefore hypothesize that Ad.mda-7 induces ER stress in HepG2 cells by activating BiP/GRP78 and caspase-12, then activating Bax and phosphorylation of p38 MAPK, inducing a translocation of Bax from the cytosol into the mitochondria and promoting the release of cytochrome c. The release of cytochrome c further activates caspase-3, eventually resulting in tumor cell apoptosis.

It remains an open question whether the Ad.mda-7-mediated mitochondrial apoptosis pathway is regulated by ER stress or vice versa, or whether the two pathways stimulate or otherwise interact with each other. Ad.mda-7 has been reported to up-regulate the expression of a protein related to ER stress, called GADD, in melanoma cells (6). Some recent studies have shown that Bim is essential for the ER stress pathway (27). Our laboratory is currently investigating the relationship between the mitochondrial pathway and ER stress, and one of the aims is to determine whether other ER stress-associated proteins are involved in the apoptotic effect of Ad.mda-7 in liver cancer cells.

In summary, the present study shows that Ad.mda-7 induces growth inhibition and apoptosis of liver cancer cells without harming normal cells. We found that Ad.mda-7 can inhibit the growth, proliferation and tumor angiogenesis of liver cancer cells *in vivo*. We also found that Ad.mda-7 induces apoptosis of HepG2 cells primarily by activating ER stress. This study offers hope that mda-7/IL-24 may prove to be a useful gene therapy in the treatment of HCC.

Acknowledgements

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