Abstract. mda-7/IL-24 has tumor-suppressor activity in a broad spectrum of human cancer cells. However, the therapeutic effect of the recombinant human IL-24 protein on human gallbladder carcinoma has rarely been explored. In this study, we used a human gallbladder carcinoma cell line (GBC-SD) to explore the effect of adenovirus-mediated IL-24 (Ad-IL24) gene therapy on GBC-SD cells. We show that Ad-IL24 treatment of GBC-SD cells in vitro conspicuously induced apoptosis of GBC-SD cells. We also demonstrate that the in vivo treatment of GBC tumor-bearing athymic nude mice intratumorally injected with Ad-IL24 significantly suppressed GBC growth. To further explore the mechanism that mda-7/IL-24 utilized in tumor cell apoptosis, we examined molecules and pathways involved in apoptotic regulation and found that Ad-IL24 induced the down-regulation of anti-apoptotic gene Bcl-2 and the release of cytochrome c, which subsequently activated caspase-9, caspase-3 and PARP to induce apoptosis. In summary, adenovirus (AdV)-mediated IL-24 overexpression exerted potent antitumor activity via stimulating mitochondrial apoptotic pathway in GBC-SD. Therefore, mda-7/IL-24 has the potential to serve as a tool for targeted gene therapy in the treatment of gallbladder cancer.

Introduction

Gallbladder carcinoma (GBC) is the fifth most common malignant tumor of the digestive system and the most common malignant neoplasm of the biliary tract. Although rarely reported in Europe and the United States, GBC is relatively common in South America, India and Poland (1). Over the past decades, the incidence rate of GBC has increased in China. The survival rate has not improved even after radical operations were performed (2) because of late diagnosis, high incidence of post-surgical regional recurrence and distant metastases. Additionally, chemotherapy and radiotherapy have limited efficacy on GBC and generally GBC is considered as a ‘chemo-resistant’ tumor, which was evidenced by low response rates and no demonstrated survival benefit (3). Many researches have been trying hard to find approaches to benefit patients with GBC but only achieved limited progress. It is imperative to identify novel therapeutic measures to enhance the therapeutic effect and improve the survival of patients with GBC.

The melanoma differentiation associated gene-7 (mda-7 gene) [renamed interleukin 24 (IL-24)] was isolated from human melanoma cells induced to terminally differentiate by treatment with fibroblast IFN and mezerein (4). This novel cytokine is classified as a member of the interleukin-10 (IL-10) gene family (5). Overexpression of mda-7/IL-24, by use of a recombinant adenovirus Ad.mda-7, inhibits the growth and kills numerous types of human tumor cell lines including melanoma, pancreatic, glioblastoma, gastric carcinoma, and laryngocarcinoma resulting in tumor-specific growth arrest, without exerting deleterious effects in normal melanocytes, astrocytes and human epithelial or fibroblast cells (6-11). It also showed potent cancer-specific apoptosis-inducing ability and tumor growth-suppressing properties in multiple human tumor xenograft animal models. mda-7/IL-24 is under evaluation in a phase I clinical trial in patients with advanced melanoma cancers (12).

Nevertheless, the detailed apoptotic pathways in Ad.mda-7 causing tumor cell death are not fully understood. Moreover, recent studies showed the mechanism of Ad.mda-7 induced apoptosis varies in different tumor cell lines. It causes some cancer cells specific apoptosis through the autocrine regulation of mda-7 (13), chronic lymphocytic leukemia B cells through phosphorylation of STAT3 (14), ovarian cancer cells mainly through the fas/fasL pathway (15), malignant cerebral glioma through the caspase-, cathepsin- and PERK-dependent pathway (16), melanoma cells through class I interferon-regulated signaling pathways (17) and malignant renal carcinoma cells through ceramide/CD95/PERK-dependent mechanism (18). 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 (19). Thus, mda-7/IL-24 lethality seems to occur by multiple distinct pathways in different cell types. We hypothesized that AdIL-24 could induce apoptosis of GBC in vitro and inhibit grafted tumor growth in vivo. In addition, we further investigated the mechanism of AdIL-24 induced apoptosis in GBC-SD cells.

Materials and methods

Cell culture and treatment. The human gallbladder carcinoma cell line GBC-SD was purchased from the Cell Bank of the Chinese Academic of Sciences (Shanghai branch). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI-1640 medium (Gibco BRL, Carlsbad, CA) with 2 mM glutamine. The medium was supplemented with 10% fetal bovine serum (Gibco BRL), 100 IU/ml penicillin and 100 μg/ml streptomycin. After 24 h at cell density, the dish, the recombinant adenovirus vector at different multiplicities of infection (MOI) transfected the GBC-SD cells. At 30 min before the recombinant adenovirus vector transfection, the pancaspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK; Alexis Biochemicals, San Diego, CA) 30 μM was added.

Construction of recombinant adenovirus. Replication-defective adenovirus-5 (Ad5) encoding mda-7/IL-24 (Ad.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 the human embryonic kidney 293 (QBI-293A) cells by Lipofectamine™ (Invitrogen, San Diego, CA) 30 μM was added.

MTT assay. Cytotoxic activity of AdGFP/IL-24 was determined based on cytotoxicity to GBC-SD cells, using the MTT assay. Briefly, cells were harvested and dispensed in 96-well culture plates in 100 μl medium at a concentration of 10⁴ cells per well. After 24 h, Ad.IL-24, Ad.GFP, Ad.JL-24 or PBS was added to MOI 10 and cells were cultured for another 3 days. Ten microliter 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Roche Diagnostics GmbH Co., Germany) was added to each well. After 4 h incubation at 37°C, supernatants were removed and replaced by 150 μl dimethyl sulfoxide. The optical density at 490 nm (OD490) of each well was measured using an automated microplate reader (Bio-Rad Model 550, Microplate Reader, Hercules, CA).

RNA isolation and RT-PCR. After cells infected with Ad.GFP and Ad.IL-24 (MOI=10), respectively, cells were harvested at 72 h after infection, total RNA was extracted from cells using the Takara A mini kit (Japan) according to the manufacturer’s protocol. Primers used in PCR were designed according to the reported IL-24 cDNA sequence. The primer sequences were 5'-CGTGTCACAACTGCAACC-3' (forward) and 5'-CTGGGCCCAAGATGGG-3' (reverse). The primer sequences of β-actin were 5'-CTTCTCCTGGAATGGAGT CCT-3' (forward) and 5'-GGAACAAATGATCTTATCTT-3' (reverse). The reaction mixture with corresponding primers was amplified 38 cycles, each cycle consisting of denaturation at 94°C for 30 sec, primer annealing at 58°C for 30 sec, and extension at 72°C for 1 min. Cycles were preceded by incubation at 94°C for 2 min, an extra incubation at 72°C for 5 min to ensure full extension of the product. The products of PCR were analyzed on 10 g/l agarose gel electrophoresis.

Western blot analysis. Cells were harvested from the plates and resuspended in cold RIPA lysis buffer with freshly added 0.1 mg/ml phenylmethylsulfonyl fluoride and 1 mg/ml aprotonin. The mixtures were lysed on ice for 30 min and were spun at 12000 g for 15 min at 4°C. Aliquots of cell extracts containing 35 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) on a 10-12% gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat dry milk and then incubated with corresponding primary antibodies overnight at 4°C and secondary antibodies. ECL was performed according to the manufacturer’s protocol. For all Western blots, equal loading of protein was verified by re-blotting of membranes for β-actin protein. For determination of translocation of cytochrome c from mitochondria to cytosol, cells were washed once with PBS and incubated with lysis buffer. The cells were lysed on ice for 30 min and were spun at 500 g for 5 min at 4°C to eliminate nuclei and unbroken cells. Then the sample was centrifuged at 12000 g for 15 min at 4°C to obtain the mitochondrial fraction. The primary antibodies: Bcl-2, Bax, caspase-3, caspase-9, IL-24, PARP, cytochrome c were purchased from Santa Cruz Biotechnology (Santz Cruz, CA, USA).

Apoptosis and necrosis assay (Annexin-V-PI assay). Cells were trypsinized (washed twice with cold PBS. Aliquots of cell (1x10⁶) were resuspended (400 μl) and stained with Annexin-V-FITC (Becton-Dickinson, San Jose, CA, USA) and stained with Annexin-V-PI (Becton-Dickinson, San José, CA, USA) according to the manufacturer’s instructions. Propidium iodide (PI) was added to the samples after staining with Annexin-V to distinguish late apoptotic and necrotic cells. Flow cytometry (Becton-Dickinson, San Jose, CA, USA) was performed immediately after staining.

Xenograft growth inhibition assay. BALB/c nude mice (4 weeks old, 20-25 g) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. The use and care of animals followed the guidelines of the Ethics Committee of Xinhua Hospital, School of Medicine, Shanghai Jiaotong University. Twenty-four nude mice whose tumors were similar in size (4-5 mm in diameter) were chosen and equal numbers were assigned to four groups PBS, Ad.JL-24, Ad.GFP and control group. Ad.IL-24 or Ad.GFP was administered by means of intratumor single point injection at a dose of 2x10⁸ pfu/100 μl for each tumor separated every 3 days.
The animals were sacrificed 3 weeks after the first injection. The tumor was measured for i) the maximum diameter and ii) the minimum diameter. Tumor volume was calculated according to the formula: tumor volume = a x b^2/2.

Statistical analysis. The data are expressed as means ± SD and either ANOVA or Student's t-test was used for the analysis. P<0.05 was considered statistically significant.

Results

Expression of IL-24 in GBC-SD cells. Before the start of the experiment, the endogenous level of IL-24 and the transduction efficiencies of Ad-IL24 were determined. RT-PCR and Western blot analysis demonstrated there were no endogenous IL-24 mRNA or protein expressed in GBC-SD cells. More the transfection efficiency was over 85% when we infected cells with 10 vp/cell of Ad-IL24 (data not shown) and IL-24 mRNA and protein were stably expressed in GBC-SD cells but not in cells treated with Ad.GFP or PBS (Figs. 1 and 2).

IL-24 inhibits gallbladder cancer cell proliferation by inducing apoptosis. To investigate the cytotoxic effects of Ad-IL24 on gallbladder cancer cells in vitro, the GBC-SD cells growth were examined using the MTT assay 3 days after virus transduction. Ad-IL24 treatment significantly inhibited GBC-SD cell growth in a dose-dependent manner, compared to the Ad.GFP-treated and PBS-treated control groups (P<0.05) (Fig. 3). These results showed that Ad-IL24 significantly suppressed GBC-SD cells growth in vitro. The mechanism that Ad.mda-7 applied to inhibit proliferation was further investigated. Associated with the induction of apoptosis in Ad-IL24-treated tumor cells, a great number of apoptotic GBC-SD cells were visible among the cells infected with Ad-IL24, compared to those infected with Ad.GFP or PBS cells (P<0.05). There were no significant differences between the cells infected with Ad.GFP and treated with PBS (P>0.05). The data indicated that Ad-IL24 induced apoptosis in GBC-SD cell, as shown in Fig. 4. The ability of Ad-IL24 to induce
apoptosis is similar to those observed by others in some other tumor types (20).

**IL-24-induced mitochondrial apoptosis pathways correlate with the down-regulation of Bcl-2 and cytochrome c release.** Bcl-2, Bcl-xL, and other apoptosis-preventing members of the family inhibit the activation of mitochondria, whereas the pro-apoptotic Bcl-2 homologs Bax and Bak and Bok promote the release of cytochrome c and initiate the mitochondrial permeability shift transition (21,22). We therefore tried to determine whether the infection of gallbladder tumor cells with Ad-IL24 altered the expression of the proteins associated with apoptosis pathway. As shown in Fig. 5, expression levels of Bcl-2 were markedly decreased after Ad-IL24 infection comparing with control cells. These results showed that the Ad-IL24 caused down-regulation of Bcl-2 expression. We also examined Bax expression levels and Ad-IL24 seemed not to have any effect on Bax expression. In addition, the release of cytochrome c from the mitochondria into the cytoplasm was observed in Ad-IL24 infected GBC-SD cells. These results indicated that Ad-IL24 might induce the apoptosis of GBC-SD via the activation of mitochondria-mediated apoptotic pathway by inhibiting Bcl-2, but not Bax.

**IL-24 activates caspases to induce apoptosis and is inhibited by pancaspase inhibitor in gallbladder cancer cells.** Cytochrome c along with Apaf-1 activates caspase-9, and caspase-9 can activate downstream caspases (23). To further confirm that expression of IL-24 is capable of inducing the mitochondrial apoptosis pathway; the activation of caspases was studied by Western blot analysis. Pro-caspase-3 and pro-caspase-9 were decreased and the cleavage of caspase-3 was detected after Ad-IL24 infection. Cleavage of PARP, a substrate for the caspases, was also observed in Ad-IL24 infected cells (Fig. 6). To determine the role of caspases in IL-24-mediated tumor cell death, we used pancaspase inhibitor, Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK; Alexis Biochemicals), to examine if the blocking of the activation of capase could compromise Ad-IL24-induced apoptosis. As shown in Fig. 8A, the caspase inhibitor was capable of partially inhibiting apoptosis after Ad-IL24 infection (Fig. 7). These results indicated that the classic caspase cascade is mainly responsible for IL-24-induced cell death. To verify the role of Bcl-2 in the Ad-IL24 induced mitochondrial apoptosis pathway, GBC-SD cells were infected with Ad-IL24 after treatment of GBC-SD cells with Z-VAD-FMK. Seventy-two hours after infection, cells were harvested and mitochondrial proteins and cytoplasmic proteins were extracted. As shown in Fig. 8B, we observed great reversion of Bcl-2 in cells treated with Z-VAD-FMK, whereas no reversion of Bcl-2 was found in tumor cells. After Z-VAD-FMK treatment, the release of cytochrome c into the cytoplasm was blocked, but cytochrome c in mitochondria was increased.

**IL-24 suppresses tumor growth in vivo.** Treatment of nude mice bearing GBC-SD tumor cells with Ad-IL24 induced a signi-
Significant reduction in tumor growth as compared with mice treated with AdGFP or PBS (Fig. 8C) (P<0.05). These results showed that Ad-IL24 can efficiently induce tumor growth suppression in vivo.

Discussion

Gallbladder carcinoma is the fifth most common gastrointestinal malignancy, with an incidence of 2.5 and 10 per 100,000 persons in the United States and Japan, respectively (24), the disease is only considered curable at its early stages (25). Owing to the lack of effective examination measures and early symptoms, most patients with gallbladder cancer are diagnosed at late stages. Despite recent advance in surgical resection techniques and innovation of chemotherapeutic agents, only a marginal advantage is seen in a limited number of patients. Most patients will progress to recurrence and metastasis which ultimately succumb to this disease. The long-term survival rate for most patients is <5% (26). Given the fact that gallbladder cancer is very aggressive in clinical behavior, most patients have metastatic disease at the time of diagnosis. Thus, novel treatment modalities are imperative to improve treatment result and to prolong survival. Since gallbladder cancer is considered to be the result of multiple genetic alterations, molecular therapy including gene therapy would be a promising new therapeutic option in the treatment of gallbladder cancer. Our study using Ad-IL24 as a potential therapeutic agent for the treatment of gallbladder cancer verified that Ad-IL24 could inhibit the growth and proliferation of GBC-SD cells in vitro and tumor-bearing mice in vivo.

In this study, the replication-incompetent adenovirus vector carrying mda-7/IL-24 was successfully transfected into human gallbladder carcinoma cell line GBC-SD. In accordance with other reports that IL-24 could inhibit the growth of several other human tumor cell types (27,28), the antitumor effects of Ad-IL24 were observed in vitro on GBC-SD cells and in vivo in human gallbladder tumor xenograft mouse models. However, there was no obvious inhibitory effect of Ad-IL24 on the growth of GBC-SD cells and human gallbladder tumor xenograft by Ad-GFP or PBS, which indicated that the observed inhibitory effect on gallbladder cancer was due to the presence of IL-24.

Apoptosis reflects a balance between molecular and signaling events that either triggering programmed death or promotes survival. Most anti-cancer agents can activate the mitochondrial pathway to induce apoptosis, which is tightly controlled by proteins of the Bcl-2 family (29). Additionally, infection with Ad-IL24 induced changes in the ratio of pro-apoptotic (Bax and Bak) to anti-apoptotic (Bcl-2 and/or Bcl-xL) members of the Bcl-family, promoting a shift from survival to programmed cell death (30). To understand the underlying mechanism of tumor killing mediated by Ad-IL24 in GBC-SD cells, we analyzed the effects of Ad-IL24 expression on the known anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax. After infection with Ad-IL24, a significant down-regulation of Bcl-2 expression was detected comparing with PBS or Ad.GFP infected cells (Fig. 5). These results suggested that IL-24 expression could down-regulate Bcl-2. It has been reported that Bax plays an important role in apoptotic pathway (31). The pro-apoptotic Bcl-2 homologs Bax, Bak and Bok promote release of cytochrome c and initiate the mitochondrial permeability shift transition (29).

In contrast to our expectations, the level of Bax expression showed no obviously change after infection of Ad-IL24.
compared with PBS or Ad.GFP infected cells. This indicated that Ad-IL24-induced apoptosis in GBC-SD cells may activate the apoptotic pathway by blocking Bcl-2 expression but not Bax. Because Bcl-2 is the key regulator of mitochondrial apoptotic pathway and is involved in the regulation of several important factors, either pro-apoptosis or anti-apoptosis including, XIAP, AIF or Endo G, we further examined which molecules were downstream effectors of Ad-IL24 and Bcl-2.

Our study showed that expression of Ad-IL24 induced the release of cytochrome c and the activation of caspase, including the activation of procaspase-9 and cleavage of caspase-3 and PARP, a substrate for the caspases (Fig. 6). The elevated cytochrome c was observed after infecting GBC-SD with Ad-IL24 (Fig. 5). Furthermore, the release of cytochrome c was inhibited after the treatment with Z-VAD-FMK which is a known cytochrome c inhibitor (Fig. 8C). In summary, these data suggested that overexpression of Ad-IL24 resulted the down-regulation of Bcl-2, but not Bax, which resulted in the release of cytochrome c. In addition, the presence of Z-VAD-FMK the inhibitor of cytochrome c, antagonizing apoptosis and the inhibitory effect of Ad-IL24 on the growth of GBC-SD, which further confirmed that caspases are required for the induction of apoptosis mediated by Ad-IL24. Our study proved that the mitochondrial apoptosis pathway plays an important role in apoptosis of GBC-SD cells induced by Ad-IL24.

It has been reported that Ad-IL24 could up-regulate the expression of a protein related to ER stress and kill cancer cells by causing endoplasmic reticulum (ER) stress (13,32-34), which raised questions regarding the relationship of the mitochondrial apoptosis pathway and ER stress; whether the Ad-IL24 mediated mitochondrial apoptosis pathway is regulated by ER stress and how they interacted with each other, and how the expression of Ad-IL24 could downregulate Bcl-2. Our current study investigated the relationship between the mitochondrial pathway and ER stress in gallbladder cancer cells.

In conclusion, Ad-IL24 could induce myelocyte c release and subsequent caspase activation resulting mitochondrial apoptosis in GBC-SD cells and growth inhibition of gallbladder cancer xenograft mice. As a result, Ad-IL24 might be a potential candidate to be used as an novel anti-tumor therapeutic agent in the treatment of gallbladder cancer.

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