Inhibitory effect of recombinant adenovirus carrying melittin gene on hepatocellular carcinoma

C.-Q. Ling*, B. Li, C. Zhang, D.-Z. Zhu, X.-Q. Huang, W. Gu & S.-X. Li

Department of Chinese Traditional Medicine, Changhai Hospital, Second Military Medical University, Shanghai, China

Received 20 March 2004; revised 19 August 2004; accepted 26 August 2004

Objectives: To search for a new clinical application of melittin (Mel): treating hepatocellular carcinoma with Mel gene.

Methods: Recombinant adenoviruses carrying the Mel gene and α-fetoprotein (AFP) promoter (Ad-rAFP-Mel) were constructed through a bacterial homologous recombinant system. The efficiency of adenovirus-mediated gene transfer and the inhibitory effect of Ad-rAFP-Mel on the proliferation of hepatocarcinoma cells were determined by X-gal stain and MTT assay, respectively. The tumorigenicity of hepatocarcinoma cells transfected by Ad-rAFP-Mel and the antitumor effect of Ad-rAFP-Mel on transplanted tumor in nude mice were detected in vivo.

Results: The Mel mRNA was transcribed in BEL-7402 hepatocellular carcinoma cells transduced by Ad-rAFP-Mel. The efficiency of adenovirus-mediated gene transferred to BEL-7402 cells was 100% when the multiplicity of infection of Ad-rAFP-Mel was 10 in vitro, and was also high in vivo. The inhibitive rates of Ad-rAFP-Mel and Ad-rAFP for BEL7402 cells were 66.2 ± 2.7% and 2.9 ± 2.3% (t = 30.83, P = 6.6 × 10^-6) by MTT assay. The inhibitive rates of Ad-CMV-Mel for BEL7402, SMMC7721 and L02 cells were 58.9 ± 9.6%, 65.9 ± 3.8% and 31.7 ± 1.2%, respectively, and of Ad-rAFP-Mel were 66.2 ± 2.7%, 16.1 ± 6.6% and 7.5 ± 3.3%, respectively (t = 1.27, P = 0.27; t = 11.31, P = 3.5 × 10^-4; and t = 12.12, P = 2.7 × 10^-4 versus the Ad-CMV-Mel group in the same cells). The tumorigenicity rates of hepatocarcinoma cells transfected by Ad-rAFP-Mel were decreased. A significant antineoplastic effect was detected on transplanted tumor in nude mice by intratumoral injection of Ad-rAFP-Mel.

Conclusions: Ad-rAFP-Mel can inhibit specifically proliferation of AFP-producing human hepatocarcinoma cells in vitro and in vivo. This suggests that animal toxin gene can be used as an antitumor gene.

Key words: α-fetoprotein, gene therapy, hepatocellular carcinoma, melittin gene, recombinant adenovirus

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, especially in several areas of Asia and Africa [1]. Despite advances in therapy for HCC such as recent modifications in chemotherapy and modern surgical innovations, overall patient outcome has not substantially improved. The low rate of success is largely attributable to the high rate of multiplicity and intrahepatic recurrence [1–3]. In addition, most HCCs develop in cirrhotic livers infected with hepatitis virus; therefore, liver dysfunction results in many inoperable cases. For these reasons, potential gene therapies for HCC are worthy of study [4, 5].

Many of the strategies developed in the last few years to treat cancer by gene therapy are based on putative killer–suicide genes, the products of which convert a prodrug into a toxic compound. When the therapy is applied to humans, a vector carrying the killer gene is first inoculated into the tumor of the patient, who, 1 week later, receives the corresponding prodrug that will selectively kill the cells able to process it to its toxic derivative. In gene therapy, in an attempt to increase the treatment index, efforts must be made to ensure that expression of therapeutic genes is restricted exclusively to the tissue of interest. This is particularly important for suicide gene strategies, in which low level expression of toxic genes in normal tissues may lead to severe toxicity [6]. Therefore, further safeguards must be put in place to ensure that gene
delivery to these tissues does not result in significant gene expression and its resulting toxicity. One attractive approach to this problem relies on the ability to control gene expression very tightly at the transcriptional level.

Many cancers often re-express fetal or embryonic genes, and α-fetoprotein (AFP) gene expression is reactivated in HCC cells [7]. There has been much progress in the characterization of cis- and trans-acting elements regulating human AFP gene expression [8–10]. To improve the selectivity of the antitumor effect in gene therapy for HCC, most investigators have used human AFP 5′-flanking sequences including enhancers, or both enhancers and a silencer [11–14].

A strategy that obviates the need for a prodrug to destroy the cancer cells would be preferable, because the patient would only need one treatment instead of two consecutive ones. Some biotoxins, including diphtheria toxin and ricin, are now being used for gene therapy [15–17]. Melittin (Mel) is the main component of bee sting toxin. It is an alkaline polypeptide consisting of 26 amino acids. Mel has a fast and potent action of killing a variety of tumor cells in vitro [18, 19]. As the gene sequence of encoded Mel protein is short (78 bp), synthesis and transfection of Mel is relatively easy for target gene therapy of tumors. The present study used Mel as the target gene, AFP gene as the promoter and replication-defective adenovirus as the vehicle to construct recombinant adenovirus in an attempt to achieve a specific killing action on liver cancer.

Materials and methods

Construction of recombinant adenovirus

Mel gene consisted of 78 bp, plus Kozak conservative sequence on 5′-end, terminator codon on 3′-end, XbaI and HindIII linker for subcloning, for a total of 100 bp. The sequence was: ggctgac cacc atg gga att gga gct gtg ctg aag tgt cag acc aca ggc ctc ccc gcc ctg atc agc tgg atc aag agg aag aga cca cag tag aag ctt, synthesized and subcloned into the XbaI–HindIII site of pUCGT plasmid to form pUCGT–Mel by Shenergy Biocolor Biological Science and Technology Company (Shanghai, China).

The 1.2 kb rAFP (AFP transcription regulating element) gene was released from the pAdrAFPK adenovirus plasmid (Shanghai Institute of Biochemistry of the Chinese Academy of Sciences) by XbaI–HindIII restriction enzyme digestion, and inserted into the same site of plasmid-Shuttle (pShuttle), resulting in plasmid pShuttle-rAFP. The Mel gene was obtained by SalI–HindIII restriction enzyme digestion from the pUCGT–Mel, and cloned into pShuttle-CMV to creat plasmid pShuttle-CMV-Mel, from which SalI–Pmel segment [containing SV40 poly(A) signal, 2.65 kb] was digested by SalI–Pmel and cloned into pShuttle-rAFP to form pShuttle-rAFPK-Mel (total length 8.15 kb).

pShuttle-rAFPK-Mel was linearized with PmeI and transfected into Escherichia coli BJ5183 cells together with pAdEasy-1 (Stratagene Holding Corporation, La Jolla, CA, USA) by electroporation, and the recombinants were selected with kanamycin. The clones were picked, grown, and then plasmids were extracted, screened and analyzed by agarose gel electrophoresis, and one named pAd-rAFPK-Mel selected. The target segment was amplified for 34 cycles by PCR (one pair of primers: 5′-ATCGGATCTTCTAGTACC-3′ and 5′-GGTCGATGGCGTTGTATGATCG-3′). The amplification product was analyzed by agarose gel electrophoresis and sequenced.

The construction of recombinant adenovirus Ad-rAFPK-Mel was performed as described by Tran et al. [20]. Infectious viruses were purified by plaques. Ad-rAFPK, Ad-CMV-Mel, Ad-CMV and Ad-CMV-LacZ were constructed by a similar method. All recombinant adenoviruses were amplified on human embryonic kidney cell line 293 and purified by double cesium chloride density gradient ultracentrifugation. Titers of the adenoviral stocks were determined by plaque assay on 293 cells.

Cell culture

The AFP-positive human hepatoma cell lines (BEL-7402), AFP-negative human hepatoma cell line (SMMC-7721) and normal human liver cell line (L02) were grown in RPMI-1640 (Gibco) containing 10% fetal calf serum (FCS; Gibco), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, respectively. All cells were kept at 37°C in a humidified incubator with 5% CO₂ and 97% relative humidity.

RT–PCR analysis of Mel gene transcription

BEL-7402 and L02 cells were plated into a six-well plate (5 × 10⁵/well), respectively. Twenty-four hours later the BEL-7402 cells were infected by Ad-rAFPK-Mel or Ad-rAFPK [multiple of infection (MOI) 250] for 1 h, and the L02 cells were infected by Ad-rAFPK-Mel (MOI 250) for 1 h. After incubating 48 h with RPMI-1640 containing 10% FCS at 37°C, cell RNA was extracted for routine RT–PCR (35 cycles, one pair of primers: 5′-GGTTCTAATAAGCAGGCTG-3′ and 5′-GGTTGATGCGTTATGTATCATCAG-3′). During reverse transcription, BEL-7402 cells were divided into two groups; in one group the reverse transcriptase was added and in the other it was not. The amplification product was analyzed by agarose gel electrophoresis and sequenced.

Infection rate of recombinant adenovirus

Detection of in vitro transfection rate. BEL-7402 cells were plated into a six-well plate (5 × 10⁵/well). Twenty-four hours later the cells were infected by Ad-CMV-LacZ (MOI 10, 5, 1, 0.5, 0.1) at 37°C for 1 h, and then fixed with 1.25% glutaraldehyde 24 h later and stained with X-gal dye for 3 h. Four hundred cells were counted in four independent areas under a microscope to calculate the percentage of the blue dyed cells.

Detection of in vivo transfection rate. A nude mouse bearing subcutaneous BEL-7402 liver tumor (1 cm in diameter) was killed 48 h after injection of Ad-CMV-LacZ into the tumor at a dose of 2 × 10⁹ pfu/0.2 ml. The tumor was removed, frozen sliced into 7-μm sections, fixed with 1.25% glutaraldehyde at room temperature for 10 min, X-gal stained and observed under a microscope.

Inhibitory effect of recombinant adenovirus on hepatoma cells

To test the sensitivity of recombinant adenovirus on AFP-positive human hepatoma cells, BEL-7402 cells were plated in triplicate wells into a 96-well plate at 1 × 10⁴/well, and cultured routinely for 24 h. The supernatant was discarded, and transfected with Ad-rAFPK-Mel and Ad-rAFPK (MOI 100) at 37°C for 60 min, and remained in culture with RPMI1640 + 10% FCS. Seventy-two and 96 h after culture absorbance, absorption (A) was determined by MTT at 490 nm. The inhibitory rate was calculated according to the following formula:

\[
\text{Inhibitory rate} = \left( \frac{A \text{ of the control group} - A \text{ of the study group}}{A \text{ of the control group}} \right) \times 100\%
\]

To test the specificity of recombinant adenovirus on AFP-positive human hepatoma cell, BEL-7402, SMMC-7721 and L02 cells were plated in triplicate wells into a 96-well plate at 1 × 10⁴/well, and transfected with
Ad-rAFP-Mel and Ad-CMV-Mel (MOI 100). The inhibitory rate was calculated as described above.

**In vivo studies**

Three-week-old male BALB/c-nu/nu athymic mice were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. The mice were maintained under specific pathogen-free conditions in the Experiment Animal Center of the Second Military Medical University (Shanghai, China). Under satisfactory diethyl ether anesthesia, the mice were challenged subcutaneously (s.c.) in the right flank with 0.2 ml of a single-cell suspension containing $2 \times 10^6$ BEL-7402 cells. About 10 days after tumor cell inoculation, s.c. HCCs were established.

To observe the effect of recombinant adenovirus on carcinogenicity of hepatoma cells, 18 tumor-free athymic mice were randomized into three groups, and were inoculated s.c. in the right back with $1 \times 10^6$ cells of the Ad-rAFP-Mel, Ad-rAFP (MOI 100) virus-infected or non-infected BEL-7402 cell lines. Growth of the tumors was observed 10 days after inoculation.

To ascertain the antitumor effect of recombinant adenovirus in vivo, 18 male athymic mice bearing subcutaneous BEL-7402 hepatoma (~ 5 mm in diameter) were randomized into three groups and were given intratumoral injections of Ad-rAFP-Mel, Ad-rAFP ($2 \times 10^8$ pfu/0.1 ml each) or normal saline (0.1 ml each) daily for 5 days. Serial changes in tumor volume were estimated twice a week after the start of the recombinant adenovirus treatment. The volume of the tumor was calculated according to the formula $V = ab^2/2$ [21] (where $V$ is estimated tumor volume, and $a$ and $b$ are the length and width of the transplanted tumor, respectively). The survival quality of the animals was also observed. Five weeks after treatment, the animals were killed by cervical dislocation and the tumors were removed and weighed.

**Statistical analysis**

Statistical evaluations of numerical variables were performed using Student’s $t$-test in both groups and ANOVA test in multiple groups. The multiple comparison in multiple groups was performed using Student–Newman–Keuls $q$-test. Significance was defined as $P<0.05$.

**Results**

**Results of Mel gene and pAd-rAFP-Mel sequencing**

The sequence of Mel gene and the target segment in pAd-rAFP-Mel were detected by PCR, and shown to be correct by sequencing. Results of electrophoresis on PCR amplificates of the target segment in pAd-rAFP-Mel are shown in Figure 1.

**Results of RT–PCR on Mel gene transcripts**

Electrophoresis of the Mel gene transcripts showed a clear band at ~230 bp (the lateral sequence of Mel gene plus the sequence of the terminal primer) in BEL-7402 cells infected by Ad-rAFP-Mel with reverse transcriptase, and the size of the segment was consistent with the expected length. The other three groups were negative (Figure 2). The results suggest that Mel gene was expressed in BEL-7402 cells but not in L02 cells.

**Transfection rate of recombinant adenovirus**

The recombinant adenovirus had a relatively high in vitro transfection rate on BEL-7402 hepatoma cells. The transfection
rates were 16.5, 44, 84.3, 96.3 and 100% when the MOI was 0.1, 0.5, 1, 5 and 10, respectively.

X-gal-stained sections showed that in tumors injected with the recombinant adenovirus Ad-CMV-LacZ, quite large numbers of cancer cells at the needle tip area and along the tip route were observed to be stained blue, while few surrounding cancer cells were stained blue.

**Inhibitory effect of recombinant adenovirus on hepatoma cells**

MTT results showed that the inhibitory rate of Ad-rAFP-Mel and Ad-rAFP on BEL-7402 cells was 66.2 ± 2.7% and 2.9 ± 2.3%, respectively (Figure 3). The difference between the two groups was significant ($t = 30.83, P = 6.6 \times 10^{-6}$).

Ad-CMV-Mel also had an inhibitory effect on AFP-positive, AFP-negative and normal cells; the inhibitory rates were 58.9 ± 9.6%, 65.9 ± 3.8% and 31.7 ± 1.2%, respectively. The inhibitory rate of Ad-rAFP-Mel was 66.2 ± 2.7% on BEL7402 cells (the difference was insignificant compared with Ad-CMV-Mel; $t = 1.27, P = 0.27$), 16.1 ± 6.6% on SMMC7721 cells and 7.5 ± 3.3 on L02 cells (the difference was significant compared with Ad-CMV-Mel; $t = 11.31, P = 3.5 \times 10^{-14}$ and $t = 12.12, P = 2.7 \times 10^{-4}$) (Figure 4).

**Carcinogenicity of virus-infected hepatoma cells**

The results of recombinant adenovirus on carcinogenicity of hepatoma cells showed that the formative rates of subcutaneous tumor were 0/6, 4/6 and 5/6 in the Ad-rAFP-Mel, Ad-rAFP and normal saline group, respectively.

**Tumor inhibitory effect of intratumor injection of recombinant adenovirus**

In Ad-rAFP-Mel group, the tumors began to shrink from week 1 and disappeared in all six animals at week 2.5. At week 4, the tumor re-appeared on one animal, and no recurrence was found in the other five animals at week 5. In Ad-rAFP and control groups, the tumors grew larger gradually, although the tumor subsided in one of the six animals. The size of the tumors between the Ad-rAFP-Mel group and Ad-rAFP and control groups was significant from week 1 through week 5 ($P < 0.05$ and $<0.01$). The difference between Ad-rAFP and control groups was not statistically significant ($P > 0.05$). The condition of the animals in Ad-rAFP-Mel was generally good, while the animals in the other two groups began to get thinner and thinner as the tumors grew larger. One mouse in Ad-rAFP group died at week 3. No animal died in the other two groups within 5 weeks. Comparison of changes in tumor size of the three groups is shown in Figure 5.

The average weight of the tumors were 3.7 ± 9, 167.2 ± 96.7 and 176 ± 97 mg in Ad-rAFP-Mel, Ad-rAFP and control groups, respectively. The difference in the weight of
the tumors between the Ad-rAFP-Mel group and Ad-rAFP and control groups was significant ($q=4.90$, $P=0.004$; and $q=5.42$, $P=0.002$).

**Discussion**

Because the development of HCC is strongly associated with chronic liver disease, particularly cirrhosis that occurs as a result of hepatitis B or hepatitis C virus, the close follow-up of patients with chronic hepatitis B or hepatitis C virus infection using imaging techniques and serum AFP assays has led to the detection of HCC at an early stage [22]. However, even among patients in whom HCC is detected early, there are very few candidates for surgery, because they generally lack a hepatic reserve as a result of the coexisting advanced cirrhosis [23]. Moreover, clinical observations have shown that tumor recurrence rates are very high in patients with HCC who receive medical or surgical treatments. Thus, new treatment modalities must be pursued.

Successful gene therapies for HCC in mice models that use the *herpes simplex virus thymidine kinase* (*HSV-tk*) gene followed by treatment with ganciclovir have been reported [24, 25]. The gene therapy procedure described in this study can be considered simpler than other killer–suicide systems because the tumor-bearing animals only need a single injection of producer cells at the tumor site instead of two consecutive treatments [26–28]. This system, based on the regulation of toxin gene expression, presents potentially curative applications for the treatment of human liver tumors.

The main component of bee sting venom is Mel. As the gene sequence of encoded Mel protein is relatively short (only 78 bp), it is easy to synthesize and transfet as a target gene for tumor treatment. Furthermore, as the antitumor action of Mel does not injure the immune system of the body [29], the prospect of using it in tumor patients with hypoinmunofunction is promising. Like many other animal toxin proteins, Mel is a membrane-interactive toxin [30]. As it mainly works on the cell membrane and kills the tumor cell directly and quickly, the action is superior to that of bacterial and plant toxins.

As the transfection rate of a viral vehicle is relatively higher than that of a non-viral vehicle, and expression is relatively stable, it is a more common practice to use viruses as vehicles of gene therapy, among which adenovirus is a common vehicle in gene therapy for liver cancer [31–33]. The present study used duplication-defective adenovirus as the vehicle for *Mel* gene. The results of verification showed that the *Mel* gene effectively incorporated into the plasmid of the recombinant adenovirus. When the constructed adenovirus transfected liver cancer cells, *Mel* mRNA was transcribed in the tumor cells. This recombinant adenovirus had a high transfection on BEL-7402 liver cancer cells, indicating that adenovirus is a good vehicle for *Mel*.

Using tumor embryonic antigen as the promoter to regulate expression of the target gene is a common strategy in cancer gene therapy. An example is using *AFP* gene as the promoter to regulate expression of the target gene in AFP-positive liver cancer cells for the purpose of curing liver tumors. Ishikawa et al. [34] described the construction of the retroviral vectors in which the *HSV-tk* gene is placed under the control of the AFP enhancer/promoter [LN(AFE0.3TK)R]. The retrovirus infection into HepG2 cells caused a greater cytotoxicity on ganciclovir exposure, together with a stronger ‘bystander effect’. In an animal model, intratumor injection of LN(AFE0.3TK)R with ganciclovir treatment resulted in pronounced growth inhibition of HepG2 tumor. Ohashi et al. [35] designed an adenovirus carrying *E1A* and attenuated *E1B* gene driven by the AFP promoter (Adv-AFP-E1AdB), which restricting the replication specificity in AFP-producing HCC. It efficiently destroyed HCC cells after injection. Experiments were conducted in vivo using systemic administration of Adv-AFP-E1AdB, and they observed tumor size reduction in nude mice with liver cancer. Hirano et al. [36] observed that repeated transfection of *HSV-tk* gene driven by the AFP promoter (AFPTK1) followed by ganciclovir treatment markedly suppressed growth of HUH7 tumors. After inoculation with the tumor, HVJ-liposomes containing the AFPTK1 plasmid vector were injected into the portal vein via the splenic hilum, followed by ganciclovir treatment. This gene therapy significantly inhibited the growth of tumors in the liver and markedly improved survival. Three injections of the AFPTK1 plasmid vector completely inhibited tumor growth. Arbuthnot et al. [37] constructed recombinant adenoviral vectors containing transcriptional elements from either the rat *AFP* or the human insulin-like growth factor II (*IGFII*) genes driving expression of the nuclear β-galactosidase gene (nls lacZ). *In vitro* infection revealed that the AFP but not the IGFII transcriptional regulatory sequence controlled nls lacZ expression specifically in hepatoma cells. The same specificity was obtained *in vivo* in subcutaneous human hepatic tumors generated by engraftment of Huh7 hepatoma cells in nude mice, as well as in primary liver tumors developed in rats and mice.

Our results in the transfected recombinant adenovirus carrying *Mel* gene has an action of inhibiting proliferation of liver cancer cells, and this action is positively correlated with the titer. Using macrophage virus (CMV) as a common promoter to promote expression of *Mel* gene had a killing effect on AFP-positive, AFP-negative and normal L02 cells, while using AFP-specific promoter to promote expression of *Mel* gene had a high inhibitory effect on AFP-positive liver cancer cells only, and had little inhibitory effects on AFP-negative and normal cells, suggesting that the AFP promoter had a specific action of promoting expression of the target gene in cells secreting AFP.

Modalities of *in vivo* gene therapy for liver cancer include tumor in situ injection, transportal injection, hepatic artery injection or biliary injection [38, 39]. It is a disputable fact that direct intratumor injection may induce gene transduction, which may be beneficial to suicidal gene therapy, because transfer of the suicidal gene may directly kill the tumor cell transferred by the gene and induce the bystander effect to amplify the killing effect [40, 41].
The present study used the *ex vivo* method, where *ex vivo* transfection of the recombinant adenovirus containing *Mel* gene markedly inhibited carcinogenicity of the cancer cells and no tumor formed in the nude mice. Secondly, intratumor injection of the recombinant adenovirus resulted in disappearance of the subcutaneous tumor of the tumor-bearing nude mice. These results demonstrated that *Mel* gene was expressed effectively in the liver cancer cells, thus weakening carcinogenicity and inhibiting growth of the tumor cells. Although injecting the Ad-CMV-LacZ virus into tumors showed very localized X-gal staining, the athymic mice bearing tumors were given multipoint, and often intratumoral, injection when the tumor volume was very small. Furthermore, the killing effect may be amplified by the bystander effect, leading to regression of the entire tumors. There was one case of recurrence in the study group, probably because the therapy was not complete enough, and the residual cancer cells continued to divide. As adenovirus is immunogenetic, *in vivo* injection of the virus induces production of antibody in the body, and repeated use of the drug within 2 weeks will not be of use. Therefore, other means of therapy should be considered for recurrent cases.

References


