

# Microencapsulation of recombinant adenovirus within poly-DL-lactide-poly(ethylene glycol) microspheres for enhanced gene transfection efficiency and inhibitory effects on hepatocellular carcinoma cells *in vitro*

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**Abstract.** When gene therapy is performed for the treatment of malignant tumors, gene transfer efficiency and selectivity are highly important. Polymer vehicle microspheres are a novel type of therapy, which have been developed rapidly in recent years and are able to control drug release, prolong the biological half-life of drugs, decrease side effects and achieve targeted delivery. The present study was designed to construct a polymer microsphere-encapsulated recombinant adenovirus with human tissue inhibitors of the matrix metalloproteinase-1 (TIMP-1) gene, and to discuss its characterization for the purpose of liver cancer gene therapy. The microsphere was prepared from biodegradable poly-DL-lactide-poly(ethylene glycol) (PELA) encapsulating rAdTIMP-1, the recombinant adenovirus carrying TIMP-1, by a modified double-emulsion method. The particle morphology, diameter, virus encapsulation, loading rate and release kinetics of the rAd-microspheres were determined *in vitro*. Hepatocellular carcinoma (HCC) HepG2 cells were transfected with the rAd-microsphere and the efficiency of transfection was assessed by fluorescent microscopy. The production and expression of TIMP-1 was identified by gelatin zymography and western blot analysis, and the invasiveness was detected by a matrigel matrix invasion assay. The microsphere encapsulating rAdTIMP-1 was successfully constructed with a diameter of 1.965  $\mu\text{m}$ , encapsulation efficiency of 60.0%, a viral load of  $10.5 \times 10^8/\text{mg}$ , a virus release of  $\sim 60\%$  within 120 h and a total release time of  $>240$  h. The resultant rAd-microspheres were able to efficiently transfect HepG2 cells with the transfection efficiency enhanced by  $\sim 90\%$ . As a result, the transfected HepG2 cells

had significantly increased TIMP-1 enzyme activity and the expression of TIMP-1 was detected by western blot analysis. In addition, the proliferation and invasion ability of the HCC cells was markedly inhibited by the rAd-microspheres. The resultant rAd-microspheres, PELA-encapsulated recombinant TIMP-1 adenovirus, had enhanced transfection efficiency and were able to markedly inhibit the *in vitro* biological behavior of HepG2 cells. This provides an experimental basis for this polymer application and may pave the way for prospective *in vivo* clinical trials and further comprehensive therapy for liver cancer.

## Introduction

Human hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related mortality worldwide and its poor prognosis mainly depends on the clinicopathological characteristics regarding invasion and metastasis. HCC frequently shows early intrahepatic metastases as well as blood vessel invasion followed by extrahepatic metastases at later stages. As with any other malignancy, the propensity for local invasion and distant metastasis of HCC is based on its ability to degrade the surrounding extracellular matrix (ECM) and invade the basement membrane (BM) (1).

Matrix metalloproteinases (MMPs) are a family of 24 secreted  $\text{Zn}^{2+}$ -dependent endopeptidases and are identified to be involved in the proteolysis of the ECM and establishment of metastatic deposits. The natural inhibitors of MMPs, termed tissue inhibitors of metalloproteinase (TIMPs), counterbalance the activity of MMPs *in vivo* and may have direct effects on cell proliferation (2).

Gene therapy, a modern molecular medicine strategy, holds great promise for the treatment of HCC and has the potential to revolutionize cancer treatment. However, liver gene therapy remains in the developmental stage and efficient and innocuous liver-directed gene transfer vectors are therefore urgently required. To achieve efficient cytosolic delivery of therapeutics, various nanomaterials have been developed that consider the diverse physicochemical nature of therapeutics (macromolecules to small molecules; water soluble to water insoluble) and various membrane-associated and

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intracellular barriers that these systems have to overcome to efficiently deliver and retain therapeutics in the cytoplasmic compartment. Biodegradable particles formulated from poly-DL-lactide-poly(ethylene glycol) (PELA), hydrophobic polylactic acid (PLA) and polylactide-co-glycolide (PLGA), have been extensively investigated for sustained and targeted/localized delivery of various agents, including plasmid DNA, proteins, peptides, drugs, enzymes, antibodies or nucleotides, and are able to be directed to a specific organ, tissue or tumor (3,4).

In the present study, the polymer microsphere was prepared by encapsulating the recombinant TIMP-1 adenovirus in biodegradable PELA instead of the traditional vectors. Its biomedical characteristics were discussed in regard to its application in gene therapy of liver cancer.

## Materials and methods

*Experimental animals, cell lines and culture method.* HepG2 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were used as a poorly differentiated human HCC cell line and the cells were maintained in RPMI 1640 (HyClone, Rockford, IL, USA). Low-passage cells were used in all experiments. Male Wistar rats (age, 10-12 weeks; weight, 250-300 g) were provided by the Medical Experimental Animal Centre of Luzhou Medical College (Luzhou, China). The study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Luzhou Medical College. All other chemicals and solvents were of reagent grade or better.

*Preparation of rAd-microspheres.* The previously described method for recombinant adenovirus rAdTIMP-1 construction was used and the ideal virus titer ranging from  $1.0 \times 10^{10}$ - $10^{11}$  efu/ml was obtained (5). PELA (Chengdu Institution of Organic Chemistry, Chinese Academy of Science, Chengdu, China) were used to prepare the polymer microsphere. The target microspheres were prepared by solvent extraction based on the formation of a modified double-emulsion water-1/oil/water-2 ( $W_1/O/W_2$ ) system as reported previously (6). Briefly, inner aqueous phase ( $W_1$ ) was prepared with adenovirus aqueous solution, Oil phase (O) was prepared with 20% PELA solution (200 g/l) dissolved in methylene chloride, and external aqueous phase ( $W_2$ ) was prepared with 2.0% polyvinyl alcohol aqueous solution (20 g/l). The adenovirus aqueous solution was added to PELA methylene chloride solution and stirred (890 x g) for 1 h. Subsequently, it was added to the PVA aqueous solution and stirred (890 x g) for 4 h. The organic solvent was removed with the solvent extraction method (50 g/l isopropyl alcohol solution). The solution was then centrifuged (4,360 x g) for 8 min, washed with double-distilled water three times and finally freeze-dried to acquire a powder of rAd-microspheres.

*Physicochemical characteristics and virus release curve.* rAd-microspheres powder (2 mg) was dispersed in distilled water with ultrasonication for 30 min, and the average particle size, standard deviation and distribution curves were

determined using a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK). The rAd-microspheres were hydrated and then dried. Their surface morphology and dispersed state were observed using a scanning electron microscope (SEM; Amray, Drogheda, Ireland).

The virus titer in the remaining liquid following encapsulation was determined and compared with the antecedent titer. The encapsulation efficiency and virus loading rate were calculated as previously described (6). To measure the viral release from rAd-microsphere preparations, the virus titers at 0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h were determined by assessing their fluorescence, and virus release curves were plotted.

*Toxicity testing.* A total of 20 Wistar rats were randomly divided into an experimental group and a control group, which were administered a single intraperitoneal injection of blank microspheres suspension ( $3 \times 10^{11}$  efu/ml) or normal saline (Chengdu Bao Yikang Medicines and Health Products Co., Ltd, Chengdu, China), respectively. Rats were fed separately under a 12-h light/dark cycle in order to observe the general condition and survival period over three months. All rats were sacrificed by cervical dislocation.

*Transfection efficiency and growth curve.* HepG2 cells in the logarithmic growth phase were conventionally digested using 0.25% trypsin (Fuzhou Maixin Biotechnology Development Co., Ltd, Fuzhou, China) and incubated overnight in a 24-well culture plate ( $1 \times 10^5$  cells/well). Thereafter, 0.01, 0.1, 1, 10 or 100 mg rAd-microspheres, were added into the 24-well culture plates. The culture medium was removed at 48 and 120 h, respectively. Thereafter, the number of green fluorescent cells (containing green fluorescent protein) and total cells in inverted culture plates were counted under a BX61 fluorescent microscope (Olympus Corp., Tokyo, Japan). The ratio between them indicated the transfection efficiency.

The HepG2 cells transfected with rAd-microspheres and blank microspheres as well as the control group were inoculated onto 24-well plates ( $1 \times 10^5$  cells/well). For each group, cells were digested and suspended for analysis daily. The number of cells was counted and the mean was recorded for six consecutive days. The cell growth curve was plotted as cell growth versus time.

*Gelatin zymography.* Analysis of TIMP-1 in rAd-microspheres-transfected HepG2 cells was performed on SDS-polyacrylamide gels impregnated with 0.1% gelatin (w/v) and 10% polyacrylamide (w/v) as described previously (7). Culture supernatants were grown in 100-mm<sup>2</sup> tissue culture plates in Dulbecco's modified Eagle's medium (DMEM; HyClone)/Ham's F-12 containing 10% fetal calf serum (FCS) until they reached 80% confluence. Cells were washed and placed in serum-free medium, and the conditioned medium was collected following 48 h. Four parts of medium containing equal quantities of protein were mixed with one part of sample buffer prior to electrophoresis. Gels were run at a constant current and then washed twice for 30 min in 50 mmol/l Tris-HCl, pH 7.5, with 2.5% Triton X-100 and incubated overnight at 37°C in 50 mmol/l Tris-HCl, pH 7.6.

Gels were stained with Coomassie Brilliant Blue R-250 and then destained.

**Western blot analysis.** Posttransfection (72 h), 5  $\mu$ l of the supernatant of the cultured cells was collected, subjected to 12% SDS-PAGE, and electrically transferred to nylon membranes. Nonspecific binding was blocked with Tris-buffered saline (TBS) containing 5% (w/v) skimmed milk for 2 h at room temperature, and then filters were stained with an affinity-purified mouse anti-human TIMP-1 polyclonal antibody (1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2 h at room temperature followed by incubation with a goat anti-mouse immunoglobulin G secondary antibody (1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h. Following further washing with PBS, the blot was incubated in 3,3'-diaminobenzidine (DAB) and captured on photographic film (5).

**Matrigel matrix invasion assay.** The cell invasion assay was performed by the invasion of cells through Matrigel<sup>TM</sup>-coated Millicell Chamber (Millipore, Billerica, MA, USA) inserts according to a procedure described previously (8). Briefly, 5-mm diameter polycarbonate filters (pore size, 8  $\mu$ m) were coated with Matrigel, dried, and reconstituted at 37°C with RPMI 1640 prior to use. The lower chambers were filled with supernatant of NIH3T3, cultured in serum-free DMEM for 24 h, as a chemotactic factor. HepG2 cells were divided into three groups: Cells treated with rAd-microspheres and normal cells served as experimental as well as control group, respectively; PBS-treated cells acted as the blank control group and the filter was without Matrigel. The cells were pre-transfected 48 h prior to the assay, and then added to the upper chamber at  $1 \times 10^5$  cells per chamber in RPMI 1640 containing 5% FCS. Following 12 h of incubation at 37°C, the suspended media in the lower chamber were removed, fixed and stained. The cells that passed through the filter into the lower chamber were stained with Hema-3 and counted under a phase contrast microscope (five random fields per chamber). Each invasion experiment was performed in duplicate and repeated at least twice.

**Statistical analysis.** SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. One-way analysis of variance was used for comparisons among multiple groups by randomization. The t-test was used for comparison between groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Physicochemical properties and virus release curve.** Fig. 1 shows the SEM images of rAd-microspheres prepared by a double emulsion ( $W_1/O/W_2$ ) based on the solvent evaporation method. The resulting microsphere was smooth and spherical with a uniform size, regular shape, good dispersion between microspheres and no apparent evidence of collapse. As shown in Fig. 2, the mean particle size has a normal distribution as assessed using a laser diffraction particle size analyzer: Of all particles, 50% were 1.965  $\mu$ m, <10% were 1.250  $\mu$ m and >40% were 3.320  $\mu$ m in size. The mean spacing of microspheres was 1.360  $\mu$ m.

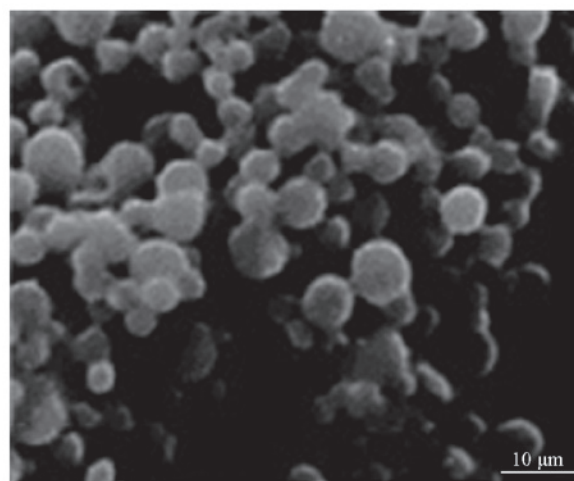


Figure 1. Dispersion pattern and morphology of the rAd-microspheres determined by SEM (magnification,  $\times 2,500$ ). rAd, recombinant adenovirus; SEM, scanning electron microscopy.

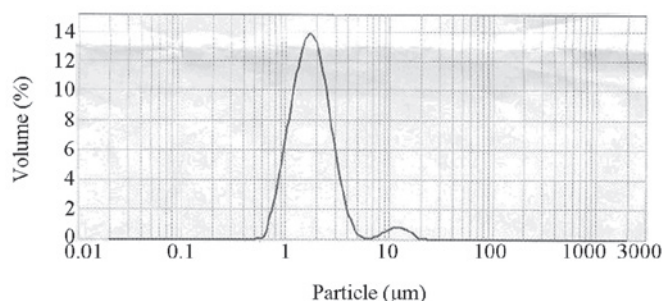


Figure 2. Particle-size distribution of the rAd-microsphere. rAd, recombinant adenovirus.

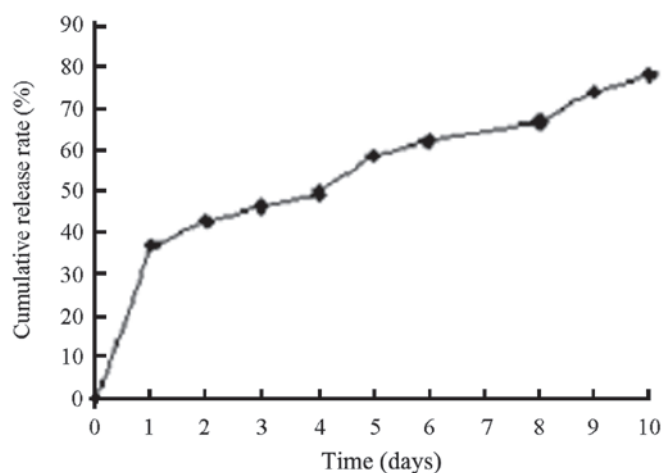


Figure 3. Percent release of viable virus from the rAd-microspheres. rAd, recombinant adenovirus

The virus titer of the stock solution prior to encapsulation was  $3.59 \times 10^{11}$  efu/ml. With additional 5 ml virus stock solution during encapsulation, the virus titer of the residual liquid was  $1.4 \times 10^9$  efu/ml following encapsulation, with residual liquid of 500 ml. PELA (1,000 mg) was added during encapsulation. Therefore, the encapsulation efficiency was 60.0% and the



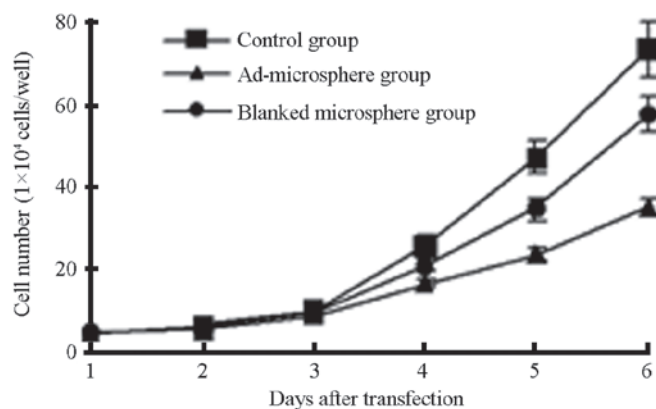


Figure 4. Growth curve of HepG2 cells with or without rAd-microspheres transfection ( $P < 0.05$ ). rAd, recombinant adenovirus.

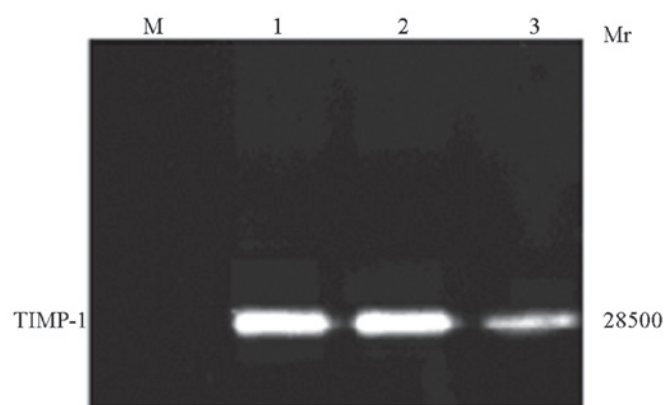


Figure 5. Gelatin zymography of TIMP-1. TIMP-1, tissue inhibitors of the matrix metalloproteinase-1.

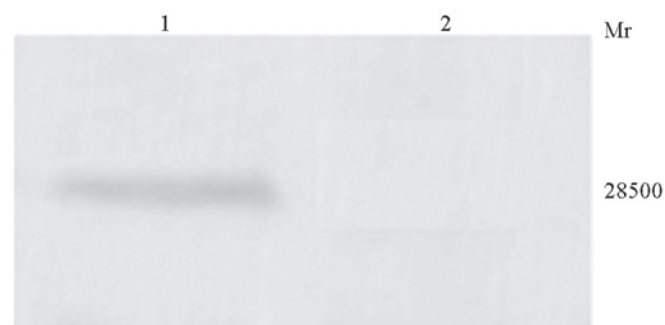


Figure 6. Western blot analysis of TIMP-1. TIMP-1, tissue inhibitors of the matrix metalloproteinase-1.

virus loading rate was  $10.5 \times 10^8$ /mg. The viable virus release of rAd-microspheres in the DMEM medium at  $37^\circ\text{C}$  was almost 60% within 120 h, with a total release time  $>240$  h (Fig. 3). A toxicity test of blank microspheres demonstrated that the microspheres were nontoxic with few side effects in accordance with the requirements for microspheres for *in vivo* application (6).

From the aforementioned results, the conclusion was drawn that rAd-microspheres may function as a suitable gene-delivery system with appropriate particle size, particle-size distribution and high gene-loading efficiency.

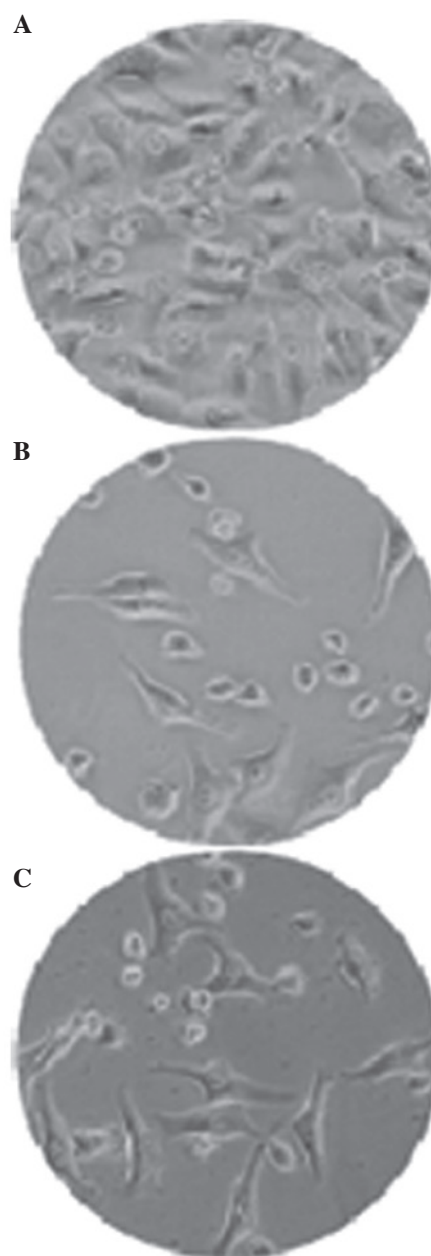


Figure 7. Inhibition of TIMP-1 overexpression attenuates HepG2 cells invasiveness mediated by rAd-microspheres under a light microscope (magnification,  $\times 400$ ). (A) Blank control group (PBS-treated HepG2 cells crossing through the blank filter); (B) experimental group (rAdmicrospheres-transfected cells crossing through the Matrigel-coated filter); (C) control group (normal cells crossing through the Matrigel-coated filter). TIMP-1, tissue inhibitors of the matrix metalloproteinase-1; rAd, recombinant adenovirus.

*Transfection efficiency and inhibition on in vitro growth of HepG2 cells.* With the viral multiplication, the transfection efficiency of HepG2 cells was also increased. When the quantity of virus was  $>10$  mg, the transfection efficiency was able to reach  $>90\%$ , indicating the enhanced gene transfection activity.

Fig. 4 shows the release profiles over a six-day period. The cells in the three groups, days 1-3, were at the stationary phase. With increasing time, the cell counts in the rAd-microspheres group were significantly lower than those in the blank microsphere and control groups, suggesting that the rAd-microspheres carrying TIMP-1 were able to inhibit the proliferation of the HepG2 cells ( $P < 0.05$ , data not shown).

**Toxicity of the microspheres *in vivo*.** A total of 20 Wistar rats were injected with either a blank microspheres suspension ( $3 \times 10^{11}$  efu/ml) or normal saline in order to determine the toxicity of the microspheres *in vivo*. Rats were kept under good general condition, with normal activities but poor mental state, decreased appetite and depilation. Following observation for 4 weeks, all the animals were still alive and there were no significant difference observed between the experimental and control groups. This indicated that the microspheres were non-toxic as they had no observable side effects under these conditions *in vivo* (data not shown).

**TIMP-1 activity determined by gelatin zymography.** Previous studies by our group have reported *in vivo* overexpression of endogenous TIMP family members inhibits tumor-induced, MMP-dependent matrix proteolysis under pathological conditions (5,9,10). The functional activity of TIMP-1 encapsulated in the rAd-microspheres was detected using the gelatin zymography assay on HepG2 cells. As shown in Fig. 5, the expression and activity of TIMP-1 were low in rAd-microspheres-transfected HepG2 cells, which indicated that TIMP-1 overexpression was able to downregulate the activity of MMPs by inhibiting its cleavage activation without suppressing its expression in HCC cells.

**TIMP-1 western blot analysis.** HepG2 cells were transfected with rAd-microspheres for 72 h, and the supernatant was collected. The TIMP-1 protein was subjected to SDS-PAGE and transferred to the membrane for western blot analysis. A major band exhibiting a molecular weight of 28.5 kDa was specifically detected in rAd-microspheres-transfected HepG2 cells, but not in the control, indicating that normal HepG2 cells are not able to express TIMP-1 protein and that, following transfection, rAd-microspheres-transfected HepG2 cells are able to express TIMP-1 protein, and even secrete it (Fig. 6).

**Matrigel matrix invasion assay.** Matrigel-coated Millicell chambers were used in a standard test to investigate whether transfection by rAd-microspheres suppresses the invasion of HepG2 cells. The results demonstrated that the percentage of cells invading the Matrigel-coated filter in experimental group (rAd-microspheres-transfected cells) and control group (normal cells) was  $12.4 \pm 3.5\%$  and  $36.5 \pm 4.3\%$ , respectively, as compared with the PBS-treated HepG2 cells crossing through the blank filter set as 100%. This demonstrated that virally induced TIMP-1 secretion markedly inhibited HCC cell invasive migration (Fig. 7). Furthermore, the results of the present study confirm that increased expression of TIMP-1 by rAd-microspheres is responsible for the inhibitory effect on cell motility and invasiveness of HCC cells.

## Discussion

HCC is the sixth most common type of cancer worldwide in terms of incidence, accounting for approximately 630,000 newly diagnosed cases per year. In addition, HCC is the third leading cause of cancer-related mortality. Around 80% of HCC cases occur in developing countries, with the areas of high incidence being sub-Saharan Africa and Eastern and Southeast Asia, particularly China. However, the incidence

of HCC is low in numerous developed countries, as well as in Latin America and South-central Asia (11,12).

HCC has a high degree of malignancy, a high metastasis rate and unfavorable prognosis. Generally, metastasis of HCC involves multi-step processes and various cytophysiological changes, including local invasion, entering the lymphatic and blood vascular system, surviving in the bloodstream, extra-vascularization from the microvessels and colonization at the secondary site. The key step in these processes may be the degradation of the ECM causing destruction of the BM. MMPs and TIMPs are two groups of functionally antagonistic proteases. A previous study suggested that perturbing the balance of MMPs and TIMPs may lead to direct inhibition of MMPs and increase of TIMPs in cancer and may be a particularly attractive target for therapeutic intervention in tumor invasion and metastasis (13). Therefore, perturbing the MMP-TIMP balance may cause degradation of ECM and destruction of BM following tumor metastasis. TIMPs have been observed to be synthesized by the same cell secreting MMPs, which may specifically close the catalytic active site and have an important role in ECM remodeling as well as the invasion and metastasis of tumors. At present, four TIMPs have been identified, TIMP-1-4; however, only TIMP-1 and -2 were found to be expressed in the liver. TIMP-1 is a secreted glucoprotein with a relative molecular weight of 28.5 kD. Its activity was able to be inhibited by forming complexes with almost all collagenases at ratios of 1:1. Upregulation of TIMP-1 was shown to suppress the tumor invasion and metastasis in various types of human cancer, which may be correlated with its inhibition of MMP-2/9 (14).

The use of recombinant adenoviral vectors as an alternative delivery method for genes or combinations of genes to tumor cells is being investigated (6,15-17). Since adenoviruses are able to efficiently enter replicating and quiescent cells, it may be used as a prospective mediator for macromolecular transport into cells. Furthermore, recombinant adenoviruses have numerous advantages, including transfection ability, high titer, efficient multicopy, no insertional mutagenesis and no genetic toxicity. In addition, it was not possible to integrate the free vector into the host DNA. Recombinant adenoviruses were able to infect a wider range of hosts, particularly cells in copy division phase. However, the relatively short expression time may evoke an immune response in the host upon repeated application (18). The two aims to improve the effectiveness of adenoviral-mediated gene transfer for HCC therapy are to increase the efficiency of gene transfer and to reduce the requirement for frequent re-dosing regimens. Theoretically, the medical microspheres produced in the present study, rAd-microspheres, PELA encapsulated recombinant TIMP-1 adenovirus, with enhanced transfection efficiency and sustained release capability, were expected to achieve these requirements.

Pharmaceutical research has led to the identification of numerous reagents compatible with controlled delivery of drugs enterically and systemically. These include particles, nanoparticles, microemulsions, submicron emulsions and liposomes (19-23). Therapeutics may require efficient cytosolic delivery if the receptors for those drugs are located in the cytosol or their site of action is an intracellular organelle that requires transport through the cytosolic compartment.

Biodegradable microspheres formulated from biodegradable PELA, hydrophobic PLA and PLGA have been successfully used to deliver drugs at a controlled rate to target specific organs including the liver (3,24).

PELA is a type of degradable polymer, obtained by polymerization of PLA and hydrophilic polyvinyl alcohol (PEG). It is hydrophilic and nontoxic, with no immunogenicity but high encapsulation efficiency, and it was also able to improve the stability and adjustability of the encapsulation contents. It is a focus of recent research of materials and has been applied in encapsulating albumin, DNA and vaccines (24,25). Ren *et al* (26) investigated the partial characteristics of a microsphere vaccine prepared by encapsulation of recombinant outer membrane protein K (OmpK) of *Vibrio harveyi* with PELA in crucian carp inoculated orally. The study indicated the feasibility of PELA as a system for oral vaccine delivery to fish. Ruan *et al* (27) investigated the effect of different polymers used for the preparation of human serum albumin (HSA)-loaded microparticles and suggested that the HSA encapsulation efficiency value of PELA microparticles was ~10% higher than that of PLGA microparticles. Yang *et al* (28) incorporated bovine serum albumin (BSA) into porous PLGA scaffolding containing microspheres of PELA and observed that the microsphere-incorporated scaffold prolonged BSA release, and the cumulative release on day 10 reached 85% of total encapsulated BSA. Wei *et al* (29) compared PELA microspheres with narrow size distribution for sustained release of recombinant human growth hormone (rhGH) with PLA and PLGA microspheres to determine the difference in encapsulation efficiency, initial burst release, high burst levels and integrity of rhGH, and concluded that PELA was an effective polymer for rhGH encapsulation and stabilization. Compared with the commonly used PLA and PLGA, PELA microspheres showed potential as delivery systems for macromolecular drugs (including protein and peptide drugs), which may be due to the amphiphilic structure of the block copolymer. PELA microspheres undergo slow degradation by hydrolysis of ester linkages to yield lactic and glycolic acid. Additionally, it is able to control the rate of release of entrapped antigens and therefore, offers potential for the development of single-dose drugs. Accordingly, previous studies performed by our group demonstrated the feasibility of encapsulating recombinant adenovirus into PELA microspheres with retention of virus viability (6).

Among the microspheres described in this study, >90% were <3  $\mu\text{m}$  in size, with a mean particle size of 1.965  $\mu\text{m}$ . They exhibited good dispersion, with a mean spacing of 1.360  $\mu\text{m}$  and a virus loading rate of  $10.5 \times 10^8/\text{mg}$ . A previous study (30) has shown that decreased sphere size results in improvements in the encapsulation yield. However, to the best of our knowledge, no studies to date have been performed to optimize the encapsulation of live viral vectors. Given the relatively large size of the adenovirus (~100 nm), and consideration of mechanical forces upon encapsulation, gentle methods for encapsulation were used, which resulted in a large sphere size (>10-20  $\mu\text{m}$ ). This size may be advantageous when delivering the antigenic adenovirus. A study that directly compared the immune response to antigens in 1-10- versus 10-110- $\mu\text{m}$  spheres demonstrated a 20-fold reduction in immunogenicity when encapsulated in larger particles (31).

The present study suggests that the viral release in the initial 24 h was faster than that after 24 h, and the cumulative release percentage was close to 60% within 120 h. As a previous study suggested (32), the release is able to be characterized by at least two phases. The first phase, usually comprising the initial 24 h, is a rapid release of the compound as a result of diffusion from the surface of the microspheres. The second phase is a relatively slow release with the erosion of the polymer through hydrolysis. This solves the problem of a rapid *in vivo* clearance of the drugs, which hinders drugs from acting efficiently over a long period of time.

Previously, acute toxicological experiments of blank microspheres showed that the microsphere vectors themselves were nontoxic with no side effects in accordance with the requirements of *in vivo* applications (6). In the present study, the HCC cell line HepG2 was transfected with rAd-microspheres, which displayed the highest transfection efficiency (~90%) compared with other microspheres. The MTT experiment and cell growth curve confirmed that rAd-microspheres carrying TIMP-1 were able to inhibit the *in vitro* proliferation of HepG2 cells. Based on the efficient, high-loading, sustained-release rAd-microspheres, the TIMP-1 gene was stably expressed in HCC cells over a long time, and the biological activities of HCC cells were inhibited through upregulation of TIMP-1 expression and downregulation of the activity of MMPs (6,33). The expression of the TIMP-1 protein was steadily detected by western blot analysis, and the overexpression of TIMP-1 was able to downregulate the activity of MMPs by effectively inhibiting gelatinase degradation as indicated by the results of the gelatin zymography assay. The cell invasion assay confirmed migration *in vitro*, and the invasion capacity was markedly inhibited by transfection with rAd-microspheres, which is consistent with the concept that the inhibition of invasion by TIMPs is mediated via the prevention of tissue-remodeling.

In conclusion, the present study revealed that PELA-encapsulated adenoviral-mediated TIMP-1 gene transfer is efficient for the treatment of HCC and may pave the way for application in prospective *in vivo* trials and further comprehensive therapy of liver cancer. In addition, different polymers should be probed in regard to their ability to perform sustained release of recombinant viral vectors. Furthermore, other methods including hydrogels, or self-diffusion and self-regulated systems may be applicable. The formulation of viral vectors for gene delivery may improve their applicability for the treatment of HCC, and may have wide-spread application in human disease (34-36).

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