RESEARCH ARTICLE

Preparation of Microspheres Encapsulating a Recombinant TIMP-1 Adenovirus and their Inhibition of Proliferation of Hepatocellular Carcinoma Cells

Dong Xia, Hui Yao, Qing Liu, Liang Xu*

Abstract

Objective: The study aim was to prepare poly-DL-lactide-poly (PELA) microspheres encapsulating recombinant tissue inhibitors of metalloproteinase-1 (TIMP-1) in an adenovirus to investigate its inhibition on the proliferation of hepatocellular carcinoma cells HepG2. Methods: Microspheres were prepared by encapsulating the recombinant TIMP-1 adenovirus into biodegradable PELA. The particle size, viral load, encapsulation efficiency and in-vitro release were measured. Microspheres were used to infect HepG2 cells, then infection efficiency was examined under a fluorescent microscope and ultrastructural changes assessed by TEM. Expression of TIMP-1 mRNA in HepG2 cells was examined by semi-quantitative RT-PCR and proliferation by MTT and cell growth curve assays. Results: We successfully prepared microspheres encapsulating recombinant TIMP-1 adenovirus with a diameter of 1.965μm, an encapsulation efficiency of 60.0%, a viral load of 10.5×10^8/mg and approximate 60% of virus release within 120 h, the total releasing time of which was longer than 240 h. The microspheres were confirmed to be non-toxic with blank microspheres. Infected HepG2 cells could stably maintain in-vitro expression of TIMP-1, with significantly effects on biological behaviour Conclusion: PELA microspheres encapsulating a recombinant TIMP-1 adenovirus can markedly inhibit the proliferation of HepG2 cells, which provides an experimental basis for polymer/chemistry-based gene therapy of hepatocellular carcinomas.

Keywords: HCC cells - tissue inhibitors of metalloproteinase - adenovirus - microsphere - gene therapy

Introduction

Human hepatocellular carcinoma (HCC) is one of the most common causes of cancer death worldwide because it is highly proliferative, invasive, and metastatic. Efforts to prolong the survival of such patients have been weakened by the high incidence of metastasis. HCC frequently shows early invasion into blood vessels as well as intrahepatic metastases and later shows extrahepatic metastases. Like other malignancies, the propensity for local/regional invasion and distant metastasis of HCC is based on its ability to invade the basement membrane (BM) and degrade extracellular matrix (ECM). Since degradation of the surrounding ECM seems to be an important step in tumor invasion and metastasis, targeting matrix proteolysis and cell invasion to achieve an accumulative benefit has also been proposed as a promising therapeutic anticancer strategy.

Matrix metalloproteinases (MMPs) are a family of 24 secreted Zn^{2+}-dependent endopeptidases with a diverse hydrolytic spectrum of ECM proteins. MMPs are identified to be involved in proteolysis of ECM and establishment of metastatic deposits, and neovascularization required for a tumor blood supply to develop. MMPs' natural inhibitors, known as the tissue inhibitors of metalloproteinase (TIMPs), counterbalance in vivo the activity of MMPs and may have direct effects on cell growth.

Gene therapeutic approaches use gene delivery systems (vectors) to introduce target DNA constructs as therapeutic agents into living cells. The genetically engineered vector of viral or non-viral origin promotes the transfer of the transgene expression unit into the intracellular compartment and allows its transient or stable expression. For the past decade, various viral and non-viral vectors have been engineered for improved gene delivery. In the present study, the microsphere was prepared by encapsulating the recombinant TIMP-1 adenovirus into the biodegradable PELA instead of traditional viral or non-viral vectors (Xia et al., 2010). HepG2 cells were infected and in vitro expressed. Observation of how the up-regulated expression of TIMP-1 affected the in-vitro biological behavior of HepG2 cells could provide an experimental evidence for the polymers/chemistry and gene therapy of hepatocellular carcinoma.

*Department of Gastrointestinal Surgery, Affiliated Hospital of Luzhou Medical College, Luzhou, Sichuan, China *For correspondence: liangxucn@126.com*

Materials and Methods

Experimental animals

Wistar rats (male adult rats, weighing about 250~300 g) were provided by the West China Medical Experimental Animal Center of Sichuan University. This study was carried out 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 has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Affiliated Hospital of Luzhou Medical College.

Construction of rAdTIMP-1

Fresh HCC tissue was taken for total RNA extraction, RT-PCR and amplification of TIMP-1 cDNA. After being demonstrated by DNA sequencing, the TIMP-1 cDNA and pAdTrack-CMV were respectively given double digestion with Kpn I and Hind III at the same time. TIMP-1 was forward cloned into pAdTrack-CMV to construct a recombinant shuttle plasmid pAdTrack-CMV-TIMP-1, which was then digested and linearized with Pme I. It was transformed into the competent BJ5183 containing plasmid pAdEasy-1 for homologous recombination. The generated recombinant pAdTIMP-1 adenovirus was digested and linearized with Pac I and then transfected into 293 cells with liposomal transfection. The GFP expression was timely monitored. 48~72 h later, the recombinant AdTIMP-1 adenovirus would be generated. 6~7 d latter, the supernatant was collected (containing AdTIMP-1). 293 cells were repeatedly infected until the ideal virus titer was obtained (Xia et al., 2005).

Preparation of adenovirus microspheres

The microspheres were prepared in double-emulsion systems ($W_1/O/W_2$) with improved solvent evaporation method. W_1 (inner aqueous phase) was prepared with adenovirus aqueous solution. O (oil phase) was prepared with 20% PELA solution (200g/L) dissolved with methylene chloride. And W2 (external aqueous phase) was prepared with 2.0% polyvinyl alcohol (PVA) aqueous solution (20g/L). The adenovirus aqueous solution was added into PELA methylene chloride solution and stirred (890×g) for 1h and then added into the PVA aqueous solution and stirred (890×g) for 4h. The organic solvent was removed with the solvent extraction method (50g/L isopropyl alcohol solution). Then the solution was centrifuged (4360×g) for 8min and washed with double-distilled water for three times and finally freeze-dried to obtain the powder of adenovirus microsphere.

Physicochemical properties and virus release curve

2 mg microsphere powder was ultrasonic dispersed for 30 min in distilled water, whose average particle size, standard deviation and distribution curve were determined by a laser diffraction particle size analyzer. The microsphere was hydrated and then dried. Its surface morphology and dispersed state were observed by scanning electron microscope (SEM). The virus titer in the remaining liquid after encapsulating was determined

and compared to the antecedent titer. The entrapment efficiency and virus load rate were calculated as follows. Encapsulation efficiency (%) = [(the virus titer of the virus stock solution × amount of the liquid) - (the virus titer of the residual solution × amount of the liquid)] / (the virus titer of the virus stock solution × amount of the liquid) × 100%.

Virus load rate (%) = [(the virus titer of the virus stock solution × amount of the liquid) - (the virus titer of the residual solution × amount of the liquid)] / microspheres mass × 100%.

The adenovirus microsphere was taken and dissolved in DMEM solution (containing 10% fetal bovine serum and 100 IU/ml penicillin/streptomycin) and stirred at 37°C. It was centrifuged at room temperature with a speed of 1000r/min. The supernatant was removed into new culture medium. The virus titers at 0, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 h were respectively determined by fluorescent notation and then the virus release curve were drawn.

Toxicity test

10 Wistar rats were randomly divided into two groups, which were respectively given intraperitoneal injection of blank microspheres suspension ($3.0×10^{11}$ efu/ml) and injectable physiological saline. They were fed separately to observe the general condition and survival period.

Cellular morphology

The HepG2 cells in logarithmic growth phase were conventionally digested and inoculated in a 24-well culture plate ($1×10^5$ cells/well) for overnight. Thereafter, 0.01, 0.1, 1, 10, 100 mg recombinant adenovirus microspheres were respectively added into the 24-well culture plate. The cytopathic effects appeared in HepG2 cells at 36~48 h. The culture medium was removed at 48 and 120 h respectively. Thereafter, the numbers of green fluorescent cells (namely GFP positive cells) and total cells in inverted culture plate were counted under a fluorescent microscope. And the ratio between them was the infection efficiency. TIMP-1 mRNA was detected to express at 48, 120 and 240 h by semi-quantitative RT-PCR detection (β-actin as the internal reference). $1×10^6$ cells were collected and centrifuged at 48 h after infection. After removing the fluid, it was conventionally fixed, dehydrated and embedded with epoxy resin. The ultrathin sections were made to be observed by transmission electron microscopy (TEM).

Growth curve

The HepG2 cells infected with adenovirus microspheres and blank microspheres and the HepG2 cells in the control group were respectively inoculated into 24-well plates ($1×10^5$ cells/well). 4-well cells in each group were regularly taken every day to be digested and made into cell suspensions. The number of cells was counted on the improved Neubauor board and the mean was recorded for consecutive 6 days. The cell growth curve was drawn counting the time as the abscissa axis and the cell count as the ordinate axis.
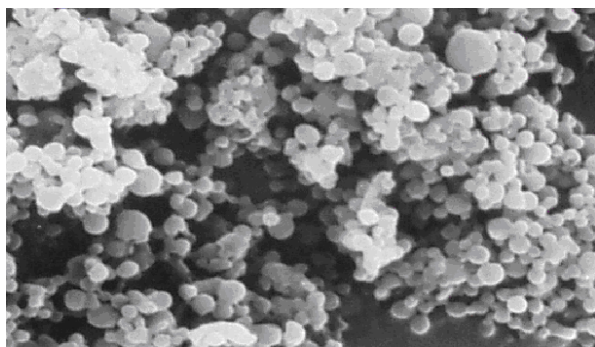
Figure 1. Ultrastructural Analysis of the Recombinant Adenoviral Microsphere (SEM, ×2 500)

MTT

The HepG2 cells were respectively infected with adenovirus microspheres ($3.5×10^{11}$ efu/ml) and blank microspheres. They were digested and inoculated in 96-well plates ($1×10^5$ cells/well) the next day. The duplicate wells were located. The blank group was the culture medium excluding cells and the control group was non-infected HepG2 cells. MTT was added to a final concentration of 0.5mg/ml and cultured with 5% CO_2 at 37°C for 24 h. The reaction was terminated and DMSO was added. The value of D was measured at 490 nm under a enzyme-linked immunosorbent detector. The cell proliferation rate was measured as the following formula: (D of the experimental wells - D of the blank wells/D of the control wells - D of the blank wells) ×100 %.

Statistical analysis

The SPSS (version 11.5) software was used for statistical analysis. One-way ANOVA was used for comparison among multiple groups by randomization. The t-test was used for comparison between groups. Values of $P < 0.05$ were interpreted as significant.

Results

Virus titer

The overall length of TIMP-1 cDNA was synthesized by RT-PCR, with a consistent sequencing result with GenBank. It was positively cloned to pAdTrack-CMV for homologous recombination with pAdEasy-1 in the BJ5183 recipient bacterium and generated AdTIMP-1 carrying TIMP-1 cDNA. After being packaged in 293 cells, the virus titer reached $5×10^{11}$ efu/m.

Physicochemical properties and virus release curve

Under the SEM observation, the adenovirus microsphere was spherical with a uniform size, regular shape; good dispersion between microspheres and no obvious synechia (Figure 1). The mean particle size was shown a normal distribution by a laser diffraction particle size analyzer: 50% was1.965μm; <10% was 1.250 μm; and >40% was 3.320 μm. The mean spacing of microspheres was 1.360 μm. The virus titer of the stock solution before encapsulation was $3.59×10^{11}$ efu/ml. With additional 5ml virus stock solution during encapsulation, the virus titer of the residual liquid was $1.4×10^9$ efu/ml after encapsulation, with residual liquid of 500ml. 1 000mg PELA was added
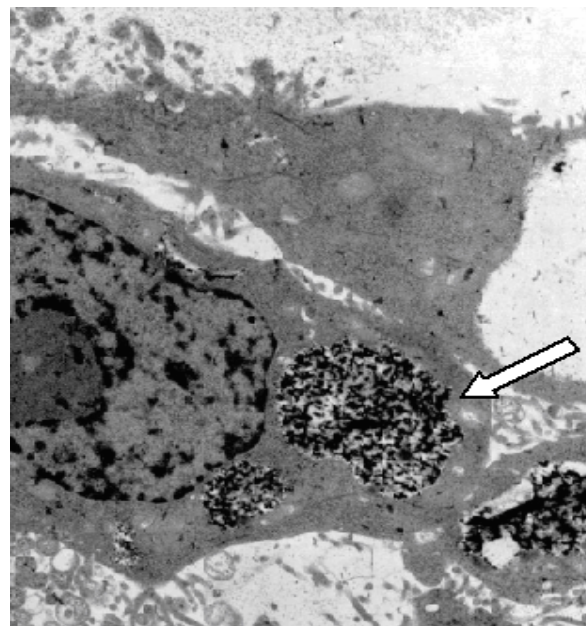

Figure 2. Microsphere Granules (↓) in Cytolymph of Infected HepG2 Cell (TEM, ×8 000)

during encapsulation. Therefore, the encapsulation efficiency was 60.0% and the virus load rate was $10.5×10^8$/mg. The virus release of adenovirus microsphere in the DMEM medium at 37°C was nearly 60% within 120h, with a total release time longer than 240 h.

Toxicity of blank microspheres

20 Wistar rats were given intraperitoneal injection of blank microspheres suspension and injectable physiological saline respectively. They were in good general conditions, with normal activities but poor mental diet and depilation, etc. After observation for 4 weeks, there was no animal died. There was no difference between the experimental group and the control group, indicating that the microspheres were nontoxic with little side effects and in accordance with the requirements of microsphere preparation in vivo application.

Infection and appraisal of HepG2 cells

HepG2 cells were infected with adenovirus microspheres. They appeared atrophy and fall off at 12~24 h and even complete lesion at 36~48 hl. With the viral multiplication, the infection efficiency of HepG2 was also increased. When the amount of virus was more than 10 mg, the infection efficiency could reach more than 90%. Under the TEM observation, compared to the HepG2 cells non-infected with virus microspheres, there were a large number of black particles with high electron density in cytoplasm, that was the successfully transfected adenovirus (Figure 2).

Expression of TIMP-1 mRNA

Semi-quantitative RT-PCR showed that the expression strength of TIMP-1 mRNA was just modestly declined from 24 to 240 h, indicating that the TIMP-1 gene carried on the recombinant adenovirus microsphere could be stably expressed in a certain time (Figure 3). It could be consistent with the release time of adenovirus
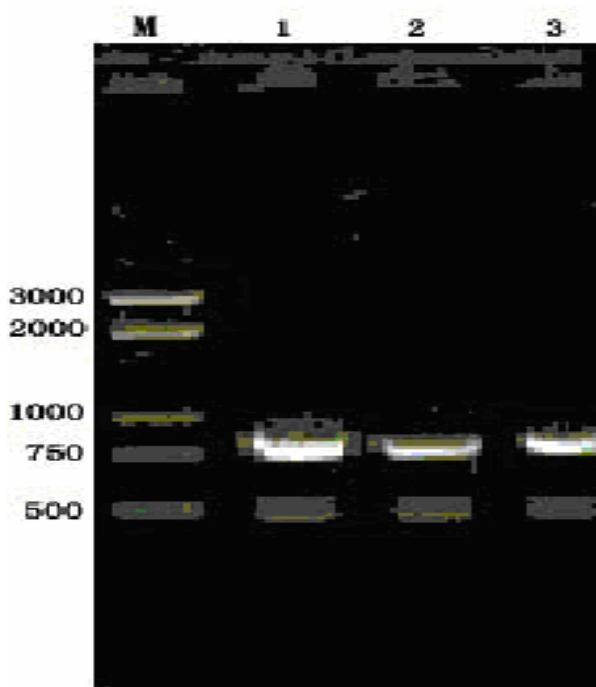
Figure 3. TIMP-1 mRNA Expression was Detected by Semi-quantitative RT-PCR in Various Phases. M: DL 3000 marker; 1: 24 hours after infection; 2: 120 h; 3: 240 h
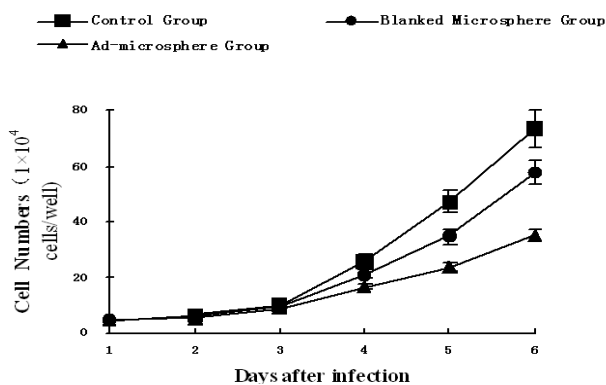

Figure 4. Growth Curve of HepG2 Cells with or Without Recombinant Adenoviral Microsphere Infection (P <0.05)

microspheres, which was better able to ensure the long-term effects and slow release of microspheres.

Inhibition on in vitro growth of HepG2 cells

From the cell growth curve, cells in three groups, d3 to d6, were in the logarithmic growth phase. The time of cells multiplication was 18~24h. The cell count in adenovirus microsphere group was significantly lower than those in the blank microspheres group and the control group, which indicated that the recombinant adenovirus microspheres carrying TIMP-1 could indeed inhibit the proliferation of the hepatoma cell line HepG2 (P <0.05, Figure 4). The cell proliferation rate of the blank microspheres was 100%, but just 53% in adenovirus microspheres group, suggesting that the adenovirus microspheres carrying TIMP-1 could inhibit the proliferation of HepG2 cells (P <0.05).

Discussion

The incidence of liver cancer around the world was on the fifth place of malignancy tumors and the third place of the death rate every year (EI-Serag et al., 2007), 55% of which occurred in China and HCC was accounted for 90% of the hepatic carcinomas. HCC was with a high malignant degree, a high transfer rate and a poor prognosis. And the key step in the infiltration and metastasis might be exactly ECM degradation causing BM destruction. MMPs and TIMPs were two groups of functionally antagonistic proteases. The balance between the two groups was the determinant factor to maintain the stability of the internal environment of ECM. Therefore, MMPs-TIMPs out of balance could cause ECM degradation and BM destruction thereafter tumor metastasis (Xia et al., 2004; Clark et al., 2008; Das et al., 2008; Lee et al., 2009). TIMPs were often synthesized by the same cell secreting MMPs, which could specifically close the catalytic active site and play an important role in ECM remodeling and the invasion, metastasis of tumors. At present there were four TIMPs known, respectively named TIMP-1, -2, -3 and -4, in which only the expressions of TIMP-1 and TIMP-2 were found. TIMP-1 was a secreted glucoprotein with a relative molecular weight of 28.5kD. Its activity could be inhibited by formating complexes with almost all collagenase with ratios of 1:1.

Adenovirus vector was a common virus vector in gene therapy (Benlahrech et al., 2009; Jiang, 2009). It had many advantages, such as strong infection ability, high titer, efficient multicopy, no insertional mutagenesis, no genetic toxicity, etc. It existed in the free type and could not be integrated into the host DNA. It could infect a wider host range, especially the cells in copy division phase. But the relatively short expression time could make its repeated application easy to cause the host immune response. We hoped to find a kind of vector, combined with the ability of virus into the host cells, which could extend the time of viral infection and reduce its repeated application which could lead to the body's immune response. Theoretically, the targeting microspheres with the matrix of biodegradable polymer materials were expected to achieve this requirement.

The existing vectors in sustained release drug delivery system included particles, nanoparticles, micro emulsion, submicron emulsion, liposomes, etc. Combining with the physicochemical and biological characteristics of adenovirus, the microspheres were selected to be the vectors in adenovirus sustained release drug delivery system was based on: (1) adenovirus was an uniform regular icosahedron with a particle size of about 70~90 nm, with nanoparticles as the vectors; the lower encapsulation efficiency and drug loading made it very difficult to obtain effective administration; (2) the adenovirus particle size of micro emulsion or submicron emulsion could influence the loading rate and a large amount of additional emulsifiers would reduce the activity of adenovirus; (3) the liposome, especially the positive liposome, was widely used in the genetic engineering as a DNA vector for its phospholipid bilayer with an affinity with bio-membrane, but it had not the sustained-release function and its transfection efficiency was far lower than adenovirus; additionally, the liposome itself was not stable and needed to be prepared fresh, so it was also unfavorable to be chosen; (4) there were only a few reports about microspheres

encapsulating virus gene vectors (Beer et al., 1998); these experiments showed that the microspheres could reduce the immunogenicity of the virus vectors and played a sustained-release effect by regulating the microspheres sizes to control the release rate of virus.

PELA was a kind of degradable polymer, polymerized by hydrophobic polylactic acid (PLA) and hydrophilic polyvinyl alcohol (PEG). It was hydrophilic and nontoxic, with no immunogenicity but high encapsulation efficiency, and it also could improve the stability and adjustability of the encapsulation contents. It was a hot spot in the recent researches of materials and it had been applied in encapsulating albumin, DNA and vaccines, etc (Zhou et al., 2001; Tyagi et al., 2008).

In this study, the improved solvent evaporation method was used to prepare microspheres in double emulsion system ($W_1/O/W_2$). The organic solvents were not needed to be stirred for a long time for volatilization. The residual rates of organic solvents were low and the viral activity influenced little. The encapsulation yield could reach as high as 60% and the cumulative release percentage was also close to 60% within 120h. The in vitro release of the recombinant adenovirus microspheres was researched by isotope labeling method, but the release of live adenovirus was difficult to be reflected due to the lack of bioactive isotopes (Schalch et al., 2004; Steel et al., 2004); although the determination of cell fluorescence intensity of 293 cells infected with adenovirus released from microspheres was more complex with a poorer precision compared to the isotope labeling method, the release of live adenovirus could be directly measured, so that the result was more reliable instead.

In this study, the particle sizes of 90% microspheres were within 3μm, with a mean particle size of 1.965μm. They were in good dispersion, with a mean spacing of 1.360μm and virus load rate of 10.5×10^8/mg. Human hepatoma cells HepG2 were infected with the recombinant adenovirus microspheres and the viral transduction was observed to be successful under a fluorescence microscope and a electron microscope. Semi-quantitative RT-PCR detection indicated that TIMP-1 mRNA could be stably expressed within a certain time. MTT experiment and cell growth curve confirmed that the recombinant adenovirus microspheres carrying TIMP-1 could inhibit the in-vitro proliferation of HepG2 cells. Recombinant adenovirus was released with a larger proportion in the first 120 h, with a high activity. And then it was released with a slower speed, with a total release time longer than 240 h. This solved the problem that drugs were in the rapid in-vivo clearance so that they could not play their roles for a long time and with a high efficiency. However, the acute toxicological experiments of blank microspheres showed that these microspheres vectors themselves were nontoxic with no side effects, which could meet the requirements of in vivo applications. In this study, based on the efficient, high-load, sustained-release recombination microspheres, the TIMP-1 gene was stably expressed in HCC cells for a long time, and the biological activities of HCC cells were inhibited through up-regulation of TIMP-1 expression and down-regulation of MMPs activity (Elezkurtaj et al., 2004; Rahbari et al., 2011; Schwettmann et al., 2011;

Sela-Passwell et al., 2011; Hou et al., 2011; Van, 2011; Bandapalli et al., 2012; Zhu, 2012). But the effective time of the recombinant microspheres was a bit longer, need to be improved in the follow-up test.

It had been confirmed that the up-regulated expression of TIMP-1 of the recombinant adenovirus microspheres could affect the in vitro biological behavior of HepG2 cells, which would be applied in the in vivo experiments of gene therapy of HCC. Compared with the commonly used PLA and polylactide–co-glycolide (PLGA), PELA showed several potentials in macromolecular drugs (such as protein, peptide drugs) delivery systems, which may be due to the block copolymer have its capability to provide a biomaterial having a broad range of amphiphilic structure. PELA microspheres can control the rate of release of entrapped antigens and therefore, offer potential for the development of single-dose drugs. The PELA microspheres have shown great potential as a carrier for gene therapy. In this study, the recombinant adenovirus microspheres had been successfully constructed and stably expressed and its influence on the in-vitro biological behavior of HCC had been researched.

Acknowledgements

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