Polycarbonate Microspheres Containing Tumor Necrosis Factor-α Genes and Magnetic Powder as Potential Cancer Therapeutics

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ABSTRACT: Amphiphilic polycarbonate copolymers including methoxy-terminated poly(ethylene glycol)-*co*-poly (5,5-dimethyl trimethylene carbonate) [Poly(PEG-*b*-TMC)] and poly(ethylene glycol)-*co*-poly(trimethylene carbonate) [Poly(PEG-*b*-DTC)] were synthesized. The water-in-oil-in-water (W/O/W) solvent evaporation technique was adopted to produce anticancer magnetic Poly(PEG-*b*-DTC) microspheres containing tumor necrosis factor-α (TNF-α) genes and Fe_3O_4 magnetic ultrafine powder. Drug release studies showed that the microspheres can sustain a steady release rate of TNF-α genes in 0.1*M* phosphate buffer saline solution *in vitro* for up to 60 h. *In vitro* cytotoxicity assays demonstrated that the microspheres have high inhibition and antitumor action to human hepatocellular carcinoma (Bel-7204) cells *in vitro*. *In vivo* inhibition on the growth of hepatic carcinomas and histopathologic observation indicated that the microspheres possess a markedly high antitumor activity to human hepatocellular carcinoma (Bel-7204). © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 3343–3349, 2008

Key words: microspheres; drug controlled release; cancer; polycarbonate; poly(ethylene glycol); tumor necrosis factor-α

INTRODUCTION

Over recent years, gene therapy has been explored as a cancer treatment by delivering DNA, RNA, or antisense sequences. The goal of this approach is to alter gene expression within tumor cells thereby generating an antitumor response and suppressing the tumor growth. The tumor necrosis factor-α (TNF-α) gene expresses a cytokine that possesses a wide variety of biological activities including potent antitumor action and immunomodulatory properties. However, the broader application of TNF-α has been hampered by high dose used, nearly close to lethal concentrations.[1–3]

Consequently, TNF-α gene has been cloned and ligated into *Escherichia coli* expression plasmid pcDNA3 vector and transformed into *Escherichia coli* to give the recombinant plasmid TNF-DNA, which is developed to a potential immunotherapeutic agent against cancer.[4,5] However, plasmid DNA can be readily degraded by endonucleases present in the extracellular space, or can be taken up by the liver via scavenger receptors and drained into the lymphatics in other tissue, and hence, may be rapidly cleared from the circulation. Thus one of the challenges of *in vivo* gene therapy is to develop safe and efficient vectors.[6,7]

Recently, an intelligent process for cancer gene therapy was offered by coupling plasmids DNA and oligonucleotides to biodegradable synthetic polymer microspheres, which can protect DNA against degradation and aprecisely control the release rates of DNA. The microspheres can readily enter some tumors, regions of inflammation, and infection sites, where the endothelium is more permeable. However, they cannot cross normal continuous capillary endothelium. Accordingly, the microspheres are expected to direct DNA to tumors and to enhance tumor cellular uptake.[8–10]

The amphiphilic copolymers of poly(ethylene glycol) (PEG) and a hydrophobic block would seem to be the most suitable choice to make microspheres, since the hydrophilic nature of PEG excludes (or inhibits) plasma protein adsorption, avoids recognition by phagocytic cells, and prolongs the circulation time *in vivo*.[11–14] Moreover, microspheres based on the amphiphilic copolymers can create circulating reservoirs with a high capacity for drugs. Nanoparticles and microspheres prepared from copolymers

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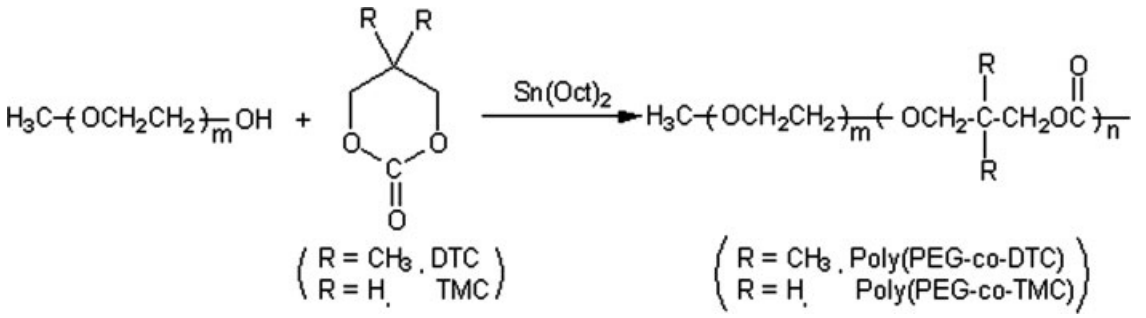

Figure 1 Synthetic route to Poly(PEG-*b*-DTC) and poly(PEG-*b*-TMC).

of poly(lactic acid) (PLA), poly(lactic acid)-*co*-(glycolic acid) (PLGA), and poly(ε-caprolactone) (PCL) with one or more PEG chains of molecular weight 2–20 kDa have been extensively studied.[15–21]

Some magnetic materials, such as magnetite and Fe_3O_4 magnetic ultrafine powder, have also been incorporated into microspheres to produce magnetic particles for specific cancer therapies. Magnetic microspheres can be activated by a magnet applied outside the body and accumulate drugs into localized tumors. Synthetic polymers including PLA, poly(vinyl alcohol) (PVA), and polyalkylcyanoacrylate have been used as matrix materials for magnetic microspheres in the current preclinical trials and clinical cancer therapy.[22–24]

Biodegradable polycarbonates, for example, homopolymers and copolymers of 1,3-dioxan-2-one (trimethylene carbonate, TMC) and 5,5-dimethyl-1,3-dioxan-2-one (5,5-dimethyl trimethylene carbonate, DTC), have been widely used in drug delivery, soft tissue implant, and tissue regeneration because of their good biodegradation, biocompatibility, elasticity, and low toxicity.[25–28]

In this work, we synthesized two amphiphilic polycarbonate copolymers poly(ethylene glycol)-*co*-poly (5,5-dimethyl trimethylene carbonate) [Poly(PEG-*b*-DTC)] and poly(ethylene glycol)-*co*-poly(trimethylene carbonate) [Poly(PEG-*b*-TMC)] (Fig. 1). Subsequently, Poly(PEG-*b*-DTC) copolymer was used to prepare microspheres which contained TNF-DNA and Fe_3O_4 magnetic ultrafine powder. We also evaluated the SEM morphology, release properties, *in vitro* cytotoxicity assay to hepatocellular carcinoma cells, magnetic targeting therapy to hepatic carcinoma in nude mice, and histopathologic image pattern analysis.

MATERIALS AND METHODS

Instruments and reagents

The compounds prepared were characterized using a Spectrum One infrared spectrophotometer (Perkin Elmer, Waltham, MA) and a Varian Mercury-VX300 NMR spectrometer (Varian, Columbia, MD). The molecular weight was measured by GPC (Waters, MA) [Waters 2960D separations module, Waters 2410 Refractive Index Detector, Shodex K802.5 and K805 with Shodex K-G Guard Column, Polystyrene Standard, Tetrahydrofuran (THF)/Chloroform solvent (v/v 1 : 1), 1.0 mL/min flow rate, 323K Column temperature and 318K Detector temperature]. The SEM morphology of microspheres was studied using a scanning electron microscope (SEM, Hitachi-X650, Japan), and specimens were coated with gold in SEM coating equipment. The male BALB/C nude mice (5–7 weeks old, weight: 20.40 ± 1.80 g) were provided by the Department of Pharmacy (School of Medicine, Huazhong University of Science and Technology, China) and were raised according to the method described in the literature.[29] Human hepatic carcinoma cell line (Bel-7204) was provided by the China Center for Type Culture Collection of Wuhan University, China. A DG-3022A ELISA-Reader (Hercules, CA) a JEOL (Japan) JSM-T300 Electron Microscope, a JY92-II Ultrasonic Homogenizer (Scientz Bio-technology Co., Ltd., Ningbo, China), a Pharmacia-Biotech Gene Quant DNA Calculator (Amersham Pharmacia Biotech Inc., Piscataway, NJ), a MPIAS-500 Multimedia Color Histopathologic Image Pattern Analysis System (pixels 0.785 μm, metrical matrix 1.718E + 05, color mode; Qing Ping Inc., Wuhan, China) were also used.

All chemicals and solvents were of analytical grade. Methoxy-terminated poly(ethylene glycol) (MePEG) (M_n = 2000), Tin (II) 2-ethylhexanoate ($Sn(Oct)_2$), and Fe_3O_4 magnetic ultrafine powder (average diameter 19.1 nm, surface area ≥97 m^2/g, material coercive force 250Q) were purchased from Sigma-Aldrich. The empty pcDNA3 vector and recombinant plasmid cloned with tumor necrosis factor alpha (TNF-α) gene into pcDNA3-tnf vector (TNF-DNA) were provided by the Chinese Academy of Preventive Medicine (Wuhan, China). The growth medium was the RPMI-1640 media [10% fetal bovine serum (Gibco), 100 U/mL penicillium, 100 μg/mL streptomycin]. DTC and TMC were prepared according to the literature (21).

Synthesis of copolymers

DTC (4.0 g, 30.8 mmol) and MePEG (2.0 g, 1.0 mmol, 0.325 equiv.) was added to a polymerization

tube and then dried by several cycles of argon purging followed by exposure to high vacuum. A solution of $Sn(Oct)_2$ in dry toluene (0.25 mol/L, 50 μL, 1/2500 equiv.) was added to the dried mixture via a syringe. After further drying under high vacuum, the tube was sealed and immersed into a thermostatically controlled oil bath at 170°C for 12 h. The resultant solid residue was dissolved in THF (15 mL) and then reprecipitated using acetone/water (v/v 1 : 1, 80 mL). The precipitated solid was filtered, washed by ethanol and diethyl ether (v/v 1 : 1), and dried under vacuum for 48 h to yield a white powder of Poly(PEG-*b*-DTC) (5.3 g, 88%). ^{1}H NMR ($CDCl_3$, δ, ppm): 4.2–4.1 (s, $CH_2OC{=}O$), 3.6 (t, CH_2O), 1.1 (s, CH_3); IR (KBr, cm^{-1}): 2961, 2883 (C—H), 1744 (C=O), 1466–1406 (CH_3) 1244 (C—O—C=O), 1111–1033 (C—O); the molecular weight (M_n) was 1.06×10^4 as determined by GPC and the average copolymer composition of PEG (mol %) was 42.9 as determined from ^{1}H NMR.

The polycarbonate copolymer Poly(PEG-*b*-TMC) was synthesized by the same method (85.7%). ^{1}H NMR ($CDCl_3$, δ, ppm): 4.3–4.1 (t, $CH_2OC{=}O$), 3.6 (t, CH_2O), 2.2–2.0 (m, CH_2); IR (KBr, cm^{-1}): 2877 (C—H), 1747 (C=O), 1264 (C—O—C=O), 1108 (C—O); the molecular weight (M_n) was 9.19×10^3 as determined by GPC and the average copolymer composition of PEG (mol %) was 41.5 as determined from ^{1}H NMR.

Degradable test

A solution of copolymer dissolved in dichloromethane was exposed to the atmosphere to allow the solvent to evaporate. The resultant films averaging 0.6-mm thick was divided into groups, weighed, and then immersed into 0.1*M* phosphate buffer saline solution (PBS, 10 mL, pH 7.4). The solution was shaken in a thermostatically controlled water bath at 37°C and a further 10 mL of PBS was added to each solution. Films generated in this manner were taken out after 2, 4, and 6 weeks, respectively, and washed thoroughly with distilled water. After drying under high vacuum, the films were weighed again and the weight loss ratios were calculated.

Preparation of microspheres

The Poly(PEG-*b*-DTC) copolymer (PC, 0.2 g) was dissolved in a 0.1% solution of Span-80 in dichloromethane (5 mL), and 0.1 mg of TNF-DNA and 100 mg of Fe_3O_4 magnetic ultrafine powder were added. The solution was homogenized by sonication for 1 min (50 W) while cooled in an ice-salt bath and then poured into a 0.1% solution of PVA in acetone/distilled water (v/v 5 : 95, 20 mL). The mixture was vortexed for 3 min, stirred rapidly for a further 30 min, and then 60 mL of water was added dropwise under vigorous stirring at room temperature. Subsequently, the magnetic stirring was continued at room temperature open to the air for a further 3 h to remove dichloromethane by evaporation. After centrifugation (10^5 rpm), the precipitate was collected, washed by distilled water, and lyophilized to give a gray powder of the microspheres containing TNF-DNA and Fe_3O_4 magnetic ultrafine powder (PC-TNF-DNA-M). The empty Poly(PEG-*b*-DTC) magnetic microspheres (PC-M) and nonmagnetic microspheres (PC-TNF-DNA) were prepared by the same method.

In vitro drug release study

The PC-TNF-DNA-M microspheres (40 mg) were suspended in 5 mL of PBS. The mixture was slowly shaken in a thermostatically controlled water bath at 37°C. After centrifugation (4000 rpm, 5 min), 50 μL of the solution was taken, and the concentration of DNA per 5 h was measured by a Pharmacia-Biotech Gene Quant DNA Calculator.

In vitro cytotoxicity assay

The human hepatic carcinoma cells (Bel-7204, 1×10^5/mL) were seeded in 96-well plates in growth medium at a density of 2×10^4 cells/well. The cells were incubated for 24 h in an incubator (37°C, 5% CO_2) and 100 μL of the growth medium containing 0.9% NaCl (Blank test), pcDNA3, TNF-DNA, PC-M, PC-TNF-DNA, and PC-TNF-DNA-M, respectively, was added (Dose of DNA was 1.0 μg/mL). After 48 h incubation, the cells were washed with growth medium. Twenty microliters of the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5.0 mg/mL) was added and the cells were further incubated for 4 h. The cells were washed again with 3% fetal calf serum and phosphate buffer saline solution (3%FCS-PBS), and 100 μL of dimethyl sulfate was added. Subsequently, the cells were shaken for 30 min at room temperature. The optical densities (OD_{570}) were measured at 570 nm and expressed as a percentage relative to control cells.

In vivo analysis of tumor cell growth

An electromagnet with a magnetic flux density of a maximum of 5000GS was used to produce an inhomogeneous magnetic field. The magnetic flux density was focused onto the region of the tumor with a specially adopted pole shoe that was placed in contact with the surface of the tumor. On the tip of the pole shoe, the gradient has its maximum that demonstrates the dependence of the magnetic flux density on the distance to the pole shoe.[30]

The Bel-7204 cells (5×10^6) were injected subcutaneously to the back of 6-week-old male nude BALB/c mice. Mice bearing a tumor of 0.6–0.8 cm in diameter were considered as positive. Thirty-six mice were divided into six groups, each mouse was injected with the certain dose of 0.9% NaCl (Blank test), pcDNA3, TNF-DNA, PC-M, PC-TNF-DNA, or PC-TNF-DNA-M solution (0.2 mL), respectively, via the tail vein (dose of DNA was 5 μg). Subsequently the mouse was anesthetized with urethane (10%, 10 mL/kg), positioned prone and fixed to a polystyrene cradle with adhesive tape to minimize motion. A magnetic flux density of 5000GS was estimated in the region of the tumor surface and at 10 mm below the tip of the pole shoe immediately after the injection with the microspheres. The mice were exposed to magnetic field for 30 min. This procedure of injection and targeting therapy was repeated every 3 days. Mice were sacrificed after 4 weeks and then the tumors were isolated and weighed accurately. The inhibition of the tumor growth was calculated compared to untreated mice.

Histopathologic observation

Tumor specimens were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm thick sections. Each specimen was stained with Hematoxylin-eosin (HE) and used for histological identification.

Statistical analysis

All results were expressed as mean differences and were tested for significance by *t*-test with $P < 0.05$ being considered as a significant difference.

RESULTS AND DISCUSSION

Preparation and characterization

Two amphiphilic copolymers [Poly(PEG-*b*-DTC) and Poly(PEG-*b*-TMC)] were synthesized by the polymerization of DTC and TMC with the hydroxyl end group of methoxy-terminated poly(ethylene glycol) (MePEG) as the initiator under the catalysis of Tin (II) 2-ethylhexanoate [$Sn(Oct)_2$]. The ^1H NMR spectra showed the characteristic peaks (3.6 ppm) of $—OCH_2CH_2O—$ group, indicating that PEG was covalently bounded to polycarbonate. The copolymers can be degraded slowly in PBS, at 37°C. The average weight losses of Poly(PEG-*b*-DTC) and Poly(PEG-*b*-TMC) after 6 weeks were 28.9% and 31.4%, respectively, indicating the rate of degradation of Poly (PEG-*b*-DTC) was slower than that of Poly(PEG-*b*-TMC) (Fig. 2). Compared with the polycarbonate homopolymers of DTC and TMC,[31] the copolymers possessed a higher rate of degradation, presumably because the hydrophilic PEG segments promote water permeation into the copolymer matrix.

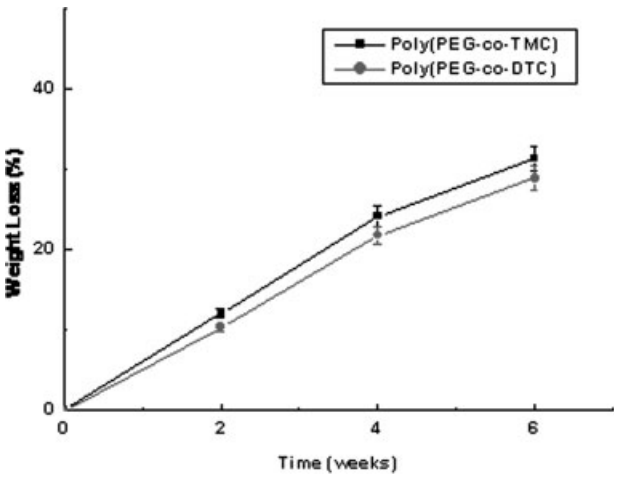

Figure 2 Weight loss (%) of the copolymers in PBS.

Amphiphilic copolymer Poly(PEG-*b*-DTC) was used to prepare the microspheres containing TNF-DNA and Fe_3O_4 magnetic ultrafine powder (PC-TNF-DNA-M), the empty magnetic microspheres (PC-M) and nonmagnetic microspheres (PC-TNF-DNA) by the modified water-in-oil-in-water (W/O/W) solvent evaporation technique. The double emulsion technique was applied for encapsulation of TNF-DNA and Fe_3O_4 magnetic ultrafine powder within the Poly(PEG-*b*-DTC) microspheres. The first W/O emulsion was obtained by sonication at ice-salt bath and then emulsified by rapidly stirring with PVA in acetone/distilled water (v/v 5 : 95) to yield the W/O/W emulsion. Finally, the W/O/W emulsion was diluted stepwise with water under vigorous stirring at room temperature. In the process of emulsion, the polycarbonate segment of copolymers formed the hydrophobic core of the micelle while the PEG segment surrounded this core as a hydrated outer shell. After evaporation, centrifugation, and lyophilization, the resultant homogenous small-size PC-TNF-DNA-M microspheres showed high water-affinity and possessed good dispersal and mobility in 0.9% sodium chloride solution suiting the experimental purpose.

The SEM morphologies of the PC-TNF-DNA-M microspheres are shown in Figure 3. The average diameter of the PC-TNF-DNA-M microspheres was 1.0 μm (ranging from 0.6 to 1.6 μm). In the microspheres, the average weight of magnetic powder accounted for 30% of the mass and the TNF-α gene loading was 0.35 μg/mg. Their mobile velocity was 21 cm/min in a magnetic field of 5000GS. Therefore, the microspheres generated have the potential to be applied in targeting therapy of human hepatic carcinomas under the influence of a controlling magnetic field.

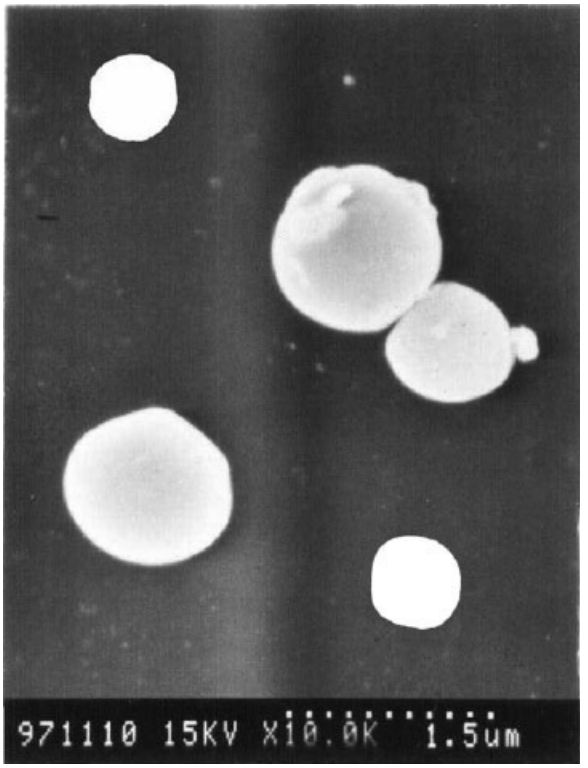

Figure 3 Electron microscope photograph of PC-TNF-DNA-M microspheres.

In vitro drug release study

The overall process of drug release from the polymeric microspheres is mostly controlled by drug diffusion, drug dissolution, and polymeric degradation (18). The DNA release profile of PC-TNF-DNA-M microspheres in PBS is shown in Figure 4. A substantial release rate from the PC-TNF-DNA-M microspheres was sustained over the 60 h of measurement. Compared with the microspheres made from polycarbonate homopolymers and copolymer of DTC and TMC,[31] the PC-TNF-DNA-M microspheres released the drug faster, presumably because of the higher rate of degradation of Poly(PEG-*b*-DTC) and the increased drug diffusion coefficient in amphiphilic microspheres.

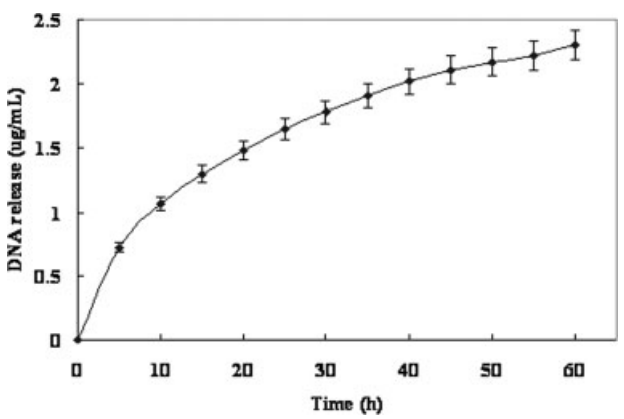

Figure 4 DNA release from PC-TNF-DNA-M *in vitro*.

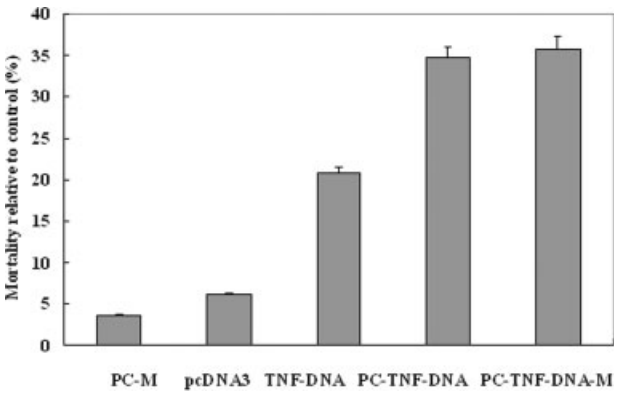

Figure 5 Antitumor activity of microspheres to Bel-7402 cells *in vitro*.

In vitro cytotoxicity assay

The effects of pcDNA3, TNF-DNA, PC-M, PC-TNF-DNA, and PC-TNF-DNA-M on cell growth and metabolism of human hepatic carcinoma cell line (Bel-7204) *in vitro* are shown in Figure 5. At the same concentration of DNA (1.0 μg/mL) in the growth medium, the Bel-7204 cells incubated with PC-TNF-DNA and PC-TNF-DNA-M produced over 30% mortalities relative to control, and markedly higher than that of the cells incubated with pcDNA3 (6.1%) and TNF-DNA (20.8%). Consequently TNF-DNA is released slowly from the microspheres, leading to increased TNF-α gene expression to produce TNF-α, and hence, killing the cells. Both PC-TNF-DNA and PC-TNF-DNA-M similarly demonstrate high inhibition and antitumor action to Bel-7204 cells *in vitro*.

In vivo inhibition of growth of hepatic carcinoma

Tumor growths after receiving injections with pcDNA3, TNF-DNA, PC-M, PC-TNF-DNA, or PC-TNF-DNA-M were monitored and the inhibitions were measured relative to control. After 4 weeks, the inhibition of PC-TNF-DNA-M on the growth of hepatic carcinoma in mice was 50.3%, and this was significantly higher than that of pcDNA3 (1.6%), TNF-DNA (5.1%), and PC-TNF-DNA (21.6%), respectively, (Fig. 6).

The vascular permeability and hydraulic conductivity of tumors in general are significantly higher than that for various normal tissues.[8,10] The TNF-DNA was distributed nonspecifically in the body postadministration, whereupon it readily loses activity via a number of mechanisms including being taken up and expressed by the muscle and epider-

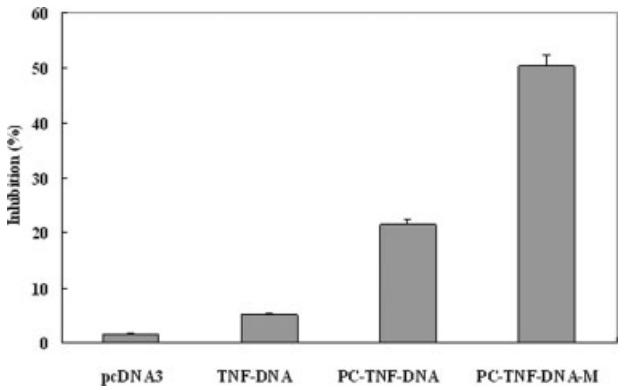

Figure 6 *In vivo* inhibitory effect of microspheres on nude mice bearing human hepatic carcinoma.

mal cells. The PC-TNF-DNA microspheres would therefore be expected to enter more easily into hepatic carcinomas than into normal cells, thereby inducing higher tumor growth inhibition than TNF-DNA. This is supported by the higher inhibition (21.6%) of PC-TNF-DNA when compared with that of TNF-DNA. However, the inhibition of PC-TNF-DNA on growth of hepatic carcinoma in mice was lower than that of PC-TNF-DNA-M, because some of PC-TNF-DNA would still be taken up and metabolized in the liver and spleen.

As a consequence, the increased drug concentration at the targeted site and an enhanced local release of the drug improves the therapeutic action on the tumors. Under a magnetic field of 5000GS, most of PC-TNF-DNA-M accumulated specifically into the hepatic carcinomas. Subsequently, the TNF-DNA which was released slowly from the magnetic polymer microspheres was endocytosed and expressed primarily by the tumor cells. The increasing level of TNF-α, generated by gene expression in the tumor, activated the immune system to the Bel-7204 cells, and hence, suppressed the growth of tumor. The targeting therapy applied to nude mice bearing Bel-7204 carcinomas, exhibited markedly tumor growth inhibition (50.3%) which was higher than the effect of single recombinant plasmid TNF-DNA or the activity of nonmagnetic microspheres (PC-TNF-DNA).

In the photograph of the hepatic carcinoma tissue section from a mouse that received the targeting treatment of PC-TNF-DNA-M, a large amount of normal Bel-7204 cells (pink) died through apoptosis and necrosis, and are invisible to HE staining when compared with control (Fig. 7). Some tumor cells have undergone condensation, loss of cytoplasmic granules, and even lysis. Induction of apoptosis was confirmed by the formation of apoptotic bodies of cell nuclei (red in HE staining). The results further demonstrated that the PC-TNF-DNA-M microspheres possess high tumor inhibition and antitumor activity.

CONCLUSIONS

Amphiphilic polycarbonate copolymers including methoxy-terminated Poly(PEG-*b*-DTC) and Poly (PEG-*b*-TMC) were synthesized. The water-in-oil-in-water (W/O/W) solvent evaporation technique was adopted to produce anticancer magnetic polymer microspheres containing tumor necrosis factor-α (TNF-α) genes and Fe_3O_4 magnetic ultrafine powder. Drug release studies showed that the microspheres can sustain a steady release rate of TNF-α genes in 0.1*M* phosphate buffer saline solution *in vitro* for up to 60 h. Moreover, the PC-TNF-DNA-M microspheres have a strong magnetic responsiveness and possess a high tumor inhibitory rate and antitumor activity to human hepatic carcinoma cells (Bel-7204) *in vitro* and *in vivo*. This study indicates a new type

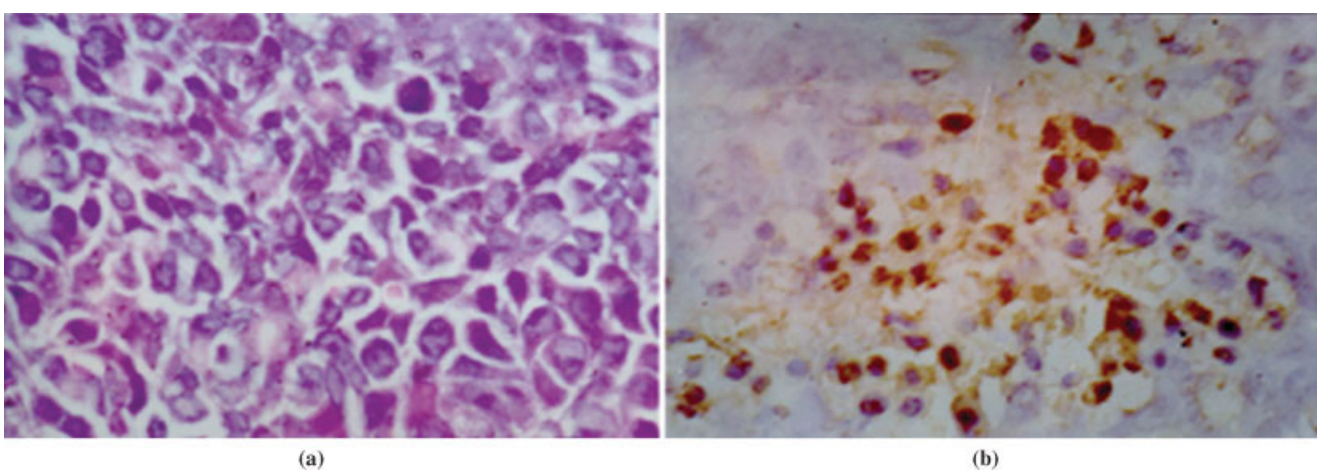

Figure 7 Photographs of carcinoma tissue section. (a) The blank group; (b) the group receiving injection with PC-TNF-DNA-M and the site-specific treatment for 4 weeks under a magnetic field of 5000GS. Each specimen was stained with Hematoxylin-eosin (HE). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of drug delivery system for liver carcinoma treatment and shows the prospects for clinical applications of the microspheres described.

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