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Dual targeting effect of Angiopep-2-modified, DNA-loaded nanoparticles for glioma

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ABSTRACT

Gene therapy offers a promising cure of brain glioma and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is able to induce cell apoptosis of glioma selectively without affecting the normal cells. In this study, the nanoscopic high-branching dendrimer, polyamidoamine (PAMAM), was selected as the principal vector. Angiopep-2, which can target to the low-density lipoprotein receptor-related protein-1 (LRP1) expressed on BCECs and glial cells, was exploited as the targeting ligand to conjugate PAMAM via bifunctional polyethyleneglycol (PEG) and then complexed with the DNA, designated as PAMAM-PEG-Angiopep/DNA nanoparticles (NPs). The cellular uptake mechanism explored in glial cells showed that the DNA of PAMAM-PEG-Angiopep/DNA NPs entered into the nuclei through the endosome/lysosome pathway. The *in vivo* biodistribution of PAMAM-PEG-Angiopep/DNA NPs in the brain especially the tumor site was higher than that of PAMAM-PEG/DNA NPs and PAMAM/DNA NPs. Furthermore, the TUNEL analysis showed a more wide-extended apoptosis in the PAMAM-PEG-Angiopep/pORF-TRAIL NPs treated group, compared to other groups including commercial Temozolomide-treated one. The median survival time of PAMAM-PEG-Angiopep/pORF-TRAIL NPs and Temozolomide treated on brain tumor-bearing mice was 61 and 49 days respectively, significantly longer than that of other groups. Besides, the NPs suggested low cytotoxicity after *in vitro* transfection. Thus, the results showed that Angiopep-2 could be exploited as a specific ligand to cross the BBB and targeted to glial cells, and PAMAM-PEG-Angiopep/DNA NPs can be a potential non-viral delivery system for gene therapy of glial tumor.

1. Introduction

Glial tumors are the most abundant of all primary central nervous system (CNS) neoplasms [1]. According to the severity of the disease, glial tumors are graded from I (benign) to IV (highly malignant) [2]. And the low-grade tumors usually evolved into a high-grade Glioblastoma multiforme (GBM). However, irrespective of their grading, primary glial tumors almost invariably display marked infiltrative growth characteristics with glial cells travelling long distances (centimeters) away from the central part into the surrounding normal brain tissue [1]. Furthermore, high-grade glioblastomas are highly angiogenic tumors and, consequently, these tumors harbor new and leaky blood vessels [3]. It could cause edema, which is the consequence of a local disruption of the BBB by impaired capillary endothelial tight junctions [4].

According to the pathological conditions of glial tumors in different periods, the different strategies should be used to the design of the drug delivery system targeting glial tumor. When the glial tumors are still low-grade, the drug delivery system should be able to cross the BBB and further target to glial cells. However, when it develops to high-grade, drug delivery system can accumulate in the glial tumors by the enhanced permeability and retention (EPR) effect.

The permeability of the BBB is regulated by the brain capillary endothelial cells (BCECs), which are closely sealed by tight junctions [5,6]. It has been reported that a series of receptors are located at BCECs that form the BBB, including the transferrin receptor, the insulin receptor, and the LRP [7,8]. And compared with the normal tissue, an increased expression of LRP1 on glial cells has been reported [9]. As a ligand of the LRP1, Angiopep-2 (TFFYGGSRGKRNNFKTEEY, molecular weight 2.4 kDa), one of the family of Kunitz domain-derived peptides, exhibited higher transcytosis capacity and parenchymal accumulation than do transferrin, lactoferrin, and avidin [10]. And NPs modified with Angiopep-2 has been proved possessing an excellent ability to cross the BBB [11]. All these results highly suggest

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that Angiopep-2 may be a potential ligand that can cross the BBB and further target the glial cells.

Comparing with the traditional chemistry drugs, gene therapy with cytotoxic gene offers a promising treatment of glial tumors. Tumor necrosis factor_ (TNF) related apoptosis-inducing ligand (TRAIL) is a signaling molecule with characteristic of TNF [12]. Most of the glial cells express the agonist TRAIL receptor but no or undetectable levels of the antagonist receptor while the normal cells have been found to express antagonist TRAIL receptor [13,14]. Thus, TRAIL is able to induce the apoptosis of glial cell selectively without affecting normal cells. In this study, pORF-TRAIL, plasmid DNA which expresses TRAIL was adopted as the therapeutic gene.

Non-viral vectors, including polyethyleneimine (PEI), poly (lactic-glycolic) acid (PLGA), poly lactic acid (PLA) and polyamidoamine dendrimer (PAMAM) have been used to deliver gene into the brain. Among those, PAMAM was proved to be taken as an effective gene vector when modified with different brain-targeting ligands, such as lactoferrin [15].

In this study, a gene delivery system was constructed via conjugating Angiopep-2 to PAMAM using bifunctional PEG and then complexed with the DNA, designated as PAMAM-PEG-Angiopep/DNA NPs. The mechanism of the cellular internalization of PAMAM-PEG-Angiopep/DNA NPs, their targeting and antitumor effect in glial cells and brain tumor-bearing mice was explored.

2. Materials and methods

2.1. Materials

PAMAM G5 dendrimer (77.35 μg/μl in methyl alcohol, containing 128 surface primacy amino groups, MW 28,826) was purchased from Dendritech, Inc (Midland, MI, USA). Angiopep-2 (TFFYGGSRGKRNNFKTEEY) was synthesized by Chinese Peptide Company (Hangzhou, China). α-Malemidyl-ω-N-hydroxysuccinimidylpolyethyleneglycol (NHS-PEG-MAL, MW 3500) was purchased from Jenkem Technology Co., Ltd (Beijing, China). The plasmid pGL2 (Clontech, Palo Alto, CA, USA) and pORF-TRAIL (InvivoGen, San Diego, CA, USA) were purified using QIAGEN Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Ethidium monoazide bromide (EMA) was purchased from Molecular Probes (Eugene, OR, USA). LysoTracker Green DND-26 was purchased from invitrogen, USA. Annexin V-FITC Apoptosis Detection Kit was obtained from KeyGEN. Temozolomide capsules were produced by Jiangsu Tasly Diyi Pharmaceutical Co., LTD (Jiangsu, China). TUNEL in situ apoptosis detection kit (TACS TdT Kit) was purchased from R&D Systems (Minneapolis, MN).

2.2. Cell line

C6 glioma cells were kindly provided by Prof. L. Y. Feng (Shanghai Institute of Materia Medica, Chinese Academy of Science). C6 glioma cells were expanded and maintained in special Dulbecco's modified Eagle's Medium (DMEM) (Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. All cells used in this study were between passage 5th to passage 15th.

2.3. Animals

ICR mice and nude mice, male, aging 4–5 wk, body weighting 18–25 g, were purchased from Sino-British Sippr/BK Lab. Animal LTD and maintained under standard housing conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

2.4. Preparation of dendrimer/DNA NPs

According to Ke WL et al. [11], PAMAM was reacted with PEG at the ratio 1:2 (mol/mol) in PBS (pH 8.0) for 2 h at room temperature. The resulting conjugate, PAMAM-PEG purified by ultrafiltration through a membrane (cutoff = 5 kDa) and redissolved in PBS (pH 7.0). Then PAMAM-PEG was reacted with Angiopep-2 at the ratio 1:1 (mol/mol) in PBS (pH 7.0) for 24 h at room temperature. PAMAM, PAMAM-PEG or PAMAM-PEG-Angiopep was freshly prepared and diluted to appropriate concentration. DNA solution (100 μg DNA/ml in 50 mM sodium sulfate solution) was added to obtain specified weight ratio (3:1, PAMAM to DNA, w/w) and immediately vortexed for 30 s at room temperature. Freshly prepared NPs were used in the following experiments.

Part of the plasmid DNA was labeled with EMA, the fluorescent photo affinity label. Plasmid DNA solution (1 mg/ml in TE buffer, pH 7.0) was diluted to 0.1 mg/ml with aqueous solution of EMA (1 mg/ml) and incubated for 30 min in dark at room temperature. The complex was then exposed to UV light (365 nm) for 1 h, and the resulting solution was precipitated by adding ethanol to a final concentration of 30% (v/v). The precipitate was collected by centrifugation and redissolved in 50 mM sodium sulfate solution. This EMA-DNA solution was used to prepare the NPs described above for some specific experiments.

2.5. Characterization of PAMAM-PEG-Angiopep and PAMAM-PEG-Angiopep/DNA NPs

The characteristics of PAMAM-PEG-Angiopep were analyzed by nuclear magnetic resonance (NMR) spectroscopy. Basically, PAMAM-PEG-Angiopep was purified by ultrafiltration through a membrane (cutoff = 5 kDa), then freeze-dried, solubilized in D_2O and analyzed in a 400 MHz spectrometer (Varian, Palo Alto, CA, USA).

The mean diameter and zeta potential of Angiopep-modified NPs with PAMAM to DNA weight ratio at 3:1 were determined by Zetasizer Nano (Marvin Instruments Ltd, UK).

Also, the morphology of Angiopep-modified NPs was examined under a high-resolution transmission electron microscope (JEM-2010, JEOL, Japan).

2.6. In vitro cytotoxicity assay

The cytotoxicity of the PAMAM-PEG-Angiopep/DNA (plasmid pGL2) NPs was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. C6 glioma cells were seeded in a 96-well tissue culture plate (Corning-Coaster, Tokyo, Japan) at 7000 cells per well in 100 μl DMEM medium containing 10% FBS, incubated at 37 °C in a humidified 5% CO_2 incubator. Cells achieving 70–80% confluence after 24 h were exposed to 40 μl of different NPs solutions with various concentrations for 2 h and 6 h. Then, 60 μl solution of MTT (50 μg MTT/well) was added to each well. After 2 h of incubation in the incubator, the medium was removed and 100 μl of DMSO was added to each well to dissolve the formazan crystals formed by the living cells. Cells without treatment were used as control. Absorbance was measured at 570 nm and corrected at 630 nm by dual wavelength detection using a Multiskan MK3 microplate reader (Thermo Scientific). Cell viability of each group was expressed as a percentage relative to that of control.

2.7. Intracellular tracking of PAMAM-PEG-Angiopep/DNA NPs

C6 glioma cells were seeded at a density of 2×10^4 cells/dish in Confocal cell culture dish (GBS-35-20, NESTBiotech). Incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator, the cells were incubated with the PAMAM-PEG-Angiopep/EMA-DNA NPs (25 μg PAMAM, 1 ml/dish) for 15 min or 60 min. Before 10 min of the preassigned time point, 0.8 μl LysoTracker Green DND-26 (1 mM) was added. After incubation, the cells were washed 2 times with Hank's solution (pH 7.4) and then observed by confocal laser scanning microscopy (Leica TCS SP2). LysoTracker Green DND-26 and EMA fluorescence was excited with the 488 nm wavelength of an argon laser, and the emission was detected in a range of 500–540 (LysoTracker Green DND-26) or 605–615 nm (EMA).

2.8. In vitro transfection experiment

C6 glioma cells were seeded at a density of 4×10^4/well in 24-well plates (Corning-Coaster, Tokyo, Japan) and growed to reach 70–80% confluence prior to transfection. Before transfection, the medium was removed and the cells were washed 2 times with Hank's solution. The cells were incubated with different NPs solutions containing 5 μg pORF-TRAIL for 2 h at 37 °C. 2 h later, the solution was removed and the cells were washed 2 times with Hank's solution. After exchanging with complete medium, the cells were further incubated 48 h for transfection. The positive control treated with 40 μg Temozolomide in 200 μl Hank's solution while the negative one incubated with Hank's solution only.

For fluorescence microscopy analysis, medium was removed and the cells were treated with the mixture of 250 μl Binding Buffer, 2.5 μl Annexin V-FITC and 2.5 μl Propidium Iodide per well for 15 min in dark condition. After that, the cells were visualized under an IX2-RFACA fluorescent microscope (Olympus, Osaka, Japan).

2.9. Tumor implantation

Male ICR or rude mice were anaesthetised by intraperitoneal injection of 10% Chloral hydrate. C6 glioma cells (1×10^5 in 4 μl Hank's solution) were implanted into the right striatum (1.8 mm right lateral to the bregma and 3 mm of depth) of the mice by using a stereotactic fixation device with mouse adaptor.

2.10. Biodistribution of dendrimer/DNA NPs in brain tumor-bearing mice

The PAMAM-PEG-Angiopep/EMA-DNA NPs was injected into the tail vein of nude mouse at a dose of 50 μg DNA at the 12th day after implantation. Then, the

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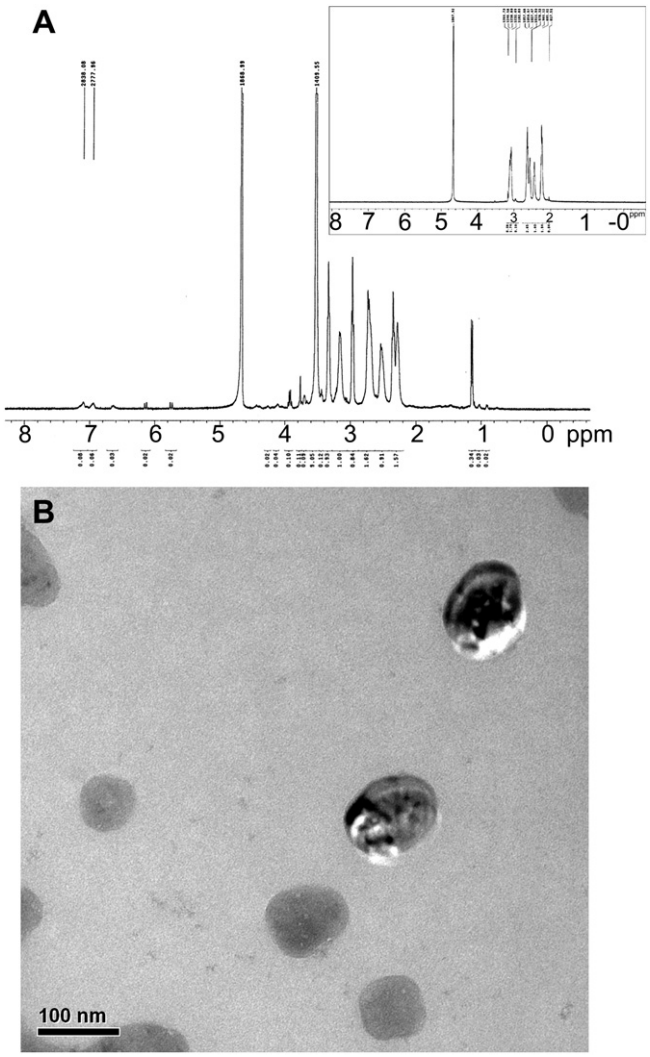

Fig. 1. Characterization of PAMAM-PEG-Angiopep and PAMAM-PEG-Angiopep/DNA NPs. NMR spectrum of PAMAM-PEG-Angiopep (A) and PAMAM (inset in A). Transmission electron micrograph of PAMAM-PEG-Angiopep/DNA NPs (B).

mouse was anesthetized. Images were taken by CRi *in vivo* imaging system (CRi, MA, USA) 2 h after injection. After that, the principal organs (including brain, heart, liver, spleen, lung and kidney) were removed and the brains were dissected carefully for comparing the relative accumulation. The PAMAM-PEG, PAMAM containing EMA-labeled DNA NPs respectively served as control.

2.11. In vivo pharmacodynamic evaluation and survival monitoring

At the day 8th, 10th and 12th after implantation, each group of mice received saline (negative control), free pORF-TRAIL, PAMAM/pORF-TRAIL NPs, PAMAM-PEG/pORF-TRAIL NPs, PAMAM-PEG-Angiopep/pORF-TRAIL NPs, or PAMAM-PEG-Angiopep/pGL2 NPs via the tail vein at a dose of 50 μg DNA/mouse respectively. While the positive control received Temozolomide i.g. at a dose of 50 mg/kg at the day 8th, 9th, 10th, 11th and 12th after implantation.

At the day 15th after implantation, mice were anesthetized with diethyl ether and killed by decapitation except for 10 mice of each group that was monitored for survival. The brain were removed carefully, fixed in 4% paraformaldehyde for 48 h, placed in 15% sucrose solution until subsidence (6 h), then in 30% sucrose solution until subsidence (24 h). After that, brains were frozen in OCT embedding medium (Sakura, Torrance, CA, USA) at −80 °C. Frozen sections of 20 μm thickness were prepared with cryotome Cryostat (Leica, CM 1900, Wetzlar, Germany). Slides were subjected to terminal deoxynucleotidyl transferase-mediated nick-end-labeling (TUNEL, TACS TdT kit) to detect the broken nuclear DNA fragments and counterstained with methyl green before observation under the fluorescence microscope.

3. Results

3.1. Characterization of PAMAM-PEG-Angiopep and PAMAM-PEG-Angiopep/DNA NPs

In NMR spectrum (Fig. 1A), the solvent peak of D_2O was found at 4.7 ppm. The methylene protons of branching units of PAMAM had multiple peaks between 2.0 and 3.2 ppm. The repeat units of PEG presented a sharp peak at 3.5 ppm. And the characteristic peak of benzene came from some amino acids that composed Angiopep-2 presented the corresponding peaks between 6.8 and 7.2 ppm. The NMR spectra result proved the existence of the conjugate structure of PAMAM—PEG—Angiopep.

The mean diameter and zeta potential of PAMAM-PEG-Angiopep/DNA NPs was 114.1 ± 7.2 nm and 16.3 ± 1.6 mV, respectively.

The result of transmission electron microscopy (Fig. 1B) showed that Angiopep-modified NPs was a well-formed spherical shape and had compacted structure.

3.2. In vitro cytotoxicity assay

The cytotoxicity of the PAMAM-PEG-Angiopep/DNA NPs at a series of concentrations was evaluated in C6 glioma cells by MTT assay. As shown in Fig. 2, after incubated 2 h with the NPs, the cytotoxicity of NPs was relatively low. And even the concentration of PAMAM reached 300 μg/ml,the cell viability was still 50% approximately. Compared with the 2 h group, after incubated 6 h, the cytotoxicity was relatively raised.

3.3. Intracellular tracking of PAMAM-PEG-Angiopep/DNA NPs

The C6 glioma cells were incubated with PAMAM-PEG-Angiopep/EMA-DNA and LysoTracker Green DND-26. Small red fluorescent dots represented EMA-labeled DNA (Fig. 3A, D) while the small green ones represented the endosomes or lysosomes that were dyed by LysoTracker Green DND-26 (Fig. 3B, E). At 15 min (Fig. 3A–C), almost all of the red dots and green ones located on the same field, which indicating that DNA was located in the endosomes or lysosomes. At 60 min (Fig. 3D–F), the red dots dispersed throughout the whole cell, including the nucleus partly. The dispersion of red dots meant that DNA had escaped from the endosomes or lysosomes and part of them had entered the nuclei.

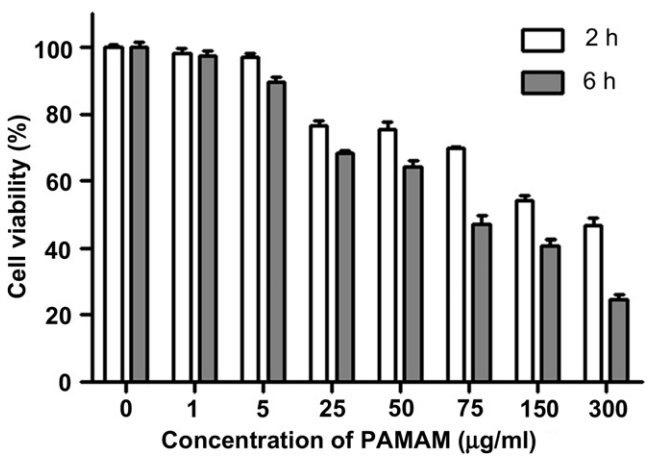

Fig. 2. Cell viability measured by MTT assay performed 2 h or 6 h in C6 glioma cells after incubated with PAMAM-PEG-Angiopep/DNA NPs at different concentrations. $n = 5$.

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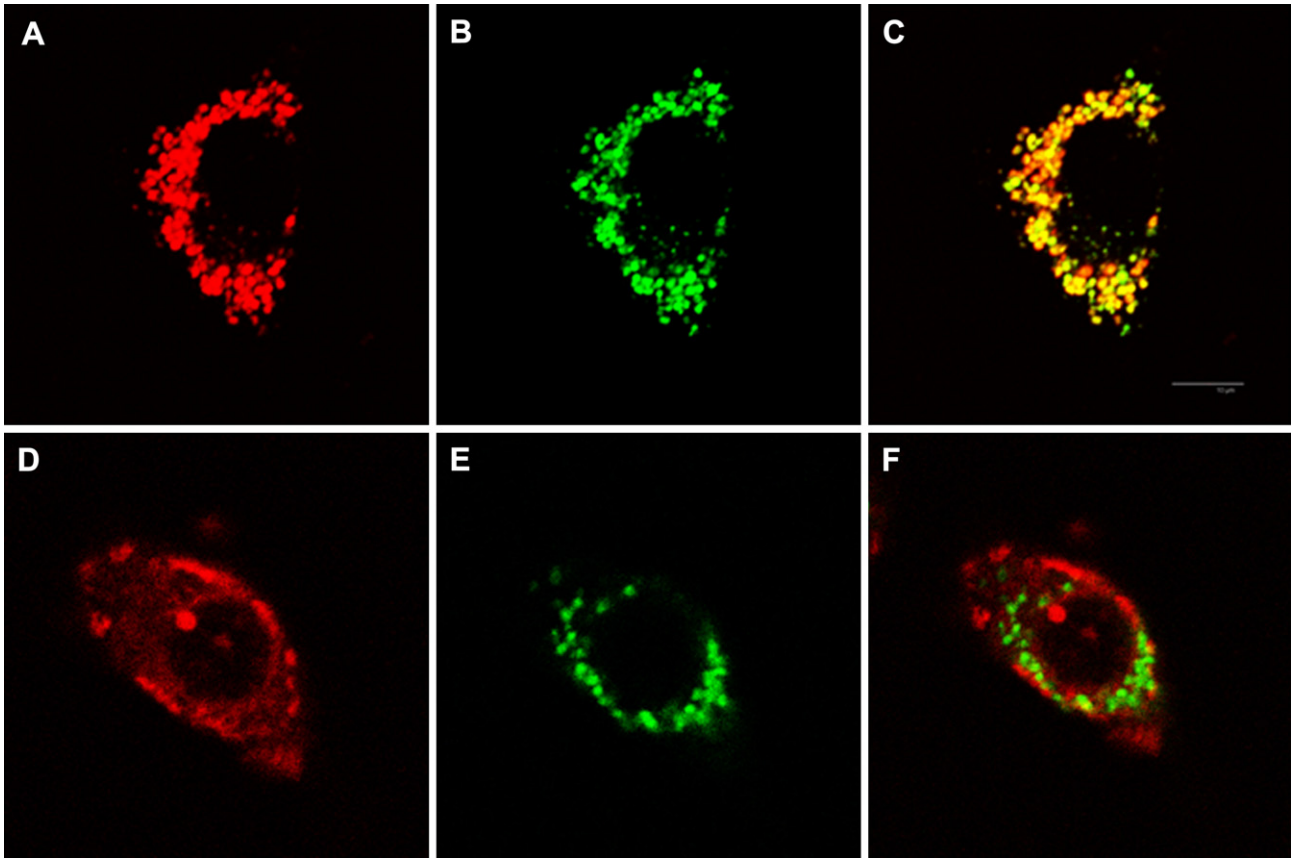

Fig. 3. Intracellular tracking of PAMAM-PEG-Angiopep/DNA NPs in C6 glioma cells. Images were taken after incubated with NPs for 15 min (A–C) or 60 min (D–F). Red: EMA-labled DNA (A, D). Green: LysoTracker Green DND-26 (B, E); Yellow: LysoTracker Green DND-26 colocalized with EMA-labled DNA (C, F). Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. *In vitro transfection experiment*

The transfection experiment *in vitro* was investigated in C6 glioma cells, which is shown in Fig. 4. Green fluorescence represented the early apoptosis cells, while the red meant the late apoptosis and dead cells. The cells treated with free pORF-TRAIL demonstrated very weak fluorescence signals (Fig. 4D–F), same as the negative ones treated with Hank's solution only (Fig. 4A–C). While the signal of the ones treated with either PAMMA-PEG-Angiopep/pORF-TRAIL (Fig. 4G–I) or Temozolomide (Fig. 4J–L) was strong.

3.5. *Biodistribution of dendrimer/DNA NPs in brain tumor-bearing mice*

Red fluorescence represented EMA-labeled DNA. As shown in Fig. 5, EMA-labeled DNA was accumulated very weakly in the brain and tumor site of the mouse treated with PAMAM/DNA NPs (Fig. 5A, B. Left), and the one treated with PAMAM-PEG/DNA NPs (Fig. 5A, B. Midst) was relatively stronger in the brain especially in the tumor site. It's much more obvious in brain especially in tumor site of the one treated with PAMAM-PEG-Angiopep/DNA NPs (Fig. 5A, B. Right). However, the accumulation in the liver of all mice was obvious but not in the other organs (spleen, lung, and kidney) except that treated with PAMAM-PEG/DNA NPs (Fig. 5C. Midst).

3.6. *In situ tumor apoptosis detecting*

Apoptosis of glial cell induced by TRAIL was assessed by TUNEL assay kit. The TUNEL kit can detect DNA fragmentation, a marker of apoptosis in nuclei of tumor cells. As shown in Fig. 6, unobvious or undetectable apoptosis phenomenon was detected in the groups that treated with saline (Fig. 6A) or PAMAM-PEG-Angiopep/pGL2 NPs (Fig. 6B), while there were a few in the group treated with free pORF-TRAIL (Fig. 6D), PAMAM/pORF-TRAIL NPs (Fig. 6E) or PAMAM-PEG/pORF-TRAIL NPs (Fig. 6F), and most of them occured on the edge of the glioma. The group that treated with Temozolomide (Fig. 6C) arouse obvious apoptosis phenomenon and the region of the apoptosis was concentrated relatively. There was very obvious apoptosis phenomenon inside the tumor site of the group treated with PAMAM-PEG-Angiopep/pORF-TRAIL NPs (Fig. 6G).

3.7. *Survival monitoring*

The survival of the mice were observed and recorded daily, and the data was analyzed by the Graph Pad Prism software (Fig. 7). The median survival time of the mice treated with the PAMAM-PEG-Angiopep/pORF-TRAIL NPs was up to 61 days that significantly longer than other groups ($p < 0.001$ compared with other groups), including the positive group treated with Temozolomide (49 days).

4. Discussion

To achieve the desired therapeutic effect of glial tumors, making more gene-loaded NPs accumulate selectively and efficiently in the tumor site, it must ensure that the drug delivery system can cross the BBB and further be uptaken by the glial cells.

LRP1, the receptor of Angiopep-2, is located on the BCECs and over expressed in the tumor cells [6,7,16]. So modifying Angiopep-2

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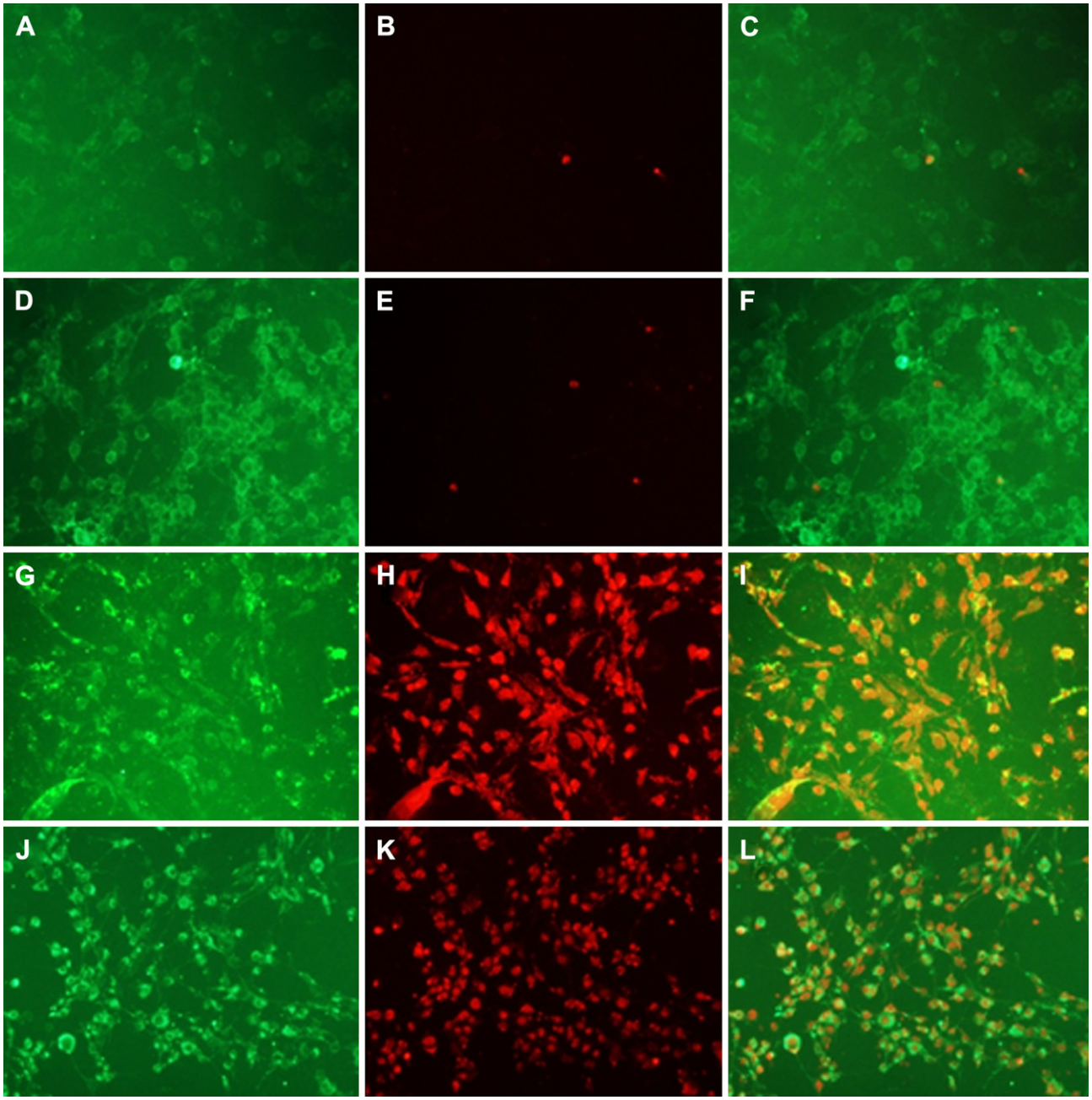

Fig. 4. *In vitro* transfection of Hank's solution (A–C), free pORF-TRAIL (D–F), PAMAM-PEG-Angiopep/pORF-TRAIL NPs (G–I) and temozolomide (J–L) were examined by fluorescent microscopy after 48 h transfection. Green: Annexin V-FITC (A, D, G, J); Red: Propidium Iodide (B, E, H, K); combined Annexin V-FITC and Propidium Iodide (C, F, I, L). Original magnification: × 200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on the surface of NPs might facilitate the uptake of NPs in the BCECs or glial cells via LRP-mediated endocytosis as it did with other NPs [17,18].

The mean diameter of the PAMAM-PEG-Angiopep/DNA NPs in this study was around 110 nm, and it has been reported that the NPs which has a mean diameter approximately 100 nm showed prolonged blood circulation and a relatively low rate of mononuclear phagocyte system (MPS) uptake [19]. The prolonged blood circulation was conductive to make more NPs reached the brain. Furthermore, as angiogenesis in brain tumor was considerably affected by the environment of CNS, which featured some tumor neovasculature with BBB properties, brain tumor vascular pore cutoff size was significantly reduced in cranial microenvironment compared with that in peripheral ones [20,21]. Therefore, the NPs with a diameter around 110 nm might give full play to the EPR effect in the brain tumor.

The biodistribution of NPs showed that the uptake of PAMAM-PEG-Angiopep/DNA NPs in brain especially tumor site was most obvious (Fig. 5). This phenomenon might benefit from the surface modification of NPs, which altered the characteristics of their distribution *in vivo*. Firstly, PEGylation could create a hydrophilic protective layer around the NPs that was able to repel the absorption of opson in proteins via steric repulsion forces and effectively prevented the protein absorbed on the NPs surface to make NPs unrecognizable by the RES as foreign bodies [22]. When the glial tumor came to high-grade, the long circulation half-life

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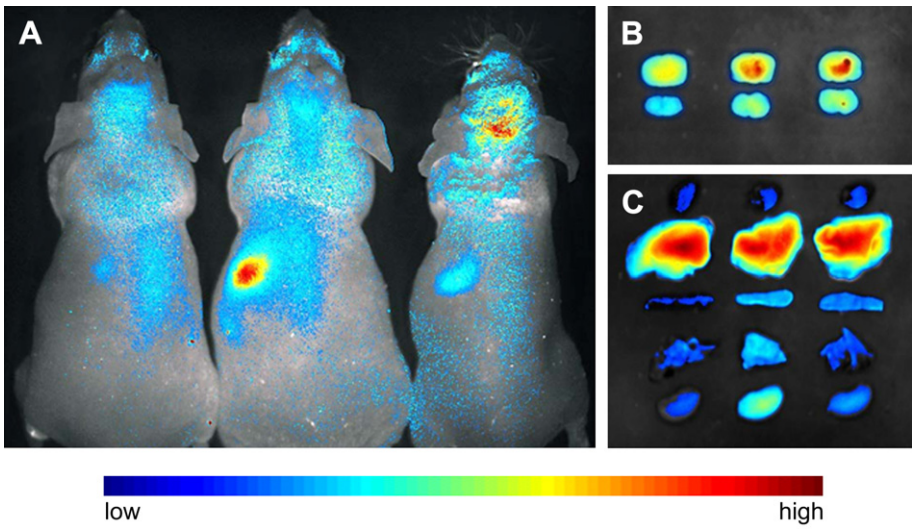

Fig. 5. *In vivo* (A) and *in vitro* (B, C) imaging of mice administrated with PAMAM/DNA NPs (Left), PAMAM-PEG/DNA NPs (Midst) or PAMAM-PEG-Angiopep/DNA NPs (Right). Images were taken 120 min after NPs administrated.

made more NPs accumulated in the glial tumor highly dependent on the EPR effect [23]. And it made the NPs possess a good passive targeting effect. Secondly, the Angiopep-2 on the surface of the NPs could further recognized and bound with LRP1 expressed on the surface of the glial cells, later triggered receptor-mediated endocytosis, resulting in an increased level of intracellular delivery of NPs in the tumor site [19]. On the other hand, it's reported that the areas that far away from the solid tumor or when the tumor was still low-grade, BBB remained relatively intact [24]. In this situation, modifying Angiopep-2 on the NPs made them possess an excellent ability to cross the BBB efficiently [11]. And it had been reported that the small molecular chemotherapeutic drugs could accumulate in brain tumor after modified with Angiopep-2 [25]. Thirdly, intracellular tracking of PAMAM-PEG-Angiopep/DNA NPs (Fig. 3) might indicate that the NPs could internalize into the glial cells through the endosome/lysosome pathway, and then DNA escaped quickly from the endosome and entered into the nuclei. So it might improve the efficiency of therapeutic gene expression. In addition, NPs would accumulate to a certain extent in other organs such as liver. However, the therapeutic gene in this study, TRAIL appeared to induce apoptosis only in tumorigenic or transformed cells but not in normal cells [26,27], and it had been reported the PEGylation of NPs could effectively reduce the toxicity in the liver [28].

In the pharmacodynamic evaluation of brain tumor-bearing mice (Figs. 6 and 7), PAMAM-PEG-Angiopep/pORF-TRAIL NPs possessed better glioma curing effect. It might be attributed to both the dual targeting effect of NPs that has been discussed above and pORF-TRAIL. TRAIL could specially recognize and induce apoptosis of tumor cells without affecting the normal cells [29], which was very different from the commercial chemotherapeutical drug, Temozolomide. So the gene therapy with pORF-TRAIL could achieve a lower side effect. And it had been reported that treatment with the TRAIL might elicit bystander effects either through interaction of cell surface TRAIL molecules with receptors on neighboring cells or through the action of soluble TRAIL from the TRAIL-expressing cells [30]. Furthermore, both the low cytotoxicity of the vector (Fig. 2) that allowed repeated i.v. administrations

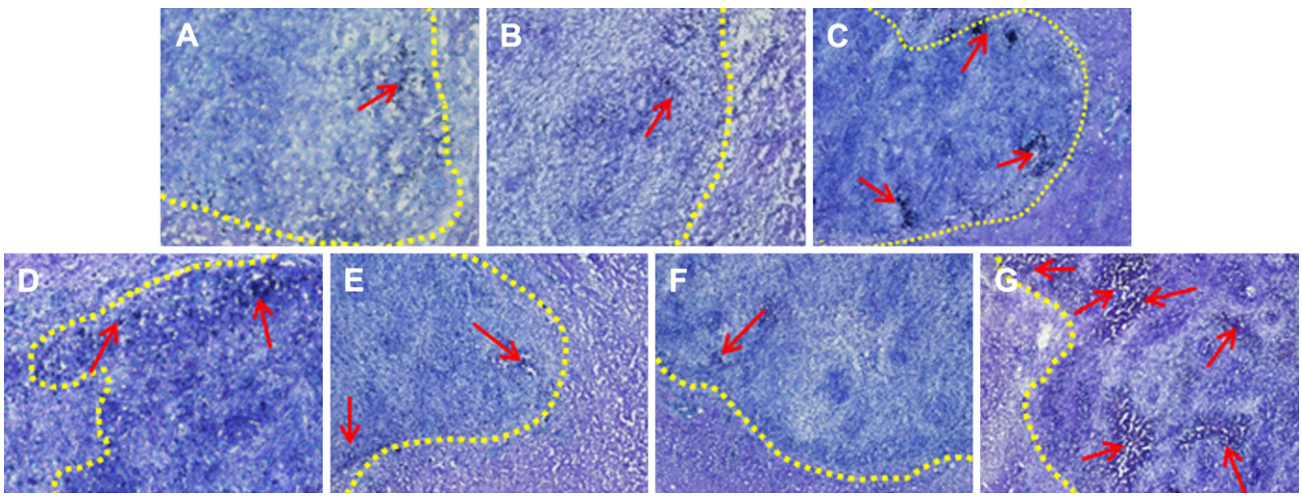

Fig. 6. Coronal frozen sections of brain tumors. A: saline; B: PAMAM-PEG-Angiopep/pGL2 NPs; C: Temozolomide; D: free pORF-TRAIL; E: PAMAM/pORF-TRAIL NPs; F: PAMAM-PEG/pORF-TRAIL NPs; G: PAMAM-PEG-Angiopep/pORF-TRAIL NPs. Yellow dashed line: border of the glial tumor. Red arrow: apoptosis cells. Original magnification: × 400. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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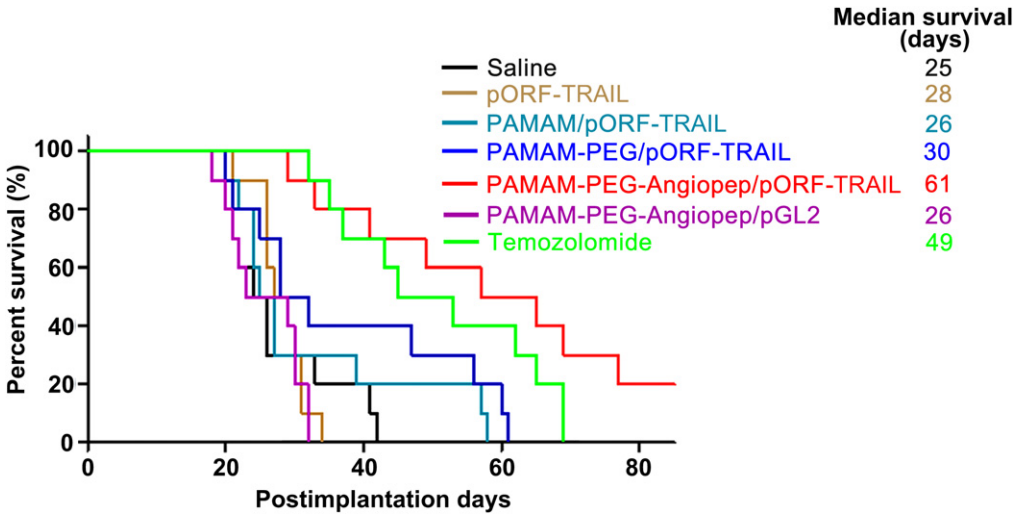

Fig. 7. Survival curve of brain tumor-bearing mice.

of PAMAM-PEG-Angiopep/pORF-TRAIL NPs and the bystander effect of TRAIL would improve the antitumor effect.

5. Conclusions

PAMAM-PEG-Angiopep was successfully synthesized and complexed with therapeutic gene, pORF-TRAIL, yielding NPs. The therapeutic effects of PAMAM-PEG-Angiopep/pORF-TRAIL NPs were systematically evaluated both *in vitro* and *invivo*, and the results showed its feasibility of systemic administration as a non-viral vector for gene therapy of glioma. The current findings encourage further studies into the application of non-viral vectors for noninvasive gene therapy of malignant glioma.

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