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ARTICLE *in* CHEMISTRY - AN ASIAN JOURNAL · JANUARY 2012

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Cyclic RGD–Polyethylene Glycol–Polyethylenimine for Intracranial Glioblastoma-Targeted Gene Delivery

Changyou Zhan,[a] Qinggang Meng,[a] Qinghua Li,[b] Linglin Feng,[c] Jianhua Zhu,[a] and Weiyue Lu*[a]

Abstract: Even though the blood–brain barrier (BBB) is compromised for angiogenesis, therapeutic agents for glioblastoma multiforme (GBM) are particularly inefficient due to the existence of a blood–tumor barrier (BTB), which hampers tumor accumulation and uptake. Integrin $\alpha_v\beta_3$ is overexpressed on glioblastoma U87 cells and neovasculture, thus making its ligands such as the RGD motif target glioblastoma in vitro and in vivo. In the present work, we have designed a modified polyethylene glycol–polyethylenimine (PEG–PEI) gene carrier by conjugating it with a cyclic RGD sequence, c-(RGDyK) (cyclic arginine-glycine-aspartic acid-D-tyrosine-lysine). When complexed with plasmid DNA, this gene carrier, termed RGD–PEG–PEI, formed homogenous nanoparticles with a mean diameter of 73 nm. These nanoparticles had a high binding affinity with U87 cells and facilitated targeted gene delivery against intracranial glioblastoma in vivo, thereby leading to a higher gene transfer efficiency compared to the PEG–PEI gene carrier without RGD decoration. This intracranial glioblastoma-targeted gene carrier also enhanced the therapeutic efficacy of pORF-hTRAIL, as evidenced by a significantly prolonged survival of intracranial glioblastoma-bearing nude mice. Considering the contribution of glioblastoma neovasculature to the BBB under angiogenic conditions, our results demonstrated the therapeutic feasibility of treating a brain tumor through mediation of integrin $\alpha_v\beta_3$, as well as the potential of using RGD–PEG–PEI as a targeted gene carrier in the treatment of intracranial glioblastoma.

Keywords: cancer · integrin $\alpha_v\beta_3$ · nanoparticles · polymers · RGD motif

Introduction

Glioblastoma multiforme (GBM) is the most frequent primary malignant brain tumor and accounts for approximately 40% of cases.[1] Treatment of GBM remains a challenge irrespective of the recent improvements, such as new therapeutic agents and strategies. Even though the blood–brain barrier (BBB) is compromised for angiogenesis,[2] therapeutic agents for GBM are particularly inefficient due to the existence of the blood–tumor barrier (BTB), which hampers tumor accumulation and uptake. Due to host-site influence, the BTB of malignant gliomas has a lower frequency of transendothelial cell fenestrations, caveolae, vesiculo–vacuolar organelles and smaller interendothelial cell gaps than that of the peripheral tumor microvasculature.[3] The pore cut-off size of different tumors implanted in the dorsal chamber is larger than that in the cranial window, and the pore cut-off size in the cranial U87 human glioma model is as small as 7–100 nm,[4] which is narrow enough to prevent the effective transvascular passage of most nanoparticles. Integrins, which comprise a large family of heterodimeric proteins, integrate extracellular matrix components with the cytoskeleton and the genome.[5] Bello et al.[6] found that both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are expressed on glioma cells and vasculature. The tripeptide Arg-Gly-Asp (RGD) motif is an essential binding motif for seven out of 24 integrins (such as $\alpha_v\beta_3$),[7] thus making it a promising ligand for GBM targeting.[8]

Among the nonviral gene-transfer agents, polyethylenimine (PEI) is one of the most effective and commercially available polymeric gene carrier, and it has been successfully used for gene transfer both in vitro and in vivo.[9] However, there are some disadvantages of PEI/pDNA polyplexes, such as their positive surface charge, which leads to undesired interactions with non-targeted cells, blood components, or vessel endothelia,[10] and their low biocompatibility as well as high cytotoxicity. Polyethylene glycol (PEG) has

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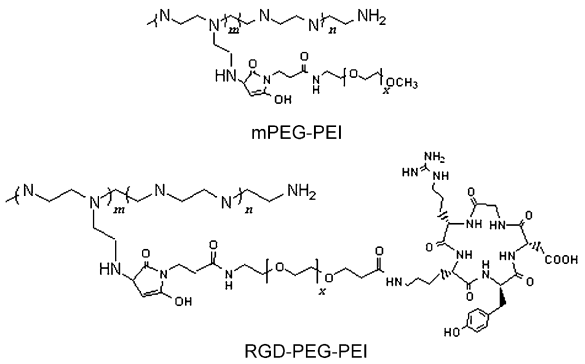
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/asia.201100570.

been widely used as a spacer, especially in targeted drug delivery systems, due to its soft chain and hydrophilicity. The presence of PEG in PEI derivatives may improve the dispersion of PEI/pDNA complexes at high concentrations, decrease their plasma protein binding and erythrocyte aggregation, prolong their blood circulation, and reduce their systemic toxicity.[11] PEI modified with the RGD motif has proven to effectively improve the gene transfection efficiency in vivo. As to the different conjugation modes, our previous work[12] indicated that RGD–PEG–PEI is more suitable than RGD–PEI–mPEG (mPEG = methoxypoly(ethylene glycol)) for subcutaneous glioblastoma-targeting gene transfection.



Scheme 1. Chemical structures of mPEG–PEI and RGD–PEG–PEI.

In the present work, we have synthesized RGD–PEG–PEI for intracranial GBM-targeted gene delivery. The results of biodistribution and gene transfection in vivo indicate that RGD–PEG–PEI/pDsRed-N1 nanoparticles can target intracranial GBM xenografts and improve gene transfection (Scheme 1).

Results and Discussion

Characterization of RGD-PEG-PEI/pDNA Nanoparticles

RGD–PEG–PEI and mPEG–PEI were synthesized, and the results of ^{1}H NMR spectrocopy (see Figure S1 in the Supporting Information) indicated that the substitution degrees of PEG and RGD were both about 7.5, close to the theoretical value 8.0.

A plasmid expressing the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), pORF-hTRAIL, was complexed with RGD–PEG–PEI or mPEG–PEI at an N/P ratio of 12:1, and characterization results are shown in Figure 1. The zeta potentials of RGD–PEG–PEI/pORF-hTRAIL and mPEG–PEI/pORF-hTRAIL were 11.29 ± 1.73 mV and 12.74 ± 2.01 mV, respectively. Both types of nanoparticles distributed homogeneously, and the diameters were 73.0 nm (PDI 0.087) and 79 nm (PDI 0.159). Electrophoretic mobility shift assay indicated the complete encapsulation of pDNA in both nanoparticles (Figure 1c). These results demonstrated no obvious difference between RGD–PEG–PEI/pORF-hTRAIL and mPEG–PEI/pORF-hTRAIL, thereby indicating that conjugation with the RGD motif does not affect the gene carrier complexes of PEG–PEI with pDNA.

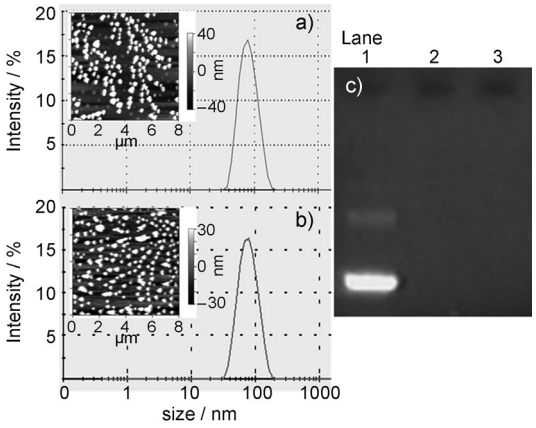


Figure 1. Characterization of the formed nanoparticles: diameter distributions and morphologies of a) mPEG–PEI/pORF-hTRAIL and b) RGD-PEG-PEI/pORF-hTRAIL nanoparticles. c) gel electrophoresis assay of naked pORF-hTRAIL (lane 1), mPEG–PEI/pORF-hTRAIL (lane 2), and RGD-PEG-PEI/pORF-hTRAIL (lane 3).

U87 Glioblastoma Cell Binding Activity

To localize the modified RGD motif in nanoparticles, we compared the binding characteristics of RGD–PEG–PEI and RGD–PEG–PEI/pORF-hTRAIL nanoparticles to U87 glioblastoma cells, which is a well-defined integrin $\alpha_v\beta_3$ overexpression cell line[13] and has been widely chosen as a model to develop gliomablastoma-targeted delivery systems for imaging, chemotherapy, and gene therapy.[14] Binding affinities were evaluated by using ^{125}I-labeled echistatin and different concentrations of materials or nanoparticles (Figure 2). No obvious difference between binding affinities of RGD–PEG–PEI and RGD–PEG–PEI/pORF-hTRAIL nanoparticles was observed, thus indicating that RGD is localized on the surface of RGD–PEG–PEI/pORF-hTRAIL

Abstract in Chinese:

随着神经胶质瘤的恶化，虽然血脑屏障局部被破坏，但血-肿瘤屏障仍然是制约药物或者给药系统发挥疗效的瓶颈。整合素$\alpha_v\beta_3$在神经胶质瘤 U87 细胞以及肿瘤新生血管上高表达，可以介导 RGD 短肽靶向治疗。本研究利用 RGD 环肽，构建了 RGD-PEG-PEI 基因载体材料。该载体材料与 DNA 质粒混合后，形成均匀分布、平均粒径为 73nm 的纳米粒。体内基因转染结果表明，RGD 修饰后显著增强基因药物在颅内肿瘤区域的表达。RGD-PEG-PEI 与治疗基因 pORF-hTRAIL 形成复合纳米粒后，体内给药显著延长原位神经胶质瘤模型裸鼠的生存期。本研究结果证明，RGD 环肽可以借助整合素$\alpha_v\beta_3$介导机制，增强 PEI 载体材料在颅内肿瘤的基因表达效果，实现脑肿瘤的靶向治疗。

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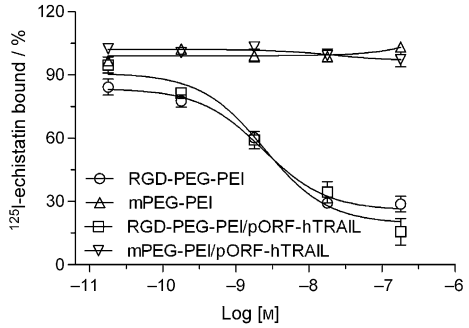

Figure 2. Inhibition of ^{125}I-echistatin (integrin $\alpha_v\beta_3$-specific) binding to integrin $\alpha_v\beta_3$ on U87 cells by RGD–PEG–PEI, mPEG–PEI, RGD–PEG–PEI/pORF-hTRAIL and mPEG–PEI/pORF-hTRAIL nanoparticles (N/P = 12). The x-axis shows the logarithm of the concentration of PEI derivatives ($n = 3$, mean ± SD). The binding data of RGD–PEG–PEI and mPEG–PEI have been shown in our previous report.[12]

nanoparticles, a result important for the targeted gene transfection in vitro and in vivo. We also investigated the U87 tumor cell-binding affinity of mPEG–PEI and its complexes. The results indicated no specific interaction.

Targeted Gene Transfection of RGD–PEG–PEI/pDsRed-N1 Nanoparticles in Intracranial U87 Tumor Xenografts In Vivo

Although both RGD–PEG–PEI and mPEG–PEI had high gene transfection efficiency in vitro (ranging from 30% to 40% as shown in Figure S2 in the Supporting Information), their in vivo transfection efficiency in intracranial U87 tumor xenografts would be a complicated issue. As shown in Figure 3, RGD–PEG–PEI/pDsRed-N1 nanoparticles had a significantly higher transfection efficiency in the intracranial U87 tumor xenograft, striatum, and cortex than mPEG–PEI/pDsRed-N1 nanoparticles. In the present study, we began to inject nanoparticles at 11 days post tumor implantation. At this time, the BBB would be compromised with the progression of intracranial glioblastoma. RGD–PEG–PEI/pDsRed-N1 nanoparticles accumulated in the intracranial tumor xenografts with overexpressed integrin $\alpha_v\beta_3$ on the tumor neovasculature and U87 cells.[15] Due to host-site influence, the special microenvironment of glioblastoma resulted in lower permeability of the tumor vasculature and lower effectiveness of accumulation through the enhanced permeability and retention (EPR) effect. However, the RGD–integrin strategy functioned and increased the entrapment of RGD–PEG–PEI/pDsRed-N1 nanoparticles in the tumor region, thereby resulting in targeted gene delivery and higher gene transfection efficiency. On the other hand, infiltrating glioma cells may possibly enhance RGD–PEG–PEI gene transfection in the striatum.

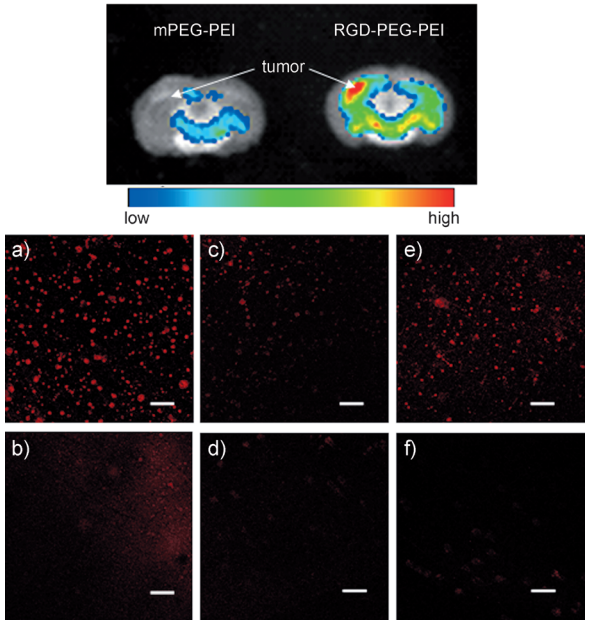

Figure 3. Optical imaging of mPEG–PEI/pDsRed-N1 and RGD–PEG–PEI/pDsRed-N1 nanoparticle transfection in brain tumor (upper panel) using an in-vivo image system. Confocal images of RGD–PEG–PEI/pDsRed-N1 (A, C, E) and mPEG–PEI/pDsRed-N1 (B, D, F) nanoparticle transfection in tumor (A, B), cortex (C, D) and striatum (E, F). Scale bar, 100 μm.

Anti-Glioblastoma Effect of RGD–PEG–PEI/pORF-hTRAIL Nanoparticles In Vivo

To study the anti-glioblastoma effect of RGD–PEG–PEI/pORF-hTRAIL in vivo, we investigated the median survival of intracranial U87 tumor-bearing mice (Figure 4a). After treatments with RGD–PEG–PEI/pORF-hTRAIL, mPEG–PEI/pORF-hTRAIL, and saline, the respective median survival times were 23.5, 20, and 19 days. Compared to mPEG–PEI/pORF-hTRAIL and saline, RGD–PEG–PEI/pORF-hTRAIL significantly ($p < 0.05$) prolonged the survival of intracranial glioblastoma-bearing nude mice. No significant statistical difference was found between the groups treated

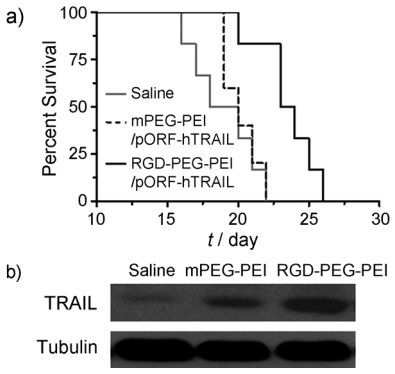

Figure 4. a) Kaplan–Meier survival curves of mice bearing intracranial U87 glioblastoma receiving the indicated treatments. b) Western blot analysis of TRAIL expression in brain tumors. Tubulin was used as a loading control.

with saline and mPEG–PEI/pORF-hTRAIL. The expression of TRAIL protein was also investigated. The result of western blotting demonstrated that RGD–PEG–PEI/pORF-hTRAIL nanoparticles had a higher transfection efficiency in tumor as compared to mPEG–PEI/pORF-hTRAIL nanoparticles (Figure 4b). RGD modification facilitates GBM-targeted delivery of nanoparticles by mediation of integrin $\alpha_v\beta_3$ overexpressed on neovasculature and U87 cells, thus circumventing the blood–tumor barrier and resulting in elevated gene transfection and increased life span.

Patients with glioblastoma, the most prevalent and aggressive glioma variant, have a median survival of only 15 months.[16] Due to the influence of the host site of gliomas, this tumor type has a distinguished specificity compared with other tumors, such as a low permeability of the tumor vasculature, resulting in ineffectiveness of the EPR effect. Several types of integrins are overexpressed on the surface of glioblastoma cells and the tumor neovasculature,[17] and many of them, such as $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$, can specifically recognize the RGD motif. Various studies have shown the potential of c(RGDyK) in the glioblastoma-targeted delivery. Even though c(RGDyK) has been widely applied for targeting of glioblastoma in vitro and subcutaneous tumor xenografts, few successful reports focused on intracranial glioblastoma. It is well demonstrated that glioblastoma is angiogenesis-dependent, suggesting the involvement of neovasculture in the BBB. To bypass the BTB, c(RGDyK) can bind to neovasculature to elevate tumor accumulation, and/or associate with glioblastoma cells to facilitate tumor uptake.

On the other hand, recent progress in molecular and cellular biology has promoted gene therapy, which has become a less risky option when compared with the conventional surgical approach,[18] as a promising strategy for the treatment of brain tumors.[19] However, the development of a highly efficient, safe, and cost-effective vector is the major challenge in gene therapy. Surface modification with a hydrophilic segment was shown necessary to design a nonviral vector for a long half-life and systematic administration.[20] Our results (Figure 1) showed that both RGD–PEG–PEI and mPEG–PEI could form stable and well-distributed nanoparticles with pDNA. mPEG–PEI/pDNA had a high gene transfection efficiency in U87 cells in vitro but failed in the gene delivery in vivo for the therapy of GBM. In this study, we investigated whether RGD–PEG–PEI could also function as a carrier for targeted gene delivery in intracranial GBM. The targeting efficiency of RGD–PEG–PEI/pDNA nanoparticles was initially confirmed in vitro and in vivo. Indeed, the RGD-modified nanoparticles not only have a high binding affinity with U87 glioblastoma cells, but also target to intracranial glioblastoma (as shown in Figure S4 in the Supporting Information) and result in higher gene transfection efficiency compared to mPEG–PEI/pDNA nanoparticles in vivo.

In the present work, we chose the TRAIL gene as the model drug to treat intracranial glioblastoma-bearing mice. Even though the survival time is statistically prolonged by the treatment with RGD–PEG–PEI/pORF-hTRAIL, the therapeutic efficacy is small because the increase in median survival is only 4.5 days as compared to the saline group. Hence, future research should be focused on: (1) increasing the anti-glioblastoma effect of TRAIL; in fact, numerous studies[21] have found that co-delivery of TRAIL and chemotherapeutics, such as paclitaxel, can extensively improve the antitumor effect of TRAIL, and (2) concurrent consideration of the BBB. Although previous works[22] have reported that the BBB would be compromised with the progression of GBM, and we have successfully prepared paclitaxel-loaded polymeric micelles, RGD–PEG–PLA–PTX (PLA = polylactic acid and PTX = paclitaxel), for the chemotherapy of GBM and prolonged the median survival time of GBM model nude mice, the BBB is an inevitable obstacle in the systematic administration of a drug delivery system. It is believed that the tumor neovasculature has not formed at the early stage of glioblastoma and that the tumor cells therefore make use of the normal brain vessels. With the progression of glioblastoma, cells can be roughly separated into an angiogenic component and an invasive or migratory component.[23] The infiltrating tumor cells around the edges of the tumor make use of the existing brain vasculature with a largely intact BBB. Vasculature in and around gliomas exhibits a wide range of permeabilities, from normal capillaries with essentially no BBB leakage to a tumor vasculature that freely passes even such large molecules as albumin.[24]

The angiogenesis-dependent nature of glioblastoma makes the tumor neovasulature a potential target for therapy and/or tumor cell-targeted delivery. Overexpressed integrins on glioblastoma cells and neovasulature ensure not absolutely but at least partially the usefulness of the RGD-modified gene carrier in the glioblastoma-targeted gene therapy.

Conclusions

We show that RGD-modified PEI as nonviral gene vector with a PEG spacer facilitates gene delivery targeted against intracranial U87 tumor xenografts, resulting in higher gene transfection efficiency and prolonging median survival of intracranial U87 tumor-bearing nude mice when complexed plasmid DNA carrying the TRAIL gene. In light of the targeted gene transfer in intracranial glioblastoma, RGD–PEG–PEI has great potential in the application of glioblastoma-targeted gene therapy or combined therapy with other therapeutics.

Experimental Section

Intracranial U87 glioblastoma-bearing nude mice

The U87 glioblastoma cell line was obtained from the Shanghai Institute of Biochemistry and Cell Biology. Cells were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Male Balb/c nude mice of 4–6 weeks old were purchased from Shanghai SLAC Laboratory Animal Co., LTD (Shanghai,

China). All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University. For the intracranial glioblastoma model, U87 cells (1×10^6 cells suspended in 5 μL PBS) were implanted into the right striatum of male Balb/c nude mice by using a stereotactic fixation device with a mouse adaptor.

Synthesis of RGD–PEG–PEI

Cyclic RGD (cyclic RGDyK) was synthesized by Fmoc solid-phase peptide synthesis, and the side-chain amino group in lysine was amidated with mal-PEG-NHS to obtain RGD–PEG-mal. RGD–PEG–PEI and mPEG–PEI were synthesized as reported previously.[12] In brief, an 8-fold molar excess of RGD–PEG-mal or mPEG-mal was added to a 5 mL solution of PEI (50 mg mL^{-1}) in PBS, pH 7.4, and the reaction was stirred overnight. The reaction mixture was then transferred into an Amicon Ultra filter tube (molecular weight cut-off of 10 kDa, Millipore) and subsequently rinsed four times with distilled water.[25] The products were lyophilized, and white powders were obtained. The products were dissolved in deuterium oxide (D_2O) and characterized by ^{1}H NMR spectrocopy at 400 MHz. The characteristic peaks of RGD (6.6 ppm, aromatic residue of D-tyrosine in the cyclic peptide) and PEG (3.5 ppm, -(CH_2CH_2O)-) were analyzed to calculate the degree of substitution.

RGD–PEG–PEI and mPEG–PEI solutions were freshly prepared and diluted to an appropriate concentration in distilled water. An equal volume of pDNA (such as pORF-hTRAIL, pEGFP-N2 or pDsRed-N1) solution (250 μg mL^{-1}) was added to obtain an N/P ratio of 12:1. The mixture was immediately vortexed for 30 s and incubated for 30 min at room temperature before use. The freshly prepared nanoparticles were used for subsequent experiments. The diameter and morphology of RGD–PEG–PEI/pORF-hTRAIL and mPEG–PEI/pORF-hTRAIL nanoparticles were analyzed by using the Malvern Zetasizer Nano-ZS System and by AFM (MFP-3D-BIO AFM, Asylum Research, CA). DNA binding of polycations was analyzed by gel electrophoresis assay.

Competitive binding assays

U87 cells (1×10^6 cells) in microcentrifuge tubes were washed twice with cell binding buffer[26] and then incubated overnight at 4 °C with ^{125}I-labeled echistatin (0.06 nM) in the presence of different concentrations of RGD–PEG–PEI or RGD–PEG–PEI/pORF-hTRAIL nanoparticles (N/P = 12). After incubation, cells were washed thrice with cell binding buffer, and the radioactivity was determined by gamma-counting. Nonspecific binding of ^{125}I-labeled echistatin to integrin $\alpha_v\beta_3$ was determined in the presence of 100 nM echistatin. The competitive binding of mPEG–PEI and mPEG–PEI/pORF-hTRAIL nanoparticles (N/P = 12) was analyzed analogously.

Tracking of gene transfer in vivo

Freshly prepared RGD–PEG–PEI/pDsRed-N1 or mPEG–PEI/pDsRed-N1 (the dosage of pDsRed-N1 was 1 mg kg^{-1} each time) was injected into the tail vein of intracranial glioblastoma-bearing mice at 11, 13, and 15 days post-implantation. At 16 days, mice were sacrificed and the coronal sections of the brain (about 0.5 cm thickness) post the optic chiasma (containing the tumor tissue) were collected. After fixation in 4% paraformaldehyde overnight, the samples were placed in 15% sucrose solution for 12 h. The sucrose solution was then replaced by 30% sucrose, and samples were incubated for 24 h. Red fluorescent protein (RFP) was detected using an in-vivo image system (FX Pro, Kodak, Rochester, NY, USA). Subsequently, the samples were embedded in Tissue Tek O.C.T. compound (Sakura, Torrance, CA, USA) and frozen at −80 °C. Frozen sections with 20 μm thickness were prepared by using a Cryotome Cryostat (CM 1900, Leica, Wetzlar, Germany) and examined under a confocal microscope (Leica, Wetzlar, Germany).

In vivo anti-glioblastoma effect

Mice were randomly divided into three groups and treated with 200 μL of RGD–PEG–PEI/pORF-hTRAIL, mPEG–PEI/pORF-hTRAIL (both at 1 mg kg^{-1} pORF-hTRAIL to body weight) or physiological saline via tail vein injection at 4, 7, 9, 12, and 14 days after implantation. The survival rates were recorded. At 15 days post-implantation, two mice of each group were randomly selected, and the tumors were isolated for analysis by western blotting.

Acknowledgements

The authors acknowledge Prof. Gaoren Zhong (Fudan University, China) for his help with radiolabeled experiments. This work was supported by the National Basic Research Program of China (973 Program 2007CB935800 and 2010CB934000) and the "Key New Drug Creation Program" 2009ZX09310-006.

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Received: June 25, 2011
Published online: November 9, 2011

