Protein-Based Drug Delivery in Brain Tumor Therapy

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Hae Hyun Hwang and Dong Yun Lee

Abstract

Despite the use of active surgeries, radiotherapy, and chemotherapy in clinical practice, brain tumors are still a difficult health problem due to their rapid development and poor prognosis. To treat brain tumors, various nanoparticles can be used to target effective physiological conditions based on continuously changing vascular characteristics and microenvironments to promote effective brain tumor-targeting drug delivery. In addition, a brain tumor-targeting drug delivery system that increases drug accumulation in the brain tumor area and reduces toxicity in the normal brain and peripheral tissues is needed. However, the blood-brain barrier is a big obstacle for drug delivery to the brain. In this chapter, we provide a broad overview of brain drug delivery and current strategies over the last few years. In addition, several questions have been reconsidered, such as whether nanoparticles believed to be delivered to the brain can pass through the blood-brain barrier, whether the drug is delivered to the target site, and what brain tumor treatment is possible.

Keywords

Brain tumor · Drug delivery · Oral administration of protein-based drug · Drug stability

13.1 Introduction

Brain diseases such as central nervous system disorders and brain cancers are the most prevalent and fatal yet untreatable diseases. Brain tumors include a variety of neoplasms that can be classed as either primary or metastatic [30, 85]. Three major types of brain tumors are known by the World Health Organization (WHO) as the classes of gliomas: astrocytomas, oligo-astrocytomas, and oligodendrogliomas [114]. These tumors are classified as subtypes (mainly astrocytomas) and are graded from I to IV, with type IV being the most aggressive, glioblastoma multiforme (GBM) [113].

Malignant astrocytoma constitutes about 50–60% of primary brain tumors [34]. The incidence of brain tumors seems to be increasing, but it is not clear whether this is because of environmental or genetic factors [46]. The standard treatment for brain tumors consists of maximal

H. H. Hwang · D. Y. Lee (✉)
Department of Bioengineering, College of Engineering, and BK21 PLUS Future Biopharmaceutical Human Resource Training and Research Team, Hanyang University, Seoul, Republic of Korea

Institute of Nano Science & Technology (INST), Hanyang University, Seoul, Republic of Korea
e-mail: dongyunlee@hanyang.ac.kr

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surgical resection, radiation therapy, and chemotherapy. However, despite ongoing research and new approaches, the prognosis for patients with malignant brain tumors is still very poor [17]. Thus, the median survival rate for GBM patients is 20 weeks with surgical resection or 36 weeks with surgical and radiation therapy, while cytotoxic chemotherapy maximizes survival and increases median survival to 40–50 weeks [9].

Despite the development and progress of anticancer drugs in the past decades, the prognosis of patients with brain cancer has remained almost unchanged [89]. These results imply that it is difficult to avoid the various resistance mechanisms, deliver the therapeutic agents across the blood-brain barrier (BBB), and reach the desired target [41, 52]. In addition, low-molecular-weight chemotherapeutic agents also have the disadvantage that they do not maintain effective steady-state concentrations in glioma cells because of their short blood half-lives [109].

Considering the high incidence of brain tumors and their poor prognosis, much effort has been made to identify the delivery of optimal drugs and valuable systems or anticancer drugs to the central nervous system (CNS). For the tumor to grow, it must develop a vascular network, and the angiogenesis system in the tumor is composed of vasculature with increased permeability due to large endothelial gaps compared to normal vessels [101]. This feature can be used in the anticancer delivery system.

This chapter deals with various approaches for the treatment of primary CNS tumors. In addition, it focuses on the recent discovery of a new strategy for delivering anticancer drugs to the CNS based on efficient targeting protein vectors (antibodies and protein carriers) or nanosystems (colloid carriers) that can cross chemical and biological barriers such as the BBB [7, 16, 58].

13.2 Barriers to Drug Delivery for Brain Tumors

Use of a drug delivery system, one of the therapies used to treat tumor progression in glioma, to reach the tumor site is complicated by many barriers. There are three major barriers to the treatment of brain tumors: the BBB, the blood-brain tumor barrier (BBTB), and the active efflux effect (Fig. 13.1). Specific brain tumor developmental stages require corresponding barrier-targeting treatment strategies.

13.2.1 Blood-Brain Barrier

The blood-brain barrier (BBB) is a diffusion barrier essential for normal functioning of the brain and regulates influx of blood into the brain to maintain homeostasis [20]. Brain capillary endothelial cells (BCECs), tight junctions (TJs), astrocytes (covering up to 90% of brain capillaries), pericytes, neurons, and basement membranes constitute physically rigid brain capillaries in the BBB [52, 99]. Unlike the peripheral microvasculature, BCECs are interconnected by tight junctions with few fenestrations that form a physical barrier to prevent diffusion from blood vessels into the brain. Interendothelial junctions severely limit penetration of water-soluble materials by connecting the endothelial cells to a continuous barrier. In addition, these junctions lead to very high trans-endothelial electrical resistance (TEER) between the blood and brain, significantly limiting the passive diffusion of compounds [3]. The interendothelial junctions are divided into adherence junctions, tight junctions, and gap junctions [64]. Primary control of the permeability of the endothelial barrier is the role of adherence junctions. Tight junctions are important in maintaining the permeability barrier of the epithelial and endothelial cells that regulate tissue homeostasis [69]. Gap junctions composed of six connexin molecules are responsible

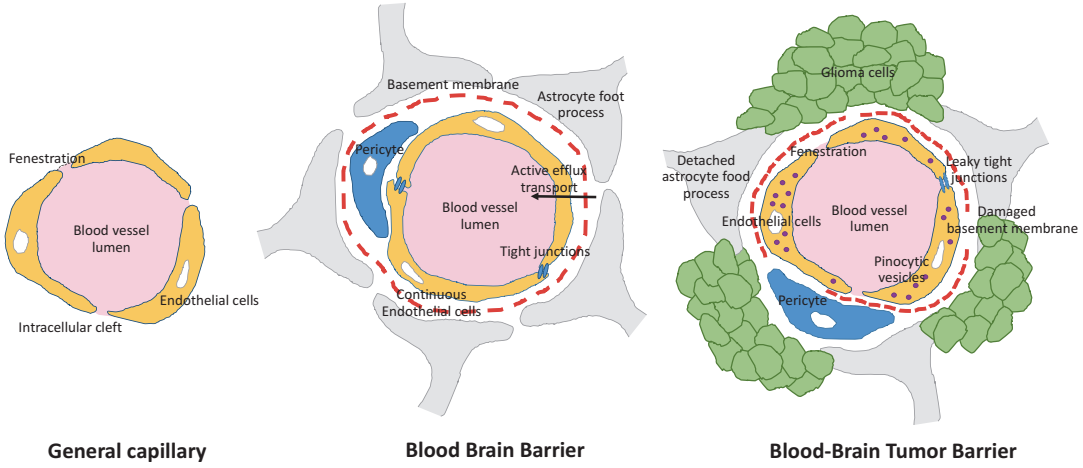

Fig. 13.1 Graphical depiction of the difference structure between normal capillary, BBB, and BBTB

for direct electrical and chemical communication between endothelial cells [42]. Pericytes, astrocytes, and basal membranes form a structure that surrounds the endothelial cells and eventually forms an impermeable BBB. Efflux transporters are located in the BCECs and provide an additional barrier to substances entering the brain (a more detailed description of efflux transporters is given in the next section). Thus, the physical barriers of the BBB significantly limit the accumulation of large molecules such as antibodies and antibody-drug conjugates, as well as small hydrophilic drugs that cannot easily traverse the plasma membranes of capillary endothelial cells [87].

However, the BBB not only has a static structure as mentioned above but also adapts continuously to various physiological changes of the brain [4, 64]. Molecules can cross the BBB by paracellular pathways or transcellular pathways. In the paracellular pathways, ions and solutes pass through the BBB by passive diffusion through a concentration gradient. The transcellular pathways involve various mechanisms such as passive diffusion, transcytosis, and receptor-mediated transcytosis [20]. Physicochemical factors affecting BBB permeability also include molecular weight, charge, surface activity, lipid solubility, and molecular size. [39]. For example, small lipophilic molecules such as carbon dioxide can pass through the BBB by passive diffusion through transcellular pathways. Hydrophilic molecules such as proteins or peptides can enter the brain through specific receptor-mediated transport mechanisms such as glucose transporter-1 (GLUT-1) and insulin transporter, and these transporters are expressed at the luminal and abluminal endothelial cell membranes [79]. Therefore, both physical and biochemical barriers within the BBB significantly limit delivery of remedial agents to the brain, which can limit treatment efficacy.

13.2.2 Blood-Brain Tumor Barrier

Brain tumor cells have a structure similar to that of the BBB in the early stage to match their rapid cell growth and migration rates. When growth of tumor cells reaches a certain level, the BBB structure is damaged, and the blood-brain tumor barrier (BBTB) is created from new blood vessels. The BBTB is located between the brain tumor tissue and the microvessels formed by endothelial cells with highly restrictive barriers [86]. Compared to peripheral tumors, the BBTB has a small pore size and represents a stronger

drug efflux pump, affecting such agents as P-glycoprotein, multidrug-resistant proteins, and breast cancer-resistant proteins [32, 60, 82, 104, 110]. This barrier also limits intercellular transport of most hydrophilic molecules to the tumor tissue. Therefore, the BBTB structure more highly limits drug distribution to brain tumors than to peripheral tumors. For example, Kunal et al. found that the metastatic breast tumor-bearing mouse model showed a lapatinib concentration in lung metastasis that was 5.15 times higher than that in brain metastasis [67]. This result assumes that the BBTB limited drug distribution from the blood to the brain tumor area [10, 38, 41]. Thus, the combination of the BBB and BBTB poses a major barrier to brain tumor drug delivery.

13.2.3 Active Efflux Transporters

Drug efflux receptors are expressed in brain capillary endothelial cells and cancer cells themselves, resulting in brain tumors that are resistant to anticancer drugs [14, 100, 107]. There are various types of efflux transporter systems, all of which belong to the multidrug resistance (MDR) family [106]. Among the MDR family, P-glycoprotein (P-gp, MDR1) is the most important active efflux transporter in drug disposition in the human body [100]. The molecular weight of P-gp is 170 kDa; it is expressed on the apical side of the BBB and actively pumps a variety of anticancer drugs into the systemic circulation [18]. This active transport process is one of the basic mechanisms of CNS anticancer drug resistance. The importance of P-gp in BBB was demonstrated using P-gp knockout mice [1, 111]. Penetration of vinblastine, a chemical analogue of vincristine, into the brain was 7- to 46-fold higher in knockout mice than in wild-type controls [124]. For this reason, many cytotoxic agents that are P-gp substrates cannot reach the tumor cells in the brain parenchyma and have no effect even if the tumor cells do not express P-gp [35, 118]. Furthermore, P-gp has been found in resistant glioblastomas, suggesting that it restricts penetration of anticancer agents into brain tumors despite the leaky nature of the glioma vasculature [6]. Therefore, inhibition of P-gp activity in brain endothelial cells is important for increasing antitumor effects.

13.3 Drug Delivery Strategies in Brain Cancers

As mentioned above, unlike other peripheral tissues, a brain tumor involves many barriers to transmission of anticancer drugs such as the BBB, BBTB, and efflux transporters. However, drug delivery systems for overcoming these problems and treating brain tumors have been actively studied. There are a number of overexpressed receptors and carriers that can act as channels through which the BBB can mediate the transport of certain ligands and cargo, even under intact conditions [102]. The BBB membrane has a negative charge, so it has a high affinity for positively charged compounds and can induce cell internalization. Low-molecular-weight, fat-soluble, and neutral drugs can pass through the BBB via passive diffusion [36]. In a brain tumor, nanoparticles of a certain size or less can pass through the gap between the endothelial cells due to the enhanced permeability and retention (EPR) effect caused by collapse of blood vessels due to solid tumor formation. In addition, drug delivery systems that target specific receptors and specific structures overexpressed in the BBTB, which is a structure independent from the BBB, have been studied.

13.3.1 EPR Effect

As brain tumors develop, they exhibit the EPR effect, though it is much weaker in the brain microenvironment than in peripheral tumors. The EPR effect allows a nanosystem with an appropriate particle size to enter the brain tumor through the microvascular endothelial cleft of the brain tumor. In addition, tumoral masses accumulate macromolecules larger than about 40 kDa in the microenvironment because of poor lymphatic drainage [49, 74, 134]. Nanoparticles use

this feature to target solid tumors. The ideal size range for achieving the benefits of EPR is 10–200 nm. Outside this range, small particles are removed by the kidneys to prevent them from accumulating at the tumor site, and particles larger than this range cannot adequately penetrate the tumor vasculature and interstitial space.

Therefore, some nano-sized drug delivery systems have been developed to use the EPR effect for brain tumor targeting. Huang et al. have developed a tumor-targeting nanoparticle system with passive tumor targeting based on the EPR effect. This system was able to extend the survival time of U87MG tumor-bearing nude mice [49]. There have also been attempts to increase the efficiency of the EPR effect by induction of hypertension, repair of abnormal vascular systems, or targeting of peripheral blood cells [51].

13.3.2 BBTB Targeting Delivery

The blood-brain tumor barrier (BBTB) is located between the microvessels and brain tumor tissues and is formed by highly specialized endothelial cells, limiting paracellular delivery of hydrophilic molecules to tumor cells [86]. The blood tumor barrier structure that grows in the peripheral tissues is generally more permeable than that in the brain [84, 104]. As brain tumors deteriorate, tumor neovascularization becomes more active and the BBB structure becomes damaged, creating a new structure called BBTB. This structure supports the growth of glioma. Abnormality of tumor vasculature increases the permeability of the BBTB, while the cranial microenvironment reduces the permeability of glioma area [112, 133]. Thus, BBTB can limit glioma-targeted transport of chemotherapeutic agents [133].

Therefore, some receptors present on the BBB/BBTB provide an opportunity for glioma-targeted drug delivery at this stage. Several studies proposed a strategy for BBTB targeting based primarily on the receptors expressed at high levels in tumors, such as epidermal growth factor receptors and integrins [128]. The adhesion receptor integrin is overexpressed in the tumor neovasculature and glioblastoma U87MG cells and was identified as a marker of angiogenic blood vessel tissue. The integrin αvβ3 expression is overexpressed in malignant glioma but not in normal brain cells. As ligands for integrins, cyclic arginine-glycine-aspartic acid (RGD) peptides and their analogues have been extensively studied for glioma-targeted drug delivery [11, 63, 73]. Therefore, integrin and RGD interactions are promising drug delivery strategies that target the BBTB. Zhan et al. developed c-RGDyK-modified polyethylene glycol-polyethylenimine nanoparticles (PEG-PEI NPs) for glioma-targeted gene delivery [136]. These NPs showed high binding affinity with U87MG cells and promoted target gene delivery to intracranial glioblastoma in vivo compared to PEG-PEI gene carriers without RGD modification. The therapeutic efficacy of this gene transducer has been demonstrated by significantly prolonging the survival rate of nude mice with intrathecal glioblastoma. These results demonstrated the therapeutic potential of the gene delivery system for the treatment of brain glioma cells using integrin αvβ3 [136]. Zhan et al. reported cyclic RGD peptide-conjugated PEG-PLA micelles for chemotherapy of intracranial glioma. The median time of intracranial U87MG tumor xenograft survival was significantly prolonged after treatment with c(RGDyK)-PEG-PLA-PTX micelles, indicating that the RGD motif is effective in drug delivery targeted to glioblastoma overexpressing integrin αvβ3 [134].

Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase expressed in epithelial cells, mesenchymal cells, and neuronal tissues [116, 131]. Overexpression of EGFR for the BBTB is a promising target for treatment [88]. Epidermal growth factors (EGF) and anti-epidermal growth factor ligand (anti-EGFL) monoclonal antibodies are commonly used EGFR ligands in glioma-targeted therapy. Fondell et al. have adopted EGF to target EGFR and have recently developed a strategy for delivering recently synthesized daunorubicin derivatives to cancer cell nuclei using PEG-stabilized targeted liposomes called “nuclisome-particles” [33]. Tsutsui et al. established a new drug delivery system using hybrid bionanocapsules (BNCs)

coupled with anti-human EGFR antibodies and confirmed the specific delivery of BNCs to brain tumors in in vivo brain tumor animal models [123].

13.3.3 Receptor-Mediated Transcytosis

Many receptors, including the transferrin (Tf) receptor, the nicotinic acetylcholine receptor (nAchR), and the insulin receptor, are overexpressed in the BBB [43, 137]. These receptors specifically bind to corresponding ligands and can cause cellular internalization. Thus, these receptors and their corresponding ligands can be functionalized into the nanoparticle phase to mediate transport through the BBB. Due to the specificity of the interaction between the receptor and ligand, receptor-mediated delivery was the most commonly used and most successful strategy for delivering NPs to the brain via the BBB. The contents of the receptor-mediated transcytosis-related studies will be discussed in more detail in the next section.

13.4 Protein-Based Drug Delivery to Brain Tumors

A variety of proteins and peptides have been studied as promising therapeutic agents for brain pathologies [5, 96] (Table 13.1). Proteins and peptides are present in the entire nervous system with a unique distribution pattern. They have numerous biological actions in the brain, such as controlling the brain's internal environment, controlling cerebral blood flow, controlling the permeability of the BBB to nutrient supply, neurotransmission, neuromodulation, and the various roles of the immune system [22, 96]. This suggests that the diversity of the biological actions of proteins could be used in the treatment of brain and spinal cord disorders. But like all potential neuroleptics, proteins must be transportable from the blood to the brain. Protein nanocarriers are now drawing great interest as drug delivery systems targeting brain tumors [26, 27]. The unique biodegradability and high drug binding capacity of protein drugs indicate them as good alternatives to synthetic polymer nanoparticles. In addition, the available functional groups present in the proteins, such as amino and carboxyl groups, can be derivatized to specific ligands for drug delivery targeted to brain tumors [25, 29].

13.4.1 Transferrin

The transferrin (Tf) receptor, composed of two 90 kDa subunits, is an iron-binding, single plasma glycopeptide that controls the concentration of free iron in biological fluids. Many reports have shown that Tf can target Tf receptors (TfR) that are overexpressed in cancer cells and brain capillary endothelial cells of the BBB, and TfRs have been shown to pass through the BBB and cancer cell membranes [102]. Thus, modification of nanocarriers with Tf is a typical pathway of receptor-mediated delivery, one of the major mechanisms by which various mediators can cross the BBB [94, 95]. In several studies, Tf-modified NPs (Tf-NPs) showed good affinity for endothelial cells of the brain capillaries and could deliver much more cargo to the brain than unmodified NPs. Linuma et al. modified cisplatin (Cis)-liposomes (Tf-Cis-lipo) to enhance transport across bEnd3 cells as a model of the BBB using the TfR [53]. They also identified Tf-Cis-lipo endocytosis through recognition of Tf receptors on the surface of C6 glioma cells. Tf-modified liposomes encapsulating vincristine and tetracene (TFT) have been developed to overcome the multidrug resistance (MDR) that causes glioma treatment failure. Similarly, Tong et al. studied the decoration of artesunate (ART)-loaded liposomes containing Tf-ART-LPs and found that the absorption rate of U87 glioma cells increased from 18.7% for ART-LPs (not modified with Tf) to 59.8% for Tf-ART-LPs [122]. Song et al. conjugated the liposome surface to the Tf via acylation, in which one of the amino groups of Tf coupled with the *N*-hydroxysuccinimide (NHS)

Table 13.1 List of protein- or peptide-modified nanocarriers for the treatment of brain tumor

Material	Modified agents	Drugs	References
Transferrin	Liposome	Tf-Cisplatin-liposome	[53]
		Tf-ART-LPs	[122]
		Tf-PEG-DSPE	[115]
	Monoclonal antibody		[92]
		PDMS-b-PMOXA conjugated to 83-14 mAb	[19]
	Gene	Tf-PEI2-ChA	[23]
	Inorganic NPs	TPGD	[21]
		C-Dots-Tf-DOX	[72]
		Tf-PLCaPZ NPs	[108]
	CPP	Tf3.4 K-CPP2K-liposome	[76]
	Dendrimer	G4-DOX-PEG-Tf-TAM	[71]
Lactoferrin	Tumor-homing peptide	tLyP-1/Lf-NPs	[83]
	Folic acid	Lf/FA/PLGA NPs	[68]
	Polymersome	Lf-PO-DOX/TET	[93]
		Lf_H-NPs	[119]
	Peptide	Urocortin-loaded Lf-NPs	[48]
		S14G-humanin/Lf	[55]
	Magnetic NPs	Lf-M-PAEEPPLLA-NPs	[78]
		Cy5.5-Lf-SPIO micelles	[138]
		Lf-CUR-PDNC	[31]
Albumin	Glucose derivatives	c/m-HSA NPs	[12]
	Folic acid	FA-BSA-SPIO NPs	[127]
	Self-assembled NPs	LMWP-BSA-NPs	[73]
		HSA-Ce6@HSA-RGD NPs	[13]
Peptides	Small peptide	SynB1	[105]
		ANG1005	[120]
		T7-modified dendrimer	[70]
		CDX	[135]
	CPP	AngioPep-2	[126]
	Glycoprotein peptide	RVG29	[50, 75]
		RVG79-modified poly(mannitol-co-PEI) vector	[97]
	Apolipoprotein	ApoA and ApoE	[103, 132]
		Polysorbate 80-coated NPs	[37, 130]
		Polysorbate 60/80	[81]

ART artesunate, *LPs* liposomes, *NP* nanoparticle, *PDMS-b-PMOXA* poly(dimethylsiloxane)-block-poly(2-methyl-2 oxazoline), *mAb* monoclonal antibody, *PEI* polyethyleneimine, *ChA* cholic acid, *TPGD* transferrin-DOX-loaded PEGylated graphene oxide nanoparticles, *DOX* doxorubicin, *C-Dots* carbon-dots, *CaP* calcium phosphate, *PLCaPZ* CaP NP was complexed with zoledronic acid (ZOL), mixed with PEGylated cationic liposomes, *CPP* cell-penetrating peptide, *G4* fourth generation, *TAM* tamoxifen, *Lf* lactoferrin, *FA* folic acid, *PLGA* poly(lactide-co-glycoride), *PO* polymersome, *Lf_H-NPs* PEGylated DOX was converted to Lf, *S14G-humanin* a humanin analogue peptide drug, *PAEEP-PLLA* poly(aminoethyl ethylene phosphate)/poly(L-lactide), *Lf-M-PAEEPPLLA-NPs* OAM-MNP-loaded PAEEP-PLLA NPs modified with Lf, *OAM-MNPs* oleylamine (OAM) coating for Fe3O4 magnetic NPs, *SPIO* superparamagnetic iron oxide, *CUR* curcumin, *PDNC* polydiacetylene nanocarriers, *HAS* human serum albumin, *c-HSA* cationic HSA, *m-HSA* mannose-modified albumin, *BSA* bovine serum albumin, *LMWP* low-molecular-weight protamine, *Ce6* chlorin e6, *RGD* Arg-Gly-Asp, *T7 peptide* HAIYPRH, *RVG29* rabies virus glycoprotein peptide

group of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)-conjugated polyethylene glycol with an active succinimidyl ester (DSPE-PEG-NHS) [115]. Tf-modified liposomes (Tf-PEG-DSPE) can enhance transport through the BBB, increase cellular uptake, and inhibit MDR. Therefore, liposomes accumulated in brain tumors and showed high anticancer efficacy in glioma mice.

Monoclonal anti-transferrin receptor antibody (OX26) is an antibody that can recognize the Tf receptor. Pang et al. conjugated OX26 to the NP for brain-targeted delivery of the peptide NC1900, which is used to treat a neurodegenerative disorder [92]. The concentration of OX26-NP in brain tissue 2 h after intravenous injection was 2.62 times higher than that of unmodified NP. As a result, NC1900-loaded OX26-NP showed the best results for Alzheimer's disease rats, as determined by the water maze learning task using rats with scopolamine-induced learning and memory impairment [92]. In addition, the insulin receptor 83-14 mAb antibody (INSR alpha [83-14]) was about 10 times more effective than the anti-Tf receptor antibody for BBB penetration [15]. Therefore, Dieu et el. conjugated insulin receptor 83-14 mAbs to the NP surface (polymersomes composed of poly(dimethylsiloxane)-*block*-poly(2-methyl-2-oxazoline), PDMS-*b*-PMOXA) for brain target drug delivery. In vitro results showed that brain endothelial cells effectively absorbed modified NPs from insulin receptor 83-14 mAb, which could be inhibited by more than insulin receptor 83-14 mAb use alone [19].

To deliver the gene to glioma cells, Dube et el. developed a new nonviral vector based on low-molecular-weight polyethyleneimine (PEI 2 kDa) modified hydrophobically to cholic acid (ChA) to obtain PEI2-ChA [23]. Condensation of pDNA by the PEI-ChA complex protected the pDNA from enzymatic degradation and promoted absorption of the complex by the cells. Tf was also incorporated into nanopeptides to combine the high gene transfer efficiency of the PEI-ChA nanopeptides with Tf receptor (TfR)-mediated uptake. Tf facilitated the binding of pDNA nanopeptides to Tf receptors on target cells and promoted endocytosis of vesicles, escape of DNA from endosomal compartments, and entered to the nuclei. The size of tumors in the mouse brain treated with Tf-PEI2-ChA nanopeptides was five times smaller than those in the untreated animals.

Inorganic NPs can also be transformed into Tf to enhance brain tumor accumulation. Dong et al. explained that Tf was covalently bound to DOX-loaded PEGylated graphene oxide nanoparticles (TPG) [21]. Modified TPG can pass through the BBB to enhance DOX accumulation and act as dual chemo- and photothermal therapies. Targeted TPGD combination therapy increased the number of neuroblastoma lymphoma cells and prolonged the survival of glioma-bearing mice compared to single DOX or PGD therapy. Carbon dots (C-dots) and quantum-sized carbon NPs, which were smaller than 10 nm, exhibited good water solubility, excellent biocompatibility, excitation wavelength-dependent photoluminescence, and high cell membrane permeability [72]. Thus, the C-Dots-Tf-DOX covalent bond was synthesized by covalently bonding the carboxyl groups of the C-dots to the primary amines of Tf via carbodiimide coupling. C-Dots-Tf-DOX at the 10-nm size was much more cytotoxic than DOX alone, reducing the survival rate by 14–45% in many pediatric brain tumor cells [72]. Another type of inorganic nanoparticle, calcium phosphate NP (CaP NP), was complexed with zoledronic acid (ZOL), mixed with PEGylated cationic liposomes, and then transformed into Tf to generate Tf-PLCaPZ NPs for brain tumor treatment. Sequential treatment with temozolomide (TMZ) and Tf-PLCaPZ NP showed superior therapeutic activity compared to single administration. In the group treated with Tf-PLCaPZ NPs, the tumor size of mice xenotransplanted with U373MG was significantly reduced, but treatment had no effect in the free TMZ group [108].

A dual brain targeting effect was achieved by decorating the nanocarrier surface with Tf combined with other ligands for the purpose of increasing drug accumulation in tumor cells. Liu et al. conjugated Tf and cell-penetrating peptide (CPP) to PEGylated liposomes (Tf-CPP-liposomes) to bind endogenous escaping and per-

meability of CPP with Tf receptors (Tf-Rs) overexpressed in the BBB and glioma cells [76]. A "hand in hand" effect was observed in the Tf3.4 K-CPP2K-liposome and allowed longer PEG chains to nonspecifically mask CPP during blood circulation. The longer PEG chain at the tumor site promotes binding of Tf to the Tf receptor, while the flexible PEG linker is shortened, so that CPP improves cellular internalization through cell adsorption. The CPP portion was concealed by the large volume of the PEG 3.4 k linker of Tf. However, when the Tf3.4 K-CPP3.4 K-liposome was used, CPP could not be sterically hindered, demonstrating its permeation efficiency and significantly increasing normal cell uptake. Thus, to obtain maximum efficacy in target cells, PEG 3.4 k and PEG 2 k were selected to conjugate Tf and CPP with liposomes for the production of Tf-CPP-liposomes. These liposomes had the highest target efficacy for brain microvascular endothelial cells and C6 cell uptake, but absorption into normal cells was scant. Furthermore, Tf-CPP-liposomes were captured in the endosomes of C6 cells, where the complex escaped from the lysosomes and successfully released liposome-confined doxorubicin (DOX) to the cytoplasm. Li et al. conducted double modification of the fourth-generation (G4) DOX-loaded poly(amidoamine) (PAMAM) dendrimer with Tf and tamoxifen (TAM) (G4-DOX-PEG-Tf-TAM). They found that about 7 DOX molecules, over 30 PEG (1000 Da) and PEG (2000 Da) chains, and one Tf group were conjugated on the surface of each G4 PAMAM dendrimer, while 29 TAM molecules were encapsulated into one dendrimer. The result is that TAM inhibited MDR efflux transporters (e.g., P-gp, which is overexpressed in BBB and C6 glioma cells) with Tf receptor-mediated endocytosis to enhance BBB transport and accumulation of DOX in C6 cells. In addition, DOX accumulated in the C6 glioma spheroids and the tumor volume was effectively reduced [71].

13.4.2 Lactoferrin

Similar to transferrin, lactoferrin (Lf) is a mammalian cationic iron-binding globular glycoprotein belonging to the transferrin family and has a molecular weight of about 80 kDa [59]. Lactoferrin has many physiological functions such as defense against infections and severe inflammation. Lactoferrin receptors (LfRs) include low-density lipoprotein receptor-related protein 1 (LRP1) and LRP2, and LfRs induce internalization of Lf into the body. Previous studies have shown that LfRs are highly expressed in the BBB and in glioma cells. The positive charge of Lf promotes electrical attraction between the positively charged Lf-modified drug carrier and negatively charged BBB basement membrane, and this combination is absorbed through LfR-mediated endocytosis. That is, the Lf-modified nanocarrier was transported through the BBB by receptor-mediated transcytosis. Several studies have shown that the BBB permeability of Lf is better than that of transferrin (Tf) [28] because binding between Lf and its receptor is not affected by endogenous Lf. Lactoferrin receptors were not saturated under physiological conditions due to low plasma concentration of endogenous Lf. Conversely, the concentration of the intrinsic Tf in plasma is very high, so TfR is almost saturated under physiological conditions. Therefore, it could be better to use LfR as a target to modify the Lf and transmit it to brain tumors through receptor-mediated transcytosis of BBB.

However, Lf-functionalized nanoparticles for glioma treatment may still be limited because of the high interstitial pressure in cerebral blood vessels and glioma glands with reduced brain function and low efflux system from the blood vessels and low permeability to the glioma parenchyma [54]. Therefore, administering nanocarriers that target both the BBB/BBTB and glioma cells with a tumor penetration-enhancing peptide is a promising platform for antitumor brain drug delivery. For example, Miao et al. reported that lactoferrin was modified with the surface of poly(ethylene glycol)-poly(lactic acid) (PEG-PLA) NPs through a maleimide-mediated cova-

lent bond to induce BBB/BBTB and glioma cell targeting. A tumor-homing peptide, tLyP-1, was also used to mediate BBB penetration through the C-end rule sequence (CendR, R/KXXR/K) and the neuropilin-1 (NRP1) interactions, which induce tissue internalization [83]. Then, tLyP-1 was co-administered with Lf-NPs, which enhanced the accumulation and deep penetration into the glioma parenchyma. In in vitro tests, Lf-NPs showed the most increased cytotoxicity and deep penetration of 3D glioma spheroids in both brain capillary endothelial cells (BCECs) and C6 glioma cells. In vivo, Lf-NPs also exhibited the highest accumulation in the brain tumor area and deep penetration. Due to the specific expression of NRP1 in the endothelial cells of tumor vessels, the distribution of functionalized nanoparticles (Lf-NPs) was reduced in normal brain tissue. In another study, Lf and folic acid (FA) were cross-linked on poly(lactide-co-glycoride) (PLGA) NPs to carry etoposide (ETO, a chemotherapy medication used for glioblastoma) across the BBB and to treat human brain malignant glioblastoma [68]. Lf- and FA-modified PLGA NPs (Lf/FA/PLGA NPs) were infiltrated into human brain microvascular endothelial cells (HBMECs) to inhibit the proliferation of U87MG cells. The antiproliferative effects on the growth of U87MG cells were highest in the Lf/FA/PLGA NP treatment group compared with the other groups. The targeting ability of Lf/FA/PLGA NPs was proved by immunostaining of LfR on HBMECs and FA receptors on U87MG cells through endocytosis.

To create a biodegradable nanoparticle, Pang et al. co-loaded doxorubicin (DOX) and tetradrine (TET) into the Lf-modified polymersomes (PO), Lf-PO-DOX/TET. In vitro, the Lf-PO-DOX/TET NPs were absorbed into cells and exhibited the strongest cytotoxic effect in C6 glioma cells compared with other NP groups. During in vivo imaging analysis, Lf-PO labeled with near-infrared (NIR) dye was absorbed in the brain and accumulated at the tumor site. A pharmacodynamic study demonstrated that the tumor size of the Lf-PO-DOX/TET group was significantly smaller than those of other groups and the median survival time of the Lf-PO-DOX/TET group was longest compared to those of the other therapeutic groups [93]. Jiang et al. modified polymersomes using Lf to stimulate brain accumulation and to be able to administer S14G-humanin (a humanin analogue peptide drug, which has been proved to have an activity 1000-fold more powerful than humanin) to protect the brain from learning and memory damage induced by amyloid β_{25-35}. These results demonstrate that Lf can act as an active BBB target ligand that enhances drug delivery to the brain [55]. Similarly, a dual-target drug delivery system based on bovine serum albumin (BSA) NPs modified using both Lf and mPEG2000 and loaded with DOX was designed and tested for infiltration of the BBB and evaluated for glioma cell targeting properties [119]. PEGylated DOX was converted to Lf (Lf_H-NPs) based on electrostatic interactions between the cationic Lf molecules and negatively charged BSA-NPs (P_{2000}-NPs) at physiological pH. Compared to the other groups, Lf_H-NPs showed strong cytotoxicity and high uptake in both BCEC and C6 cells in vitro. In glioma model rats, the biodistribution of DOX testing showed that the Lf_H-NP group had significantly increased DOX accumulation in the brain compared with other groups, especially at 2 h post-infusion (intravenous, $P < 0.05$). Hu et al. used Lf-NPs to deliver urocortin, a peptide composed of 40 amino acids and highly expressed in the central and peripheral nervous systems, to the brain for treatment of Parkinson's disease (PD) [48]. The results showed that the urocortin-loaded Lf-NP treatment group had significantly attenuated striatum lesions induced by 6-hydroxydopamine (6-OHDA) in rats. In addition, immunohistochemistry and transmitter results demonstrated that treatment with urocortin-loaded Lf-NPs prevented the loss of transmitter contents in the brain, similar to that in normal rats, which means that the behavior of mice from the urocortin-loaded Lf-NP treatment group was significantly better than those in the control and untreated nanoparticle-infused rats.

Magnetic resonance imaging (MRI) is widely used for clinical diagnosis because it is safe to use nanoparticles for diagnostic purposes. In recent years, nano-scale contrast agents have

been developed to improve MRI diagnosis. For this, Lue et al. developed an oleylamine (OAM) coating for Fe_3O_4 magnetic NPs (OAM-MNPs), which were encapsulated in amphipathic poly(aminoethyl ethylene phosphate)/poly(L-lactide) (PAEEP-PLLA) copolymer NPs to diagnose malignant neuroma [78]. The OAM-MNP-contained PAEEP-PLLA NPs (M-PAEEP-PLLA-NPs) were further modified with Lf (Lf-M-PAEEPPLLA-NPs) for brain targeting. The Lf-M-PAEEP-PLLA-NPs showed excellent biocompatibility in cytotoxicity assays and high cell uptake in C6 cells, which indicated that Lf provided active targeting to the brain tumor site. Moreover, a significant enhancement of contrast images was obtained on MRI of Wistar rats in the glioma area in the Lf-M-PAEEPPLLA-NP treatment group. Prussian blue staining in this section also demonstrated retention of Lf-M-PAEEP-PLLA-NPs in brain tumor tissues. Zhou et al. used encapsulated hydrophobic superparamagnetic iron oxide NPs (SPIONs) in polyethylene glycol-*block*-polycaprolactone (PEG-*b*-PCL) and Cy5.5, a near-infrared fluorescent probe, to obtain optical imaging. Then, to target glioma, Lf was used with NPs as a brain MRI contrast agent [138]. The in vivo results showed that Cy5.5-SPION micelles with Lf accumulated efficiently in the C6-induced glioma region and prolonged the intensity persistence in tumor sites over 48 h in MR images compared to non-target groups. The MRI results demonstrated that the glioma margin was clearly distinguished from the fluorescence image, and the mean fluorescence intensity of the tumor was about four times higher than that of normal brain tissue. Therefore, these optical/MRI dual-functional micelles (Cy5.5-Lf-SPIO micelles) can specifically target glioma and provide guidance for surgical resection of glioma prior to and during surgery.

Polydiacetylene nanocarriers (PDNCs) exhibit higher sensitivity and color change depending on temperature and pH due to molecular perturbation [31]. Hydrophobic superparamagnetic iron oxide (SPIO) NPs were used as a nano-substrate for spontaneous assembly of 10, 12-pentacocadylic acid, a diacetylene monomer, on the surface through strong ionic and hydrogen bonds under ultraviolet (UV) irradiation. In addition, curcumin (CUR) was incorporated into the shell between SPIO and polymerized 10, 12-pentacosadiynic acid (PCDA), while self-assembled PCDA micelles were formed. PDNC-modified lactoferrin was used to improve the transport of PDNC across the BBB to track and target gliomas. As a result, improved therapeutic efficacy was obtained using Lf-CUR-PDNC, with improved retention time of the encapsulated CUR, and the number of NPs was four times higher in the brain than in the group treated with free CUR. Recent studies have also shown that lactoferrin not only is a ligand for glioma targeting but also inhibits glioblastoma cell growth. This suggests that lactoferrin may play a role in enhancing the anticancer effect in clinical uses such as temozolomide for the treatment of GBM [2].

13.4.3 Albumin

Albumin nanocarriers have been used as drug delivery systems and were successfully used to target drugs to brain tumors. The biodegradable, nonantigenic, and non-toxic characteristics of human serum albumin (HSA) make it an ideal candidate for tumor targeting [24]. The reason for this is that the secreted protein acidic and rich in cysteine (SPARC) and glycoprotein 60 (gp60), albumin-binding proteins, are highly expressed in human glioma cells. On the other hand, since normal BBB blood vessels have a very low level of albumin protein expression, the passage of natural albumin is difficult [24]. The surface of albumin NPs can be transformed into various ligands for enhanced brain targeting. For example, the surface of albumin can be cationized through the binding of ethylenediamine onto the carboxyl group of albumin, and this is an effective form for brain targeting [61]. Cell surfaces in brain endothelium are maintained with a negative charge at physiological conditions (pH). Therefore, positively charged HSA attached to negatively charged endothelial cells by electro-

static interactions, which led to absorption-mediated transcytosis [77].

Several glucose derivatives, such as mannose, galactose, and 2-deoxyglucose, can pass through the BBB via carrier-mediated delivery. For example, mannose can pass through the BBB via glucose transporter 1 (GLUT1) and GLUT3, and across the brain monolayer endothelial cells [55]. Therefore, Byeon et al. designed nanoparticles to contain naive albumin (human serum albumin, HSA), cationic HSA (c-HSA), or mannose-modified albumin (m-HSA) in doxorubicin (DOX) [12]. In vitro, c/m-HSA NPs showed the most prominent transport across the monolayer of bEnd.3 brain endothelial cells and were also absorbed into U87MG glioblastoma cells and spheroids. In vivo xenografted glioma cell-bearing mice were treated with PBS, free DOX or HSA NPs, and c/m-HSA NPs. Among them, the c/m-HSA NP-treated mice group showed significantly smaller tumor size in the brain than other groups. This improved antitumor efficacy can be explained by dual cationic absorption transformation and glucose transport by the combination of c- and m-HSA. Wang et al. used folic acid (FA), a tumor-specific ligand, to coat bovine serum albumin (BSA)-superparamagnetic iron oxide (SPIO) NPs as a contrast agent for MRI. After confirming intracellular absorption and internalization by glioma U251 cells, FA-BSA-SPIO NPs were labeled with fluorescein isothiocyanate (FITC) for intracellular visualization [127].

However, effective intratumoral penetration is another obstacle that leads to drug resistance and cancer treatment failure due to inadequate drug distribution and intracellular concentrations into the tumor hypoxic area. Lin et al. designed self-assembled NPs through hydrophobic interactions with the domains of albumin by adding hydrophobic drugs such as paclitaxel (PTX) and fenretinide (4-HPR) with a large amount of water [73]. Cleavage of the disulfide bond of albumin allowed the protein to form a linear structure, and additional disulfide bridges were formed to further stabilize the NPs. The combination of the two drugs, PTX and 4-HPR, improved the inter- and intra-molecular interactions with linear albumin, and this structure formed more stable hydrophobic cores. These NPs were further modified by low-molecular-weight protamine (LMWP), one of the cell permeability peptides (CPPs), to produce more potent nanoparticles for glioma cell penetration, because CPPs are often used as adjuvants in tumor invasion. LMWP-BSA-NPs showed 2.5-fold higher cellular uptake in U87MG cells than in unmodified BSA-NP via bEnd.3 monolayers. In addition, LMWP-BSA-NPs penetrated significantly deeper into the U87MG spheroids. Compared with the free drug, the cytotoxicity of LMWP-BSA-NP exhibited the highest antitumor activity, although a weaker inhibitory effect was observed in the PTX or 4-HPR treatment group [73]. Based on a strategy of hydrophobic drug-induced albumin self-assembly, Chen et al. also used PTX to induce aggregation of HSA into theragnostic NPs. Albumin was pre-modified using chlorin e6 (Ce6) and cyclic Arg-Gly-Asp (cRGDyK) peptides. Ce6 is a substance used as a chelating agent for Mn^{2+} to enable dual-modal magnetic resonance and fluorescence imaging, and cRGDyK peptide is a peptide capable of targeting the αvβ3-integrin upregulating endothelial cells of tumor vessels [13]. The result was that significant synergistic cancer cell death was observed using NPs under light irradiation, which means that HSA-Ce6@HSA-RGD NPs were able to target αvβ3-integrin. This signifies that HSA-Ce6@HSA-RGD NPs can be applied by combining photodynamic therapy and chemotherapy for treatment of glioma.

13.4.4 Peptide-Based Drugs for Brain Delivery

Protein ligands have several disadvantages that limit their application, including low stability, high immunogenicity, high molecular weight, and high production costs. To avoid these problems, research on peptide-based ligands, rather than proteins, has received increasing attention. There are two common strategies to generate peptide ligands: protein ligand redesign and selection from a peptide library [38]. Rousselle

et al. have shown that doxorubicin increases brain intake in rats when conjugated to a small peptide (SynB1) compared to doxorubicin alone [105]. AngioPep-2 (TFFYGGSRGKRNNFKTEEY, a cell penetrating peptide) also showed enhanced delivery of small molecules through the BBB via low-density lipoprotein receptor-related protein (LRP1) [126]. ANG1005 (also known as GRN1005) is a conjugate of three molecules of paclitaxel and one molecule of AngioPep-2 peptide and can significantly increase paclitaxel delivery in a rat brain perfusion model [120].

Phage display can select peptides capable of binding to specific receptors or cells. Using this method, the T7 peptide, HAIYPRH, was selected for specificity onto transferrin (Tf) receptors through sequential negative and positive selection [70]. T7 was decorated with peptides onto dendrimers to deliver DNA for genetic treatment of gliomas [65]. Modification with T7 significantly increased cell uptake by BCEC, and gene transfer efficiency could be reduced if Tf was exceeded, which means that the T7-modified dendrimer absorption is mediated by the Tf receptor. The T7-modified dendrimer showed 1.7-fold higher gene expression in the brain, demonstrating that T7 can act as an effective brain-targeting ligand. Rabies virus glycoprotein peptide (RVG29) is derived from a rabies virus glycoprotein capable of binding the nicotinic acetylcholine receptor (nAchR) and can enhance drug delivery to the brain [50, 75]. The apparent permeability coefficients of the RVG79-modified poly(mannitol-co-PEI) vector were 2.23 times higher than those for the vector untreated by RVG [97]. In vivo the RVG-modified vector delivered the GADPH siRNA and BACE1 siRNA to the brain more effectively than the unmodified vector.

Homeobox protein (CDX) is a peptide made from the loop II robe of candoxin and is a ligand capable of binding to nAchR. Although the binding affinity of CDX and nAchR is lower than that of candoxin, it showed significantly improved intake in BCEC cells. After being loaded with paclitaxel, CDX-modified NPs demonstrated a better antitumor effect with a prolonged median survival time of 27 days, which was longer than that for untreated NPs [135].

There are other ligands that can recruit proteins from plasmids to bind to specific receptors. Apolipoproteins (Apo), including ApoA and ApoE, are serum proteins that can be delivered to the brain via low-density lipoprotein (LDL) receptors that are highly expressed in the BBB. Thus, peptides derived from ApoA and ApoE showed the ability to mediate brain transmission of nanoparticles [56, 103, 121, 132]. Polysorbate-80, a nonionic surfactant and emulsifier often used in foods and cosmetics, was able to adsorb ApoE in serum when conjugated to NP, and there have been many studies demonstrating that polysorbate-80-coated NPs can target delivery to the brain [37, 81, 129, 130]. Martins et al. evaluated the efficiency of polysorbate-60 and 80 NPs to enhance brain targeting. The plasma area under the curve (AUC) of NPs coated with polysorbate-60 was 1.18 times higher than that of polysorbate-80-coated NPs; however, in the brain, the number of NPs coated with polysorbate-80 was 1.77 times higher than that coated with polysorbate-60 [81]. This result indicates that polysorbate-80 is a better surfactant for brain targeting. Gao et al. also found that the efficiency of brain targeting of NPs coated with polysorbate-80 was affected by the particle size [37]. Comparisons of polysorbate-80-coated NPs with particle sizes of 70, 170, 220, and 345 nm showed that 70-nm NPs delivered the cargo most effectively to the brain.

13.5 Oral Delivery of Protein-Based Drugs to Brain Tumors

It is still challenging to increase the bioavailability of therapeutic peptides and proteins that are administered orally and deliver them to the target site correctly. However, since they have many advantages, work will continue. Because of their small size and high surface area, nanoparticles used to mediate oral peptide delivery improve the bioavailability of these protein drugs (increase long-term drug exposure compared to intermittent intravenous infusion) [57]. However, biocompatibility through intraoral delivery is almost meaningless due to proteolytic degradation and

gastrointestinal (GI) barriers, as these polymers cannot penetrate the intestinal wall. For example, P-gp expressed in the luminal aspect of the plasma membranes of intestinal epithelial cells prevents P-gp substrate-based chemotherapy from adsorbing in the intestine [45]. Thus, for the past several years, various kinds of microparticles and nanoparticles have been used to modify protein and peptide drugs to overcome intestinal barriers and obtain advanced bioavailability in oral administration.

13.5.1 Current Studies on Oral Delivery of Protein Drugs in Brain Tumors

Paclitaxel is a potent chemotherapeutic agent that has been shown to have therapeutic effects on a variety of solid tumors such as breast cancer, lung cancer, and head and neck cancer [44]. Paclitaxel has also been reported to have antiangiogenic properties, and this property indicates that paclitaxel may be a good candidate for the treatment of brain tumors [8, 40, 98]. However, since paclitaxel is a P-gp substrate [80, 117, 125], it is difficult for orally administered paclitaxel to reach the tumor cells from the parenchyma [45, 62]. Therefore, as inhibition of P-pg activity is essential, Paek et al. studied the combination of P-gp inhibitor HM30181A and paclitaxel to produce oral paclitaxel chemotherapy for brain tumors [90, 91]. They have investigated the therapeutic effects of this combination method in two animal models, a melanoma brain metastasis (MBM) mouse model and an early glioblastoma mouse model. Oral co-administration of HM30181A and paclitaxel showed significant therapeutic effects in both brain tumor models.

13.6 Conclusion and Future Perspectives

This chapter highlights important advances in brain delivery of protein drugs that have been studied in recent years. There are still several limitations on cerebral delivery through administration of peptides and protein drugs. Drug delivery to GBM is difficult because the BBB provides physical and biochemical barriers that limit the penetration of most drugs. However, some strategies show significant potential for improvement in brain intake. Therefore, this approach is still under development, but it will play an increasingly important role in the treatment of central nervous system disorders.

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