Nanoparticle-Mediated Delivery of Anticancer Agents to Tumor Angiogenic Vessels

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Drug delivery of anticancer drugs to tumors is a promising approach to improve cancer therapy. Cancer chemotherapy with cytotoxic drugs that damage proliferating cells in a nonspecific manner often involves severe side effects such as bone marrow suppression. Molecular-targeted drugs have also shown unanticipated severe side effects including interstitial pneumonia. These severe side effects restrict the administration of anticancer drugs, resulting in difficulty in eradicating tumors. Nanoparticle-mediated delivery of anticancer drugs to tumors is a potential approach to reduce their side effects and maximize their efficacy. Systemic delivery of anticancer drugs using nanoparticles has been demonstrated to improve the pharmacokinetics and pharmacodynamics. Some anticancer drugs formulated in nanoparticles such as liposomes have already been launched and used in clinical cancer therapy. In addition, since nanoparticles are potential vehicles for gene delivery, small interfering RNA (siRNA) formulated in nanoparticles has been studied in clinical trials of cancer therapy. Encapsulation of anticancer agents in nanoparticles allows their half-life in the blood circulation to be prolonged and the accumulation of these drugs in tumors to be enhanced. Nanoparticles have been shown to accumulate in tumors because of enhanced permeability of angiogenic vessels and lack of functional lymphatic vessels in the tumor microenvironment, which is called the enhanced permeability and retention (EPR) effects.

Accumulation of nanoparticles in tumors is often dominated by the EPR effects. Nanoparticles modified with a specific ligand that recognizes tumor-specific receptors are also theoretically dependent on the EPR effects since they should extravasate into tumors before the ligand-receptor interaction. Hence, the tumor microenvironment such as density and leakiness of angiogenic vessels will affect the efficacy of tumor-targeted drug delivery. In other words, the extravasation of nanoparticles from the blood circulation into tumor tissues is considered to be a rate-limiting step in delivering anticancer drugs. The efficacy of nanoparticle-mediated drug delivery should be higher in vascular-rich tumors than in hypovascular ones. However, drug delivery to hypovascular tumors is an important specific requirement for clinical cancer therapy. Recently, alteration of the tumor microenvironment with certain chemotherapeutic agents such as nitroglycerin and S-1 has been investigated to enhance the EPR effects of nanoparticles, in which the accumulation of nanoparticles in the tumors was enhanced by the drug treatments. These approaches may overcome a barrier to tumor-targeting dependent on the EPR effects.

This review introduces our recent findings on nanoparticle-mediated delivery of anticancer drugs to tumor angiogenic vessels, which is a different approach to evade the rate-limiting step of tumor-targeting via the EPR effects. This strategy is theoretically able to eliminate the limitation of the EPR effects since target angiogenic endothelial cells face the circulating blood. Angiogenic vessel-targeted nanoparticles can contact target endothelial cells without extravasation into tumors. More importantly, angiogenic vessel-targeting is expected to provide potential therapeutic benefits by damaging angiogenic vessels. In this review, the advantages of angiogenic vessel-targeting are discussed to provide an insight into
why angiogenic vessels are a promising target of drug delivery systems (DDS) for cancer therapy.

2. THE CONCEPT OF ANGIOGENIC VESSEL-TARGETING DDS

Since angiogenic vessels for the supply of oxygen, nutrients, and growth factors are necessary for the expansion of solid tumors and for the hematogenous metastasis of cancer, a molecular mechanism of angiogenesis has been elucidated for drug discovery.\(^2\) As a result, inhibitors of proangiogenic factors such as vascular endothelial growth factor (VEGF) have already been approved for clinical cancer therapy. Suppression of tumor growth via angiogenesis inhibition has been demonstrated in preclinical models, and the significance of angiogenesis inhibitors has been confirmed in clinical therapy, whereas the mechanism of action of these inhibitors in cancer patients remains unclear. Certain angiogenesis inhibitors have been shown to normalize tumor angiogenic vessels, which may enhance the delivery of cytotoxic drugs to tumors in clinical combination therapy.\(^3\) Further research on the mechanism of antiangiogenic therapy is required to increase the efficacy of angiogenesis inhibitors because the limited efficacy and resistance of these inhibitors remain problems in clinical therapy.\(^4,5\) In addition, since angiogenesis inhibitors have been shown to facilitate lymphatic and distant metastasis in certain cancer models,\(^6\) the effects of these inhibitors on tumor microenvironments should be elucidated in detail.

On the other hand, we have explored a DDS targeting angiogenic vessels in which cytotoxic drugs,\(^7,8\) angiogenesis inhibitors,\(^9,10\) and nucleic acids such as siRNA\(^11,12\) have been delivered to angiogenic endothelial cells using targeted liposomes. Since proliferating cells are killed by cytotoxic drugs nonspecifically, not only tumor cells but also angiogenic endothelial cells should be attacked by these drugs. The purpose of our strategy using cytotoxic drugs is complete disruption of angiogenic vessels by inducing apoptosis of target endothelial cells, for which we have tried to develop targeted liposomes. This is a major difference compared with other antiangiogenic strategies. Cytotoxic drugs delivered to angiogenic endothelial cells are expected to show different therapeutic actions compared to angiogenesis inhibitors. Complete eradication of angiogenic vessels may lead to indirect lethal damage to tumor cells even at a distance from these vessels by depletion of oxygen and nutrients, and at the same time the route of hematogenous metastasis may be blocked. Since a relatively small number of endothelial cells exists to support large numbers of tumor cells in tumor tissues, targeting endothelial cells may be an efficient method to deliver cytotoxic drugs. Furthermore, angiogenic endothelial cells are genetically stable and less likely to acquire drug resistance, which also indicates that these cells are an ideal therapeutic target.

3. LIPOSOMAL DDS TARGETING TUMOR ANGIOGENIC VESSELS

Angiogenic endothelial cells express specific receptor molecules that are not or only rarely expressed on normal endothelial cells,\(^21\) which enables the design of targeted nanoparticles. Selective ligands for these receptors are considered to be useful for angiogenic vessel-targeting DDS. We have explored such a ligand by in vivo biopanning from a phage-displayed peptide library.\(^22\) The biopanning was performed using an in vivo tumor angiogenesis model to obtain a small peptide specific to angiogenic vessels but not to tumor cells (Fig. 2). As a result, the Ala-Pro-Arg-Pro-Gly (APRPG) peptide was successfully identified as an angiogenic vessel-targeting ligand. An M13 phage clone expressing APRPG accumulates in tumors implanted in mice independent of tumor type. Binding of APRPG to human angiogenic vessels was demonstrated by histologic staining of human tumor sections. Taken together, APRPG should have an affinity for both murine and human angiogenic vessels. These results indicate that APRPG can be evaluated in murine experimental models for preclinical study and at the same time is possibly applicable to human cancer. Our recent data have demonstrated that APRPG selectively binds to VEGF receptor-1.\(^24\)

Liposomes modified with APRPG (APRPG liposomes) were developed for angiogenic vessel-targeting DDS.\(^12\) The biodistribution of APRPG liposomes after intravenous injection was examined in Meth A sarcoma- and Colon26 NL-17 carcinoma-bearing mice. APRPG liposomes labeled with \(^3\)H showed significant accumulation in those subcutaneous tumors compared with nontargeted liposomes. Noninvasive pharmacokinetic analysis of \(^19\)F-labeled APRPG liposomes using positron emission tomography also revealed that APRPG significantly increases the tumor accumulation of liposomes in tumor-bearing mice. Next, APRPG-modified PEGylated liposomes (APRPG-polyethylene glycol (PEG) liposomes) were developed to increase their half-life in the blood circulation and opportunity for binding to angiogenic vessels. As expected, the accumulation of APRPG-PEG liposomes in subcutaneous tumors was substantially higher than that of APRPG liposomes. However, no marked difference in tumor accumulation was observed between PEGylated and APRPG-PEG liposomes.\(^26\) Both PEGylated and APRPG-PEG liposomes exhibit similar long circulation times, distribution in each organ, and overall tumor accumulation, whereas the intratumoral distribution of these liposomes differs. Immunohistochemical analysis of tumor sections clearly revealed that APRPG-PEG liposomes associate with angiogenic vessels in tumors.\(^27\) In contrast, PEGylated liposomes extravasate into tumors without binding to angiogenic vessels and then remain near these vessels.\(^27\)

The biodistribution of APRPG-PEG liposomes was also examined in an orthotopic xenograft model of SUIT-2 human
pancreatic cancer. The tumor of this orthotopic model was found to be hypovascular by histologic analysis. APRPG-PEG liposomes were also shown to associate with angiogenic vessels in orthotopic pancreatic tumors (Fig. 3), although the total amount of these liposomes accumulated in the tumor was lower than our data in the subcutaneous models. Interestingly, PEGylated liposomes are mainly detected inside blood vessels in orthotopic pancreatic tumors 2 h after intravenous injection, which is not consistent with our data in the subcutaneous models. In the case of subcutaneous tumors, a large amount of PEGylated liposomes extravasate into the tumors 2 h after intravenous injection. These results indicate that extravasation of PEGylated liposomes into tumors is affected by differences in the tumor microenvironment.

Our data from biodistribution studies suggest that passive targeting via the EPR effects can affect the total amount of PEGylated liposomes accumulated in tumors regardless of APRPG conjugation, and that APRPG contributes to intratumoral localization of PEGylated liposomes in angiogenic vessels.
4. THERAPEUTIC EFFICACY OF ANGIOGENIC VESSEL-TARGETED LIPOSOMES ENCAPSULATING CYTOTOXIC DRUGS

Doxorubicin encapsulated in the aqueous phase of APRPG liposomes (APRPG L-dox) was prepared to verify the concept of angiogenic vessel-targeting DDS. The therapeutic efficacy of APRPG L-dox was superior to that of nontargeted L-dox in Meth A sarcoma- and Colon26 NL-17 carcinoma-bearing mice (Fig. 4). APRPG L-dox was shown to damage angiogenic vessels, suppress tumor growth, and prolong survival time in tumor-bearing mice significantly without any apparent side effects.12) Importantly, APRPG L-dox suppresses tumor growth even in mice bearing dox-resistant P388 solid tumors, possibly through induction of apoptosis of angiogenic endothelial cells.15) The antiangiogenic action of APRPG L-dox is considered to be mainly exerted through enhanced internalization of APRPG L-dox into angiogenic endothelial cells, although an increase in the local concentration of free dox gradually released from APRPG L-dox may partly contribute to this action. To distinguish between angiogenic vessel-targeting and gradual drug-release effects, we developed APRPG liposomes containing a water-insoluble anticancer drug since such a drug is expected to be delivered to target cells in a liposomal form. For this purpose, \(5'-O\)-dipalmitoylphosphatidyl \(2'-\text{cyano-2'-deoxy-1-\text{beta}-D-arabino-pentofuranosylcytosine}\) (DPP-CNDAC), a lipophilic derivative of a novel anticancer drug, encapsulated in the lipid bilayer of APRPG liposomes (APRPG L-CN) was prepared.13) The therapeutic efficacy of APRPG L-CN is superior to that of nontargeted L-CN in Meth A sarcoma-bearing mice, although the local concentration of free dox does not differ greatly. The difference in therapeutic efficacy between APRPG-PEG L-dox and PEG L-dox appears to be due to the intratumoral distribution of these liposomes. APRPG-PEG L-dox binds to angiogenic vessels in the tumors, similar to APRPG L-dox. These data suggest that APRPG-PEG liposomes encapsulating dox (APRPG-PEG L-dox) were designed to prepare long-circulating APRPG L-dox.26,27) APRPG-PEG L-dox shows more potent inhibition of tumor growth than PEGylated liposomes encapsulating dox (PEG L-dox) in Colon26 NL-17 carcinoma-bearing mice, although the local concentration of these liposomes in the tumors does not differ greatly. The difference in therapeutic efficacy between APRPG-PEG L-dox and PEG L-dox appears to be due to the intratumoral distribution of these liposomes. APRPG-PEG L-dox binds to angiogenic vessels in the tumors, similar to APRPG L-dox. These data suggest that APRPG L-dox markedly inhibited tumor growth in Meth A sarcoma-bearing mice (Fig. 4) and in Colon26 NL-17-bearing mice (b) (n=6; *p<0.001). Data are presented as mean tumor volume and SD. Arrows show the day of treatment.
contributes to cellular uptake in angiogenic vessels rather than overall tumor accumulation as in the case of stealth liposomes, which results in the potent therapeutic efficacy of APRPG-PEG L-dox through damaging angiogenic vessels. Similar results were obtained using DPP-CNDAC encapsulated in APRPG-PEG liposomes.18)

We investigated whether an angiogenic vessel-targeting DDS is applicable against hypovascular cancers because the EPR effects appear to be insufficient to treat these cancers. For this purpose, an orthotopic xenograft model of hypovascular cancer was established using SUIT-2 human pancreatic cancer. APRPG-PEG L-dox significantly inhibited the growth of hypovascular tumors compared with PEG L-dox (Fig. 5), although the accumulation of APRPG-PEG liposomes in the tumors was not markedly different from that of PEGylated liposomes.17) APRPG-PEG L-dox selectively damages angiogenic vessels in hypovascular tumors, which can result in enhanced antitumor activity. Topologic change is considered to be critical for the difference in therapeutic efficacy between APRPG-PEG L-dox and PEG L-dox. Our data suggest that angiogenic vessel-targeting DDS will be effective to treat hypovascular cancers.

In these therapeutic studies, since APRPG-PEG L-dox induces apoptosis of not only angiogenic endothelial cells but also tumor cells, the actual anticancer effects appear to be achieved through total damage of these cells. Still, our data suggest that increased apoptosis of angiogenic endothelial cells by targeting DDS will lead to enhanced therapeutic efficacy.

5. PROSPECTS FOR ANGIOGENIC VESSEL-TARGETING DDS

Recent progress in cancer research has yielded anticancer drug candidates based on a new mechanism of action. These candidates include cytotoxic drugs, molecular-targeted ones, and RNA interference effectors. Most of these candidates have been designed to increase selectivity toward tumor cells, whereas drug delivery to tumors still remains a problem. Research and development of DDS technologies are a promising approach for advanced cancer therapy. We have proposed an angiogenic vessel-targeting DDS that is theoretically and practically different from previous targeting DDS. Although we have shown the concept of angiogenic vessel-targeting DDS using the APRPG peptide, targeting ability and therapeutic efficacy are anticipated to be enhanced by further research. Since molecules expressed on angiogenic endothelial cells should alter dependent on the maturity of angiogenic vessels and on cancer type, the appropriate ligand for targeting will not necessarily be the same as the ligand we have studied. To improve the targeting ability of angiogenic vessel-targeting DDS, nanoparticles modified with multiple types of ligands can be used. We found that dual-targeting liposomes modified with APRPG and Gly-Asn-Gly-Arg-Gly (GNGRG) peptides,
an aminopeptidase N (CD13) ligand that binds to angiogenic vessels in tumors, shows enhanced targeting ability compared with targeting liposomes modified with the single peptide20) (Fig. 6).

This review mainly introduces the delivery of cytotoxic drugs to angiogenic vessels; on the other hand, delivery of angiogenesis inhibitors to these vessels is also effective in enhancing their efficacy.21, 22, 23 Although angiogenesis inhibitors have a selective mechanism compared with cytotoxic drugs, the limited efficacy of these inhibitors remains a problem. To improve both selectivity and therapeutic efficacy at the same time, vascular disrupting agents (VDAs),23, 24 a new class of potential anticancer drugs, may be ideal for angiogenic vessel-targeting DDS. VDAs can destroy tumor angiogenic vessels, although their clinical development is hampered by cardiovascular and neurologic toxicities. Since liposomal DDS are expected to reduce the side effects of VDAs, the application of angiogenic vessel-targeting DDS to VDA therapy is a possible approach. Another promising approach is siRNA delivery by angiogenic vessel-targeting DDS. Our therapeutic concept can be achieved by the selective induction of apoptosis in angiogenic endothelial cells via gene knockdown. For this purpose, we developed siRNA vectors modified with APRPG and formulated therapeutic siRNA candidates in these vectors.23, 24, 29) Our results revealed that APRPG-modified vectors can be applicable to the systemic delivery of siRNA to angiogenic vessels.

In conclusion, angiogenic vessel-targeting DDS are an interesting approach to eliminate the limitations of previous DDS strategies in treating cancer. It is expected that this approach will lead to beneficial clinical anticancer effects.

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