論 文 の 内 容 の 要 旨

論文題目　Study on the Design of Antibody-Conjugated Polymeric Micelles and Their Biological Performance for Tumor Targeted Therapy
（抗体結合型高分子ミセルの設計及びがん標的治療への応用に関する研究）

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Antibody-drug conjugates (ADC) are attracting much interest in cancer therapy due to the improvement of therapeutic efficacies by selectively delivering anticancer drugs to cancer cells compared to conventional chemotherapies. The development of ADC has been one of the most active areas in recent years, and as much as thirty ADC have entered clinical evaluation in 2013 for the treatment of solid tumors and leukemia. Nevertheless, a major challenge in the development of ADC is the limited amounts of drugs that can be delivered by a single antibody, as overloading may reduce the binding affinity of the antibody or affect the pharmacokinetics. Thus, 2 to 4 cytotoxins per antibody are generally introduced in an ADC for accomplishing effective therapeutic responses without compromising the affinity of the antibody. Consequently, the drugs conjugated to the antibody must be highly cytotoxic, such as auristatins, maytansines and calicheamicins, which are 100-1,000 times higher than typical anticancer drugs, for exerting enough efficacy, although there are growing concerns over side effects from decomposition of ADC under physiological conditions. However, these obstacles of ADC may be overcame by integrating antibodies to drug-loaded nanocarriers, which are capable of delivering a significantly higher amount than ADC.

Among long-circulating nanocarriers with improved tumor extravasation and penetration, polymeric micelles offer substantial benefits as platform nanocarriers for conjugating antibodies. Polymeric micelles present high and versatile loading of bioactive molecules and their controlled release, and show prolonged blood circulation (stealth property) due to their surface coverage by biocompatible PEG strands. Besides the relatively small size of micelles ranging from 10 to 100 nm, they exhibit enhanced tumor accumulation by the enhanced permeability and retention (EPR) effect through leaky vasculatures and impaired lymphatic drainage in solid tumors. To date, a few antibody-conjugated micelles (immunomicelles) were reported for specific delivery of drugs in cancer therapy. However, no immunomicelle has proceeded to clinical evaluation so far. Therefore, using micellar platform with high

potential for clinical translation should facilitate the development of anticancer therapies based on immunomicelle.

Herein, we introduced the antibody fragments to polymeric micelles incorporating an active complex of oxaliplatin, (1,2-diaminocyclohexane)platinum(II) (DACHPt) (DACHPt/m), which have shown strong therapeutic activity against several cancer models and are being evaluated in phase I clinical studies. Moreover, because DACHPt/m can improve the efficacy of the loaded platinum drugs by enhanced delivery to solid tumors and effective intracellular drug release, by conjugating antibody fragments to these micelles, therapeutic effects could be maximized through antibody-antigen recognition and subsequent cellular uptake. Thus, the present study aimed to prepare antibody-conjugated DACHPt/m using DACHPt/m platform and confirm their potential in anticancer therapy. For the antibody introduction to the micelle surface, DACHPt/m was modified to have surface-functionality by using the maleimide end-functionalized PEG-*b*-P(Glu). The proportion of maleimide functionality on the periphery of micelles was controlled by directly adjusting the ratio of PEG-*b*-P(Glu) and Mal-PEG-*b*-P(Glu). In this way, 20% or 50% maleimide-surface functionalized DACHPt/m (Mal-DACHPt/m) was prepared, and the diameter of these Mal-DACHPt/m was approximately 30 nm as determined by dynamic light scattering (DLS) measurement. The maleimide groups on Mal-DACHPt/m allow introducing antibodies through thiol-maleimide reaction, which is one of the widely used linking systems for the antibody conjugation.

For the tumor-directed therapy, tissue factor (TF), a transmembrane receptor that is upregulated on various human cancer cell membranes or vascular endothelial cells in angiogenic vasculatures, was targeted by using anti-TF antibody fragment Fab'. Because TF is expressed both on cell surfaces and angiogenic vasculatures, the types of TF-targeting antibodies were expanded to anti-human TF (AH-TF) antibody or anti-mouse TF (AM-TF) antibody, in order to select target sites in mouse xenografts bearing human tumors such as human-TF (hTF) overexpressing tumor cells or mouse-TF (mTF) overexpressing angiogenic vasculatures. This site-specific targeting system against tumor microstructures could provide insight of the mechanism in the treatment of malignant tumors.

To decrease the size of antibody-conjugate micelles as well as reduce immunogenicity, thiol-having antibody fragment Fab' was fabricated through the reduction with dithiothreitol (DTT) and confirmed by gel permeation chromatography (GPC). The anti-TF Fab' was introduced to the surface of the micelle by conjugation of thiol-maleimide reaction. The conjugation condition was determined by monitoring the reaction between Alexa 488 fluorescence-labeled anti-TF Fab' and Alexa 647 fluorescence-labeled Mal-DACHPt/m using GPC equipped with fluorescence detectors. The colocalization of both fluorescence peaks at the same retention time revealed the successful conjugation of Fab' to DACHPt/m. Furthermore, fluorescence correlation spectroscopy (FCS) showed the direct evidence of Fab' conjugation onto the micelles, as diffusion coefficients of Fab'-installed DACHPt/m decreased from that of Fab'. In addition, it was found that one antibody was conjugated to each 50% Mal-DACHPt/m while immunomicelles that obtained from 20% Mal-DACHPt/m were a mixture of

micelles with and without Fab'. Therefore, 50% Mal-DACHPt/m was chosen for the evaluation for biological performance. The DLS measurement revealed that the diameter of obtained micelles, AH-IM/m and AM-IM/m, was not changed through whole preparation process. This relatively small size of immunomicelle (~30 nm) could be a significant advantage for effective extravasation and penetration in stroma-rich tumor tissues of pancreatic cancer.

The effect of introduced Fab' on the surface of DACHPt/m on cell binding activity was determined by flow cytometry after 1 h incubation of Alexa 647-labeled AH-IM/m, AM-IM/m and DACHPt/m against various human cancer cell lines. The average fluorescence levels in the hTF-overexpressing cells treated with AH-IM/m were more than 15-fold higher than control cell lines, indicating the promoted cellular binding of these micelles after 1 h incubation, while DACHPt/m or AM-IM/m did not show any cellular binding. However, AH-IM/m did not exhibit increased binding activity against hTF non-expressing cell line, suggesting that the AH-IM/m is immunologically specific. Additionally, competition experiments with 10 times excessive AH-TF $F(ab')_2$ showed significant decrease of binding activity of AH-IM/m to the same level of control cells. Thus, it was concluded that the interaction of antibody-antigen contributes the increase of binding activity of AH-IM/m against hTF-overexpressing cell lines. Moreover, the stability of antibody surface-introduced DACHPt/m was measured by flow cytometry by assessing the affinity of AH-IM/m that was kept at 4 ^{o}C more than 30 days. The results illustrated that AH-IM/m maintained strong affinity to hTF-overexpressing tumor cells. Considering conventional ADC lost their structures and decomposed in a few hours, this significant improvement in stability using antibody-conjugated DACHPt/m could be substantial advantages in anticancer therapy.

To confirm the internalization of immunomicelles in cancer cells, confocal laser scanning microscopy (CLSM) was performed with fluorescence-labeled AH-IM/m, AM-IM/m and DACHPt/m. Monitoring and quantification of fluorescent intensity clearly indicated the rapid binding and internalization of AH-IM/m to hTF-overexpressing BxPC3 cells. Compared with DACHPt/m or AM-IM/m, the promoted cellular uptake of AH-IM/m was verified against hTF-overexpressing PANC1 cells. These results suggest that the hTF-targeting AH-TF Fab' critically contributes to determine cellular binding and subsequent uptake efficiency. By staining the late-endosomes and lysosomes with LysoTracker, the colocalization of AH-IM/m and late-endosomes/lysosomes in BxPC3 cells was confirmed, suggesting that AH-TF Fab' promoted cellular uptake. The *in vitro* cytotoxicity of immunomicelles was studied in BxPC3 and PANC1 cells by exposing micelles for 3 h followed by post-incubation for 48 h. As a result, AH-IM/m indicates more than 5 times decreased IC_{50} value than DACHPt/m or AM-IM/m against both cell lines. It is worthy of note that, AH-TF Fab' did not show any *in vitro* cytotoxicity against BxPC3 cells under the tested conditions, indicating that the cytotoxicity of AH-IM/m can be attributed exclusively to the enhanced intracellular delivery of cytotoxic DACHPt. Consequently, these results support that, by introducing AH-TF Fab' on the surface of DACHPt/m, the

micelles could undergo the antibody-mediated rapid binding and uptake in hTF-overexpressing cancer cells. Moreover, as the release of DACHPt from the micelles is enhanced in late endosomal compartments, the colocalization of AH-IM/m with late endosomes/lysosomes may contribute to the substantial improvement of the *in vitro* cytotoxicity.

In vitro verified TF-targeting immunomicelles were challenged whether they exert therapeutic activity toward mouse xenograft bearing human pancreatic adenocarcinoma BxPC3. AH-IM/m suppressed the growth of tumors for approximately 40 days ($p < 0.01$), outperforming DACHPt/m or AM-IM/m. It is worth mentioning that, even though BxPC3 xenografts present characteristics of intractable pancreatic cancer, including poor vascularization, perycite-covered vasculature and thick fibrosis, which impede the access of therapeutic agents, polymeric micelles with the size smaller than 50 nm showed facilitated penetration and accumulation in this tumor model. Both DACHPt/m and AH-IM/m showed similar accumulation in tumor tissues, indicating the prolonged antitumor efficacy achieved by AH-IM/m could be attributed to the combined effect of TF-targeting and effective tumor penetration. Although AM-IM/m was expected to target angiogenic vasculatures or stroma where mTF are up-regulated, the therapeutic activity was not as significant as AH-IM/m. At the assessed dosage, every micelles were non-toxic according to the body weight profile. In addition, co-injection of AH-TF Fab' and Mal-DACHPt/m resulted in lower antitumor activity than AH-IM/m ($p < 0.01$), indicating that AH-TF Fab' may not exert any antitumor effect at the applied dose and that the thiol-maleimide conjugation of AH-TF Fab' on the micelles was an effective strategy for enhancing drug delivery. Recent findings in a preliminary attempt, AH-IM/m as well as AM-IM/m could excellently suppress the tumor growth in pancreatic cancer PANC1 subcutaneous tumor model, suggesting the therapeutic potentials of immunomicelles to be applied in wide range of TF-overexpressing tumor models by targeting angiogenic vasculatures.

In conclusions, the enhanced antitumor efficacy of AH-IM/m without impairing the safety of parent micelles, as at least suggested from the negligible loss in the body weight of treated mice, confirmed the advantages of *in vivo* tumor targeting by immunomicellar system loaded with platinum drugs, which are key drugs for many clinical anticancer therapies, for the first time. The strategy of one-to-one conjugation of Fab' fragment of antibody to polymeric micellar surface by maleimide-thiol coupling is applicable to a broad variety of cargo molecules and antibodies, including clinically approved tumor-directed antibodies, without substantial change in the structure and the size of parent micelles. Hence, it should provide a universality to deliver therapeutic agents into stroma-rich intractable tumors with strict limitation in extravasation of carrier systems, including pancreatic tumor as reported here, and enhance their therapeutic efficacy.