Research Article

Journal of PeptideScience

Received: 1 November 2010 Revised: 2 December 2010 Accepted: 11 December 2010 Published online in Wiley Online Library: 24 February 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1349

Characterization of antilytic peptide antibody: application for the detection of lytic-based hybrid peptide in serum samples

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We previously reported that a novel targeted drug termed hybrid epidermal growth factor receptor (EGFR)-lytic peptide, made by chemical conjugation of targeted binding peptide and cell-killing, lytic-peptide components, has selective cytotoxic activity that allows it to discriminate between normal and cancer cells. In addition, *in vivo* analysis revealed that this hybrid peptide displays significant antitumor activity in a xenograft model of human breast and pancreatic cancer in mice. Here, we characterized antilytic peptide antibody, which was raised from rabbit serum using the antigen of lytic peptide conjugated with keyhole limpet hemocyanin. It was found that antilytic peptide antibody is specific to the lytic peptide as assessed by both ELISA and surface plasmon resonance analysis and can also bind to EGFR-lytic peptide. Epitope mapping analysis using Biacore showed that two successive lysine regions in the lytic-peptide sequence are significant for recognition by this antibody. In addition, it was shown that this antibody can detect lytic-based hybrid peptide in serum samples from mouse blood and also in cultured breast cancer MDA-MB-231 cell samples by immunocytochemical staining experiments. It was found that the maximum concentrations of this peptide in serum were reached within 15–30 min of i.v. administration of EGFR-lytic peptide to mice. These results indicate that this antibody will be a useful tool for the detection of lytic-based peptides to investigate their *in vivo* stability and pharmacokinetics. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hybrid peptide; lytic peptide; tumor targeting; antipeptide antibody; Biacore system

Introduction

Therapeutic peptides are gaining increasing popularity as therapeutic agents for a variety of applications [1], including as tumor vaccines [2], in antimicrobial therapy [3], and in nucleic acid delivery [4]. In addition, research and development of new cancer therapeutics involving peptide-based drugs have been undertaken [5,6]. It is also known that peptide therapeutics are relatively easily generated using either recombinant or solid-phase chemical synthesis techniques and are generally less expensive when compared with antibody-based therapeutics. We have previously reported that hybrid epidermal growth factor receptor (EGFR)-lytic peptide, which is a chemically conjugated peptide of EGFR-binding amino acid sequence and cell-killing, lytic-peptide amino acid sequences, has selective cytotoxic activity to discriminate between normal and cancer cells to overcome the resistance of cancer cells to tyrosine kinase inhibitor drugs [7]. We also demonstrated that the hybrid peptide can penetrate the cancer cell membrane quickly by making a pore and induce cancer cell death within 20 min as assessed by confocal microscopy analysis using EGFR-lytic peptide labeled with carboxytetramethylrhodamine (TAMRA) [7]. In addition, *in vivo* analyses revealed that EGFR-lytic peptide displayed significant antitumor activity in a xenograft model of human breast and pancreatic cancer, regardless of the presence of K-ras mutation [7].

Determining the stability profile of a peptide drug administrated *in vivo* is necessary for pharmacokinetic analysis of the drug. Recently, the technique of using synthetic peptides to generate antibodies in animals has gained popularity. The advantage of using peptides instead of proteins as antigens includes ready availability [8] and the ease of producing antipeptide antibodies specifically against protein isoforms or site-specific phosphorylated proteins. Although multiple-antigen peptide systems [9–11] requiring no carrier protein have been used to generate antipeptide antibodies, for routine mainstream production of antisera, the synthetic peptides are coupled to carrier proteins to immunize animals, and these conjugations normally elicit a strong immune response. Keyhole limpet hemocyanin (KLH)- and BSA-conjugated peptides are two major kinds of antigens used to generate antipeptide antibodies [12].

In this study, lytic peptide conjugated with KLH was used to immunize a rabbit and then we successfully isolated and purified antilytic peptide antibody from rabbit antiserum. This antibody specifically recognized not only lytic peptide but also lytic-based hybrid peptide with high affinity. Here, we describe the characterization of antilytic peptide antibody by ELISA and surface plasmon resonance (SPR) biosensor technology using the Biacore system and the application of this antibody to the investigation of the stability of EGFR-lytic peptide in mouse blood and immunocytochemical staining of cancer cells.

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Table 1. Peptide sequences used in this study

Peptides	Sequences[a]
EGFR-lytic peptide	YHWYGYTPQNVIGGGKL***L***LK***L***L***KK***LLK***L***LKKK
Wild-type lytic (D, L)	KL***L***LK***L***L***KK***LLK***L***LKKK
Wild-type lytic (L)	KLLLKLLKKLLKLLKKK
Deletion 1	KL***L***LK***L***L***KK***LLK***L***L
Deletion 2	KL***L***LKLL***KK***
Deletion 3	KL***L***LK***L***L
Deletion 4	***KK***LLK***L***LKKK

[a] Italic and underlined letters indicate D-type amino acids.

Materials and Methods

Materials

BSA and KLH were purchased from Sigma (St Louis, MO, USA). PD-10 column and horseradish peroxidase (HRP)-conjugated antirabbit IgG were from GE Healthcare (Buckinghamshire, England). Alexa 488-labeled goat antirabbit IgG was from Invitrogen (Carlsbad, CA, USA). Other agents were mostly from Nacalai Tesque (Kyoto, Japan). All agents were of reagent-grade quality.

Peptide Synthesis

Peptides used in this study were synthesized by Invitrogen or Sigma. All peptides were synthesized by the use of solid-phase chemistry, purified to homogeneity (i.e. >90% purity) by reversed-phase high-pressure liquid chromatography, and assessed by mass spectrometry. Peptides were dissolved in water and buffered to pH 7.4. Peptide solutions were prepared freshly every time immediately before use and were not stored. Peptide sequences used in this study are listed in Table 1.

Isolation of Antilytic Peptide Antibody

Coupling of lytic peptide to KLH was carried out as described previously using sulfo-*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (SMBS) [8]. Antilytic peptide antibody was obtained from custom antisera by Sigma Genosys (Sigma-Aldrich Japan, Ishikari, Japan). Briefly, a rabbit was immunized subcutaneously with 1 mg of lytic peptide/carrier protein (KLH) conjugate using SMBS in 1 ml of phosphate-buffered saline (PBS) mixed 1 : 1 with complete Freund's adjuvant. The rabbit was boosted with 0.5 mg of peptide/carrier conjugate in 1 ml of PBS mixed 1 : 1 with incomplete Freund's adjuvant at Week 4, and this was repeated at Week 8. The rabbit antiserum was collected 10 days after the last immunization and then serum was tested using ELISA. Obtained antiserum was further purified using lytic peptide-SMBS-KLH-conjugated agarose column.

ELISA

ELISA using antilytic peptide antibody was performed as described previously [13]. Briefly, 1 μg of lytic peptide, lytic peptide conjugated with KLH using SMBS (lytic-SMBS-KLH), or KLH were coated on 96-well plates using Tris/HCl buffer, pH 8.5. Wells were washed using PBS containing 0.1% Tween 20, blocked with 1% skim milk in PBS for 1 h, and then several diluted antiserum samples were added to the plates. After the second antibody reaction using HRP-conjugated antirabbit IgG, enzyme activity was detected using *o*-phenylene diamine as a substrate.

Surface Plasmon Resonance

SPR experiments were performed using the Biacore biosensor 2000 or T100 system as described previously [14,15]. Briefly, antilytic peptide antibody was immobilized on the surface of CM5 sensor chips by *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide activation chemistry according to the manufacturer's instructions. As the analyte, several concentrations of peptide were injected over the flow cell at a flow rate of 30 μl/min at 25 °C. HBS-EP buffer (0.01 M Hepes/0.15 M NaCl/0.005% Tween 20/3 mM EDTA, pH 7.4) was used as a running buffer during the assay to inhibit nonspecific binding. For epitope mapping of antilytic peptide antibody using several lytic peptides with deletions (Table 1), responses obtained at a concentration of 250 nM were divided by the molecular weight of these peptides to allow a comparison of binding levels as described previously [16]. Concentrations of EGFR-lytic peptide in mouse serum samples were calculated from equilibrium binding (R_{eq}) values at a flow rate of 30 μl/min at 25 °C. All data analysis was performed using BIA evaluation version 4.1 software.

Animal Experiments

EGFR-lytic peptide (10 mg/kg) or PBS (control) was injected i.v. to BALB/cAJcl mice (Clea Japan Inc., Hamamatsu, Japan). After the mice were killed, mice sera were purified from blood by centrifugation at 3000 rpm.

Immunocytochemistry

Immunocytochemical staining was performed as described previously [17]. Briefly, MDA-MB-231 breast cancer cells treated with or without 10 μM EGFR-lytic peptide for 30 min were fixed with 4% paraformaldehyde in PBS, and then fixed cells were blocked with 1% BSA at room temperature for 1 h. The first antibody reaction was performed with antilytic peptide antibody (1 : 10 000) for 24 h, followed by Alexa 488-labeled goat antirabbit IgG (1 : 200). Propidium iodide (PI) was then added to label all the nuclei. Fluorescence images were captured using an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Results

Isolation and Characterization of Antilytic Peptide Antibody from Rabbit Antiserum

With the aim of isolating antilytic peptide antibodies, we immunized a rabbit using lytic peptide/carrier protein (KLH) conjugate. We successfully isolated rabbit antiserum, which recognized lytic peptide/carrier protein with high affinity, from immunized rabbit blood, and then further purification was performed using lytic-peptide-conjugated affinity column chromatography (data not shown). Figure 1(A) and (B) demonstrates that purified antilytic peptide antibody from rabbit antiserum could recognize lytic peptide (D, L; which contains both D- and L-type amino acids) by ELISA. Biacore biosensor analysis also indicated that the binding curves of injected lytic peptides to immobilized antilytic peptide antibody were typical sensorgrams for antibody binding to an antigen on the Biacore system, as it showed that the association occurs gradually at low concentrations of the analytes and that dissociation occurs slowly at both high and low analyte concentrations. The K_D value of the antilytic peptide antibody for lytic peptide was 3.11×10^{-9} M, whereas this antibody did not recognize KLH without lytic peptide (data not shown). These observations indicate that purified antilytic peptide antibody can recognize the

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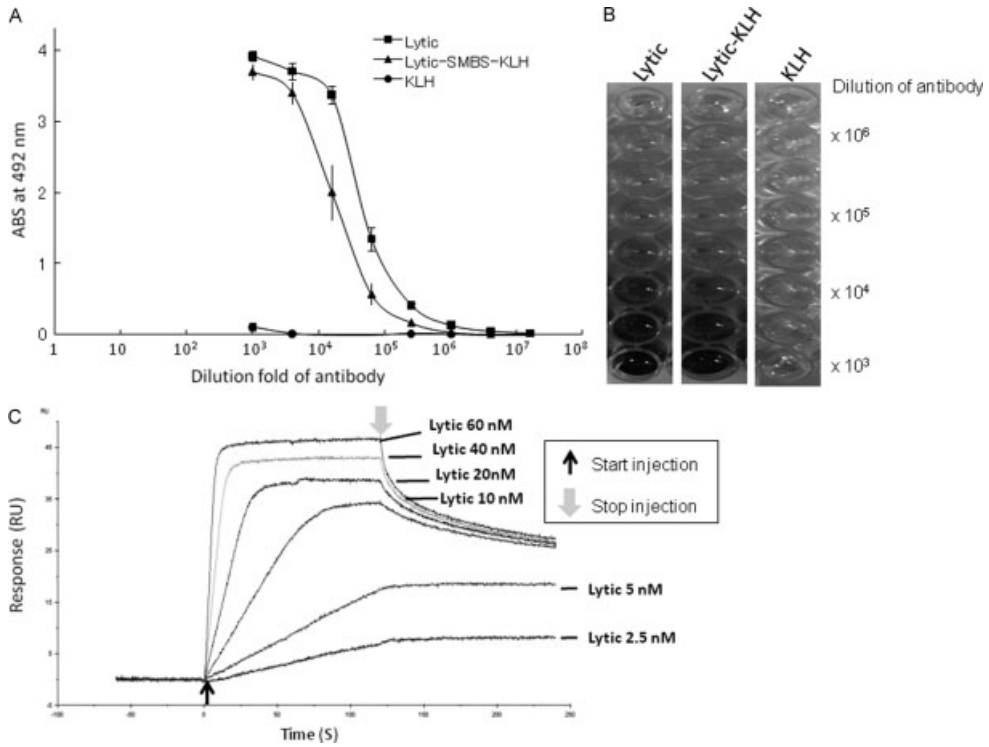

Figure 1. Sensitivity and specificity of antilytic peptide antibody purified from rabbit antiserum. (A) ELISA of lytic peptide, lytic peptide-KLH, and KLH with antilytic peptide antibody. Lytic peptide, lytic peptide conjugated with KLH by SMBS (Lytic-SMBS-KLH), or KLH alone were used to assess the specificity and sensitivity of the antilytic peptide antibody. (B) Photograms from spots with diluted antibody in the ELISA assay. (C) Sensorgrams for the binding of lytic peptide to immobilized antilytic peptide antibody by Biacore biosensor. All analytes (60, 40, 20, 10, 5, and 2.5 nM) of lytic peptide were injected over immobilized antilytic peptide antibody on the sensor chip. Progress in the binding of lytic peptide to immobilized antilytic peptide antibody was assessed by following the increase in the signal (expressed in terms of RU, resonance units). The thin and thick arrows indicate the beginning and end of the injection, respectively.

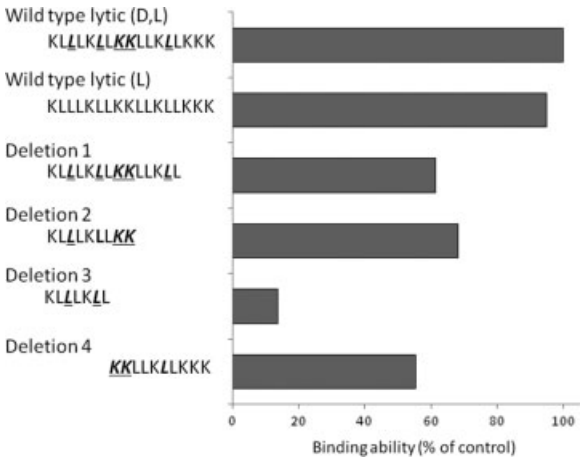

Figure 2. Epitope mapping of antilytic peptide antibody by SPR analysis. Binding ability was calculated from the responses obtained at a peptide concentration of 250 nM using the Biacore system as described in Section on Materials and Methods. The value of binding ability for the wild-type lytic peptide was set to 100%. Italic, underlined letters indicate D-type amino acids.

small lytic peptide with high affinity, which is in agreement with the ELISA results (Figure 1(A) and (B)).

Significance of Two Successive Lysine Regions for the Recognition by Antilytic Peptide Antibody

Using the Biacore system, we next assessed which regions of the lytic peptide are significant for recognition by the antilytic peptide antibody. Epitope mapping analysis of this antibody using several deletion mutants of the lytic peptide showed that two successive regions of lysines in the lytic peptide sequence are significant for recognition by the antibody (Figure 2). Interestingly, wild-type lytic peptide (L), in which all amino acids are of the L-type, was also recognized by this antibody (Figure 2). However, the association sensorgrams of this peptide to antilytic peptide antibody were different from those of wild-type lytic peptide (D, L), which contains both D- and L-type amino acids (data not shown),

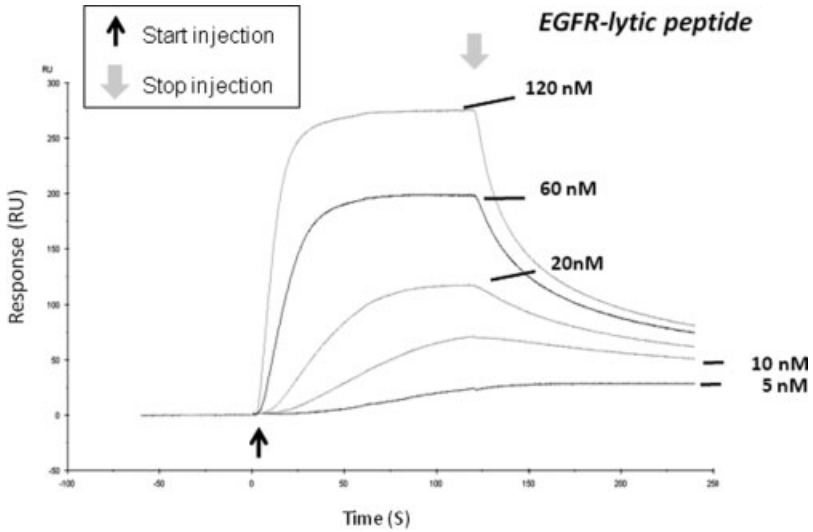

Figure 3. Sensorgrams of EGFR-lytic peptide bound to immobilized antilytic peptide antibody as determined by the Biacore biosensor. All analytes (120, 60, 20, 10, and 5 nM) of EGFR-lytic peptide were injected over antilytic peptide antibody. The progress of binding to immobilized antilytic peptide antibody was monitored by following the increase in signal (response) induced by the peptide. The thin and thick arrows indicate the beginning and end of the injection, respectively. RU, resonance units.

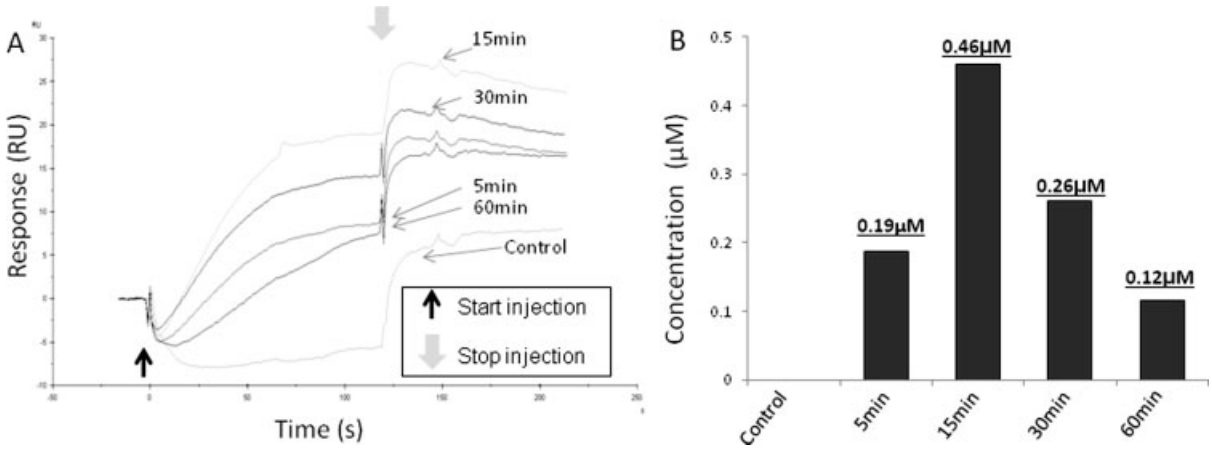

Figure 4. Detection of EGFR-lytic peptide from blood samples after i.v. injection in mice. (A) Sensorgrams of mouse sera bound to immobilized antilytic peptide antibody using the Biacore biosensor. Mouse sera purified from mouse blood samples 5, 15, 30, and 60 min after i.v. administration of EGFR-lytic peptide (10 mg/kg) were injected over immobilized antilytic peptide antibody on the sensor chip as described in the Section on Materials and Methods. Serum purified from mice that had received PBS was used as a control. The thin and thick arrows indicate the beginning and end of the injection, respectively. RU, resonance units. (B) Concentrations of EGFR-lytic peptide in mouse serum samples. Peptide concentrations in the mouse serum samples were calculated from Biacore results as described in the Section on Materials and Methods. The experiment was performed twice using mouse serum samples ($n = 3$) with similar results.

suggesting that the direction of side chains is also a significant factor for recognition by this antibody.

Interaction of Antilytic Peptide Antibody with the Hybrid EGFR-lytic Peptide

As shown in Figure 3, Biacore biosensor analysis demonstrated that hybrid EGFR-lytic peptide was also recognized by antilytic peptide antibody, and the sensorgrams were similar to those for lytic peptide to the antibody. The analytes in this reaction show a concentration-dependent relationship, as shown for the lytic peptide in Figure 1(C), although the K_D value of the antibody for EGFR-lytic peptide was 7.38×10^{-8} M, which is higher than that for the lytic peptide. These results indicate that antilytic peptide antibody can recognize not only lytic peptide but also a conjugated peptide such as hybrid EGFR-lytic peptide.

Investigation of the Stability of EGFR-lytic Peptide Administrated i.v. to Mice

The stability of a peptide in blood is an important factor in the development of peptide-based therapeutic drugs. To explore this parameter with the antilytic peptide antibody, we examined the concentration of EGFR-lytic peptide administered i.v. to mice, as assessed using the Biacore system. Figure 4(A) shows the progressive sensorgrams for the purified mouse serum samples at 5, 15, 30, and 60 min after i.v. injection of EGFR-lytic peptide (10 mg/kg), which indicate that there was a detectable peptide for antilytic peptide antibody in these samples. However, antilytic peptide antibody did not show any response in the control serum samples (Figure 4(A)). The concentrations of EGFR-lytic peptide in mouse serum samples were calculated from the Biacore sensorgrams, and the results showed that the maximum

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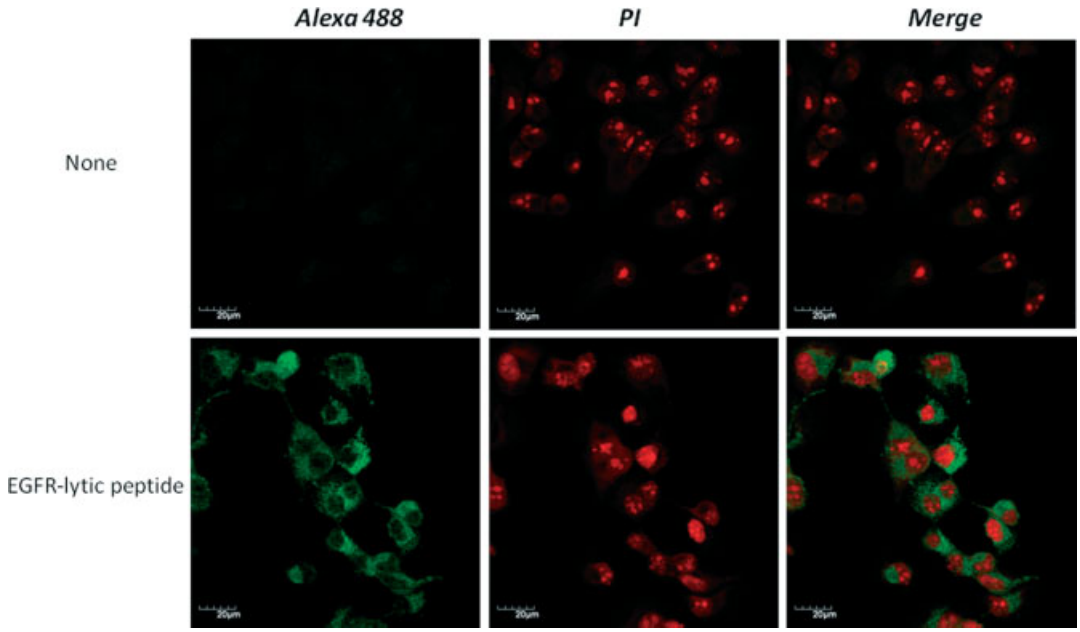

Figure 5. Immunocytochemical staining with antilytic peptide antibody to assess EGFR-lytic peptide in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were treated with or without 10 μM EGFR-lytic peptide for 30 min, and immunocytochemistry was performed using antilytic peptide antibody followed by Alexa 488-labeled second antibody and PI staining. All images were obtained using confocal microscopy as described in the Section on Materials and Methods. Scale bars, 20 μm.

concentration of injected EGFR-lytic peptide in mouse serum was reached 15–30 min after i.v. administration and that later the concentration of the peptide decreased (Figure 4(B)).

Localization of Hybrid EGFR-lytic Peptide in Cancer Cells

We previously showed that a 20-min exposure of cancer cells to hybrid EGFR-lytic peptide is sufficient to kill the cancer cells and that more than 70% of the cells lost viability [7]. We also demonstrated that EGFR-lytic peptide can penetrate the cell membrane by making a pore on the cancer cell surface, as demonstrated by the influx of calcein-labeled medium into the cytosol of cancer cells [7]. These results prompted us to perform further functional analysis of EGFR-lytic peptide in cancer cells using the newly developed antilytic peptide antibody. As shown in Figure 5, confocal microscopy images indicated that EGFR-lytic peptide that has penetrated the cell membrane accumulates in the cytosol but not in the nuclei of MDA-MB-231 breast cancer cells. However, control images did not show any accumulation in the cells. These results indicate that EGFR-lytic peptide accumulates in the cytosol after penetration of the cancer cell membrane by this peptide.

Discussion

It is known that several monoclonal and polyclonal antibodies can recognize tripeptides [18–21] and tetrapeptides [22]. It has also been reported that antiglutathione antibody scFv 20C9, which was isolated from a human synthetic phage antibody scFv library, can recognize the dipeptide γ-glutamylcysteine [23]. These findings indicate that antibodies raised from rabbit, mouse, or phage display library can recognize small epitopes, including dipeptides, and it is possible that these antibodies could be used for the development of peptide-based drugs for detailed functional assessment and characterization.

We have previously reported that chemical conjugation of targeted binding peptide and cell-killing, lytic-peptide components produces a hybrid peptide that shows selective and effective cytotoxic activity toward cancer cells both *in vitro* and *in vivo* [7]. Obtaining an antilytic peptide antibody was necessary to explore the more detailed functional mechanism of the hybrid peptide *in vitro* and *in vivo*. In this study, we successfully isolated and purified antilytic peptide antibody from rabbit antiserum after immunization of a rabbit with lytic peptide conjugated with KLH. We chose KLH as a carrier protein for conjugation with the lytic peptide. The advantage of using a carrier protein, for which KLH and BSA are commonly used, is the efficient immunization and production of antibody against small-molecule compounds such as peptides of less than 20 amino acids [12]. These techniques are useful for the isolation of phage antibodies, which can recognize small tripeptides from phage display libraries, as described previously [24].

It is generally believed that detection and calculation of peptide or peptide-compound concentrations in blood samples is difficult. In this study, we applied the purified antibody to detect and estimate the concentration of hybrid EGFR-lytic peptide in blood serum after i.v. administration of the peptide to mice using the Biacore method. The main advantages of biosensor technology using an SPR sensor are that (i) binding is monitored directly without the use of labels, (ii) sample consumption is low, and (iii) fewer false-positive results are obtained compared with ELISA. In addition, ELISA was recently shown to be less reliable than SPR in detecting low-affinity antibodies in samples [25]. Therefore, utilizing the advantages of biosensor technology, the peptide concentrations were estimated using antilytic peptide antibody. Interestingly, the maximum concentration of injected EGFR-lytic peptide was observed in mouse serum within 15–30 min of i.v. administration (Figure 4), indicating that this peptide does not exist for a longer period of time in the blood. As we previously showed that EGFR-lytic peptide can penetrate the cancer cell

membrane quickly [7], it is suggested that once in the blood, EGFR-lytic peptide might enter the tumor quickly or be processed for prompt degradation. These traits of the EGFR-lytic peptide in the blood might be favorable because it may mean that the patient experiences less-severe side effects caused by the lytic moiety.

Detection of EGFR-lytic peptide in cancer cells treated with this peptide was successfully shown by immunocytochemical staining with antilytic peptide antibody. It was observed that EGFR-lytic peptide mainly accumulates in the cytosol of cancer cells after penetration across the cell membrane; the peptide was not found in the nuclei of cancer cells (Figure 5). This observation was consistent with our previous confocal microscopy results using EGFR-lytic peptide labeled with TAMRA [7]. It is interesting that EGFR-lytic peptide can quickly penetrate the cancer cell membrane but accumulate in the cytosol without translocating to the nucleus. The functional mechanism of lytic-type peptides in cancer cells following their cytosolic accumulation still remains obscure. However, the elucidation of how the hybrid peptide works in cancer cells, such as the signal pathways that it triggers, might provide us with a more detailed anticancer mechanism for the hybrid peptide.

Acknowledgements

We thank Nana Kawaguchi and Kumi Kodama (Department of Pharmacoepidemiology, Kyoto University, Japan) for technical assistance with IHC staining and tissue culturing. The study was sponsored by a grant from Olympus Co.

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