CANCER BIOTHERAPY & RADIOPHARMACEUTICALS
Volume 24, Number 1, 2009

DOI: 10.1089/cbr.2008.0537

In-Vitro Internalization and *In-Vivo* Tumor Uptake of Anti-EGFR Monoclonal Antibody LA22 in A549 Lung Cancer Cells and Animal Model

Zhaofei Liu,[1] Zilin Yu,[1] Weiwei He,[1] Shujun Ma,[2] Le Sun,[2] and Fan Wang[1]

Abstract

Purpose: Internalization is one of the key steps for anticancer immunoconjugates to deliver the drugs inside of cancer cells. Herein, the internalization property of antiepidermal growth factor receptor (EGFR) monoclonal antibody (mAb) LA22 was evaluated. ***Materials and Methods:*** The binding and internalization properties of LA22 on A549 cells were investigated by using ^{125}I-LA22. *In vitro* internalization was also confirmed by indirect fluorescent staining. *In vivo* tumor targeting and internalization of ^{125}I-LA22 were evaluated in the A549 nude mice model. ***Results:*** The mAb LA22 showed a high affinity to EGFRs expressed on A549 cells (Kd = 0.69 ± 0.13 nM). The *in vitro* internalization of LA22 was time- and temperature dependent. The cell-surface-bound LA22 was rapidly internalized at 37°C. The experimental results of LA22 internalization obtained from radioassay and fluorescent staining were consistent with a good linear correlation. At 72 hours postinjection, a clear γ-image of tumor was obtain in A549 tumor xenografts, and the tumor uptake of ^{125}I-LA22 was 8.00 ± 0.61 percent injected dose per gram (%ID/g) (2.19 ± 0.37 %ID/g for ^{125}I-mIgG). Similar to the *in vitro* observation, 64.06% of the cell-bound mAb LA22 was internalized into the tumor cells *in vivo*. ***Conclusions:*** The mAb, LA22, is a rapid, high-internalizing antibody, and this property makes it a promising vehicle for tumor-targeted drug delivery.

Key words: epidermal growth factor receptor (EGFR), monoclonal antibody LA22, internalization, immunoscintigraphy, tumor targeting

Introduction

Epidermal growth factor receptor (EGFR), a 170-kDa transmembrane glycoprotein overexpressed in a variety of human solid tumors,[1,2] can be activated by receptor-specific ligands, such as epidemal growth factor (EGF), transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF), and others.[2,3] Once EGFR is activated by the specific ligands, the ligand-receptor complex undergoes dimerization and internalization, which in turn, activates the intracellular protein kinase via autophosphorylation, leading to signal transduction and cellular functions. There is evidence that EGFR is correlated with tumor proliferation, invasiveness, distant metastasis, and angiogenesis.[4]

The critical role of EGFRs in cancer has led to an extensive search for selective inhibitors of the EGFR-signaling pathway.[5] One of the most promising strategies in clinical application is to develop blocking monoclonal antibodies (mAbs) to prevent ligand binding. Erbitux (cetuximab, IMC-C225) is the first EGFR-targeted antibody approved by the Food and Drug Administration (FDA) for cancer therapy.[6] In addition to Erbitux, other neutralizing mAbs have also been developed against the EGFR.[7] As the clinical application of "naked" anti-EGFR antibodies may often be limited by their modest cancer therapeutic effects—and Erbitux was approved only for use in combination with chemotherapy—the approach to enhance the anticancer activity of naked antibodies by arming them with cytotoxic effector molecules, such as chemical drugs, cytotoxin, radionuclides, and so forth, has become practical. Antibody-drug conjugates de-

[1]Medical Isotopes Research Center, Peking University, Beijing, China.
[2]Welson Pharmaceuticals, Inc., Ellicott City, MD.

Address correspondence to: Fan Wang; Medical Isotopes Research Center, Peking University; 38 Xueyuan Road, Beijing 100191, China; Tel. and Fax: 86-10-82801145
E-mail: **wangfan@bjmu.edu.cn**

signed to selectively eliminate cancer cells have been explored extensively in recent decades.[8] Several conjugates are currently in clinical evaluation,[9] and one conjugate, gemtuzumab ozogamicin, is licensed for the treatment of refractory acute myeloid leukemia.[10] Anti-EGFR antibodies and antibody fragments as the vehicles to deliver drugs for tumor-targeted therapy were also investigated.[11,12]

We have previously reported that a novel anti-EGFR antibody-drug conjugate (LA22-MMC), which consists of an anti-EGFR mAb, LA22 (mAb LA22), and an antitumor chemical drug, mitomycin (MMC), showed a significant antitumor effect in the nude mice model.[12] In the *in vitro* cytotoxicity assays, LA22-MMC killed the tumor cells more efficiently than naked mAb LA22 and Erbitux-MMC in both A431 and A549 tumor cells. It was expected that the significant antitumor effect of the antibody-drug conjugates was due to the rapid internalization property of mAb LA22, which carried the cytotoxic antibiotic MMC into the tumor cells and then released it, eventually resulting in the death of tumor cells.

As the excellent internalization property of an antibody is necessary not only for antibody-drug conjugates, but also for other antibody-based toxicity deliveries (e.g., radioisotope) to reach a high retention of the toxicity in the tumor cells, in the present report, we characterize the *in vitro* internalization property of mAb LA22 to explain the previous experimental results, and also provide direct evidence to prove the *in vivo* tumor targeting and *in vivo* internalization property of mAb LA22 itself, in order to develop mAb LA22-based drug or radioisotope conjugates for tumor-targeted therapy.

Investigators have previously described the methods to characterize the *in vitro* internalization property of anti-EGFR antibodies.[13–16] In general, the internalization of antibody was determined by either the direct labeling method with radioisotope[13–15] or fluorescent dye,[15,16] or indirect fluorescent staining.[14] In this study, we used both the radioassay method and the indirect fluorescent staining method to evaluate the internalization property of mAb LA22 and compared the results from two different methods.

Materials and Methods

Preparation of mAb LA22 and ^{125}I-LA22

The anti-EGFR mAb, LA22, was prepared as previously reported.[12] For radiolabeling, 100 μg of mAb LA22 and 37 MBq of Na^{125}I (Beijing Atom High Tech, Beijing, China) in 0.2 M (pH 7.4) of phosphate-buffered saline (PBS) were added to a vial coated with 40 μg of Iodogen (Sigma, St. Louis, MO). After 10 minutes of reacting at room temperature, the reaction mixture was purified by a PD-10 column (Amersham, Piscataway, NJ). The radiochemical purity (RCP) of ^{125}I-LA22 after purification was higher than 98%. The radioimmunoreactive fraction of ^{125}I-LA22 was determined to be more than 80%, using the method established by Lindmo et al.[17] Murine IgG (mIgG, isotype-matched control; Sigma) was also labeled with ^{125}I, using the same procedure described above as the negative control.

Cell culture and animal model

The A549 human non-small-cell lung cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT) at 37°C in a humidified atmosphere with 5% CO_2. The BALB/c nude mice (4–5 weeks of age; Department of Experimental Animals, Peking University Health Science Center, Beijing, China) were inoculated subcutaneously with 5×10^6 A549 cells per mouse in the right upper flanks. When the tumors reached ~0.8 cm in diameter, the mice were used for *in vivo* animal studies.

Binding specificity and affinity of ^{125}I-LA22 to EGFR

The binding specificity of mAb LA22 to EGFRs expressed on A549 cells was evaluated by using the competition binding assay. Briefly, ~200,000 counts per minute (cpm) of ^{125}I-LA22 in triplicate was added to A549 cells in the presence of increasing concentrations of unlabeled mAb LA22 or mIgG. After washing the unbound radiotracer, the specific binding of ^{125}I-LA22 to A549 cells was analyzed by measuring the radioactivity associated with the cells, using a γ-counter (Wallac 1470-002; Perkin Elmer, Finland).

The binding affinity of ^{125}I-LA22 to EGFRs was evaluated by the saturation binding assay. The A549 cells, seeded in a 48-well plate (5×10^4 cells per well), were incubated at 37°C in a humidified atmosphere with 5% CO_2 overnight to allow a firm adherence. After washing with PBS three times, increasing concentrations of ^{125}I-LA22 (from 44.4 pM to 4.44 nM) were added. The total volume was adjusted to 300 μL. For each concentration, nonspecific binding was determined in the presence of an excess (2.22 μM) of unlabeled mAb LA22. After 4 hours of incubation at 4°C, the cells were washed twice with cold PBS and then solubilized with 2 M of NaOH. The cell-associated radioactivity was determined by using the γ-counter. A saturation binding curve and Scatchard transformation were obtained by nonlinear regression analysis, and the Kd value of ^{125}I-LA22 was determined by using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Each data point represents the average value from triplicate wells.

Internalization of ^{125}I-LA22 into A549 cells

A549 cells cultured in a 24-well plate (1×10^5 cells per well) were incubated with saturating concentrations of ^{125}I-LA22 (~200,000 cpm) or ^{125}I-mIgG (~200,000 cpm) at 37°C or 4°C in culture medium for 0, 10, 20, 30, 60, 120, and 240 minutes, respectively. At the time points, cells were rinsed with PBS to remove the unbound antibodies, and treated with 5-minute acid washing twice (50 mM glycine-HCl/100 mM NaCl; pH 2.8) to remove the surface-bound antibodies. Cells were solubilized with 2 M of NaOH at 37°C for 1 hour. The internalized radioactivity was collected and measured in the γ-counter. Each data point represents the average counts (cpm) from triplicate samples.

Internalization rate and ratio of the cell-surface-bound ^{125}I-LA22 were determined, as previously described, with some modifications.[18,19] Briefly, A549 cells grown in 24-well plates were incubated with ~200,000 cpm of ^{125}I-LA22 for 2 hours at 4°C to allow the maximum surface binding. After three times of washing with ice-cold PBS, warmed culture medium was added, and cells were incubated at 37°C for various time periods (0, 2, 5, 10, 20, 30, and 60 minutes) in order to allow for internalization. Cell-surface-bound ^{125}I-LA22 was removed by 5-minute acid washing twice (50 mM glycine-

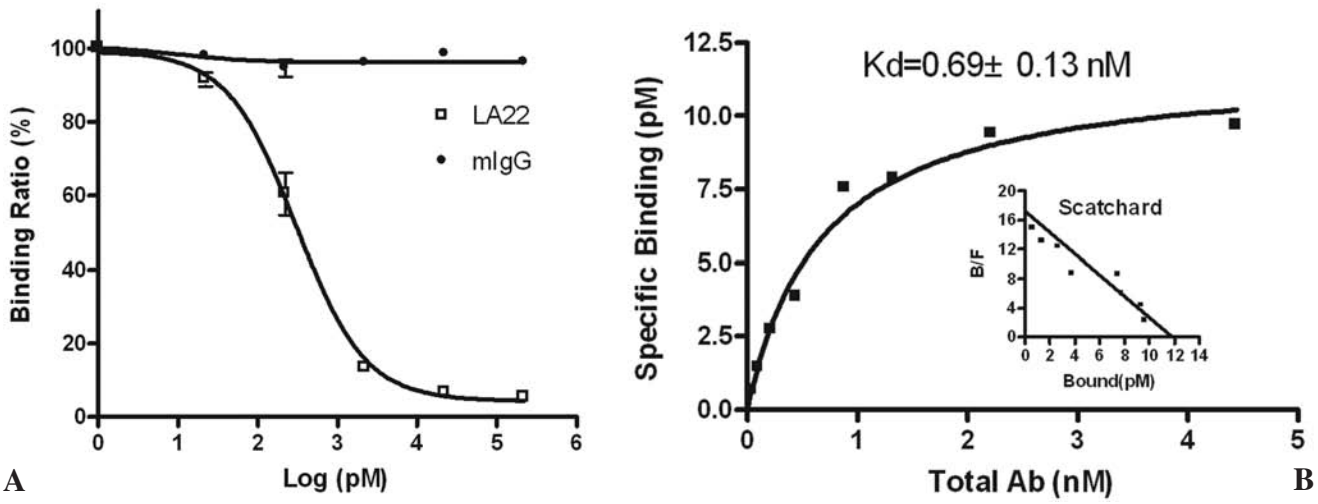


FIG. 1. Binding specificity and affinity of ^{125}I-LA22 to epidermal growth factor receptors (EGFRs). (**A**) The binding of ^{125}I-LA22 to EGFRs on A549 tumor cells in the presence of increasing concentrations of unlabeled mAb LA22 or mIgG. (**B**) Saturation binding of ^{125}I-LA22 to EGFRs on A549 tumor cells. Data are expressed as the mean ± standard deviation (SD) ($n = 3$).

HCl/100 mM NaCl; pH 2.8) at room temperature. Cells were solublized with 2 M of NaOH, and then the internalized radioactivity and acid-washed radioactivity were measured in the γ-counter. The internalization ratio was expressed as a percentage of internalized radioactivity to total radioactivity associated with the cells (acid-washed radioactivity + internalized radioactivity). Experiments were carried out twice with triplicate samples.

Indirect immunofluorescent staining of mAb LA22 internalization

The procedure was based on a previous report, with some modifications.[20] A549 cells were harvested and plated into 35-mm dishes containing sterile, 12-mm, round coverslips. Cells were allowed to attach and spread on the coverslips for at least 24 hours before use in the experiments. The cells on the coverslips were rinsed in PBS and then incubated with mAb LA22 (50 μg/mL) and mIgG (50 μg/mL), respectively, for various time points at 37°C. Coverslips were then washed in PBS and cell-surface-bound proteins were removed by 5-minute acid washing twice (50 mM glycine-HCl/100 mM NaCl; pH 2.8) at room temperature. After neutralization with 0.5 M of Tris-HCl (pH 7.4) for 5 minutes, the cells were fixed with 4% (w/v) paraformaldehyde for 20 minutes, washed with PBS, permeabilized with 0.2% Triton X-100 for 10 minutes, and blocked with 3% bovine serum albumin (BSA) for 1 hour at room temperature. After a brief wash with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-coupled goat antimouse IgG (1:100; Santa Cruz, CA) for 30

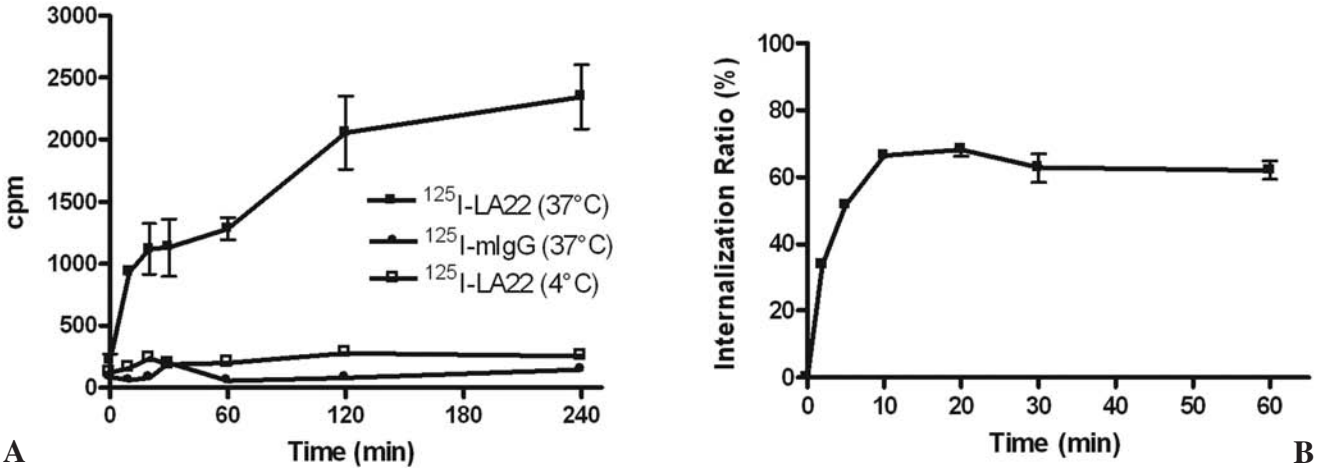


FIG. 2. *In vitro* internalization of ^{125}I-LA22. (**A**) A549 tumor cells were incubated with ^{125}I-LA22 for various times at 4°C or 37°C. The internalized radioactivities were measured and expressed as a function of time. ^{125}I-IgG was used as a control. Data are expressed as the mean ± standard deviation (SD) ($n = 3$). (**B**) A549 tumor cells were first incubated with ^{125}I-LA22 for 2 hours at 4°C, and then the cell-surface-bound ^{125}I-LA22 was allowed to internalize at 37°C for various times. Data are expressed as the mean ± standard deviation (SD) ($n = 3$).

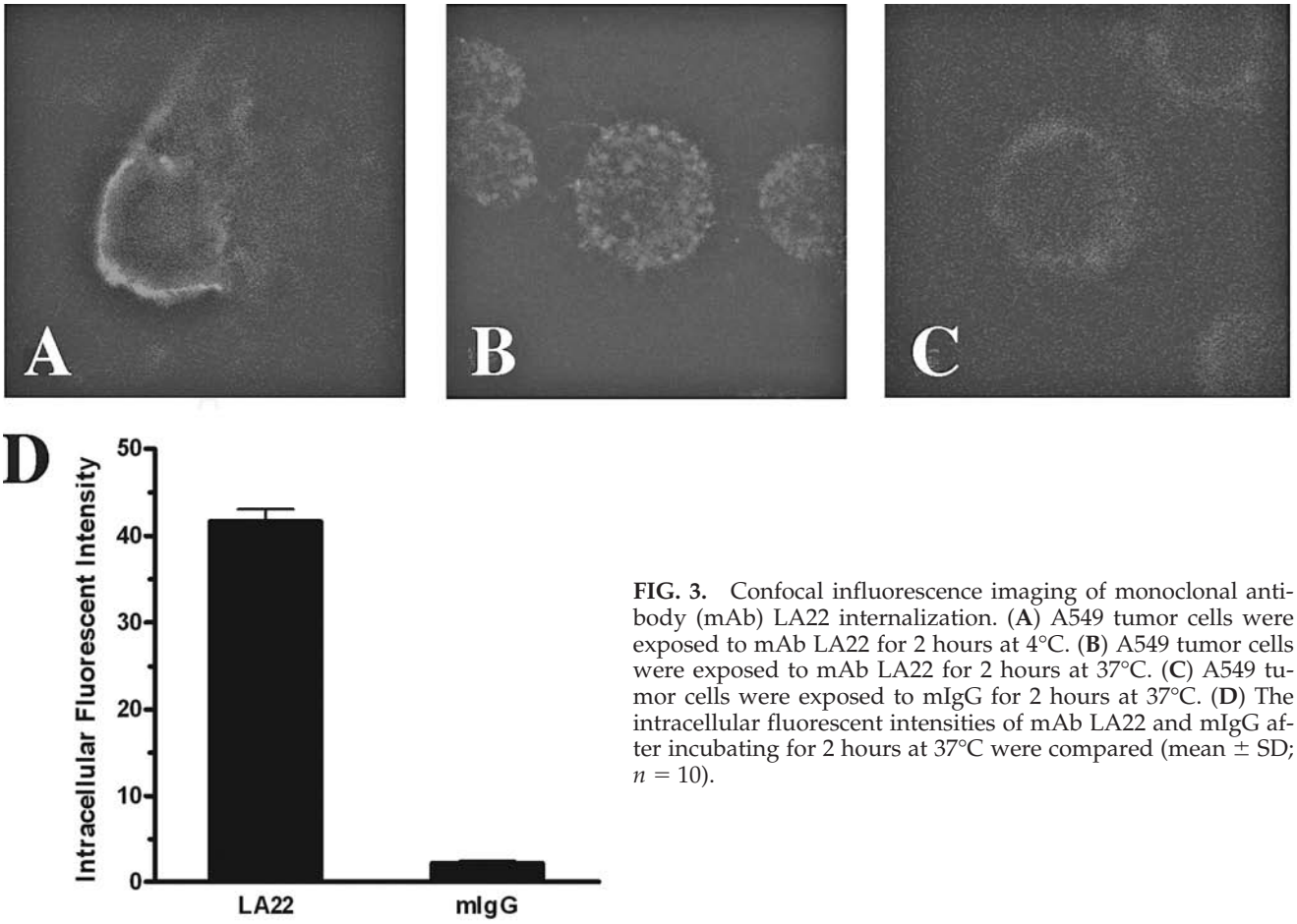


FIG. 3. Confocal influorescence imaging of monoclonal antibody (mAb) LA22 internalization. (**A**) A549 tumor cells were exposed to mAb LA22 for 2 hours at 4°C. (**B**) A549 tumor cells were exposed to mAb LA22 for 2 hours at 37°C. (**C**) A549 tumor cells were exposed to mIgG for 2 hours at 37°C. (**D**) The intracellular fluorescent intensities of mAb LA22 and mIgG after incubating for 2 hours at 37°C were compared (mean ± SD; $n = 10$).

minutes at 37°C. Unbound secondary antibody was removed by rinsing three times with PBS containing 0.1% Tween 20 and then a final wash of PBS. The coverslips were inverted on glass slides on mounting medium and analyzed with a Leica TCS-NT confocal microscope (Wetzler, Heidelberg, Germany). The images were observed and obtained under identical conditions (i.e., exposure time, plane, and magnification). To quantitate, average fluorescent intensities inside the cells were measured by using Leica confocal software. For each image, 10 cells were randomly selected and the mean was calculated. The mean background obtained by the measurement of five randomly selected areas with no cells in the same image was then subtracted from the calculated values. For the cell-surface-binding assay, mAb LA22 was incubated with A549 cells at 4°C for 2 hours. Unbound antibodies were removed by PBS rinsing and an FITC-coupled second antibody was added, incubated, washed, and analyzed by using a confocal microscope, as described above.

In vivo *tumor targeting of ^{125}I-LA22*

All animal experiments were performed in accordance with the guidelines of the Peking University Health Science Center Animal Care and Use Committee. The *in vivo* tumor targeting and uptake of ^{125}I-LA22 were evaluated by both immunoscintigraphy and biodistribution. With respect to immunoscintigraphy, 3 nude mice bearing EGFR-positive A549 human lung cancer xenografts were injected intravenously (i.v.) with 14.8 MBq of ^{125}I-LA22 (20 μg, 0.3 mL), then the mice were placed prone on a two-head γ-camera (E-Cam; Siemens), equipped with a parallel-hole, low-energy, high-resolution collimator. Planar images were acquired at 4, 24, 72, and 120 hours postinjection and stored digitally in a 128 × 128 matrix. The acquisition count limits were set at 100 k. For the biodistribution study, ^{125}I-LA22 (185 kBq, 5 μg) was administered i.v. to one group of 4 tumor-bearing mice, and the mice were sacrificed at 72 hours postinjection. Tumor tissues and other interested organs, including blood, heart, liver, spleen, kidney, and muscle, were harvested, weighed, and measured for radioactivity in a γ-counter. The tumor uptake was calculated as a percentage of the injected dose per gram of wet tissue mass (%ID/g). As a control experiment, the same dose of ^{125}I-mIgG (185 kBq, 5 μg) was administered i.v. to another group of 4 tumor-bearing mice and the biodistribution study was performed, as described above.

^{125}I-LA22 internalization into tumor cells in vivo

^{125}I-LA22 and ^{125}I-mIgG were injected i.v. into nude mice bearing A549 tumor xenografts (3 mice per group) at a dose of 185 kBq (5 μg), respectively. At 72 hours postinjection, mice were sacrificed, and tumor tissues were excised, weighed, and disaggregated in 0.1% (w/v) collagenase IV

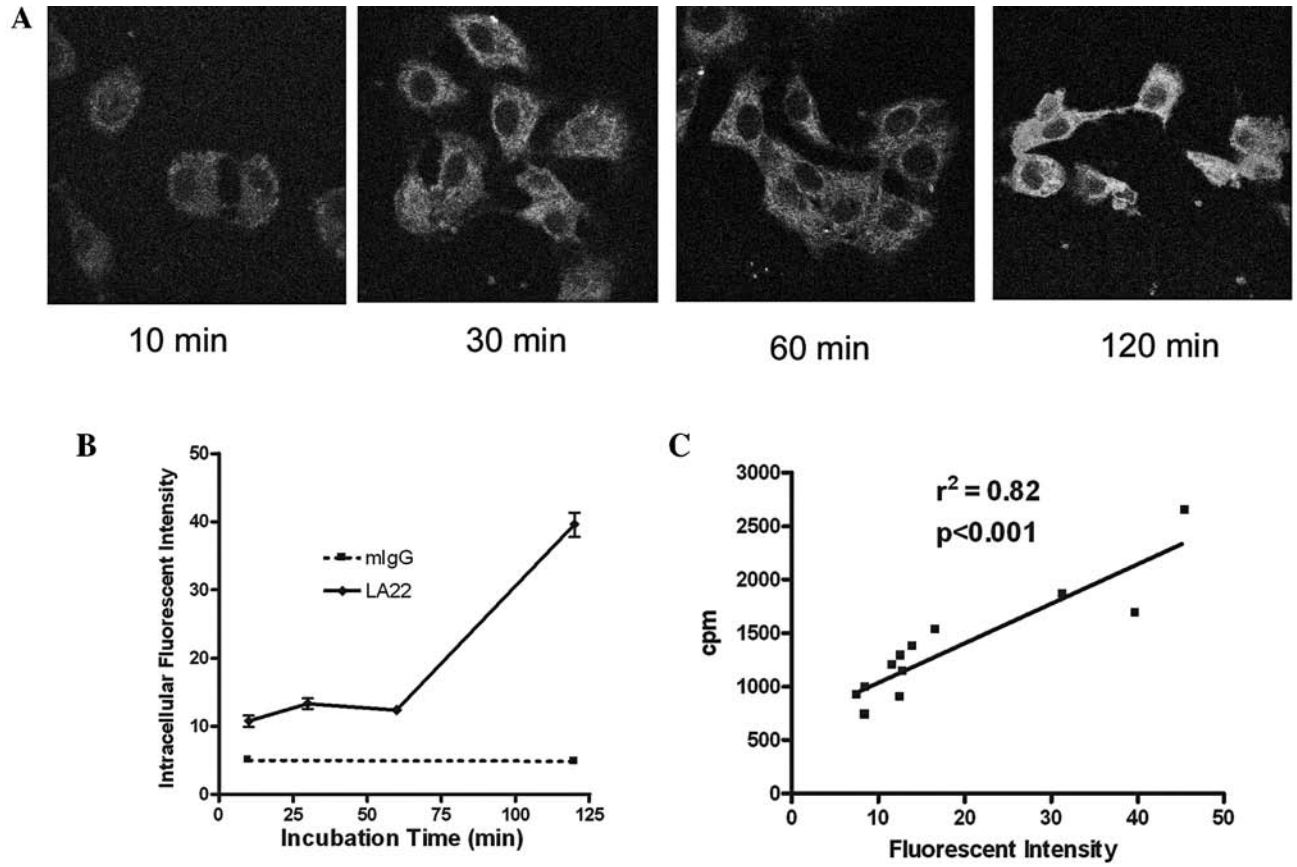


FIG. 4. Internalization course of monoclonal antibody (mAb) LA22. (**A**) The internalization course of mAb LA22 from 10 to 120 minutes at 37°C. (**B**) The intracellular fluorescent intensity of mAb LA22 is expressed as a function of time (mean ± SD; $n = 10$). (**C**) The correlation of mAb LA22 internalization measured by radioassay and fluorescent staining.

and 0.003% DNaseI for 2 hours at 37°C with slow agitation.[11,21] After these procedures, the tumor suspensions were homogeneous without macroscopic lumps. The suspensions were then passed through Nitex cloth (mesh size, 150 μm), and the single-cell suspensions were collected by centrifugation at 800 cpm for 5 minutes. After washing carefully with PBS, the cells were then treated with an acid wash, as described above, to distinguish the cell-surface-bound and internalized activities. The total cell-associated radioactivities were calculated as the sum of the acid removed and the intracellular radioactivities. The results were expressed as the percentage of the injected dose per gram of tumor tissue (%ID/g tumor), which represent the average of triplicate samples.

Results

In vitro *binding assay*

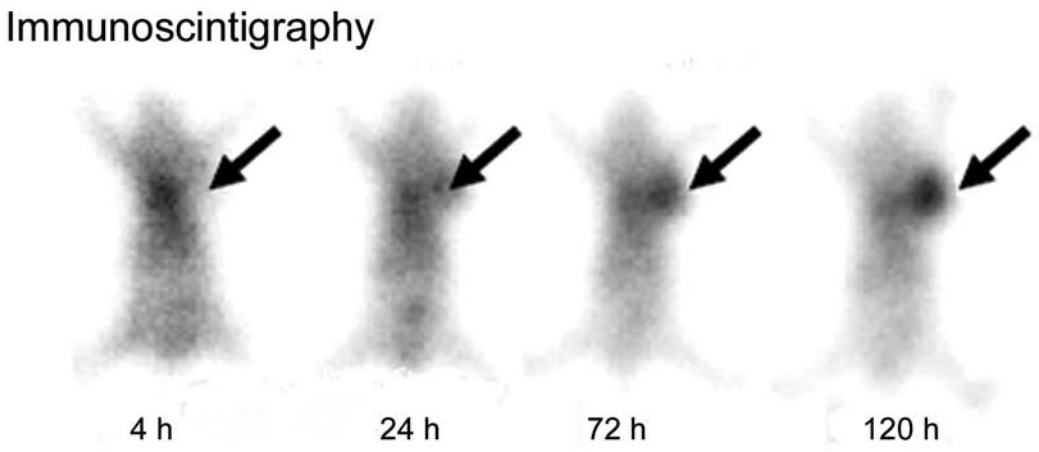
The ^{125}I-LA22 bound to EGFRs decreased along with the increasing concentrations of unlabeled mAb LA22, and the cell-associated ^{125}I-LA22 was less than 6% when 200-fold unlabeled mAb LA22 was added, indicating the high binding specificity of ^{125}I-LA22 to EGFRs expressed on A549 cells. In contrast, the negative control mIgG showed no significant block for ^{125}I-LA22 binding (Fig. 1A).

The affinity of ^{125}I-LA22 to EGFRs expressed on A549 cells was determined by Scatchard analysis. The 5×10^4 cells per well in triplicate were incubated with increasing amounts of ^{125}I-LA22 at 4°C for 4 hours. A second set of samples was incubated in the presence of a 500-fold excess of unlabeled mAb LA22 to block the specific binding and allow the determination of nonspecific binding of radioactivity. The specific binding of radioactivity was defined as the total binding radioactivity subtracting the nonspecific binding radioactivity. The specific saturation binding curve and the Scatchard plot are shown in Figure 1B. mAb LA22 shows a very high affinity to EGFRs on A549 cells, with the Kd value determined to be 0.69 ± 0.13 nM, using GraphPad Prism 4.0 software.

Internalization of ^{125}I-LA22 into A549 cells

The internalization of ^{125}I-LA22 was determined by incubating A549 cells with ^{125}I-LA22 at 37°C or 4°C for various time periods. The internalization of ^{125}I-LA22 was time- and temperature dependent. ^{125}I-LA22 was internalized rather rapidly in the first 10 minutes and remained with increasing internalization until 2 hours after incubation (Fig. 2A). In comparison of incubating at 4°C, ^{125}I-LA22 did not show significant internalization and the intracellular antibodies remained at a low level throughout the entire incubation period (Fig. 2A). The negative control, mIgG, also did not show

A



B Biodistribution

%ID/g (n=4)	LA22	mIgG
Blood	11.42 ± 0.79	8.62 ± 0.31
Heart	0.83 ± 0.10	0.54 ± 0.06
Liver	2.57 ± 0.18	1.60 ± 0.05
Spleen	2.20 ± 0.32	1.38 ± 0.33
Kidney	2.43 ± 0.37	1.04 ± 0.23
Muscle	2.67 ± 0.05	1.72 ± 0.13
Tumor	8.00 ± 0.61	2.19 ± 0.37

C

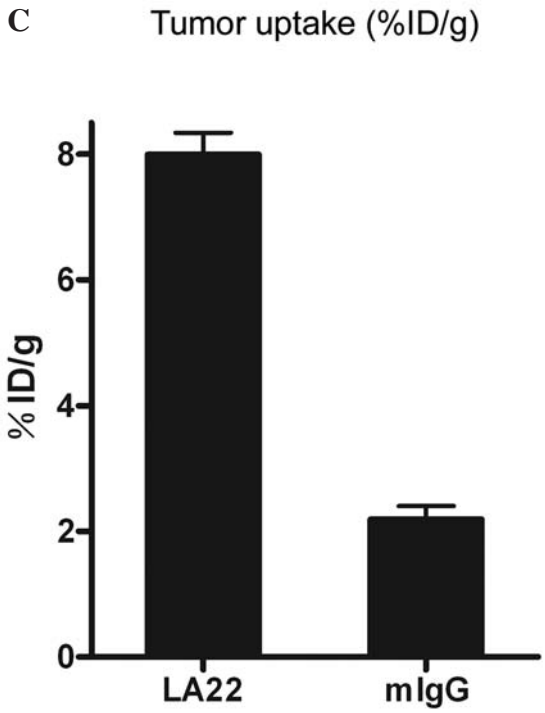


FIG. 5. *In vivo* tumor targeting of ^{125}I-LA22. (**A**) Nude mice bearing A549 tumor xenografts were injected intravenously (i.v.) with 14.8 MBq of ^{125}I-LA22, and then planar images were acquired at 4, 24, 72, and 120 hours postinjection. Arrows indicate the tumor locations. (**B**) ^{125}I-LA22 was administered i.v. to one group of 4 A549 tumor-bearing mice. Animals were sacrificed at 72 hours postinjection. Tumor tissues and other interested organs were harvested, weighed, and measured for radioactivity in a γ-counter. The percent injected dose per gramof tissue of each organ was presented. ^{125}I-mIgG was used as a negative control (mean ± SD; $n = 4$). (**C**) Tumor uptake of ^{125}I-LA22 and ^{125}I-mIgG in nude mice bearing A549 tumor xenografts was compared (mean ± standard deviation; $n = 4$).

significant internalization into A549 cells, indicating that the internalization of ^{125}I-LA22 is EGFR dependent (Fig. 2A).

The internalizing ratio and rate of the cell-surface-bound ^{125}I-LA22 were evaluated. A549 cells were first incubated with ^{125}I-LA22 at 4°C for 2 hours, to allow the maximum cell-surface binding, and then incubated with prewarmed culture medium at 37°C for various time periods to allow the internalization of the surface-bound antibodies. As shown in Figure 2B, the internalization of surface-bound ^{125}I-LA22 is time dependent. The intracellular antibodies sharply in-

crease during the first 10 minutes, reaching the maximum internalization level of 65.8%, and it remained at a relatively high plateau for at least 60 minutes.

Confocal influorescence imaging of mAb LA22 internalization

The binding of mAb LA22 to EGFRs expressed on A549 cells and internalization of mAb LA22 into A549 cells were visualized by a laser-scanning confocal microscope. A549 cells were incubated with mAb LA22 for 2 hours at 4°C to allow the surface binding and minimize the internalization, then FITC-coupled goat antimouse IgG was used to stain the cells. mAb LA22 was not internalized into the cells at 4°C and totally bound to the cell surface (Fig. 3A), which was similar to the result of the internalization study using ^{125}I-LA22. After incubating at 37°C for 2 hours, mAb LA22 showed clear internalization (Fig. 3B). The high-powered imaging revealed that the internalized antibodies accumulated in the cytoplasm. The fluorescence in the cytoplasm was punctate instead of dispersed, indicating that the internalized antibodies most likely accumulated in some specific organelles. In contrast, mIgG did not show significant internalization (Fig. 3C). The intracellular fluorescent intensity of mAb LA22 was significantly higher than that of mIgG after incubating at 37°C for 2 hours (Fig. 3D), which was calculated by using Leica confocal software.

The internalizing visualization of mAb LA22 depends on the incubation time at 37°C (Fig. 4A). Internalized mAb LA22 was not very clear within the incubation time from 10 to 60 minutes, whereas after 120 minutes of incubation, the intracellular antibodies sharply increased (Fig. 4A). Figure 4B shows that the internalization occurs mainly during the period from 60 to 120 minutes.

The internalization course detected by indirect fluorescent staining was similar to that detected by using ^{125}I-LA22 (Fig. 2A). More important, the analytic results from two different methods showed a highly significant ($p < 0.001$) linear correlation (Fig. 4C; $r^2 = 0.82$). Both of the two different methods confirmed the *in vitro* internalization of mAb LA22 into A549 cells.

In vivo *tumor targeting of ^{125}I-LA22*

The *in vivo* tumor targeting and uptake of ^{125}I-LA22 was evaluated by both the immunoscintigraphy and biodistribution studies in nude mice bearing A549 human lung cancer xenografts. In the earlier period, the uptake of ^{125}I-LA22 in blood-rich organs, such as the heart, was higher than tumors, but along with the time, the accumulation of ^{125}I-LA22 in tumors was higher than other tissues. At 72 hours postinjection, the tumor uptake was significantly higher against the background, and at 120 hours postinjection, the tumor image had a much better contrast with the clearance of radioactivity from normal tissues (Fig. 5A). The quantitative tumor uptake of ^{125}I-LA22 at 72 hours postinjection is summarized in Figure 5B. The tumor uptake was 8.00 ± 0.61 %ID/g ($n = 4$), and radioactive ratios of tumor to nontumor were more than 1.0 for all of tissues (data not shown), except for the ratio of tumor to blood (due to the slow blood clearance of intact antibody). In the control experiment, the tumor uptake of isotype-matched ^{125}I-mIgG was 2.19 ± 0.37 %ID/g ($n = 4$) (Fig. 5B and 5C).

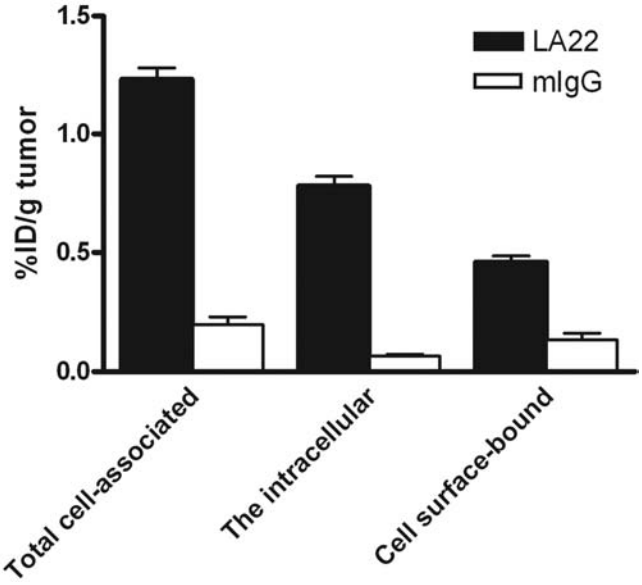


FIG. 6. *In vivo* uptake and internalization of ^{125}I-LA22 in tumor cells. ^{125}I-LA22 or ^{125}I-mIgG (isotype-matched control) was injected intravenously into nude mice bearing A549 tumor xenografts (3 mice per group) at a dose of 185 kBq (5 μg). At 72 hours postinjection, mice were sacrificed, and tumors were excised, weighed, and disaggregated. Cell suspensions were collected, washed, and then treated with acid wash. Total cell-associated, acid-removed, and intracellular radioactivities were measured in a γ-counter and are expressed as the percentage of injected dose per gram of tumor tissue (mean ± standard deviation (SD); $n = 3$).

In vivo *internalization of ^{125}I-LA22 into tumor cells*

To investigate the *in vivo* tumor-cell uptake of mAb LA22, ^{125}I-LA22 was injected into nude mice bearing A549 tumor xenografts, then the tumor tissues were excised and disaggregated, and the related tumor cells were collected. The total cell-associated, intracellular, and surface-bound radioactivities were measured. The results were expressed as the %ID/g tumor. As shown in Figure 6, the tumor-cell-associated, intracellular, and surface-bound ^{125}I-LA22 are 1.23 ± 0.08, 0.78 ± 0.08, and 0.46 ± 0.05 %ID/g tumor, respectively ($n = 3$), and the internalization ratio (percent of intracellular radioactivity/total cell-associated radioactivity) was calculated to be 64.06%. As a control study, the tumor-cell-associated, intracellular, and surface-bound ^{125}I-mIgG are all much lower, with 0.20 ± 0.06, 0.06 ± 0.01, and 0.14 ± 0.05 %ID/g tumor, respectively.

Discussion

The internalization of antibody into the cells is required for many targeted therapeutics, such as immunotoxins, immunoliposomes, and antibody-drug conjugates.[22,23] The mechanism of the internalization may be that some antibodies can mimic the natural ligands of the receptors to mediate receptor dimerization and then stimulate the endocytosis.[24] So, the internalization of antibodies may differ significantly, depending on both the antibodies themselves and the epitopes they recognize.

Many anti-EGFR antibodies have been investigated for

cancer-targeted therapy, such as IMC-C225, ABX-EGF, and mAb LA22.[2,25,26] mAb LA22 (IgG2a) recognizes the epitope from Ala-351 to Asp-364 in the mature EGFR,[27,28] and shows high affinity to EGFRs expressed on A549 cells (Kd = 0.69 ± 0.13 nM). The internalization assay of mAb LA22 was performed by both radiolabeling and indirect fluorescent staining methods. The results showed that the internalization of mAb LA22 was time- and temperature dependent. When A549 cells were incubated with mAb LA22 at 4°C, the mAb LA22 was not internalized into the cells (Fig. 3A). However, when the cells were incubated with mAb LA22 at 37°C, the intracellular fluorescent density measured by using confocal microscopy and the internalized radioactivity detected by γ-counter showed the consistent trend of increasing internalization with time. More important, after binding to the cell surface, mAb LA22 was internalized into the cells rather rapidly (~10 minutes) and highly (65.8%).

Most of the therapeutic mAb approved for use in oncology belong to the IgG1 subclass, which has the potential to trigger the immunofunctions, such as complement-dependent cytotoxitity (CDC), complement-dependent cell-mediated cytotoxicity (CDCC), and antibody-dependent cellular cytotoxicity (ADCC).[29–31] The mAb LA22 belongs to the IgG2 subclass, so the naked antibody itself has no advantage over the other anti-EGFR antibodies, such as cetuximab, for tumor therapy. However, the rapid and high internalization property of mAb LA22 does provide a potential application of using it as a vehicle for cytotoxicity delivery.

The excellent *in vitro* characteristics (e.g., high antigen-binding affinity, as well as high and rapid internalization) of mAb LA22 cannot directly predict the *in vivo* characteristics, because there are many other factors (such as the metabolism, stability, tumor vascular permeability, and antigen shedding) that affect the *in vivo* behavior of the antibody. Therefore, the *in vivo* tumor targeting specificity and internalization preperty of mAb LA22 were also evaluated in nude mice bearing EGFR-positive A549 tumors. Clear tumor images were obtained at 72 hours postinjection by immunoscintigraphy; moreover, the tumor uptake of ^{125}I-LA22 was significantly higher than that of control ^{125}I-mIgG, indicating the specific binding between ^{125}I-LA22 and EGFRs in A549 xenografts. As for the relative higher uptake of ^{125}I-mIgG in tumor than that of the normal organs, it may be due to the enhanced permeability and retention (EPR) effect, as tumors have abnormal and leaky vasculature and lack lymphatic drainage.[32]

Since most of the intact antibodies (molecular weight, ~150 kDa) exhibit the highest tumor uptake between 48 and 120 hours postinjection in nude mice models, so we harvested the tumor tissues at 72 hours after the injection of ^{125}I-LA22, when the antibody concentration in the tumor site would be higher and the more internalization would occur. The intracellular radioactivity of ^{125}I-LA22 accounted for more than 66% of total cell-associated radioactivity, which is much higher than that of ^{125}I-mIgG. More important, the *in vivo* internalization of ^{125}I-LA22 into tumor cells was consistent with the *in vitro* internalization data.

Conclusions

In this study, we validated the *in vitro* specific binding and internalization property of anti-EGFR mAb LA22 to A549 human lung cancer cells, which confirmed the expectation for our previous study. The *in vivo* studies also showed specific tumor targeting and high internalization of mAb LA22 in EGFR-positive tumor. The specific binding to EGFRs with a high affinity, as well as rapid and high internalization of mAb LA22, make it a potential vehicle to deliver the cytotoxic agents for tumor-targeted therapy.

Acknowledgments

This study was supported, in part, by the following research grants: the Beijing Science and Technology Program (Z00004105040311 and D0206001041991) and the 863 Project (2007AA02Z467).

References

1. Singh AB, Harris RC. Autocrine, paracrine, and juxtacrine signaling by EGFR ligands. *Cell Sign* 2005;17:1183.
2. Mendelsohn J, Baselga J. The EGF-receptor family as targets for cancer therapy. *Oncogene* 2000;19:6550.
3. Emir H, Michael S, Stefan H, et al. EGF-stimulated migration in ovarian cancer cells is associated with decreased internalization, increased surface experession, and increased shedding of the urokinase plasminogen activator receptor. *Gynecol Oncol* 2006;101:28.
4. Grandis JR, Sok JC. Signaling through the epidermal growth factor receptor during the development of malignancy. *Pharmacol Ther* 2004;102:37.
5. Mendelsohn J, Baselga J. Status of epedermal growth factor receptor antagonists in the biology and treatment of cancer. *J Clin Oncol* 2003;21:2787.
6. Rabindran SK. Antitumor activity of HER-2 inhibitors. *Cancer Lett* 2005;227:9.
7. Speake G, Holloway B, Costello G. Recent developments related to the EGFR as a target for cancer chemotherapy. *Curr Opin Pharmacol* 2005;5:343.
8. Wu AM, Senter PD. Arming antibodies: Prospects and challenges for immunoconjugates. *Nat Biotechnol* 2005;23:1137.
9. Lambert JM. Drug-conjugated monoclonal antibodies for the treatment of cancer. *Curr Opin Pharmacol* 2005;5:543.
10. Bross PF, Beitz J, Chen G, et al. Approval summary: Gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clin Cancer Res* 2001;7:1490.
11. Mamot C, Drummond DC, Noble CO, et al. Epidermal growth factor receptor–targeted immunoliposomes significantly enhance the efficacy of multiple anticancer drugs *in vivo*. *Cancer Res* 2005;65:11631.
12. Shao W, Zhao S, Liu Z, et al. Inhibition of human tumor xenograft growth in nude mice by a conjugate of monoclonal antibody LA22 to epidermal growth factor receptor with antitumor antibiotics mitomycin C. *Biochem Biophys Res Commun* 2006;349:816.
13. Eiblmaier M, Meyer LA, Watson MA, et al. Correlating EGFR Expression with receptor-binding properties and internalization of ^{64}Cu-DOTA-cetuximab in 5 cervical cancer cell lines. *J Nucl Med.* 2008;49:1472.
14. Reist CJ, Archer GE, Kurpad SN, et al. Tumor-specific antiepidermal growth factor receptor variant III monoclonal antibodies: Use of the tyramine-cellobiose radioiodination method enhances cellular retention and uptake in tumor xenografts. *Cancer Res* 1995;55:4375.
15. Nordberg E, Friedman M, Göstring L, et al. Cellular studies of binding, internalization, and retention of a radiolabeled EGFR-binding affibody molecule. *Nucl Med Biol* 2007;34:609.

16. Perera RM, Zoncu R, Johns TG, et al. Internalization, intracellular trafficking, and biodistribution of monoclonal antibody 806: A novel antiepidermal growth factor receptor antibody. *Neoplasia* 2007;9:1099.
17. Lindmo T, Boven E, Cuttitta F, et al. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J Immunol Meth* 1984;72:77.
18. Chen X, Park R, Hou Y, et al. MicroPET and autoradiographic imaging of GRP receptor expression with ^{64}Cu-DOTA-[Lys^3]bombesin in human prostate adenocarcinoma xenografts. *J Nucl Med* 2004;45:1390.
19. Bella RL, Garcia-Garayoa E, Langer M, et al. *In vitro* and *in vivo* evaluation of a ^{99m}Tc(I)-labeled bombesin analogue for imaging of gastrin releasing peptide receptor-positive tumors. *Nucl Med Biol* 2002;29:553.
20. Rosenblum MG, Cheung LH, Liu Y, et al. Design, expression, purification, and characterization, *in vitro* and *in vivo*, of an antimelanoma single-chain Fv antibody fused to the toxin gelonin. *Cancer Res* 2003;63:3995.
21. Kirpotin DB, Drummond DC, Shao Y, et al. Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. *Cancer Res* 2006;66:6732.
22. Altenschmidt U, Schmidt M, Groner B, et al. Targeted therapy of Schwannoma cells in immunocompetent rats with an erbB2-specific antibody-toxin. *Int J Cancer* 1997; 73:117.
23. Poul MA, Becerril B, Nielsen UB, et al. Selection of tumor-specific internalizing human antibodies from phage libraries. *J Mol Biol* 2000;301:1149.
24. Heldin CH. Dimerization of cell-surface receptors in signal transduction. *Cell* 1995;80:213.
25. Ciardiello F, Tortora G. A novel approach in the treatment of cancer: Targeting the epidermal growth factor receptor. *Clin Cancer Res* 2001;7:2958.
26. Speake G, Holloway B, Costello G. Recent developments related to the EGFR as a target for cancer chemotherapy. *Curr Opin Pharmacol* 2005;5:343.
27. Wu D, Wang L, Sato GH, et al. Human epidermal growth factor (EGF) receptor sequence recognized by EGF competitive monoclonal antibodies: Evidence for the localization of the EGF-binding site. *J Biol Chem* 1998;264:17469.
28. Wu D, Wang L, Chi Y, et al. Human epidemal growth factor receptor residue covalently cross-linked to epidermal growth factor. *Proc Natl Acad Sci USA* 1990;87:3151.
29. Astsaturov I, Cohen RB, Harari PM. EGFR-targeting monoclonal antibodies in head and neck cancer. *Curr Cancer Drug Targets* 2006;6:691.
30. Imai K, Takaoka A. Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer* 2006;6:714.
31. Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. *Nat Biotechnol* 2005;23:1147.
32. Maeda H, Fang J, Inutsuka T, et al. Vascular permeability enhancement in solid tumor: Various factors, mechanisms involved, and its implications. *Int Immunopharmacol* 2003;3:319.