Integrinβ6-Targeted Immunoliposomes Mediate Tumor-Specific Drug Delivery and Enhance Therapeutic Efficacy in Colon Carcinoma

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Abstract

Purpose: Adjuvant chemotherapy is one of the significant treatments for colon cancer in clinic. However, it does not achieve the desired therapeutic efficacy, largely due to chemotherapeutic resistance. Integrinβ6 (ITGB6) is expressed in malignant colonic epithelia, but not in normal epithelia, and is associated with the progression, metastasis, and chemotherapeutic resistance of colon cancer. Accordingly, it is necessary to design therapeutic approaches for efficient and targeted drug delivery into ITGB6-positive cancer cells to improve chemotherapeutic efficacy in colon cancer.

Experimental Design: PEGylated liposomes were employed to design ITGB6-targeted immunoliposomes, which have ITGB6 monoclonal antibodies (mAbs) conjugated. We evaluated the ITGB6-targeted immunoliposomes internalization into colon cancer cells and examined 5-fluorouracil (5-FU)–induced cellular apoptosis produced by ITGB6-targeted immunoliposomes + 5-FU. In addition, the biodistribution and antitumor efficiency of ITGB6-targeted immunoliposomes were observed in vivo.

Introduction

Colon cancer is the third most common cancer and the fourth leading cause of cancer-related deaths worldwide (1). Although surgery remains the preferred treatment, 5-fluorouracil (5-FU)–based adjuvant chemotherapy is the conventional care for stage III (lymph node–positive) patients and can reduce mortality by 25% compared with surgery alone (2). However, because the response rate of chemotherapy is only 10% to 20%, the treatment of advanced and metastatic cases remains a challenging problem (3). Therefore, it is urgent to explore novel therapeutic strategies for colon cancer treatment to overcome chemotherapeutic drug resistance and to improve chemotherapeutic efficacy.

The targeted delivery of anticancer drugs to tumors has been broadly recognized as an important method for improving chemotherapeutic efficacy and attenuating chemotherapeutic side effects. Sterically stabilized liposomes with a polymeric PEG coating, which have prolonged circulation times, lower reticuloendothelial system (RES) uptake and higher tumor accumulation, were thought to be an ideal drug delivery system (4, 5). However, these liposomes passively interact with tumor cells, in vitro or in vivo, resulting in nonspecific drug release that leads to the eventual diffusion of the drugs into some normal tissues or cells rather than tumors (6).

Immunoliposomes, which are conjugated with monoclonal antibodies (mAb), are capable of both drug delivery and molecular targeting (7, 8). By combining the specific targeting properties of mAbs and drug delivery advantages of liposomes, immunoliposomes are a promising approach for the targeted delivery of anticancer drugs to tumors (9). To date, various tumor-associated antigens have been validated as targets for antibody-based immunoliposomes in cancer therapeutics. Immunoliposomes targeting CD30, HER2/neu, EGFR, and VEGFR, which are expressed on various tumor cell types, have been developed and thoroughly characterized (10–13). On the basis of encouraging preclinical data and advances in large-scale production processes, EGFR-specific immunoliposomes are already being used in clinical trials (14).
Translational Relevance

The targeted delivery of anticancer drugs into tumors is an important factor for improving the therapeutic efficacy of cancer treatments. By combining specific targeting and drug delivery, immunoliposomes are a promising approach for the targeted delivery of anticancer drugs to tumors. Our group has confirmed that integrin α6 (ITGB6) is associated with the invasion, metastasis, and chemotherapeutic resistance of colon cancer. In this study, we describe preclinical data that support the therapeutic efficacy of ITGB6-targeted immunoliposomes against colon cancer.

Integrin α6 (ITGB6) is a subtype of integrin that is expressed exclusively on the surfaces of epithelial cells and is a receptor for extracellular matrix proteins (15). ITGB6 expression is upregulated during embryogenesis, oncogenesis, and epithelial repair, whereas it is generally undetectable in healthy epithelial tissues (16). In colon cancer, ITGB6 is specifically expressed in tumor tissues and is rarely present in tissues adjacent to the tumor (17). In addition, we previously reported that ITGB6 was associated with colon cancer pathology, malignancy, and TNM stage and could act as a prognostic indicator in aggressive colon carcinomas (18). Our research previously confirmed that ITGB6 contributed to chemotherapeutic resistance in colon cancer; ITGB6 protected colon cancer cells from 5-FU-induced growth inhibition and apoptosis (19). Unsurprisingly, the exclusive expression of ITGB6 and its influential effects in colon cancer make it a novel therapeutic target for colon cancer treatment.

ITGB6 has previously been employed as a clinical biomarker for early cancer detection (20, 21). Moreover, ITGB6 signaling axis-blocking agents have also been designed to exploit this receptor as a therapeutic target for cancer treatment (22, 23). However, research concerning ITGB6-targeted drug delivery for colon cancer chemotherapy has not been reported. In the current study, we describe the design, preparation, and characterization of ITGB6-targeted immunoliposomes, and explore their antitumor efficacy against colon cancer in vitro and in tumor xenograft models using 5-FU–loaded ITGB6-targeted immunoliposomes.

Materials and Methods

Materials

Hydrogenated soy phosphatidylcholine (HSPC) and cholesterol were purchased from Nippon Fine Chemical. Distearoylphosphatidylethanolamine (DSPE)-mPEG (2000) and DSPE-Peg (2000)-NH2 were purchased from Avanti Polar Lipids. The homobifunctional crosslinker bis (sulfosuccinimidyl) suberate (BS3) was from ProteoChem. 5-FU, organic solvents, and other reagent purity chemicals were from Sigma. The cytochrome C apoptosis assay kit and caspase fluorometric assay kit was obtained from Biovision. Phosphorylated ERK1/2 (p-ERK1/2), caspase-3/9, cleaved PARP and GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc., and cleaved caspase-3 and β-actin antibodies were from Cell Signaling Technology, Inc. The monoclonal antibody that detects the extracellular domain of human ITGB6 (E7P6) was prepared and provided as a gift from Michael Agrez (The University of Newcastle, Newcastle, Australia). The Annexin V–FITC/propidium iodide (PI) cell apoptosis assay kit was purchased from Merck Millipore. The cell counting kit-8 (CCK-8) was from DOJindo Molecular Technologies. The In Situ Cell Death Detection Kit was obtained from Roche.

Cell lines

Human colon cancer cell lines, SW480 and HT-29, were obtained from the ATCC. SW480 colon cancer cells lack constitutive ITGB6 expression. SW480B6 cells that were stably transfected with pcDNA1neo constructs containing the ITGB6 gene and SW480 mock cells expressing only plasmid were prepared as previously described (24). HT-29 cells constantly express ITGB6. HT-29 cells with siRNA suppressed ITGB6 expression were also prepared as reported previously (25). The cells were maintained as monolayers in medium comprising DMEM (HyClone) containing 10% heat inactivated FCS (Gibco) and supplemented with 20 mmol/L HEPES, 100 IU/ml penicillin, and 100 mg/ml streptomycin. The cells were incubated in 37°C, 5% CO2, and saturated humidity.

Liposome preparation

The nontargeted liposomes that were to be loaded with 5-FU were composed of HSPC, cholesterol, and DSPE-Peg2000 at a molar ratio of 2:1:0.1. The liposomes were prepared using pH gradients combined with the reverse-phase evaporation method. Briefly, lipids were dissolved in a mixture of chloroform and methanol (9:1 v/v). After addition of 0.3 mol/L sodium citrate buffer (SCB, pH = 4.0) of 5-FU-containing DSPE-Peg2000, the mixture in a ratio 4:1 (v/v) between organic and aqueous phase was sonicated at room temperature, and the chloroform and methanol were evaporated using a rotary evaporator. The pH was then adjusted to 7.0 using 0.4 mol/L sodium phosphate dibasic buffer. The liposomes were shaken in a vortex to form an aqueous suspension and were subsequently extruded 10 times through polycarbonate filters with a defined pore size of 400–100 nm. The liposomes encapsulating 5-FU were separated from free 5-FU by using a Sephadex G50 column. The concentrations of liposomal and free 5-FU were determined by spectrophotometry. The encapsulation efficiency (EE%) of drugs was calculated from the equation:

\[ EE\% = \frac{W_{\text{encapsulated}}}{W_{\text{encapsulated}} + W_{\text{free}}} \times 100\% \]

ITGB6-targeted immunoliposomes were composed of HSPC/Chol/DSPE-Peg2000/DSPE-Peg2000-NH2 at a molar ratio of 2:1:0:08:0.02. These liposomes were prepared and loaded with 5-FU as described above. Then ITGB6-specific antibody E7P6 was coupled to liposomes using the previously reported method (26). The cross-linker BS3 was applied to activate the DSPE-Peg2000-NH2 in the liposomes, which were then conjugated to the amino groups of the ITGB6 antibody. Briefly, BS3 was added to the liposomes at a final concentration of 5 mmol/L and was incubated for 1 hour at room temperature. The excess untreated cross-linker was removed via ultrafiltration using an Amicon Ultra-0.5 3K (Millipore). The activated liposomes were collected and incubated with ITGB6 antibody for 2 hours at room temperature. To quench the reaction, 50 mmol/L Tris was added at room temperature for 15 minutes. The immunoliposomes were separated from the unconjugated ITGB6 antibody using Sepharose CL-4B columns. For the cellular uptake studies, liposomes containing coumarin-6 were prepared by the same method.
For the in vitro release studies, 1 mL of 5-FU liposomes was placed into a dialysis bag and dialyzed against phosphate buffer (200 mL, pH 7.4). The medium was stirred at 37°C. At predetermined time intervals, 2 mL of the medium was removed and replaced with an equal volume of phosphate buffer. The released 5-FU was quantified using spectrophotometry. Drug release studies in the presence of serum were conducted in a similar manner. In brief, 200 µL of the liposomes was mixed with 800 µL of PBS and was then dialyzed against buffer at pH 7.4 at 37°C. Samples were removed at specified time points and the concentrations of 5-FU were determined. Release of 5-FU from immunoliposomes at various values of pH was investigated with buffers (pH = 6.5, 7.5, and 8.5) using the same method.

Characterization of liposomes

The morphologies of liposomes and ITGB6-targeted immunoliposomes were examined by transmission electron microscopy (TEM; JEM-100CX II, Japan). The samples were placed onto a copper grid and air-dried, followed by negative staining with a drop of 2% phosphotungstate solution.

The mean particle size and distribution of liposomes, as well as zeta-potential values, were determined by Malvern Zetasizer Nano ZS90.

Cellular uptake and internalization studies

The cellular uptake of liposomes was examined using a fluorescence microscope. Cells were seeded on chambered coverslips in 24-well culture plates (2 × 10^4 cells/well) and were cultured for 24 hours. Then, coumarin-6 loaded liposomes, ITGB6-targeted immunoliposomes, or free coumarin-6 were added to each well at 24 hours. Then, coumarin-6 loaded liposomes, ITGB6-targeted immunoliposomes, or free coumarin-6 were added to each well at a final coumarin-6 concentration of 2 µg/mL. Cells cultured with only medium served as blank controls. After a 1-hour incubation, the medium was removed and the cells were washed three times with PBS. Then, the cells were fixed with 4% paraformaldehyde for 15 minutes. After being washed twice with PBS, the cell nuclei were stained with DAPI for 10 minutes. The fluorescence images were analyzed using a fluorescence microscope (OLYMPUS IX81, Japan). The cellular internalization of the various liposomes was also analyzed using flow cytometry. HT-29 and SW480β6 cells were seeded onto 6-well plates (1 × 10^5 cells/well). The cells were cultured and treated as described above. The cells were trypsinized and washed three times with PBS. Then, the cells were collected via centrifugation and were resuspended in 500 µL of PBS. Cellular uptake was analyzed by flow cytometry.

Cytotoxicity studies

The cytotoxicity studies were performed using the CCK-8 assay. Cells were seeded in 96-well cell culture plates (1 × 10^4 cells/well) and incubated for 24 hours. Then, the medium was exchanged with 100 µL fresh DMEM containing free 5-FU, liposomes + 5-FU, or immunoliposomes + 5-FU ([5-FU] = 0.2, 1, 5, 25, 125 µg/mL). Cells treated with serum-free medium were used as a control. After incubation for predetermined times, the cell viability was determined using a CCK-8 assay kit according to the manufacturer's instructions. After adding 10 µL CCK-8 to each well, followed by a 2-hour incubation, the absorbance of each sample was measured at a wavelength of 450 nm using a microplate reader (RT-2100C, China). The 5-FU concentration producing a 50% inhibition of proliferation (IC_{50}) in HT-29 or SW480β6 cells for the different experimental formulations was calculated.

Flow cytometry apoptosis analysis

Cells were plated in 6-well culture plates (1 × 10^5 cells/well) and incubated for 24 hours. Then, the medium was exchanged with DMEM containing various formats of 5-FU, while cells treated with only medium were used as control. After incubation for 24 hours, cells were harvested, washed in PBS, and resuspended in Annexin V binding buffer. Following the instructions provided by the manufacturer, Annexin V–FITC was added to the cell suspensions and the cells were incubated for 15 minutes at 4°C. Then, PI was added and incubated for 5 minutes at 4°C. The fluorescently labeled cells were tested using a flow cytometer, and the results were analyzed by Flowjo software.

Western blotting

Cytochrome C release was analyzed by Western blotting. The cytosol lysate was extracted using the cytochrome C apoptosis assay kit by the method described before (19). Cytosolic proteins at a final protein amount of 20 µg were loaded onto an SDS-PAGE gel and were electrophoresed. Subsequently, the separated proteins were transferred onto polyvinylidene difluoride membranes and were immunoblotted using the cytochrome C primary antibody overnight at 4°C, followed by incubation with an HRP (horseradish peroxidase)-labeled secondary antibody. The protein bands were visualized using the ECL method.

The levels of caspase-3/9, cleaved caspase-3, cleaved PARP, and phosphorylated ERK1/2 (p-ERK1/2) were also determined using Western blotting following the same procedures that were described above.

Caspase activity assay

A caspase activity assay was carried out using a caspase fluorometric assay kit following the instructions provided by the manufacturer. In brief, cells were homogenized on ice with lysis buffer. An aliquot of 50 µL of supernatants was incubated with an equal volume of the reaction buffer containing fluorogenic peptide substrate at 37°C for 1 to 2 hours. Enzymatic release of free fluorogenic moiety was measured by a fluorometer.

Tissue distribution study

BALB/C female nude mice bearing HT-29 of which the tumor size was larger than 100 mm^3 were randomly assigned to three groups and injected intravenously through tail veins with a dosage of 5-FU (20 mg/kg), liposomes + 5-FU (20 mg 5-FU/kg), or ITGB6-targeted immunoliposomes + 5-FU (20 mg 5-FU/kg). Blood samples were obtained from the retro-orbital plexus at predetermined time points, and the plasma was collected and stored. The mice were sacrificed at the indicated time points (0.5, 2, 4, 8, 12, and 24 hours after the drug was administered i.v.) to collect tissue samples. The tissues (heart, liver, spleen, lung, kidney, and tumor) were removed, washed, weighed, and homogenized in physiologic saline. 5-FU was extracted using ethyl acetate. The amount of 5-FU in tissues was analyzed by the HPLC assay. The data were normalized to the tissue weight. The main pharmacokinetic parameters were calculated via the statistical moment method using DAS 2.0 software.

In vivo therapeutic efficacy

BALB/C female nude mice were subcutaneously implanted with HT-29 or SW480β6 human colon cancer cells at a final concentration of 1 × 10^7/200 µL. Mice with tumor volumes of...
approximately 50 mm$^3$ were selected and randomly assigned to three treatment groups. Free 5-FU, 5-FU-loaded liposomes, or ITGB6-targeted immunoliposomes were intravenously injected twice a week at a 5-FU dosage of 20 mg/kg. Blank immunoliposomes or physiologic saline (N.S.) were also injected as controls. The tumor size and body weight were measured every other day during the treatment period. The tumor volume was calculated from the following formula: $(W^2 \times L)/2$. After 3 weeks, the mice were sacrificed and the tumors were dislodged and weighed. All of the animal studies were conducted using a protocol approved by the Institutional Animal Care and Use Committee at the School of Medicine, Shandong University (Jinan, Shangdong, P.R. China).

TUNEL staining assay

Mice were sacrificed, and sections of tumor tissues in each group were prepared. The TUNEL assay was carried out using an In Situ Cell Death Detection Kit according to the manufacturer’s instructions. Finally, the apoptotic index was calculated by counting the number of TUNEL-positive nuclei visible on 40× high-power-field objective in at least 3 fields/sample and expressing the results as percentage of the total number of cells in the same fields. Apoptotic cells were recognized by the appearance of brown or tan-stained nuclei.

Statistical analysis

Data were presented as the mean ± SD and all measurements were performed from at least three independent experiments. The statistical significance was determined by the Mann–Whitney test or Student t test. $P < 0.05$ was considered statistically significant. The statistical analyses were performed using the GraphPad Prism software (GraphPad Software, Inc.).

Results

Characterization of liposomes

The morphology of liposomes and ITGB6-targeted immunoliposomes were spherical or ellipsoidal, as we could directly observe by TEM (Fig. 1B). Size distribution of both liposomes was spherical or ellipsoidal, as we could directly investigate. The zeta potential of the liposomes was $-2.74$ nm. After mAb conjugation, the zeta potential further decreased to $-2.23$ nm. These indicated that immunoliposomes stability was associated with the mAb conjugation may have a significant impact on immunoliposomes size. The zeta potential of the liposomes was $-23.77$ ± 2.35 mV. After mAb conjugation, the zeta potential further decreased to a value of $-8.18$ ± 1.83 mV. Furthermore, as the amount of conjugated mAbs increased, the immunoliposomes zeta potential gradually decreased (data not shown), which most likely indicated that immunoliposomes stability was associated with the amount of mAbs conjugated. The 5-FU encapsulation efficiencies (EE%) of the liposomes and immunoliposomes were 78.43% ± 2.23% and 75.95% ± 2.30%, respectively. Figure 1C showed the in vitro 5-FU release profiles from both liposomal formats in the PBS or serum condition. ITGB6-targeted immunoliposomes and liposomes had similar trends of 5-FU release, indicating that the ITGB6 mAbs conjugated onto the liposomes had no influence on the kinetic properties. In addition, compared with release profiles in PBS, serum could significantly retard the release of 5-FU in liposomes. In addition, ITGB6-targeted immunoliposomes showed no greatly sensitivity to different pHs on the release of 5-FU (Fig. 1D).

Uptake of ITGB6-targeted immunoliposomes in ITGB6 expressing colon cancer cells

Before researching the cellular association aspects of ITGB6-targeted immunoliposomes, we assessed the ITGB6 expression level in HT-29 and SW480 cells colon cancer cells by flow cytometry. As shown in Fig. 1E, both HT-29 and SW480 cells obviously expressed ITGB6, whereas HT-29 treated with β6-siRNA and untreated SW480 had low or negative ITGB6 expression. SW480 mock cells had lower ITGB6 expression, partially due to the nonspecific binding; however, the fluorescence values were within the experimental error. ITGB6-targeted immunoliposomes and liposomes were then loaded with fluorescent coumarin-6 to investigate the interactions between the liposomes and the target cells. The specific cell binding and cellular uptake of ITGB6-targeted immunoliposomes were visualized by fluorescence microscopy (27). The fluorescence images of HT-29 and SW480 cells were shown in Fig. 2A. The fluorescence intensities of ITGB6-targeted immunoliposomes in both HT-29 and SW480 cells were significantly greater than the liposomes, suggesting that ITGB6-targeted immunoliposomes had higher cellular accumulation than liposomes. However, free coumarin-6 had high cellular accumulation, partially due to its highly hydrophobic nature; as a result, it was chosen as the positive control (28). A flow cytometry analysis was also performed to quantify the cellular internalization of ITGB6-targeted immunoliposomes. As illustrated in Fig. 2B, slight fluorescence was detected in HT-29 and SW480 cells incubated with liposomes + coumarin-6, whereas much higher fluorescence intensities were measured in both cell lines treated with ITGB6-targeted immunoliposomes + coumarin-6. All above results indicated that ITGB6-targeted immunoliposomes could enhance the cellular internalization compared with liposomes, which probably resulted in improved anticancer efficacy in colon cancer cells.

To evaluate the correlation between ITGB6-targeted immunoliposomes cellular uptake and ITGB6 expression and to confirm the specificity of ITGB6-targeted immunoliposomes toward ITGB6-expressed colon cancer cells, HT-29 cells of which ITGB6 was suppressed by siRNA or IGTB6-negative SW480 cells were also both treated with ITGB6-targeted immunoliposomes + coumarin-6. Figure 2A also showed that fluorescence in ITGB6-suppressed HT-29 or ITGB6-negative SW480 cells was significantly weaker compared with HT-29 or SW480 cells after ITGB6-targeted immunoliposomes + coumarin-6 treatment. The differences observed between ITGB6-targeted immunoliposomes internalization in cells that expressed different levels of ITGB6 may testify the specific cell binding of ITGB6-targeted immunoliposomes and indicate that the cellular uptake was dependent on the ITGB6 expression on cellular surface.

Cytotoxicity and proliferation inhibition on colon cancer cells

To evaluate the antitumor activity of ITGB6-targeted immunoliposomes, colon cancer cells were exposed to 5-FU, liposomes + 5-FU, and ITGB6-targeted immunoliposomes + 5-FU, with 5-FU at an equivalent dose from 0.2 to 125 μg/mL. The cytotoxicity and growth inhibition of the cells was then evaluated by the CCK-8 assay. As shown in Fig. 2C, all treatments demonstrated a clear 5-FU dose-dependent cytotoxicity in the experimental cell lines. However, for HT-29 and SW480 cells, the ITGB6-targeted immunoliposomes + 5-FU inhibited the cellular growth more greatly than liposomes + 5-FU ($P < 0.01$). Interestingly, the difference in growth...
Figure 1.
A, typical particle size and distribution of nontarget liposomes (Ls) and ITGB6-targeted immunoliposomes (IL). B, transmission electron photomicrograms of nontarget liposomes and ITGB6-targeted immunoliposomes. Bar = 100 nm. C, in vitro release profiles of 5-FU from nontarget liposomes (●) and ITGB6-targeted immunoliposomes (■) in PBS and serum. Data represent mean ± SD (n = 5). D, the release profiles of 5-FU from ITGB6-targeted immunoliposomes in different pH conditions (pH = 6.5, 7.5, and 8.5). E, FACScan analyzed ITGB6 expression in HT-29 and SW480 colon cancer cells.
Figure 2.
A, fluorescence microscopy images showed the internalization of ITGB6-targeted immunoliposomes (IL) and liposomes (Ls) in HT-29 and SW480β6 colon cancer cells, and the internalization of ITGB6-targeted immunoliposomes in HT-29 β6-siRNA or SW480 cells on which ITGB6 was low expressed or negative. Cell nuclei were stained blue with DAPI and coumarin-6 was shown as green fluorescence. (Continued on the following page.)
inhibition between these two types of liposomes was almost undistinguishable in the ITGB6-negative SW480 cells (Fig. 2D), which implied that the improved antitumor activity of ITGB6-targeted immunoliposomes may be attributed to the specific target binding to ITGB6 on cellular surface. The IC_{50} values of 5-FU at 24 and 48 hours for HT-29 and SW480/C6 cells were presented in Fig. 2E. The ITGB6-targeted immunoliposomes produced a more than 90% decrease in 5-FU IC_{50} relative to that of liposomes, which suggested that the ITGB6-targeted immunoliposomes enhanced the cytotoxicity of 5-FU against these colon cancer cells.

Also in Fig. 2C and E, compared with free 5-FU, liposomes+5-FU was more cytotoxic against both HT-29 and SW480/C6 cells (P < 0.05) and reduced the 5-FU IC_{50} values over 50%. These results were in accordance with previous studies which demonstrated that the in vitro cytotoxicity of liposomal 5-FU was higher than that of the free drug (29, 30). In addition, the blank nontargeted liposomes and ITGB6-targeted immunoliposomes had no significant effects on cell growth (data not shown).

**Efficacy of ITGB6-targeted immunoliposomes on the 5-FU-induced apoptosis of colon cancer cells**

ITGB6 could protect HT-29 and SW480/C6 colon cancer cells from 5-FU-induced apoptosis (19). Therefore, to confirm the 5-FU-induced apoptosis efficacy of ITGB6-targeted immunoliposomes, HT-29, and SW480/C6 cells were treated with 5-FU, liposomes+5-FU, and ITGB6-targeted immunoliposomes+5-FU for 24 hours, with final 5-FU concentrations of 10 μg/mL in SW480/C6 cells and 40 μg/mL in HT-29 cells. Subsequently, the cells were analyzed using flow cytometry. As presented in Fig. 3A and B, in the ITGB6-targeted immunoliposomes groups, the apoptotic rate of HT-29 cells was 21.91% ± 1.62%; the rates in the free 5-FU and liposome groups were 8.99% ± 1.00% and 14.53% ± 0.99%, respectively. It was evident that the ITGB6-targeted immunoliposomes caused more apoptosis in HT-29 cells than the liposomes (P < 0.01). For SW480/C6 cells, the apoptotic rates in the free 5-FU, liposome, and ITGB6-targeted immunoliposome groups were 12.77% ± 1.21%, 16.61% ± 1.23%, and 26.18% ± 2.43%, respectively. There were also significant differences between the liposome and immunoliposome groups (P < 0.01). The apoptotic rates of both HT-29 and SW480/C6 cells in the Ls+5-FU groups were higher than in the free 5-FU groups (P < 0.05). Moreover, the blank ITGB6-targeted immunoliposomes did not affect the apoptosis in either cell line.

Because the ITGB6-mediated chemotherapeutic resistance of colon cancer cells was through the ITGB6-ERK pathway (19), it was essential to verify whether the ITGB6-targeted immunoliposomes directly affected the pathway before we speculated the reason for the enhanced 5-FU-induced apoptosis of ITGB6-targeted immunoliposomes. Therefore, we examined the levels of phosphorylated ERK1/2 (p-ERK1/2) in HT-29 and SW480/C6 cells treated with ITGB6-targeted immunoliposomes or liposomes using the Western blot method. As shown in Fig. 3C, there were no obvious differences in p-ERK1/2 levels between the immunoliposomes and liposomes, which could indicate that ITGB6-targeted immunoliposomes had no influence on the ITGB6 pathway. In conclusion, we confirmed that in comparison with liposomes, ITGB6-targeted immunoliposomes promoted 5-FU-induced apoptosis probably by enhancing cellular internalization rather than inhibiting the ITGB6 pathway.

**Efficacy of ITGB6-targeted immunoliposomes on the apoptosis-associated proteins in colon cancer cells**

It was evident that 5-FU-induced apoptosis in colon cancer cells was mediated predominantly via the mitochondrial apoptotic pathway, which involves two crucial processes: cytochrome C release and caspase-3 activation (31). As we previously demonstrated, ITGB6-mediated colon cancer cells’ 5-FU resistance was associated with this apoptotic pathway (19). Therefore, to investigate whether the ITGB6-targeted immunoliposomes enhanced 5-FU sensitivity in colon cancer cells via this apoptotic pathway, HT-29 and SW480/C6 cells were grouped and treated as described in the section above, and the levels of mitochondrial apoptosis-related proteins were then measured by Western blotting. As shown in Fig. 3D–G, in HT-29 cells, cytosolic cytochrome C level was evidently higher in the ITGB6-targeted immunoliposome group than the liposome group (P < 0.01). Consequently, the ITGB6-targeted immunoliposomes group had higher cleaved caspase-3 and cleaved PARP levels compared with the liposome group (P < 0.01), indicating the upregulation of the mitochondrial apoptotic pathway. However, there were no significant differences between the liposome and free 5-FU groups. Similar results were obtained in SW480/C6 cells: cytosolic cytochrome C, cleaved caspase-3, and cleaved PARP remained at higher level in the ITGB6-targeted immunoliposome group than the liposome group (P < 0.05). Unlike HT-29 cells, the liposome groups in SW480/C6 cells had higher cytochrome C, cleaved caspase-3, and cleaved PARP than the free 5-FU groups (P < 0.05), most likely due to different cell characteristics. However, there were no significant differences in the expression levels of capase-9 and capase-3 between free 5-FU and liposomal 5-FU groups in both cell lines. In addition, the activities of capase-9 and capase-3 were also determined. In both HT-29 and SW480/C6 cells, the caspase-9 and caspase-3 activities were higher in ITGB6-targeted immunoliposomes than liposome groups (P < 0.05). In conclusion, considering the result that there were no obvious differences in cytochrome C expression and caspase-9/3 activities between the blank immunoliposomes and control groups, the potential mechanism underlying the promoted mitochondrial apoptotic pathway may include enhanced 5-FU accumulation in the target cells due to higher ITGB6-targeted immunoliposomes intracellular uptake.

**Pharmacokinetic studies and biodistribution of ITGB6-targeted immunoliposomes**

The main pharmacokinetic parameters of both 5-FU liposomal formulations were evaluated in HT-29 human colon cancer xenograft models. The areas under the curve of blood 5-FU (Continued) Green fluorescence intensity in the immunoliposomes group was higher than in the liposomes group. There was no increase of fluorescence intensity by immunoliposomes in HT-29 β6-siRNA and SW480 cells. HT-29 con-siRNA and SW480 mock transfectant cells treated with immunoliposomes +coumarin-6 served as control. Bar = 20 μm. B, internalization of liposomes were evaluated by flow cytometry in HT-29 and SW480/C6 cells. Growth inhibition in cells was evaluated by the CCK-8 assay. All format of 5-FU inhibited colon cancer cells growth in a dose-dependent way; ITGB6-targeted immunoliposomes enhanced 5-FU-induced growth inhibition compared with liposomes in HT-29 and SW480/C6 cells (C), whereas there was no evident difference between ITGB6-targeted immunoliposomes and liposomes (Ls) in SW480 cells (D). *P < 0.05 vs. 5-FU; **P < 0.01 versus liposomes + 5-FU. E, IC_{50} of 5-FU in HT-29 and SW480/C6 cells at 24 and 48 hours. Data represent mean ± SD (n = 5). ***P < 0.01 versus 5-FU; ****P < 0.01 versus liposomes + 5-FU.
Figure 3.
Efficacy of ITGB6-targeted immunoliposomes (IL) on the 5-FU-induced apoptosis in colon cancer cells. A, apoptosis of HT-29 and SW480β6 cells induced by free 5-FU, liposomes (Ls)+5-FU and immunoliposomes+5-FU. And quantification of cell apoptotic rates was shown (B). In both cell lines, no significant differences were observed in apoptosis between control and blank immunoliposomes groups. (Continued on the following page.)
Compared with liposomes, ITGB6-targeted immunoliposomes observably increased the 5-FU accumulation in tumor. Distribution of 5-FU in mean tissues. Concentration of 5-FU in the heart, liver, and kidney, which would be expected to reduce 5-FU side effects. The tissues 5-FU concentrations versus time were determined to estimate the biodistribution and tumor localization of the ITGB6-targeted immunoliposomes and liposomes (Fig. 4). The 5-FU concentrations were high in heart, liver, and kidney tissues after free 5-FU injection, which probably could indicate 5-FU side effects. In addition, because 5-FU was mainly metabolized in the liver (32), a high liver accumulation of 5-FU may indicate accelerated drug depletion. On the contrary, both liposomal formulations decreased the 5-FU concentration in the heart, liver, and kidney, which would be expected to reduce 5-FU side effects and clearance. In tumor tissues, ITGB6-targeted immunoliposomes and liposomes had a higher 5-FU concentrations than free 5-FU (P < 0.01; Figs. 4 and 5). However, as shown in Fig. 5, the 5-FU concentration for ITGB6-targeted immunoliposomes in the tumor steadily increased and climaxed after 12 hours, whereas 5-FU concentrations for liposomes decreased as time progressed. In addition, the AUC0–24 values of ITGB6-targeted immunoliposomes in tumor tissues were 39.28 ± 0.80 μg/g·h, higher than that of liposomes (10.42 ± 0.62 μg/g·h), indicating that ITGB6-targeted immunoliposomes enhanced tumor-targeting efficiency compared with liposomes.

Therapeutic efficacy of ITGB6-targeted immunoliposomes in vivo

To evaluate the therapeutic efficacy of ITGB6-targeted immunoliposomes, free 5-FU, liposomes + 5-FU, and immunoliposomes + 5-FU at the dose of 5-FU 20 mg/kg was intravenously injected in mice bearing HT-29 and SW480/J6 human colon cancer xenografts. The antitumor effect, indicated by tumor growth, was shown in Fig. 6A and C. In comparison with ITGB6 mAbs remained the sustained release and prolonged half-life pharmocokinetic properties of the liposomes.

For effective tumor-targeted drug delivery, the ITGB6-targeted immunoliposomes should be more inclined to localize in the tumor compared with the nontargeted liposomes, which would result in high drug concentrations in tumor cells and spare normal tissues from unnecessary toxicity. The tissues 5-FU concentrations versus time were determined to estimate the biodistribution and tumor localization of the ITGB6-targeted immunoliposomes and liposomes (Fig. 4). The 5-FU concentrations were high in heart, liver, and kidney tissues after free 5-FU injection, which probably could indicate 5-FU side effects. In addition, because 5-FU was mainly metabolized in the liver (32), a high liver accumulation of 5-FU may indicate accelerated drug depletion. On the contrary, both liposomal formulations decreased the 5-FU concentration in the heart, liver, and kidney, which would be expected to reduce 5-FU side effects and clearance. In tumor tissues, ITGB6-targeted immunoliposomes and liposomes had a higher 5-FU concentrations than free 5-FU (P < 0.01; Figs. 4 and 5). However, as shown in Fig. 5, the 5-FU concentration for ITGB6-targeted immunoliposomes in the tumor steadily increased and climaxed after 12 hours, whereas 5-FU concentrations for liposomes decreased as time progressed. In addition, the AUC0–24 values of ITGB6-targeted immunoliposomes in tumor tissues were 39.28 ± 0.80 μg/g·h, higher than that of liposomes (10.42 ± 0.62 μg/g·h), indicating that ITGB6-targeted immunoliposomes enhanced tumor-targeting efficiency compared with liposomes.

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Figure 6.

Efficacy of ITGB6-targeted immunoliposomes (ILs) in the ITGB6-expressing HT-29 and SW480β6 colon tumor xenograft models. A and C, free 5-FU, liposomes (Ls) + 5-FU, and immunoliposomes (ILs) + 5-FU were administered by intravenous injection at the dose of 5-FU 20 mg/kg on the indicated day after tumor implantation. Saline and blank immunoliposomes were used as control. (Continued on the following page.)
free 5-FU, both liposomal 5-FU formulations obviously suppressed tumor growth during the treatment period (P < 0.05 in HT-29 models and P < 0.01 in SW480/B6 models). No antitumor effects were observed in the N.S. and blank immunoliposome groups. Moreover, the tumor suppression of ITGB6-targeted immunoliposomes + 5-FU was significantly stronger than liposomes + 5-FU (P < 0.01). Consistent with the above result, both liposomal 5-FU formulations greatly reduced the final tumor weight in comparison with 5-FU and the average tumor weight in mice treated with ITGB6-targeted immunoliposomes + 5-FU was approximately 3-fold lower than that in mice treated with 5-FU alone (P < 0.01; Fig. 6B and D). In addition, the TUNEL assay was done to correlate the in vivo antitumor activity of ITGB6-targeted immunoliposomes with its apoptosis-inducing activity. As shown in Fig. 6E and F, compared with liposomes + 5-FU, ITGB6-targeted immunoliposomes + 5-FU induced apoptosis in tumor cells by a factor of 1.5- to 1.7-fold (P < 0.05).

The changes in mice body weights were monitored to evaluate the side effects of the different treatments. As shown in Fig. 6G, an obvious loss of body weight was observed in mice treated with 5-FU compared with mice treated with 5-FU-loaded liposomes or immunoliposomes (P < 0.01). Body weight gain of liposomes and immunoliposomes groups that was similar within the margin of error, was not as robust as that of control, partially due to the 5-FU release in blood and slight accumulation of 5-FU in other tissue. Figure 6G also demonstrated that body weight changes in these three types of 5-FU treatment groups were inconspicuous during the first 8 days of treatment, but differences were observed after that time point and subsequently becoming larger as time passed by. In addition, the mice treated with free 5-FU had poorer body conditions after the 9th or 12th day during the treatment period.

Discussion

The development of multidrug resistance (MDR) in cancer cells and the systemic toxic side effects are major obstacles to successful chemotherapy for colon cancer treatment. One of the approaches for simultaneously overcoming MDR and reducing systemic toxicity is to incorporate therapeutic drugs into nanocarriers, such as liposomes (33), for drug delivery into tumor sites. Liposomes carrying PEG phospholipid derivatives on their surfaces have prolonged blood circulation times and facilitate the tumor tissue accumulation via the enhanced permeability and retention effect (34, 35). However, there is concern that liposomes accumulation in the tumor area may not guarantee the bioavailability of the encapsulated drug in cancer cells or MDR cancer cells, immunoliposomes have been used to increase the efficiency and specificity of intracellular drug uptake by targeted delivery and ultimately to improve chemotherapy efficacy (36).

ITGB6 has the necessary characteristics to be a target for liposomal drug delivery, that is, abundant expression in solid tumors and limited expression in normal epithelial tissues (17). Furthermore, ITGB6 has specific tumor-related properties that make it a potential targeting receptor for tumor chemotherapy, particularly for colon cancer. We previously verified that ITGB6 was associated with chemotherapeutic resistance in colon cancer and that ITGB6 overexpression protected colon cancer cells from 5-FU-induced growth inhibition and apoptosis (19). Therefore, we used ITGB6-targeted immunoliposomes in this study as a drug delivery to investigate whether ITGB6-targeted immunoliposomes can selectively and specifically deliver increased drug to ITGB6-positive colon cancer cells to overcome MDR and to improve chemotherapeutic efficacy in colon cancer.

Immunoliposomes enhance the cellular internalization via ligand–receptor interactions compared with normal liposomes (37). In this study, the specific cell binding and internalization of ITGB6-targeted immunoliposomes in colon cancer cells were also observed. We determined that ITGB6-targeted immunoliposomes had significantly higher cell associations in HT-29 and SW480/B6 cells compared with liposomes. However, the cellular internalization of ITGB6-targeted immunoliposomes was prominently suppressed in ITGB6-negative or low-expression cells, which verified the specific cellular internalization of ITGB6-targeted immunoliposomes and indicated the fact that ITGB6-targeted immunoliposomes cellular internalization depended on the ITGB6 expression level on the cell surface. According to these results, it could be concluded that ITGB6 targeted binding was proved to enhance the cellular uptake of liposomes in ITGB6-positive colon cancer cells, and even speculated that a higher level of ITGB6 could possibly result in more cellular internalization of ITGB6-targeted immunoliposomes. In addition, it is well known that the interaction between ligand-targeted liposomes and target cells is mediated by endocytosis (38). Therefore, as a result of the endo/exocytic cycling procedure of ITGB6 in the colon cancer cells (39), we hypothesize that upregulated cellular internalization of ITGB6-target immunoliposomes is also associated with this procedure of ITGB6, which we will validate in future studies.

As a hydrophilic drug, 5-FU is absorbed in low efficiency just mainly dependent on cell membrane transporters, while the entry of liposomal 5-FU into the cells is relatively efficient by endocytosis-mediated internalization of liposomes (40, 41). This could potentially explain the fact that liposome-5-FU exerted greater cellular toxicity than free 5-FU (Figs. 2C and 3A). The ITGB6-targeted immunoliposomes, which had increased cellular internalization compared with liposomes, could consequently enhance 5-FU accumulation in colon cancer cells. Then, the increased 5-FU uptake would result in higher cellular toxicity against colon cancer cells, as what we determined in cellular toxicity and apoptosis studies. Furthermore, because of the fact that ITGB6-targeted immunoliposomes cellular internalization was associated with the ITGB6 expression level on cellular surface, the higher ITGB6-expressed colon cancer cells, which were more likely to produce 5-FU resistance, would probably have a higher level of 5-FU cellular accumulation. In addition, it has previously been reported that the suppression of the mitochondrial apoptotic pathway was the preferred mechanism of chemotherapeutic resistance for the majority of anticancer drugs, including 5-FU (42, 43). Because

(Continued) Both liposomes +5-FU and immunoliposomes +5-FU were significantly superior to free 5-FU and tumor suppression of immunoliposomes +5-FU was greater than liposomes +5-FU. B and D, tumors were dislodged and weighed. Immunoliposomes +5-FU groups had a lower tumor weight than liposomes +5-FU groups. Data represent mean ± SD (n = 5). P < 0.05 versus 5-FU, P < 0.01 versus 5-FU, P < 0.01 versus liposomes +5-FU. HT-29 and SW480/B6 xenographs were done with TUNEL staining (E), and quantification of percentage of apoptotic cells was calculated (F). *P < 0.05 versus 5-FU, **P < 0.05 versus liposomes +5-FU, G, body weight changes during the treatment. Free 5-FU had more serious effects on body weight than liposomes +5-FU or immunoliposomes +5-FU, while there was no difference between liposomes +5-FU and immunoliposomes +5-FU. Data represent mean ± SD (n = 5). *P < 0.01 versus 5-FU.
we demonstrated that the expression levels of the mitochondrial apoptotic-related proteins (cytochrome C, cleaved caspase-3, and cleaved PARP) and activities of caspase-9/3 were increased after ITGB6-targeted immunoliposomes + 5-FU treatment, we speculated that ITGB6-targeted immunoliposomes may overcome drug resistance by facilitating mitochondrial apoptotic pathway activation.

These promising in vitro results were further evaluated in vivo. The antitumor efficacy of ITGB6-targeted immunoliposomes was significantly superior to that of liposomes, which was probably caused by increased drug accumulation in tumor tissues. Although we determined that both types of liposomal 5-FU had similar pharmacokinetic properties in vivo, the 5-FU concentration in the tumor tissues of mice treated with ITGB6-targeted immunoliposomes was consistently higher than liposomes. These increased 5-FU level observed in the tumor tissues was probably achieved by the following potential mechanism. Both liposomes are able to accumulate in tumor tissues, ultimately reaching high levels in the tumor tissues. The nontargeted liposomes passively interact with the tumor cells, eventually resulting in the delivery of the majority of drug into some normal tissues or cells rather than the tumor (44). Conversely, ITGB6-targeted immunoliposomes are internalized more efficiently due to target binding and ITGB6 endocytosis, which greatly increase the accumulation of the loaded drug in cancer cells. The body weight was additionally observed to evaluate the side effects. Although body weight changes in immunoliposomes and liposomes groups were unobvious, which was likely due to the drug release. Biochim Biophys Acta 1992;1109:203–88:11460.

Conclusively, this study described a targeted drug delivery system for highly efficient and selective delivery of anticancer drugs in colon cancer overexpressing ITGB6. The ITGB6-targeted immunoliposomes achieved favorable antitumor efficacy and insignificant systemic toxicity, followed by specific binding and internalization in colon cancer cells. As a result, this approach may be useful for the delivery of various drugs for enhanced therapeutic index against colon cancer. Moreover, because ITGB6 is also specifically expressed in a variety of epithelial carcinomas (45–48), ITGB6-targeted immunoliposomes may represent a potential strategy for the clinical treatment of additional cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Integrinβ6-Targeted Immunoliposomes Mediate Tumor-Specific Drug Delivery and Enhance Therapeutic Efficacy in Colon Carcinoma

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