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• *COLORECTAL CANCER* •

Antitumor activity of anti-type IV collagenase monoclonal antibody and its lidamycin conjugate against colon carcinoma

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Abstract

AIM: Type IV collagenase including MMP-2 and -9 plays an important role in cancer cell invasion and metastasis and is an attractive target for mAb-directed therapy. The immunoreactivity of mAb 3G11, a mAb directed against type IV collagenase in human colorectal carcinomas, was studied by immuno-histochemical (IHC) staining. mAb 3G11 was conjugated to an antitumor antibiotic lidamycin (LDM). The antitumor activity of 3G11-LDM conjugate against colon carcinoma was investigated in mice.

METHODS: ELISA, gelatin zymography, and Western blot assay were used for the biological characterization of mAb 3G11. The immunoreactivity of mAb 3G11 with human colorectal carcinomas was detected by IHC staining. The cytotoxicity of LDM and 3G11-LDM conjugate to human colon carcinoma HT-29 cells was examined by clonogenic assay and MTT assay. The therapeutic effect of conjugate 3G11-LDM was evaluated with colon carcinoma 26 in mice.

RESULTS: As shown in ELISA, mAb 3G11 reacted specifically with type IV collagenase, while 3G11-LDM conjugate also recognized specifically its respective antigen. In IHC assay, mAb 3G11 showed positive immunoreactivity in most cases of colorectal carcinoma, and negative immunoreactivity in the adjacent non-malignant tissues. By gelatin zymography, the inhibition effect of mAb 3G11 on the secretion activity of type IV collagenase was proved. In terms of IC_{50} values in MTT assay, the cytotoxicity of LDM to human colon carcinoma HT-29 cells was 10 000-fold more potent than that of mitomycin C (MMC) and adriamycin (ADM). 3G11-LDM conjugate also displayed extremely potent cytotoxicity to human colon carcinoma HT-29 cells with an IC_{50} value of 5.6×10^{-19} mol/L. 3G11-LDM conjugate at the doses of 0.05 and 0.1 mg/kg inhibited the growth of colon carcinoma 26 in mice by 70.3 and 81.2%, respectively.

CONCLUSION: mAb 3G11 is immunoreactive with human colorectal carcinoma and its conjugate with LDM is highly effective against colon carcinoma in mice.

INTRODUCTION

There were 622 000 deaths of colorectal cancer globally in 2002 according to the World Health Report 2004 of WHO. Colorectal cancer can be considered as a complex disease, with a combination of predisposing genetic variants and environmental factors that contribute to the illness as a whole. Since colorectal carcinoma is a leading cause of cancer death in the world, many therapeutic strategies are being investigated, among which the recent major achievements are Avastin and Erbitux, as antibody therapeutics approved for treatment of refractory and advanced colorectal carcinoma by the FDA of USA in 2004. This indicates that antibody-based drugs are promising for colorectal cancer therapy.

Lidamycin (LDM), also called C-1027, is a member of enediyne antitumor antibiotics family and binds to the minor groove of DNA, causing double-strand breaks and apoptosis. LDM consists of an apoprotein (LDP) of 10.5 ku and an enediyne chromophore (LDC) of 843 Da. These two parts of the molecule, connected each other through non-covalent binding, can be dissociated and reconstituted[1,2]. Because of its extremely potent cytotoxicity against cancer cells and its remarkable activity of anti-angiogenesis and anti-metastasis, LDM can serve as an "effector" agent or "warhead" molecule to construct immunoconjugates[3-5]. In addition, as a potential chemotherapeutic agent for cancer treatment, LDM has recently entered phase I clinical trials.

Type IV collagenase (also called gelatinases including MMP-2 and -9), as the main member of MMPs family, is of particular interest in the study of antibody-based drugs because the enzyme plays an important role in cancer invasion and metastasis. Using type IV collagenase as a molecular target, mAb 3G11 directed against MMP-2 and -9 was herein produced, analyzed, and evaluated for targeting

cancer therapy. Previous studies have demonstrated that 3G11-LDM immunoconjugate remarkably suppresses the growth of hepatoma 22 (H22) and increases the survival time of tumor bearing mice. Moreover, the antitumor efficacy of the conjugate was higher than that of free LDM or mAb 3G11 alone[6]. In order to reduce the molecular size of agent, e.g. from the mAb to Fab' and even to the Fv, a series of antibody-based drugs have been studied in our laboratory[7,8]. Utilizing anti-tumor and anti-metastasis mAb 3G11 as a therapeutic agent or as a carrier in cancer treatment may enhance the selectivity of chemotherapy.

In this study, we observed the immunoreactivity of mAb 3G11 with 32 cases of human colorectal carcinoma specimens by IHC staining. For further characterization of the mAb, Western blot assay, ELISA, and gelatin zymography were performed. mAb 3G11 was used as a targeting carrier for colon cancer as it demonstrated a highly specific immunoreactivity to human colorectal carcinoma. 3G11-LDM immunoconjugate was prepared and its antitumor efficacy against colon carcinoma was examined.

MATERIALS AND METHODS

Materials
Highly purified LDM was prepared in our institute. MTT was obtained from Sigma Chemical Co., (St. Louis, MO, USA). Mitomycin C (MMC) was purchased from Kyowa Hakko Kogyo Co., Ltd.

Preparation of monoclonal antibody and conjugate
mAb 3G11, a murine IgG1-type mAb, was prepared using type IV collagenase (SIGMA Inc.) to immunize BALb/c mice. According to our previous methods, mAb 3G11 was purified by sequential affinity and size-exclusion chromatography on protein G and Superose-12 columns and then used for conjugation with LDM immediately. The 3G11-LDM conjugate was formed by heterobifunctional crosslinking reagent *m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester (MBS) through linkage of the amino group of LDM apoprotein with the 3G11 molecule at molecular ratio of 1:1 in our laboratory.

Cells and cell culture
The following cancer cell lines were used: human colon carcinoma HT-29 cells, human fibrosarcoma HT-1080 cells, human breast carcinoma MCF7 cells, mouse colon carcinoma 26 cells, mouse hepatoma 22 cells. All the cell lines were cultured in RPMI 1640 medium (Gibco BRL Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL Inc.), 0.03% L-glutamine, 100 μg/mL streptomycin and 100 IU/mL penicillin at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2. For use in experiments, cells grown in exponential phase were disaggregated to single cells by treatment with trypsin/EDTA for 2 min.

Specimens of human colorectal carcinomas
Tumor tissue specimens collected by standard surgical oncology procedures were obtained from the Pathology

Department of Friendship Hospital in Beijing. A total of 32 cases of colorectal carcinoma were examined. The specimens were fixed in 40 mL/L formaldehyde in PBS (pH 7.0) and embedded in paraffin.

Immunohistochemical staining
Paraffin-embedded tissue sections of 4 μm thickness were placed on APES-coated slides. Immunohistochemical (IHC) staining of the sections was performed after dewaxing and rehydrating. Then the sections were incubated in 0.3% H_2O_2 for 15 min, blocked by normal mouse serum for 30 min, and incubated with primary mouse mAb (3G11) overnight at 4 ℃. IHC staining was performed by the labeled streptavidin–biotin immunoperoxidase technique with SABC kit (Bostern Inc.). After each step, the sections were washed thrice with PBS. Finally, 3,3'-diaminobenzidine-hydrogen peroxide (DAB, Bostern Inc.) was used as a chromogen for visualized reaction. For negative control, PBS and irrelevant mAb F9 were used instead of the primary antibody.

The IHC intensity of various colorectal carcinoma cases was assessed by image analysis and semiquantitative scoring, respectively. The sections were observed under a microscope. Manual evaluation of staining results was performed according to the general methods by semiquantitative scoring[9]. For quantitative evaluation of mAb 3G11 staining, each tumor section was examined by the image progressing and analysis system (Leica Inc.) in 20 representative high-power fields (×400).

Western blot assay
Total cell extraction from human colon carcinoma HT-29 cells, human fibrosarcoma HT-1080 cells and human breast carcinoma tissue lysate (Prosci Inc.) was performed by Western blot assay. The samples in the cold lysate buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 2 mg/L aprotinin, 2 mg/L lupeptin, 2 mmol/L AEBSF, pH 8.0) were electrophoresed on 10% gradient SDS-polyacrylamide gels in the presence of β-mercaptoethanol. The proteins were transblotted onto a PVDF membrane, blocked with 5% BSA/TBS for 2 h and incubated with primary antibody (mAb 3G11) overnight at 4 ℃ and then with peroxidase-conjugated goat anti-mouse IgG (Zhongsan Inc.) at room temperature for 1 h. The membrane was washed and the antibody reaction was visualized using Western blot luminol reagent: sc-2048. The membrane was scanned and the data were fed into the computer by image analysis system (AIO Inc.).

Gelatin zymography
Gelatinolytic activity of HT-29 cells was analyzed according to the method described previously[10]. The cells at the concentration of 1×10^6/mL were incubated for about 18-24 h with mAb 3G11 in serum-free 1640 medium and PBS. After low-speed centrifugation at 3 000 r/min to remove cellular debris, the condition medium (CM) was collected. All sample volumes were adjusted with PBS to 1 g/L of total protein to obtain a uniform protein content of 20 μg per sample. Then 20 μL of CM was mixed with 10 μL of 3× sample buffer containing 10 mmol/L Tris-HCl (pH 6.8), 20% glycerin, 2% SDS, 0.1% bromophenol blue. Samples

(30 μL) were then separated by electrophoresis on 10% polyacrylamide gel containing 0.1% SDS and 1% gelatin as a substrate. Thereafter, gels were washed in the reaction buffer (50 mmol/L Tris-HCl, pH 7.6, 0.15 mol/L NaCl, 10 mmol/L CaCl$_2$, 0.02% NaN$_3$) containing 2.5% Triton-X 100 for 1 h to remove SDS. During this process, pro-gelatinases A and B were autocatalytically activated *in situ*. Gels were then incubated for 24 h at 37 ℃ in the reaction buffer and stained with 0.1% Coomassie-brilliant blue R-250. The location of gelatinolytic activity was detectable as a clear band in the background of uniform staining. The blank bands were scanned and saved by the image system (AIO Inc.).

Enzyme-linked immunosorbent assay

Above-mentioned single-cell suspension volume was placed into the poly-L-lysine-coated 96-well ELISA plates (Costar Inc.) containing 1×10^5 cells/100 μL per well at 4 ℃ overnight. After being blocked with a solution of BSA (1 mg/100 mL in PBS) and washed thrice with 0.05% Tween-20 in PBS (PBST), plates were incubated with primary antibody (mAb 3G11 or 3G11-LDM conjugate) and goat-anti-mouse IgG-conjugated horseradish peroxidase for 1 h at 37 ℃, respectively. Following six washes with PBST, *o*-phenylenediamine-hydrogen peroxide (OPD-H$_2$O$_2$) substrate was used as a chromogen for visualization. Finally, the reaction was stopped by the addition of 100 μL of 0.1 mol/L H$_2$SO$_4$, and the absorbance readings (490 nm) were taken using a microplate reader (Bio-Rad Inc.).

MTT assay

Cells were detached by trypsinization and seeded at 3 000 cells/well in a 96-well plate (Costar, Cambridge, MA, USA) overnight. Then different concentrations of LDM, adriamycin (ADM), and MMC were added and incubated for an additional 48 h. The effect on cell growth was examined by MTT assay. Briefly, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated at 37 ℃ for 4 h. The supernatant was aspirated, and the MTT formazan formed by metabolically viable cells was dissolved in 150 μL of DMSO, and then monitored by a microplate reader (Bio-Rad) at a wavelength of 560 nm.

Clonogenic assay

Cancer cells at the concentration of 50 cells/well were seeded in 96-well plates and cultured for 24 h, then treated with LDM or 3G1-LDM at 37 ℃ for 1 h. Subsequently they were incubated for 5-7 d. Colonies formed were scored microscopically and the survival fractions (% control) were calculated using the following formula: survival fraction = (colony number of control well-colony number of treated well)/colony number of control well.

In vivo therapy studies

Female BALB/c mice (20±2 g, 7 wk of age, obtained from the Institute for Experimental Animals, CAMS) were inoculated subcutaneously with murine colon carcinoma 26 cells (1.5×10^6 cells/mouse). Mice were divided randomly into untreated control group and six treatment groups, and carefully monitored for general well-being, body weight, and tumor size. Diameter of the tumor was measured twice a week with a caliper. Tumor volume was calculated with the following formula: $v = 1/2ab^2$, where *a* and *b* are the long diameter and its perpendicular short diameter of the tumor, respectively. The data were presented as mean±SD. Student's *t*-test was used to determine statistically significant differences.

RESULTS

Immunohistochemical staining of tumors with mAb 3G11

MAb 3G11 showed positive immunoreactivity in 81.3% (26/32) of cases of colorectal carcinoma, 43.8% (7/16) of cases of mammary carcinoma, 63.6% (7/11) of cases of gastric carcinoma, and 66.7% (4/6) of cases of esophageal carcinoma, respectively. Cytoplasmic staining tended to be strongest in the cells at the periphery or invasive margin of the tumors and in the cells at the margin of tumor nests, whereas adjacent non-neoplastic tissues showed negative staining (Figure 1).
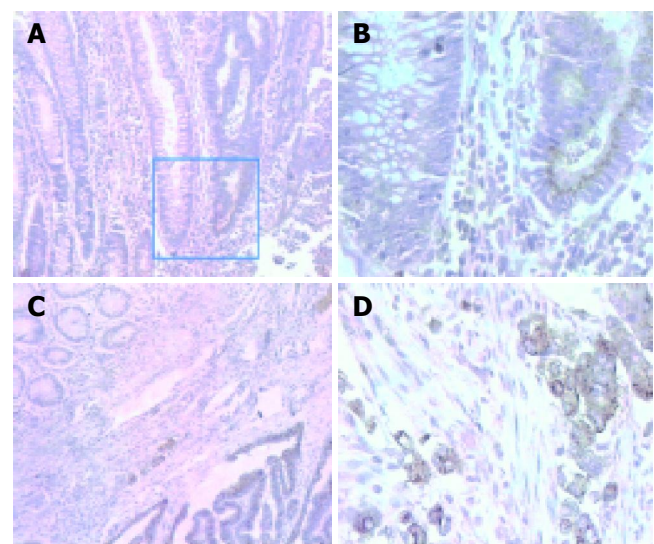

Figure 1 Immunohistochemical staining of mAb 3G11 in human colon carcinoma section. **A:** Colon carcinoma (100×, the bar represents 400 μm in length.); **B:** the same case of A (400×, the bar represents 100 μm in length.); **C** (100×) and **D** (400×): another case of typical colon adenocarcinoma.

Moreover, we found a significant correlation between the evaluation method of 3G11 immunostaining by computer image analysis and by semi-quantitative scoring (Figure 2). The consistent results assessed by both manual methods and quantitative image analysis system proved that 3G11 showed highly specific immunoreactivity with human colon carcinomas and that quantitaive estimation might also serve as an useful approach for IHC analysis.

Western blot analysis

MAb 3G11 was characterized by Western blot analysis and two specific electrophoretic bands of 72 and 92 ku in the cell or tissue lysates are shown, supporting the IHC results (Figure 3). The appearance of two bands indicated that other matrix metalloproteinase present in the samples did
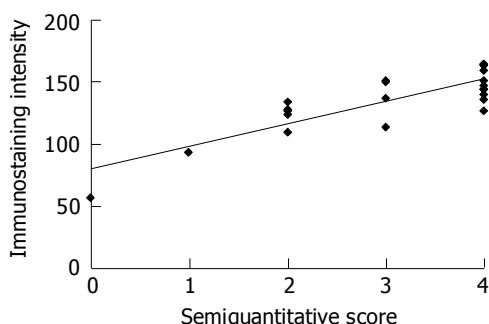
Figure 2 Immunohistochemical intensity of mAb 3G11 in various cases of human colorectal carcinoma assessed by image analysis and semiquantitative scoring.
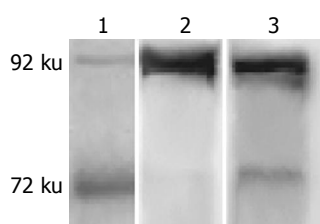
Figure 3 Immunological property of mAb 3G11 by Western blot analysis. Lane 1: human colon cancer HT-29 cell lysate; Lane 2: human fibrosarcoma HT-1080 cell lysate; Lane 3: human breast cancer tissue lysate.
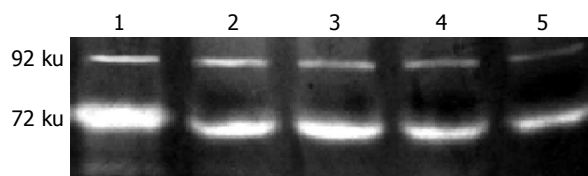
Figure 4 Gelatin-zymography assay of HT-29 cells after treatment with mAb 3G11 at different concentrations.

not cross-react with mAb 3G11. The intensity of two bands of 72 and 92 ku was different in various cell lines and tissue lysates. The results indicated that the expression level of gelatinase was different in various tumors.

Gelatin zymography assay

Both active and latent species could be visualized by using this technique. It showed that the clearance of the gelatin substrate by gelatinases with 72 and 92 ku was detected as a negative staining band, respectively. As shown, both the secreted activity of 72 and 92 ku gelatinases in human colon carcinoma HT-29 cells was inhibited by mAb 3G11 in a dose-dependent manner (Figure 4).

Immunoreactivity of mAb 3G11 and immunoconjugate 3G11-LDM

The binding of mAb 3G11 to antigen-related cancer cells including HT-29, H22, and C26 cells, which expressed type IV collagenases including MMP-2 and -9, is shown in a concentration-dependent manner ranging from 0.01 to 0.3 μmol/L of 3G11 (Figure 5A). The immunoconjugate 3G11-LDM still retained the immunoreactivity at a range
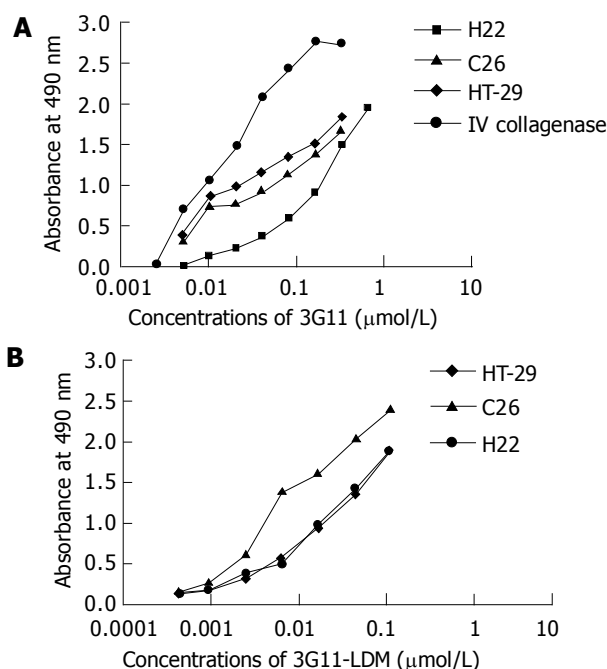
Figure 5 Immunoreactivity of 3G11(**A**) and 3G11-LDM (**B**) with type IV collagenase and various cancer cells in ELISA.
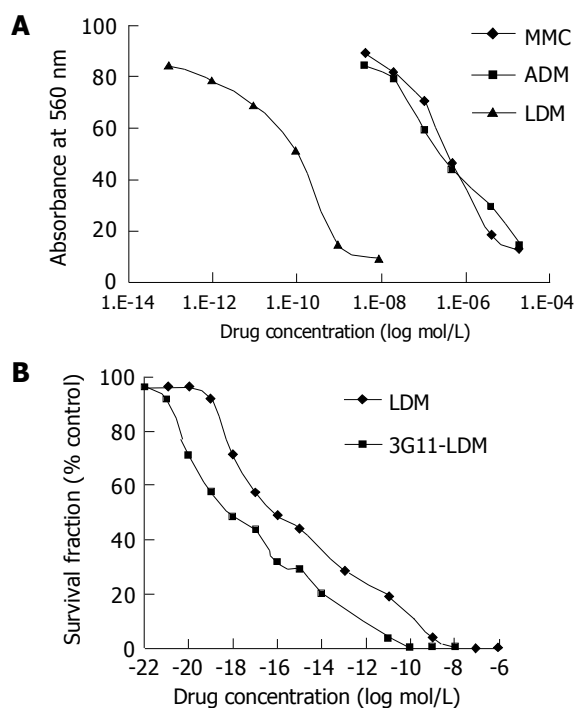
Figure 6 Cytotoxicity of LDM to human colon carcinoma HT-29 cells in comparison to ADM and MMC (**A**) and 3G11-LDM (**B**).

of concentrations between 0.01 and 0.1 μmol/L (Figure 5B).

Cytotoxicity of LDM to human colon carcinoma cells

As determined by MMT assay, the IC_{50} values for LDM, ADM, and MMC to human colon carcinoma HT-29 cells were 0.042 ± 0.007, 528.3 ± 97.7, and 992.0 ± 93.0 nmol/L, respectively (Figure 6A).

In terms of IC_{50} values, the effect of LDM on HT-29 cells

Table 1 Growth inhibition of colon carcinoma 26 by immunoconjugate 3G11-LDM in mice

Groups	Doses (mg/kg)	Mice number (begin/end)	BWC (g)	Tumor volume (cm³) mean±SD	Inhibition rate (%)
Control		10/10	+0.5	1.0±0.20	
3G11	0.75	10/10	+0.6	0.8±0.20	25.7 [b]
LDM	0.05	10/10	-0.2	0.4±0.16	56.4[b]
3G11+	0.75	10/10	-0.3	0.7±0.13	38.6[b]
LDM	+0.05				
3G11-LDM	0.025	10/10	-0.4	0.5±0.09	54.5[a,b]
3G11-LDM	0.05	10/10	-0.3	0.3±0.06	70.3[a,b]
3G11-LDM	0.1	10/10	-0.3	0.2±0.06	81.2[a,b]

[a]$P<0.05$ *vs* LDM; [b]$P<0.01$ *vs* control; BWC = body weight change.

was 10 000-fold more potent than that of ADM and MMC.

Cytotoxicity of 3G11-LDM to human colon carcinoma cells

By clonogenic assay, the conjugate 3G11-LDM displayed extremely potent cytotoxicity to human colon carcinoma HT-29 cells (Figure 6A). The IC_{50} value of 3G11-LDM for HT-29 cells was 5.6×10^{-10} nmol/L and that of the free LDM was 3.48×10^{-9} nmol/L.

In vivo therapy with immunoconjugate 3G11-LDM

According to the results on d 10 (Table 1), the tumor growth of colon carcinoma 26 in mice treated with 3G11-LDM was significantly inhibited by 54.5%, 70.3%, and 81.2% at the dose of 0.025, 0.05, and 0.1 mg/kg, respectively. It proved that 3G11-LDM showed higher anti-tumor efficacy against colon carcinoma in mice, as compared to the results of either free LDM or mAb 3G11.

DISCUSSION

Though numerous patients with alimentary tract carcinoma could be successfully induced into first remission by chemotherapy or radiotherapy, most of these patients ultimately have a relapse within 3-5 years. In addition, chemotherapy is accompanied with substantial side effects because of the non-specificity of the cytotoxic drugs. Therefore, it is clear that novel or additional therapy approaches are required. A promising therapy is antibody-targeted chemotherapy, which allows the specific delivery of cytotoxic drugs to the tumor tissues and is likely to have minimal toxic side effects[11,12]. Owing to its high potency cytotoxicity and molecular constitution, LDM may be successfully applied as a promising "warhead" in the production of antibody-based targeting therapeutics. The FDA-approved Zevalin and Bexxar are radioimmunotherapeutics derived from murine mAb. Their clinical application indicates that murine mAb may be used for cancer therapy under certain circumstances.

MMPs are now known to contribute tumor progression in addition to invasion, including tumor promotion, angiogenesis, and the establishment and growth of metastatic lesions in distant organ sites[13]. There is evidence that the expression of MMP-2 and -9, two of the most important MMPs, are linked to enhanced tumor angioge-nesis, tumor invasion, and metastasis[14-16]. By IHC staining in our studies, mAb 3G11 showed significant immunoreactivity with gelatinases in various human tumor tissues, especially in human colon carcinoma specimens, and no cross-reaction

with adjacent non-neoplastic tissues was found. The IHC staining of mAb 3G11 was found to localize in most colon carcinoma cells and a few of surrounding fibroblasts. Our IHC results are in accordance with some previous reports[17]. MMP-2 and MMP-9 expression is more frequently found at the lateral and deep margins of the tumors as observed by immunohistological staining. Among the examined human cancer specimens, mAb 3G11 had the strongest staining intensity and the highest positive rate in colon carcinomas, suggesting that mAb 3G11 has highly specific immunoreactivity with human colon carcinoma and this is the key reason for the use of mAb 3G11 as a carrier for antibody-based therapy.

Inhibitors of MMPs (MMPIs) can be used to halt the spread of cancer. MMPIs do not directly kill cancer cells, but instead targets processes such as cancer cell invasion and metastasis. However, many initial clinical trials using MMPIs proved to be disappointing[18]. 3G11-LDM is a conjugate composed of a mAb directed against type IV collagenase, including MMP-2 and -9, and LDM displays extremely potent cytotoxicity. This antibody can inhibit the enzyme activity and selectively bind to the target enzyme in tumor tissues. 3G11-LDM conjugate has potent antitumor efficacy both *in vivo* and *in vitro*.

In conclusion, 3G11-LDM is a promising agent for targeted cancer chemotherapy, especially for colorectal carcinoma.

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