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Cluster of differentiation 24 monoclonal antibody induces apoptosis in the osteosarcoma cells

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Abstract. – OBJECTIVES: Cluster of differentiation 24 (CD24) was overexpressed in osteosarcoma and positive CD24 expression correlates significantly with distant metastasis invasion and poor survival in osteosarcomas. We, therefore, suggested that CD24 would be a new molecular target for therapeutic strategies. In the present study, we aimed to investigate the effects of CD24 down-regulation using monoclonal antibodies (mAb) on apoptosis in osteosarcoma cells *in vitro* and *in vivo*.

MATERIALS AND METHODS: Osteosarcoma MG-63 cells were treated with Anti-CD24 mAb, and the effects on growth and apoptosis were evaluated *in vitro* and *in vivo*.

RESULTS: Anti-CD24 mAb could induce the apoptosis of cultured MG63 cells and anti-CD24 mAb treatment inhibited the tumor growth after cancer cell grafting and enhanced the cell apoptosis inside the tumor tissue.

CONCLUSIONS: The findings showed that anti-CD24 mAb targeting therapy provides a new avenue toward treating osteosarcoma.

Key words:
Osteosarcoma, CD24, Monoclonal antibody, Apoptosis.

Introduction

Cluster of differentiation 24 (CD24) is a small glycosylphosphatidylinositol-linked cell surface molecule that is expressed in a variety of human carcinomas with poor prognosis, such as extrahepatic bile duct cancer[1], nonmelanoma skin cancer[2], ovary cancer[3], pancreatic cancer[4,5], laryngeal squamous cell carcinoma[6], breast cancer[7] hepatocellular carcinoma[8], gastric cancer[9] and osteosarcoma[10]. Experimentally, the over-expression or depletion of CD24 alters cell proliferation, adhesion, and invasion *in vitro* and tumor growth *in vivo*[11,12]. In ovarian cancer, transfection of CD24-shRNA effectively down-regulated CD24 expression *in vitro* and *in vivo*. Administration of CD24-shRNA into nude mice bearing ovarian cancer significantly suppressed tumor volume growth[13]. Similarly, Smith et al[14] have shown how transient down-regulation of CD24 expression in human carcinoma cell lines resulted in growth inhibition and reduced clonogenicity and cell migration through a change in the actin cytoskeleton.

Anti-CD24 monoclonal antibodies (mAb) induced growth inhibition in lymphocytes precursors[15,16]. The growth of human colon and pancreatic cancer cell lines is also inhibited in response to CD24 mAb in a dose-dependent and time-dependent manner and in a close association with their CD24 expression level. This growth inhibition was a consistent finding and reproducible with three different mAbs[17-19].

It has shown CD24 up-regulation was significantly correlated with pathological high stages and distant metastasis in osteosarcoma[10]. Osteosarcoma patients with high CD24 expression should be closely monitored for recurrence following resections[10]. We, therefore, suggested that CD24 expression could provide a new molecular target for therapeutic strategies. The current study shows that down-regulation of CD24 expression *in vitro* and *in vivo* using CD24 monoclonal antibody, clone ALB9, decreases osteosarcoma cell tumorigenicity.

Materials and Methods

Cell culture

Osteosarcoma cell line MG63 was obtained from American Type Culture Collection (ATCC, Shanghai. China). It was cultured in RPMI-1640 (Life

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Technologies, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mg/ml sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37°C in 5% CO_2.

Regents

Human-specific Anti-CD24 mAb (ALB9) was purchased from AMS/Immunokontact, Shanghai, China. Anti-CD24 mAb was dissolved in phosphate buffered saline (PBS) and stored at 4°C. Primary anti-CD24 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (1:200).

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was used to measure cell viability. Cells were plated in 96-well plates and incubated with and without Anti-CD24 mAb (60 ug/ml) for 48 hours. Then MTT solution (5 mg/mL in PBS, 150 μL per well) was added and the plates were incubated for a further 2 hours. Finally, the supernatant was removed and discarded and formazan crystals dissolved in dimethyl sulfoxide (DMSO) were added (150 μL per well). The absorbance at 570 nm of each sample was measured using a special plate reader HZ320X (BIOEER, Zhengzhou, China). The experiments were repeated five times for each condition.

Flow cytometry assay

An apoptosis detection kit (BD Biosciences, San Jose, CA, USA) was used to determine apoptotic cells. Cells were plated at a density of 1×10^5 cells/ml. After allowing 24 hours for cell adherence, cells were exposed to 60 ug/ml anti-CD24 mAb for 48 h and were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) following the manufacturer's instructions and analyzed by flow cytometry on a BD Biosciences LSR II software.

Xenografts model in mice for measuring in vivo tumor development

A total of 24 nude mice were used in the animal study. Nude mice were housed in sterile cages and were handled with aseptic precautions, supplemented with *ad libitum* nutrition. Exponentially growing MG63 cells were harvested with brief treatment of 0.25% Tryspin-EDTA solution and resuspended at a final concentration of 5×10^5 cells per 0.15 mL PBS per injection as indicated. The cells were injected s.c. into the site on the back of the mice. Mice were randomized and split into 2 cohorts. On palpation of tumor formation (7 days after inoculation), human-specific anti-CD24 mAb was delivered i.v. in a bolus dose of 5 mg/kg/d (0.1 mg in 100 μL), every 4 days, for 3 total treatments. Control animals were given 0.9% saline. This treatment course was adapted from prior trials of this same mAb in mice[20]. The tumor growth was measured twice a week; tumor volumes were calculated as $4\pi ab^2/3$. Finally, the animals were sacrificed at 28 days after the graft and Paraffin-embedded tissues were cut at 4 μm for TUNEL assay, and hematoxylin and eosin staining was also used to count the number of apoptotic cells. Each assay was revised at least twice.

In vivo TUNEL assay

Apoptosis was evaluated by terminal transferase dUTP nick end labeling (TUNEL) staining using the Apoptag Peroxidase *In Situ* Detection Kit S7100 (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. Briefly, histologic sections were deparaffinized, hydrated in deionized water and then rinsed with PBS. The sections were treated with 20 ug/mL of proteinase K for 15 min to digest protein, and with 3% H_2O_2 for 5 min to quench endogenous peroxidase activity. After washing with PBS, the equilibration buffer was added. The slides were then treated with 10 uL working strength TdT enzyme at 37°C for 60 min. Subsequently, the sections were incubated with preheated working strength Stop solution for 10 min, with anti-digoxigenin-POD for 30 min and with Pierce Metal Enhanced diaminobenzidine (DAB) for 3-6 min, and washed with PBS after each incubation. Finally, the sections were counterstained with methyl green [Vector stock solution] or Mayer's hematoxylin and, then, mounted. Control slides were ordered from Serologicals Corporation (San Diego, CA, USA). The results were observed with an optical microscope. The percentage of apoptotic cells was calculated as the number of apoptotic cells per number of total cells × 100%.

Statistical Analysis

Cell proliferation data were compared using 2-way ANOVA. Apoptosis cells were compared using a 2-tailed Student'*t* test, as were comparisons of s.c. tumor growth across mice cohorts. Statistical analyses were conducted in SPSS11.0. The results were presented as means ± SD of three replicate assays. A p value of <0.05 was considered to indicate statistical significance.

Results

Effect of anti-CD24 mAb on cancer cell viability

We found that anti-CD24 mAb treatment significantly decreased MG63 cell viability, with the number of viable cells significantly decreasing after 48 hours. In the control group, 99.4% ± 0.1% of the cells were viable, while in the treated group, the percentage of viable cells was only 32.6 % ± 5.5% ($p = 0.0026$) (Table I).

Effect of anti-CD24 mAb on cancer cell apoptosis in vitro

Anti-CD24 mAb was found to lead to MG63 cell apoptosis, as determined by flow cytometry assay**.** In the control group, only 4.4% ± 0.3% of cells were apoptotic, while in the anti-CD24 mAb groups, after 48 h for 60 ug/ml anti-CD24 mAb treatment, 47.4% ± 10.3% of cells were apoptotic ($p = 0.002$) (Table I).

Therapeutic targeting of CD24 inhibits growth of Xenografts model and promotes apoptosis

The Xenografts model in mice grew slowly after anti-CD24 mAb treatment. Mice inoculated with 5×10^5 MG63 cells treated with anti-CD24 mAb resulted in lower mean tumor volume (240 mm^3±26 mm^3) compared with control saline (1280 mm^3±110 mm^3) by 28 days after treatment initiation ($p = 0.0043$) (Table I). Anti-CD24 mAb treatmithent significantly decreased tumor graft growth *in vivo*. Additionally, within smaller tumors, the number of apoptotic cells was found to have increased. In tissues obtained at 28 days, the percentage of TUNEL-positive cells harvested from tumors in the mice was 1.6% ± 0.2% in the saline group and 11.4% ± 1.8% ($p = 0.0014$) in the anti-CD24 mAb group (Table I). Similar changes were also found in tumor tissues harvested from other time points (data not shown).

Discussion

We found that the newly developed CD24 monoclonal antibody ALB9 could induce apoptosis of cultured osteosarcoma cells. Additionally, ALB9 treatment inhibited tumor growth after cancer cell grafting and enhanced cell apoptosis inside the tumor tissue. This is consistent with previous studies that have shown that antagonizing CD24 activities reduces tumor cell proliferation and inhibits tumor xenograft growth[13].

The potential for targeting CD24 in cancer therapy seems promising, as CD24 is overexpressed in many human cancers whereas it is barely detectable in normal tissues[18,21]. Most remarkably, cells with CD24 membrane expression using anti-CD24 monoclonal showed decreased tumorigenicity and increased apoptosis.

Although several anti-CD24 mAbs exist, we used ALB9 because it targets the LAP sequence present in human, but not the murine homolog CD24[22], which allowed for treatment of human xenografts in murine hosts. Moreover, ALB9 was evaluated in human clinical trials for the treatment of B-cell lymphoproliferative disease, where it was efficacious in promoting complete remission in a majority of patients[23,24]. Other studies have used a similar peptide core targeting anti-CD24 antibody that was delivered coincidently with s.c. implantation of HT-29 colorectal carcinoma cells, which reduced subsequent tumor growth in nude mice[25]. Our study is the first to evaluate anti-CD24 mAb as a treatment for established tumors in osteosarcoma. This observation sets the stage for use of such antibodies in the adjuvant for osteosarcoma patients.

Conclusions

CD24 targeting therapy offers a potential new method for treating osteosarcoma. We are planning to combine ALB9 with other chemotherapy drugs in future studies. In addition, it will be necessary to examine the effects of ALB9 on established tumors to validate this treatment as a therapy.

Table I. Effect of anti-CD24 mAb on MG63 cell

	In vitro		*In vivo*	
MG63	**Cell viability**	**Cell apoptosis**	**Tumor volume**	**Cell apoptosis**
anti-CD24 mAb	32.6 % ± 5.5%	47.4% ± 10.3%	(240±26) mm^3	11.4 % ± 1.8%
PBS	99.4% ± 0.1%	4.4 % ± 0.3%	(1280±110) mm^3	1.6 % ± 0.2 %
	$p = 0.0026$	$p = 0.002$	$p = 0.0043$	$p = 0.0014$

Competing of interest

The authors have declared that no competing interests exist.

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