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Production, characterization, and *in vitro* effects of a novel monoclonal antibody against Mig-7

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ABSTRACT

Development of new cancer therapies based on specific recognition of molecules in cancer cells is a significant challenge, as this requires identification of such molecules (molecular targets) and subsequent development of high-affinity, selective binders (targeting molecules). While several molecular targets for cancer therapies are currently under evaluation in clinical trials, greater selectivity for cancer cells over normal cells is required to enhance efficacy. Migration-inducing gene 7 (Mig-7), a membrane protein found in various types of carcinoma cells, is a cancer-specific biomarker and a promising molecular target for targeted cancer therapies. The purpose of this study was to produce and characterize a novel monoclonal antibody (mAb) raised against an N-terminal peptide of human Mig-7 (Mig-7(1–30)). The Mig-7(1–30) peptide was conjugated with a KLH carrier protein for immunization, and the mAb specific to Mig-7 (STmAb-1) was produced using hybridoma technology. Western blot analysis showed that STmAb-1 specifically reacted with a 23-kDa Mig-7 protein expressed in cancer cell lines, and, crucially, not with primary human fibroblasts. The affinity constant (K_{aff}) of STmAb-1, as measured by non-competitive enzyme immunoassay, was 1.31×10^9 M^{-1}, indicating high mAb affinity against Mig-7. Immunofluorescence assays demonstrated that STmAb-1 could specifically recognize Mig-7 expressed in cancer cell lines, but not in primary human fibroblasts and keratinocytes. Moreover, STmAb-1 inhibited the growth of MCF7 and HeLa cell lines in contrast to primary human fibroblasts, highlighting its potential usefulness in the development of new cancer therapeutics.

1. Introduction

Cancer is recognized as a dominant cause of death worldwide, and therefore more effective treatments are urgently needed in order to increase survival rates. Chemotherapy is a universal treatment strategy involving the use of medicines to stop or slow tumor growth. In addition to destroying fast-proliferating cancer cells, chemotherapy is often not selective and other rapidly dividing cells, such as blood-producing cells in bone marrow, skin cells, and hair follicles are also destroyed, resulting in side-effects. Recently, cancer therapies geared towards targeting specific molecules have been introduced. These treatments are more selective for cancer cells, resulting in lower mortality rates in normal healthy cells. To date, several targeting molecules have been developed which are capable of facilitating selective drug-delivery to cancer cells through targeted binding to surface-associated marker molecules [1–4]. However, many of these molecular targets are expressed in both tumor, and normal cells so the development of targeting molecules having higher specificities and affinities against cancer-specific biomarkers will enhance the potency of such targeted therapies.

Migration-inducing gene 7 (Mig-7), a cysteine-rich protein, is found in the cell membrane and cytoplasm of carcinoma cells [5]. In previous studies, it was revealed that Mig-7 mRNA levels increase in embryonic cytotrophoblast cells during placenta development and in more than 80% of tumors, but no such elevations occur in normal tissue samples or in blood from normal subjects [5–7].

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Furthermore, *in vitro* studies have demonstrated that expression of Mig-7 protein contributes to cancer cell invasion and vascular cell mimicry [7–10]. The molecular mechanisms underlying the inhibition of cancer invasion and metastasis by Mig-7 remain largely unexamined. It has been reported that silencing Mig-7 expression inhibited early tumor growth *in vivo* presumably, in part, by deactivation of MT1-MMP, ERK1/2, Akt, and S6 kinase [11]. Recently, Mig-7 was also proposed as a potential therapeutic target for Akt/GSK-3β-, and COX-2/PGE2-mediated cancer metastasis [12–14]. In this study, we report the successful production, characterization, and *in vitro* cytotoxic activity of a novel anti-Mig-7 monoclonal antibody having potential applications in cancer diagnosis and therapy.

2. Materials and methods

2.1. Cell culture

Cancer cell lines (MCF7 breast adenocarcinoma, HeLa cervical adenocarcinoma, HL60 promyelocytic leukemia), normal human fibroblasts, and normal human keratinocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. MCF7, HeLa, and normal human fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM), and HL60 was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Normal human keratinocytes were cultured in DMEM supplemented with 1% human keratinocyte growth supplement (HKGS) and 10% heat-inactivated FBS. All media and supplements for culture were purchased from Gibco, Thermo Fisher Scientific Inc. (Waltham, MA).

2.2. Preparation of Mig-7 peptide conjugates

The N-terminal amino acids 1–30 of the Mig-7 protein sequence (Mig-7(1–30)) (Accession DQ080207), a putative extracellular domain of Mig-7, was chosen as the immunogen for monoclonal antibody (mAb) production. The carboxyl group of the Mig-7(1–30) peptide was conjugated with Keyhole Limpet Hemocyanin (KLH) using an Imject® Immunogen EDC Conjugation Kit (Thermo Fisher Scientific Inc.). Conjugation of Mig-7(1–30) to ovalbumin (OVA) utilized an identical protocol as used for KLH, with the conjugate being subsequently used for screening of Mig-7 specific monoclonal antibodies. All peptides used in the study were purchased from GenScript (Piscataway, NJ).

2.3. Production of the anti-Mig-7 monoclonal antibody

Four BALB/c mice were each immunized by injecting intraperitoneally at monthly intervals for 4 months with 50 μg of KLH-conjugated Mig-7(1–30) emulsified in Freund's complete adjuvant. After each immunization, the mice were bled to monitor levels of Mig-7 specific antibodies in serum using OVA-conjugated Mig-7(1–30). The mice showing the highest titers as determined by ELISA were boosted intraperitoneally with 50 μg of KLH-conjugated Mig-7(1–30) without adjuvant 3 days before cell fusion. After sacrificing, the spleens were removed and the splenocytes were fused with the X63-Ag8.653 mouse myeloma cell line. Culture supernatants from individual hybridoma clones were collected after fusion and initially screened against OVA-conjugated Mig-7(1–30) and KLH by ELISA, then against the MCF7 cancer cell line by dot-blot assay. An antibody isotype was identified with rabbit anti-mouse isotype-specific antibodies, and the mAb was purified by precipitation with 50% (v/v) saturated ammonium sulfate followed by affinity chromatography based on its isotype. The concentration of mAb was determined using the Bradford protein assay (Bio-Rad, Philadelphia, PA).

2.4. Antiserum titer determination by ELISA

Immunoplates (Thermo Fisher Scientific Inc.) were coated with 2 μg/ml of OVA-conjugated Mig-7(1–30) or KLH and kept overnight at 4 °C. After washing five times with PBS containing 0.05% tween (PBST), and blocking with 5% skim-milk in PBST for 1 h at 37 °C, plates were incubated with either serum (1:500 to 1:50,000 dilution with 5% skim-milk in PBST) or individual culture supernatant (1:5 dilution with 5% skim-milk in PBST) for 30 min at 37 °C. Plates were then washed 5 times with PBST and subsequently incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgM (Thermo Fisher Scientific Inc.; 1:10,000) for 1 h at 37 °C. Peroxidase activity was detected using the TMB substrate (KPL, Inc., Gaithersburg, MD). Plates were read at an absorbance of 450 nm using a microplate reader (Bio-tek instrument, USA). Skim-milk was used as a negative control. All experiments were done in triplicate, and the data are presented as mean ± standard deviations.

2.5. Affinity constant determination

A non-competitive enzyme immunoassay [15] was used to measure the affinity constant (K_{aff}) of anti-Mig-7(1–30) mAb serially diluted to concentrations of 21, 18, 9, 6, 3, 1, 0.781, 0.390, 0.195, 0.0195, and 0.00195 μg/mL. The sigmoid curve was plotted to depict the relationship of OD_{450} versus mAb concentration. Accordingly, K_{aff} could be calculated using the following equation: $K_{aff} = (n-1)/2(n[Ab']t - [Ab]t)$, where $n = [Ag]t/[Ag']t$. [Ag]t and [Ag′]t represent antigen concentrations, with [Ab′]t and [Ab]t corresponding to antibody concentrations at the half maximum OD (OD-50) for plates coated with [Ag′]t and [Ag]t, respectively.

2.6. Western blot analysis

Cell pellets were lysed with M-PER lysis buffer (Thermo Fisher Scientific Inc.) and a mixture of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) for 10 min while standing on ice. The lysate supernatants were resolved in SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with 5% skim-milk in PBST for 1 h, the membrane was incubated with the monoclonal antibody (1:750) and mouse anti-β-actin mAb (Santa Cruz Biotechnology, Inc., Dallas, TX; 1:200; loading control), and thereafter rinsed three times with PBST. The membrane was then incubated with HRP-conjugated goat anti-mouse IgM and HRP-conjugated goat anti-mouse IgG (H + L) (Thermo Fisher Scientific Inc.; 1:10,000) prior to rinsing three times with PBST. The blot was developed using the TMB membrane peroxidase substrate (KPL, Inc.). Normal human fibroblasts served as a negative control.

2.7. Immunofluorescence

Cell pellets were diluted with PBS and smeared onto clean microscope slides. After air-drying, cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% TritonX-100/PBS. Permeabilized cells were then treated with 10% normal goat serum in PBS to block nonspecific binding sites, followed by incubation with the mAb (1:750). After washing with PBS cells were incubated with FITC-conjugated anti-mouse IgM (1:50). Nuclei were visualized after staining with Hoechst 33,342 dye (Thermo Fisher Scientific Inc.). Images of the stained cells were acquired using a Fluoview FVi10 confocal microscope (Olympus, Japan). Cells