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Characterization and utilization of a monoclonal antibody against pancreatic carcinoma.

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Abstract

A monoclonal antibody was produced against a human pancreatic adenocarcinoma line and was found to react with several different human carcinomas by immunoperoxidase staining of fixed tissues¹. The original cells used to generate the monoclonal antibody were treated with detergent to lyse the cell membrane. A membrane associated protein of molecular weight 35kD was isolated from this detergent lysed preparation and found to be recognized by the monoclonal antibody. The binding constant of the antigen antibody reaction on the cells is 5×10^{-5} . It was further determined that there are 700,000 binding sites per cell. Kinetics of the antigen-antibody reaction under several conditions were also explored.

Introduction

Cancer of the pancreas remains one of the most incurable human neoplasms. There are approximately 23,000 new cases per year in the United States.² The expected two year survival is 4%. Currently, complete surgical extirpation is the only modality that offers hope of long-term survival in this disease. Unfortunately, at the time of diagnosis, only 10% of patients will have resectable disease confined to the pancreas.^{2,3} When metastatic disease is present, chemotherapy is largely ineffective.⁴ Newer methods of delivering radiation therapy may be effective, but only when the disease is regionally confined and can be treated with a local modality.⁵

Immunologic manipulation is a growing area of oncologic research, and the use of monoclonal antibodies directed against pancreatic cancer has been demonstrated to have some efficacy in treating patients with unresectable disease.⁶ Furthermore, newer technologic advances are enabling different chemotherapeutic molecules to be coupled to the monoclonals (e.g., ricin, adriamycin), thereby expanding the clinical usefulness of monoclonal antibodies for treatment of primary and metastatic cancer.

An IgG1 mouse monoclonal antibody, Chip, was developed against a cultured human pancreatic adenocarcinoma cell (SHAW). The spectrum of cancerous and noncancerous tissues that this antibody recognizes has been elucidated elsewhere.¹ The purpose of this study was to study the binding characteristics of the antibody and to elucidate the nature of the recognized antigen. Herein, we describe the binding characteristics of Chip antibody directly radiolabeled with iodine and Chip conjugated to DTPA which is labeled with ¹¹¹Indium. Additionally, a preliminary description of the antigen recognized by Chip is given.

Materials and Methods

Monoclonal Isolation

Chip monoclonal antibody was produced against a human adenocarcinoma line (SHAW).¹ The hybridoma line was kept in continuous culture in serum-free medium supplemented with glutamine, pyruvate, and gentamicin. Supernatant was collected from the cells, spun down to remove cellular debris, and concentrated under 60 psi of nitrogen using a filter and the Amicon apparatus. Concentrated supernatant was then passed over a Protein A Sepharose column, and the monoclonal antibody eluted.⁷ The antibody isolated was found to be an IgG1,¹ and purity was established by SDS 10% polyacrylamide gel electrophoresis.

SHAW and ZRYD cell lines which were originally initially isolated from a human source have been kept in continuous culture in RPMI Media supplemented with 10% fetal bovine serum, MEM amino acids, antibiotic-antimycotics, glutamine and MEM vitamins. Cells were harvested for passage with 0.25% trypsin, and by mechanical scraping for antigen isolation.

Confluent flasks of the two pancreatic cell lines were washed two times with PBS. The cells were then mechanically scraped from the plates, and washed twice further with PBS. 1 ml of 0.5% NP40 detergent was then added to the cell pellet, and the mixture vortexed for 3 minutes. The slurry was placed on ice for 15 minutes, and then centrifuged using the Beckman centrifuge for 3-4 minutes to remove particulate matter and unlysed cells. 100 ug of protein from the crude cell extracts were placed on 7.5% SDS polyacrylamide gels, and run at 4 mA overnight. The gel was separated into sections for silver nitrate staining,⁸ and electrophoretic transfer.⁹ The nitrocellulose paper containing the transferred immobilized protein using the immunoperoxidase method,^{10,11} An aliquot of the supernatant resulting from the above soft spin centrifugation

was centrifuged at 40,000g to further remove finer particulate matter. This supernatant was then passed over an AH-Sepharose affinity column washed with 400 ul PBS, and the antigen eluted with Citrate buffer at pH 2.8. The collected samples were immediately buffered by collection into tubes containing 2M Tris to correct the pH to 6.8.

B. Iodination

13 x 75 mm tubes were treated with 10 ug of Iodo-Gen™ dissolved in chloroform, and evaporated under nitrogen gas.¹² The Ab and Na¹²⁵I were added and allowed to react at room temperature for 15 minutes. The reaction mixture was then placed on a PD-10 column and eluted with PBS. Aliquots of labeled protein solution were then counted on the gamma counter. An identical aliquot was mixed with 0.3 ml of 1% BSA in PBS and precipitated in 10% Trichloroacetic acid. The pellet was spun in the Beckman microfuge and the supernatant collected. The pellet was resuspended in 10N NaOH for 1 hour at 37° C., and both pellet and supernatant counted.

C. Protein Isolation

In order to examine the effect of the harvesting technique on antigen integrity, flasks of SHAW cells were either scraped to release cells, or treated with 0.25% trypsin until the cells were released in single cell suspension. The cells were then lysed as described above.

To test for glycosylation of the antigen, confluent cells were treated with either PBS or 5 units of neuraminidase.¹³ The cells were then scraped off the plates and treated as above.

D. Affinity Chromatography

Affinity columns were prepared using both CNBr activate Sepharose¹⁴ and AH Sepharose.¹⁵ Elution buffers used included SDS, and 0.1 M Citrate buffer at pH 2.8. NP40 cell extracts as described were used on the columns.

E. Binding Studies

10,000 SHAW or ZYRD cells per well were placed in Removawel® plates and allowed to adhere overnight in the incubator at 37° C. under 5% CO₂. The plates were then spun at 1000 RPM (148 g) in the RT 6000 centrifuge. Non specific binding sites in the well were blocked with PBS/1% BSA for 1 hour at room temperature. The blocking solution was removed, and the cells washed with PBS. The radiolabeled antibody was then applied. Following incubation with the antibody, the plates were washed twice with 200 uL PBS per well, and the wash solutions and cell counted separately. Protein determination for antibody concentration was made using the Bradford protein dye reagent¹⁴; an IgG was used for calibration of protein curves.

F. Chelation¹⁵

The cyclic anhydride of DTPA (material section) was dissolved in DMSO freshly distilled from calcium hydride to insure it's dryness. The Chip antibody (5 mg/ml) was dialyzed into 50 mM bicarbonate buffer, pH 8.5, and a fifty fold molar excess of the anhydride was added directly to it while vortexing. After 1 hour, the reaction mixture was put into dialysis against 50 mM citrate buffer, pH 5.5. The dialysate was changed three times. Finally, the protein was dialyzed into 20 mM MES (abbrev.), pH 6.0, and this solution was used for radiolabeling with metals.

¹¹¹In was neutralized with 1 M sodium acetate to pH 4.5 and the antibody was immediately added and vortexed. After one half hour, the reaction mixture was purified by passage through an HPLC equipped with a size exclusion column. Only the peak at molecular weight 150,000 was collected for use in later experiments. Quality of the radiolabel as assessed by TCA precipitation.

G. Kinetics

1) For generation of binding curves, 10,000 SHAW or ZRYD cells per well were treated with radiolabeled Chip antibody, and allowed to react at room

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resulted in inactivation of the Chip antibody. Consequently, we used AH-Sepharose which has a chemically milder binding step. With SDS as the eluting buffer, a single band of protein was demonstrated on silver nitrate stained 7.5% SDS-PAGE gels. However, no staining was demonstrated using Chip antibody on the nitrocellulose immobilized protein. When the protein was eluted from the column by pH gradient, a single band was obtained that did stain positive, demonstrating antigenic identity. The molecular weight of the protein was approx. 35kD.

Neither Trypsin nor neuraminidase treatment of the cells affected the antigenicity of the preparation as determined by Western Blot transfer.

B. Binding Studies

Binding curves were generated for both the SHAW and ZRYD cells using the 125I labeled Chip, and the 111 chelated Chip (figures 2 and 3). Scatchard plots of these binding curves determined a K_a of 10^{xx} (kd _____). The number of binding sites per cell was calculated to be 700,000, and was _____ $\times 10^6$ by Scatchard analysis. The binding sites were saturated at 1 hour incubation (figure 4). The bound 125I labeled Chip was competed off the cells using unlabeled Chip (Figure 5). Rate of competition at 37° C. was increased when compared to room temperature, and was considerably lower at 4° C. The rate of antibody dissociation from the cells at room temperature is shown in figure 6.

Chip is an IgG1 monoclonal antibody that was generated against SHAW cells. This antibody reacts specifically with these cells in vitro, when compared to ZYRD cells or a number of other cell extracts tested. The time for antibody saturation of monolayer cells is 1 hour. The reaction is specific, and can be competed with unlabeled antibody. The K_a of the reaction is _____ x 10^x , and there are approx. xxx binding sites per cell. The antigen recognized by this antibody has a molecular weight of 35 kD. The antigen is either not glycosylated, or the glycosylated regions are not needed

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