The Use of Specific Monoclonal Antibodies to Target Immunogenic Tumor Membrane Proteins in Patients with Recurrent Pancreatic and Colon Cancer

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Abstract: Tumor associated antigens from pooled allogeneic membrane proteins were isolated, partially purified and tested as a possible vaccine in patients with stage II and III colon cancer. The vaccine, when given in combination with an adjuvant following surgical resection, resulted in marked improvement in survival compared to control patients having only undergone surgical resection of their tumor. While it was possible to demonstrate that patients receiving vaccine turned on both humoral and cell mediated responses, it appears that survivors remaining free of disease at 5-7 yrs post op were able to mount a strong IgG1 response as the primary mechanism for tumor destruction. Antibodies from hybridomas made against the vaccines resulted in production of monoclonals with a high degree of ADCC. Those monoclonals targeting pancreatic cancer and in particular the MUC5ac mutated antigen representing tumor immunogen were studied in detail. Animal models indicated rapid tumor destruction when nude mice, injected with human pancreatic cancer were then immunized with NPC-1 monoclonal antibody targeting mutated MUC5ac. FDA studies including tissue cross reactivity, biodistribution, and cytokine release assays indicated safety and efficacy of the monoclonals we have developed. Submission of the IND allowed for initiation of the Phase I trial using mAb NPC-1 targeting pancreatic cancer when that antigen was found to be expressed.

Keywords: Monoclonal antibodies, immunotherapy, pancreatic and colon cancer, immunogenic membrane proteins, ADCC.

INTRODUCTION

The research group at Neogenix Oncology has, over a several years period of time, isolated and characterized several immunogenic human tumor associated antigens Each of these tumor antigenic proteins (TAA's) was derived from fresh pooled operative tumor specimens. This allowed for a threshold level of antigen to be obtained. When used as vaccines in FDA approved clinical trials, these TAAs elicited both cellular and humoral immune responses within 4-6 months of administration [1]. For those patients with stage I and II lung cancer undergoing surgery alone, and which represented the control group, survival was approximately 35%. However, for the treatment group undergoing surgical resection of their tumors and who then received post resection immunotherapy (TAA vaccines), evaluation over a period ranging from 5-7 years showed a significant improvement in their clinical course with survival rates approximating 75% in patients receiving vaccine post surgery [2].

Failures among the vaccinated group who showed re growth of their lesions within a year or two of surgery, indicated that such recurrences were associated with the inability to induce an adequate IgG1 response against the tumor. Cell mediated immunity seemed to play a limited role in terms of tumor response in both this and other reported trials. The survivors receiving immunotherapy in our treatment groups were found to produce a significant and prolonged elevation of serum IgG1 levels targeting the tumor. These responses lasted for more than 5 years in most of the vaccinated patients [3].

Considering the importance for developing specific therapeutic IgG's, similar to those induced by TAA (vaccine) therapy, monoclonal antibodies directed against the various TAA's were developed [4]. Partially purified antigen preparations from patients with colon and pancreas cancers used in the original clinical studies were used to induce hybridomas and subsequently monoclonal antibodies that target tumor immunogens. *In vitro* studies described the ability of these antibodies to destroy tumors expressing the target at 4-6 hours. *In vivo* animal studies confirmed the ability of the humanized monoclonal antibodies to reduce tumor burden in animals treated with the antibody as compared to control groups. The FDA approved an IND for initiating antibody trials in patients with advanced pancreatic and colon cancer expressing specific target proteins and who had failed all standard forms of therapy.

METHODS AND RESULTS

Immunohistochemical (IHC) studies to define time of appearance of the immunogenic tumor associated antigens in various tumors for targeting purposes, have indicated that expression of these antigens can be demonstrated in cells having undergone genotypic changes several months before phenotypically appearing malignant.

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In an analysis of 60 colon cancer specimens and adjacent normal colon tissue, when tumor defined by H&E was examined by immunoperoxidase staining, essentially all tumor cells expressed tumor antigen. Similarly when adjacent normal tissue was stained with H &E the appearance of normal appearing colonocytes was observed. In 10 of those cases read as normal colon mucosa by H&E and where anastomosis of bowel usually followed, 10% of normal sections that were examined, displayed tumor antigen expression. Fig. (**1a,b**) In such instances, anastomotic recurrences may be anticipated.

In order to consider the therapeutic value of our monoclonal antibodies in targeting immunogenic proteins expressed in both primary and metastatic malignancies, we were interested in tumor destruction via ADCC (antibody dependent cell cytotoxicity). The NK cell activity needed, could be optimized through chimerization and or humanization of our monoclonal antibodies to make them more potent therapeutics agents In the presence of a human Fc, the NK cell receptors present, attract those NK cells needed for initiation of tumor destruction. Several different chimeric monoclonals that we developed for therapeutic purposes, were found to be expressed as IgG1's, with the murine version being an Ig2a that isotype which switched during the chimerization and or the humanization process. Only the IgG1's were shown to optimize the ADCC response.

All of our mAbs were studied in detail in terms of their bio- distribution, tissue cross reactivity and cytokine release. Chimeric versions of the mAbs as well as humanized forms were shown to exhibit ADCC activity and were able to induce apoptosis against the tumor system from which they were derived.

Fig (**2**) provides an example of ADCC activity of one of the chimerized mAbs (NPC-1) that targets a variant of MUC5ac. A33, an other monoclonal antibody that does not target MUC5ac was used as control against two human pancreatic cancer cell lines (CPAC-1 and PANC-1). A human skin melanoma cell line was employed as control. Despite slight variations in the percent killing at effector to target ratios of 100:1, only the NPC-1 monoclonal antibody exhibited greater than 40% killing of pancreatic cancer cells.

The various ratios of effector to tumor cells in the procedure, allow for optimization in the number of NK cells that would be delivered to the tumor cell population and which most probably achieve what is present in the hosts circulation at the time of immunization. The use of 100:1 effector to target ratio may be useful clinically to qualify the mAb activity.

Because of their relatively low toxicity in the murine model, rapid targeting of human tumor cell lines during *in-vitro* studies, and their ability to destroy established xenograft transplants (*in-vivo*) within days of delivery, these monoclonal antibodies appear to be promising for use in the treatment of recurrent as well as metastatic tumors [5].

Chimeric NPC-1 was the first to be produced GMP grade, for initiating clinical trials in pancreatic and colon cancer, where patients to be entered into the planned clinical trials were found to have failed all standard forms of therapy. A requirement for use of the monoclonal antibody was that +2 expression of tumor antigen was to be defined by immuno- histochemical analysis.

The protein complex that was targeted by NPC-1 was shown to be derived from the MUC (oncofetal) group of surface antigens. It was defined in more detail, by affinity purification, Western blot and Coomassie Blue staining which suggested that MUC5AC contained the target epitope. The MUC5ac protein is believed to be aberrantly glycosylated in tumor cells as well as demonstrating a core peptide

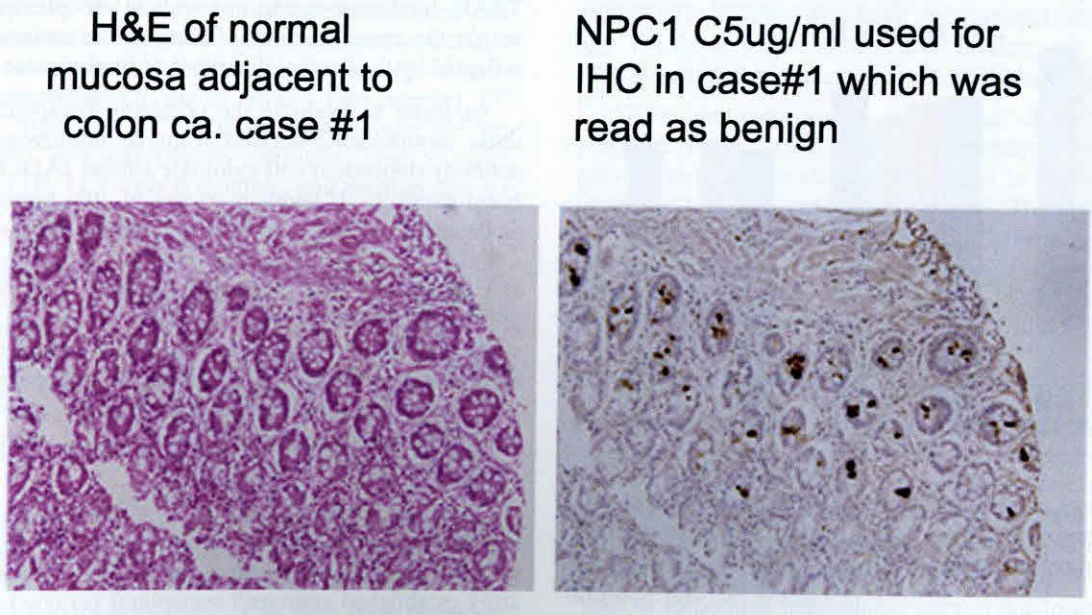

Fig. (1). (a,b) Normal colonocytes at the margin of tumor resection expressing tumor antigen.
Figure illustrates a normal mucosal glandular pattern. In panel **a)** this mucosal appearance is almost always found in the margins of resection following colectomy. Under normal circumstances the bowel would be anastomozed with this apparently normal colonocytic appearance staining with monoclonal NPC-1 in panel **b)**, demonstrates that a mutated form of MUC5ac antigen found in genotypically expressed colonocytes indicates that these cells are in the process of undergoing malignant transformation.

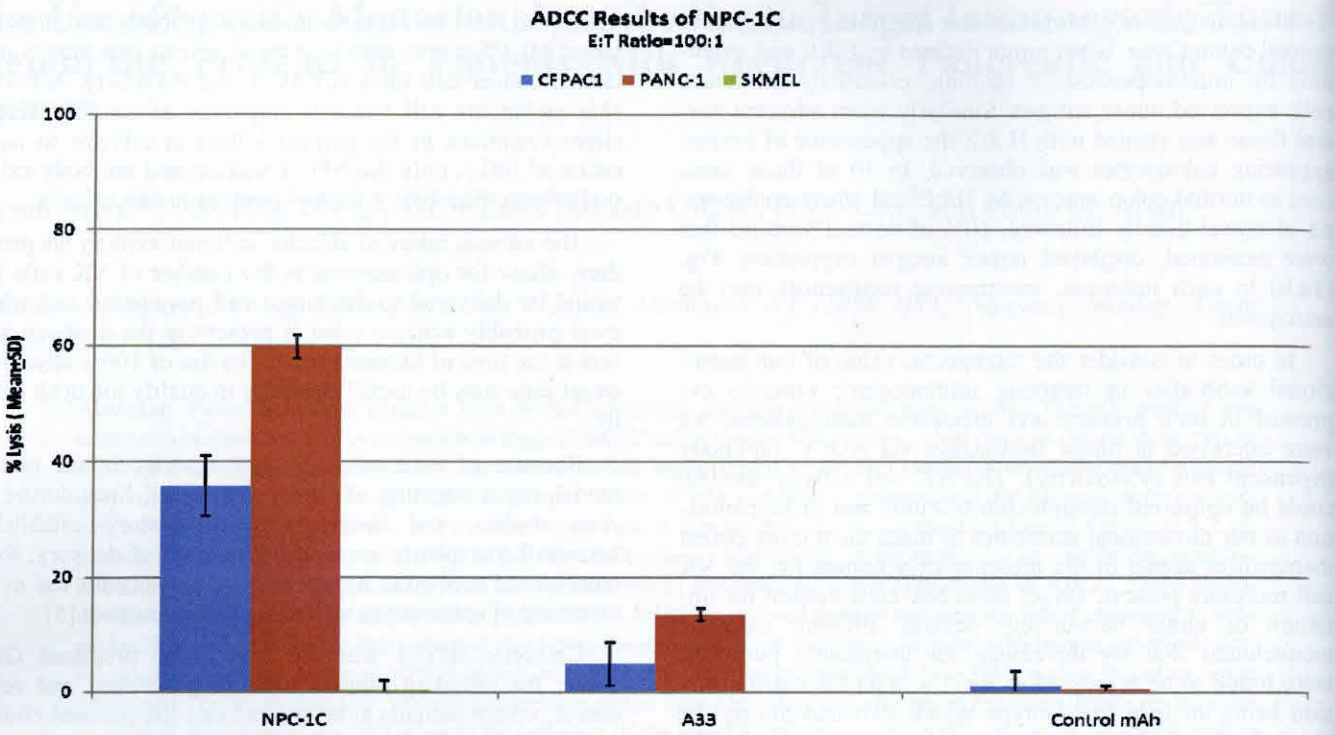

Fig. (2). Graphic display showing ADCC lysis of pancreatic cancer cells induced by mAb NPC-1 with mAb A33 as control. Slight variation in tumor destruction can be noted between the CPAC-1 and PANC -1 cell lines but the effect is still near or better than 40% ADCC.

mutation, thus creating the tumor-specific immunogenicity of the molecule.

A second monoclonal antibody, 31.1 monoclonal antibody, has been produced and found to have a further enhancement in ADCC, see Fig. (**3**). Plans for GMP production are being made.

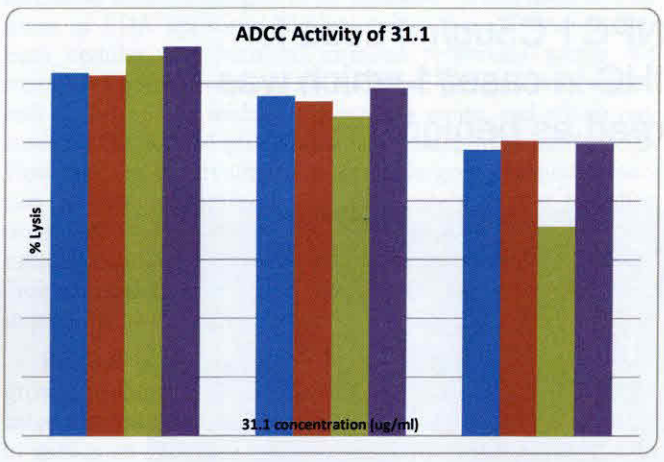

Fig. (3). Shows improvement in ADCC using monoclonal 31.1 which targets a different and distinct tumor protein in pancreatic and colon cancer.

Conversion of the murine monoclonal antibodies to their chimeric version as well as the humanized format was shown to not only enhance ADCC, but to secondarily induce apoptosis through mechanisms defined by enhanced annexin V binding. The chimerization process with transfection into CHO also stabilized the Fab so that mutations do not occur. Loss of monoclonal antibody activity is frequently seen when hybridomas are stored for a long period of time.

While the lytic effect of the antibody occurs directly on the tumor through ADCC with the monoclonal Fc carrying NK cells to the tumor target protein, additionally apoptosis separate and distinct from ADCC is initiated probably through TRAIL ligation resulting in a shift in phospholiposerine from within the inner membrane lining to the surface, where it is reflected by the degree of annexin V binding seen.

In order to evaluate the potential therapeutic effects of those monoclonals derived from an immunogenic protein, antibody dependent cell cytotoxic studies (ADCC) were analyzed carefully, If levels in excess of 40% tumor destruction as determined by a chromium or indium release assay, were defined as positive, further evaluation for *in-vivo* activity in animal models was performed. In this instance, tumor injected into the thigh of nude mice was allowed to grow and when the lesion reached a size of 2-4 cm^3, intraperitoneal administration of the therapeutic mAb was performed along with administration of human effector cells. In each instance, marked reduction in tumor size was noted within two weeks of mAb delivery.

Because of the relatively low toxicity in the murine model of these monoclonal antibodies, their rapid targeting of tumor cell lines (*in vitro*), as well as their ability to destroy established xenograft transplants (*in vivo*), these monoclonals appear ideal for use in both the diagnosis and treatment of metastatic tumors alone as naked antibodies, as well as in combination with chemotherapy.

DISCUSSION

Immunotherapy as employed today, has failed for the most part due to the inability to clearly define as well as isolate specific and well defined immunogenic tumor proteins, those only expressed in tumor cells without cross reactivity to normal tissue. As such those monoclonal antibodies derived from tumor molecules that do not induce an immune response such as growth factors are limited in their therapeutic capabilities and offer no opportunity to be employed as diagnostic reagents.

Whole cell preparations, usually with the patients own tumor, have been employed assuming that adjuvants and other additives including heat shock protein, dendritic cells and transfected GMCSF in the GVAX preparations can accomplish the desired results. In quantifying the level of antigen administration that we found to be effective, it was determined that 500 – 1000 mg of relatively pure immunogenic antigen may be needed. In those instances where whole cell preparations were delivered, it was anticipated that roughly 50 mg was present in the vaccine preparation, a level too low to induce a proper therapeutic immune response.

A number of monoclonal antibodies have appeared for therapeutic use in solid tumor malignancies. Because of their failure to induce a significant ADCC response they have been approved to be used in combination with chemotherapy for the treatment of a variety of solid tumors. In colorectal cancer, adding bevacizumab, a humanized monoclonal antibody (mAb) targeting VEGF, two regimens containing 5-FU, leucovorin (LV), and irinotecan or oxaliplatin improve outcomes. However, these advances have come with a cost of treatment-related side effects, including bleeding, hypertension, bowel perforation, and thromboembolic events. In the first study of bevacizumab plus irinotecan and bolus 5-FU/LV (IFL), the objective response rate was 49 percent [6]. Grade 3 diarrhea developed in 15 of 87 cases, and there were 37 bleeding events, and nine venous thromboembolic events (four pulmonary emboli). Benefit was confirmed in a trial of 813 patients who were randomly assigned to IFL with or without bevacizumab (5 mg/kg every two weeks) [7]. Bevacizumab improved the objective response rate (45 versus 35 percent), and significantly improved time to tumor progression (TTP, 11 versus 6 months), and median survival (20 versus 16 months). Gastrointestinal (GI) perforation (1.5 percent) and grade 3 hypertension (11 percent) were more common with bevacizumab, while the incidence of venous thrombotic events was similar with and without bevacizumab (19 versus 16 percent), as was the fraction of patients who developed grade 3 or 4 bleeding during therapy (3.1 versus 2.5 percent). Following publication of these data, bevacizumab received broad approval in the US in combination with 5-FU for first-line treatment of metastatic colorectal cancer.

Trastuzumab is a humanized monoclonal antibody that binds to a specific epitope of the HER2 protein on the cell surface and inhibits signal transduction induced by other peptide growth factors interacting with their own receptors. The net result is cellular growth inhibition. Other proposed mechanisms of antitumor activity include augmentation of humoral and cellular immunity, and reversal of resistance to both endocrine therapy and certain chemotherapies [8-10]. Preclinical studies suggest additive or synergistic interactions between trastuzumab and multiple cytotoxic drugs, including platinum analogs, taxanes, anthracyclines, vinorelbine, and cyclophosphamide [11]. For the 18 to 20 percent of patients with breast cancer whose tumors overexpress HER2, molecularly targeted therapy with trastuzumab is an important option for treatment of metastatic and locally advanced disease. In addition, multiple trials now demonstrate a survival benefit when trastuzumab is integrated into adjuvant chemotherapy for early stage localized HER2-overexpress-ing breast cancer [12].

Unfortunately, the limitations of many current therapeutic products for pancreatic cancer are widely recognized. Despite the development of several new treatment regimens for pancreatic cancer, little if any benefit has been appreciated. Twelve randomized trials of various FDA-approved drug combinations (including avastin + gemcitabine) have failed to extend survival in this patient population. Only one drug combination (gemcitabine + erlotinib) has been approved by the FDA for the therapy of pancreatic cancer, and it extended survival by just 0.4 months. In addition, the majority of patients with pancreatic cancer are diagnosed with advanced disease and thus the opportunity for drugs to be effective when there is little or no tumor burden is diminished considerably. This is exemplified in a clinical trial recently conducted with the pox vector based PANVAC vaccine platform (CEA-MUC-1-TRICOM), that was given to 255 patients with metastatic pancreatic cancer who had already failed prior gemcitabine therapy [13]. The trial failed to meet its primary endpoint of overall survival. This trial thus exemplifies an inappropriate patient population to evaluate an immunotherapeutic approach as a monotherapy. Thus, for the treatment of most advanced solid tumors, biologic therapy should be considered in combination with cytotoxic or other therapeutic maneuvers (i.e. radiation therapy). The appropriate population to consider using these drugs, includes (a) patients who have had minimal prior chemotherapy, (b) those with a lower tumor burden, and (c) and those with a predicted life span to permit multiple rounds of therapy.

When the commercial monoclonal antibodies that are available today, were used to clinically target the tumor growth, most preparations were found to be relatively ineffective in terms of improving survival. This can be seen for those mAbs developed against growth factor molecules on the surface of the tumor that are also expressed in normal tissue. Although progression can be blunted, actual tumor shrinkage is infrequently noted, as exemplified by those molecules targeting the vascular endothelial and epidermal growth factors expressed on tumors as well as many normal tissues.

Monoclonals that were developed at Neogenix targeting each of the specific immunogenic tumor proteins (TAA's) from colon/pancreatic cancer as well as squamous cancer of the lung/cervix, have now been studied in detail. In over 30 different normal human tissues obtained from necropsy, minimal if any evidence of cross reactivity was noted. In addition when radiolabeled antibody was given to xenograft models with human pancreatic cancer, the tumor was found, to contain 10-15 times the concentration of radiolabeled antibody as found in circulation, which is known as a localization index of 10-15:1. This suggests that if a radiolabeled gamma emitter is given to a patient to define the presence of

malignancy, then a high quality image could be expected. Furthermore, when animals were given 100 more fold monoclonal antibody that would be delivered at a maximum dosage to the patient, somewhere between 2-8 mg/kg, no evidence of a toxic cytokine reaction was noted, suggesting that these reactions would not be expected in a clinical phase I trial.

Xenograft studies with transplanted human colon and pancreas cancer revealed the ability of mAb NPC-1 and two of our other antibodies, 31.1 and 16C3, to diminish and in many cases eliminate growth of established tumor that was expanding in the animals. While specific mAbs directed against the target tumor protein were essential, human effector cells containing NK cells were also required. This of course represented the ADCC (antibody dependent cell cytotoxicity) effect where the human Fc of our IgG1 contained NK cell receptors, delivering the NK cells to the tumor surface where they were able to initiate tumor cell destruction selectively. When effector cells were not delivered with the monoclonals, we still were able to see some apoptotic effects that appeared related to studies revealed by Annexin V binding. Here it is believed that apoptosis, independent of ADCC, may be initiated by antibody binding to the TRAIL receptors on the surface of the tumor cell.

The monoclonals that have been developed at Neogenix Oncology against immunogenic proteins expressed on the surface of the tumor cells have a unique feature in that they may be used for both diagnosis and therapy. We are now in the process of defining those immunogenic molecules (molecular markers), both, proteins and their corresponding monoclonals, for use in serum ELISA, diagnostic IHC and immunocytology as a complement to their therapeutic uses.

CONCLUSIONS

Monoclonal antibodies derived from immunogenic tumor glycoproteins have the capability of inducing apoptosis in both recurrent and metastatic settings for many of the adenocarcinomas. This form of tumor destruction occurs in contrast to what one sees with those monoclonal antibodies attacking growth factors. These protein derived monoclonal antibodies that we have developed against tumor specific antigens also appear to define the presence of malignant transformation in many normal appearing cells, when the process of mutation has been initiated genotypically, several months prior to the phenotypic expression of malignancy. Utilizing the TAA's derived from colon cancer membrane protein, 3 monoclonal antibodies were developed, two of which were specific for colon and pancreatic cancers and the 3rd a more general type of antibody that appears to target an array of adenocarcinomas. Neither of the monoclonal antibodies developed by our group had any significant cross reactivity to normal tissue and essentially do not appear to elicit a cytokine response when delivered to the animal models studied. Among the several hundred patients treated in the original pooled allogeneic vaccine trials, looking at the colon cancer patients in particular, there were no instances of bowel toxicity or diarrhea suggesting that the immunogen itself whether inducing a T or B cell response, had no adverse response on normal adjacent tissue. Antibodies developed against tumor specific immunogenic proteins appear to be useful for both diagnosis and therapy. When used alone as a naked molecule, we anticipate the degree of clinical response. We are currently exploring the utility of combining them with chemotherapy and immunostimulants and developing radiolabeled antibodies to further enhance clinical efficacy.

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