Clinical Evaluation of BR96 sFv-PE40 Immunotoxin Therapy in Canine Models of Spontaneously Occurring Invasive Carcinoma

Carolyn J. Henry,1 Michael S. Buss,2 Ingegerd Hellström,4 Karl Erik Hellström,4 William G. Brewer,3 Jeffrey N. Bryan,1 and Clay B. Siegall4
1Departments of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri-Columbia, Columbia, Missouri; 2Veterinary Clinical Sciences, Washington State University, Pullman, Washington; 3Small Animal Surgery and Medicine, College of Veterinary Medicine, Auburn University, Auburn, Alabama; and 4Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington

ABSTRACT

Purpose: The immunotoxin BR96 sFv-PE40 is an effective antitumor agent against human breast and lung carcinoma xenografts in rodents. This study was designed to (a) determine the frequency with which canine carcinoma cells express Lewisβ (Leβ) antigen, thereby identifying canine carcinoma types suitable for the clinical evaluation of BR96 sFv-PE40, and (b) determine the safety and efficacy of BR96 sFv-PE40 in a canine model of spontaneously occurring cancers for investigation of targeted therapy.

Experimental Design: Carcinoma tissue samples were obtained from client-owned dogs presented for medical care. The tissues were assessed for Leβ antigen expression using immunohistochemical methods. Dogs with tumors expressing Leβ antigen were offered enrollment in a clinical trial to receive twice-weekly infusions of 4 to 12 mg/m2 BR96 sFv-PE40. Clinical toxicity and response data were assessed at each treatment.

Results: Twenty-two of 61 carcinomas evaluated were positive for Leβ expression, including mammary, prostate, lung, and rectal carcinomas, and 12 dogs were enrolled in the clinical trial. The primary side effect was transient emesis.

INTRODUCTION

The mouse monoclonal antibody BR96 recognizes a Lewisy (Leβ-related carbohydrate) antigen that is expressed by a variety of solid tumors as well as by normal gastrointestinal epithelium (1). After binding of BR96 to Leβ at the cell surface, the monoclonal antibody internalizes into endosomes and lysosomes (2, 3). Immunotoxins of BR96 and doxorubicin have been shown to possess strong antitumor activity in preclinical models (4, 5). Studies done in dogs showed that the dose-limiting toxicity of this immunotoxin as well as of unconjugated BR96 is damage to the gastrointestinal epithelium. A doxorubicin conjugate using chimeric (mouse-human) BR96 has shown biological activity in humans, but the amount of conjugate that could be safely delivered because of the gastrointestinal toxicity has not been sufficient to justify its use as a single agent (6). However, there is a renewed clinical interest in this conjugate based on the finding in preclinical models that the therapeutic efficacy can be increased when the conjugate is combined with a taxane (7), and a phase II clinical trial of a BR96-doxorubicin conjugate in patients with advanced non–small cell lung carcinoma is ongoing.

A single-chain immunotoxin, BR96 sFv-PE40, has been genetically engineered by cloning the variable regions of BR96 and assembling them as a single chain (sFv), which was then fused with a truncated, nonbinding derivative of Pseudomonas exotoxin A (PE40) (8, 9). BR96 sFv-PE40 binds to Leβ, is internalized, and translocates into the cytosol where it causes cell death via catalytic inhibition of protein synthesis. Previous studies have shown the immunotoxin to be an effective antitumor agent against human breast and lung carcinoma xenografts in rodents (8–10). The purpose of this study was 2-fold: (a) to determine the frequency with which canine carcinoma cells express Leβ antigen, thereby identifying canine carcinoma types suitable for the clinical evaluation of BR96 sFv-PE40, and (b) to determine the safety and efficacy of BR96 sFv-PE40 in the treatment of naturally occurring carcinomas in dogs. The long-term goal of this study was to establish a large animal model of spontaneously occurring cancers for investigation of therapy with this novel immunotoxin.
MATERIALS AND METHODS

Antigen Identification in Tissues

Carcinoma tissue samples were obtained from client-owned dogs presented for veterinary care at one of three referral veterinary oncology centers (Washington State University Veterinary Teaching Hospital, Auburn University Department of Small Animal Surgery and Medicine, and University of Missouri-Columbia Veterinary Medical Teaching Hospital). The biopsy specimens were immediately placed in OCT medium and frozen using liquid nitrogen. Tissue was stored (−80°C) overnight on dry ice before shipment for Le expression analysis and indirect immunostaining. The peroxidase-antiperoxidase technique of Sternberger (11) was modified as described previously (1, 12) and used for immunostaining of tissue samples. The anti-Le antibody was developed at Bristol-Myers Squibb (Syracuse, NY) and has been described previously (1). Frozen sections were prepared and treated with acetone before immunostaining. To decrease nonspecific background, sections were preincubated with normal dog serum diluted 1:5 in PBS. The mouse anti-Le (BR96) monoclonal antibody to be tested, rabbit anti-mouse IgG (Sternberger-Meyer Immunochemicals, Inc., Jarrettsville, MD), and mouse peroxidase-antiperoxidase (Sternberger-Meyer Immunochemicals) were diluted in a medium containing 10% normal dog serum and 3% rabbit serum. Rabbit anti-mouse IgG was used at a dilution of 1:50. Mouse peroxidase-antiperoxidase containing 2 mg/mL specifically purified peroxidase-antiperoxidase was used at a dilution of 1:60. Each new batch of reagent was titrated to the most appropriate dilutions. A human breast carcinoma cell line (H3396) served as the positive control. Slides were evaluated by a medical doctor with considerable experience in immunohistology under code and regularly checked (also under code) by an independent investigator. Antigen expression was scored for homogeneity using a +/− scale and for degree of positive staining using a 0 to 4+ scale (0, no staining of any cells; 1+, staining of <30% of cells; 2+, staining of ≥30% to <50% of cells; 3+, staining of ≥50% to <75% of cells; 4+, staining of ≥75% of cells). Tumors that were homogenous and had a 3+ or greater score for Le expression were considered positive for the presence of Le antigen.

Clinical Evaluation in Dogs

Client-owned dogs undergoing surgical biopsy or excision of a carcinoma at the Washington State University Veterinary Teaching Hospital were screened for the study via immunostaining techniques described above. Inclusion criteria for eligible candidates included histopathologic confirmation of Le-positive carcinoma and measurable tumor (primary or metastatic) remaining after surgery. In dogs 5 to 10, a specimen of normal colonic mucosa was obtained at the time of tumor biopsy such that immunostaining for Le could be done. This was based on the hypothesis that the degree of Le expression in normal gastrointestinal epithelium would correlate with the severity of gastrointestinal side effects associated with BR96 sFv-PE40 administration. Informed, signed owner consent and decline of standard-of-care therapy were required for study enrollment. No adjuvant therapy was permitted during study participation. Baseline evaluations included complete blood count, serum biochemical analysis, urinalysis, and complete tumor staging.

Assays for antibody formation against the immunotoxin were done using serum collected before each treatment with BR96 sFv-PE40. Serum samples from each dog were evaluated after the dog had received the first six doses of BR96 sFv-PE40 or when adverse reactions were noted and again at the time of study completion. Analysis of serum for presence of anti-immunotoxin antibodies was done by standard ELISA method as described previously (13). The study protocol did not require treatment discontinuation when antibody formation was detected unless adverse effects were clinically noted. BR96 sFv-PE40 was diluted in 0.9% NaCl to a total volume of 10 mL and infused over 20 minutes on a Tuesday/Friday schedule. The planned protocol was for dogs to receive two 5-dose courses of BR96 sFv-PE40 with a 2-week rest period between courses. Routine patient monitoring consisted of serum biochemistry evaluation, complete blood count, and urinalysis repeated before treatments 3 and 5 and with every other treatment thereafter. Concurrent immunosuppressive therapy [dexamethasone 0.5 mg/kg i.v. 30 minutes before infusion and 24 hours later or anti-CTL-associated antigen 4 immunoglobulin (CTLA4-Ig; Bristol-Myers Squibb) 10 mg/kg i.v. 30 minutes before infusion] was given to dogs enrolled later in the study after anti-immunotoxin antibody formation was noted as a consistent clinical problem developing in the first six dogs. The dose of BR96 sFv-PE40 ranged from 4 to 12 mg/m² depending on date of entry into the study and whether colon immunoreactivity was used as a dose determinant. The first dog enrolled in the study was treated with two initial doses of 6 mg/m² and then escalated to 12 mg/m² based on the lack of clinical toxicity. In dogs for which colon immunoreactivity was determined (n = 5), BR96 sFv-PE40 was dosed as follows: 3 to 4+ immunoreactivity, 4 mg/m²; 2+, 6 mg/m²; 1+, 9 mg/m²; and 0, 12 mg/m². Colonic biopsy sampling was discontinued after enrollment of these five dogs. This decision was made based on the clinical perception that Le expression in normal gastrointestinal mucosa did not predict toxicity so that the risk of morbidity associated with colonic biopsy was not warranted. The final two dogs in the study were treated with 4 mg/m² BR96 sFv-PE40 along with CTLA4-Ig as part of a protocol amendment intended to decrease the likelihood of anti-immunotoxin antibody formation. The combination protocol and dosage determinations were based on preclinical evaluation of CTLA4-Ig in laboratory monkeys and dogs.5

Response to therapy was assessed before each treatment using standard veterinary oncology response criteria (14). Tumor measurement was accomplished with techniques appropriate to tumor location. These techniques included thoracic radiography for evaluation of pulmonary metastasis, ultrasonography or computed tomography for internal masses, and caliper measurement of external masses. Complete response was defined as resolution of all clinical signs and absence of measurable tumor. Partial response was defined as a ≥50% decrease in tumor volume and no evidence of new metastases. Stable disease was defined as <50% decrease or 25% increase in tumor volume and no evidence of new metastatic lesions. Progressive disease was

5Bristol-Myers Squibb Pharmaceutical Research Institute, unpublished data.
defined as an increase in tumor volume by ≥25% or the development of new metastatic lesions. Treatment was discontinued if disease progression or undesirable side effects occurred. Survival time was defined as the time from first histopathologic diagnosis until death or euthanasia due to tumor-related disease.

RESULTS

Sixty-one carcinoma samples were evaluated for presence of Leα antigen. Twenty-two tumors were positive according to the criteria given in MATERIALS AND METHODS. Carcinoma types expressing Leα included mammary (9 of 19), prostatic (3 of 7), bronchogenic (4 of 6), rectal (2 of 4), nasal (1 of 2), hepatic (1 of 3), tonsillar squamous cell carcinoma (1 of 1), lacrimal gland adenocarcinoma (1 of 1), and nasal planum squamous cell carcinoma (1 of 1). Carcinoma types in which Leα was not detected (score <3+) included thyroid (n = 5), transitional cell carcinoma of the urinary bladder (n = 3), biliary carcinoma (n = 2), perianal adenocarcinoma (n = 2), oral squamous cell carcinoma (n = 2), sweat gland carcinoma (n = 1), cutaneous carcinomatosis (n = 1), and carcinoma of unknown primary site (n = 1).

Of 22 dogs with carcinomas that expressed Leα, 10 met the eligibility criteria for clinical study enrollment. Tumor types in these 10 dogs included inflammatory mammary carcinoma (n = 3), rectal carcinoma (n = 2), bronchogenic carcinoma (n = 2), tonsillar squamous cell carcinoma (n = 1), nasal adenocarcinoma (n = 1), and lacrimal gland adenocarcinoma (n = 1). In addition to the dogs that met the inclusion criteria, two dogs (4 and 10) had tumors with heterogeneous or weak positive staining (1+) for Leα antigen and thus were ineligible for study enrollment. However, they were treated with BR96 sFv-PE40 on a compassionate-use basis at the owners’ request. Their data were included in this report as the development of anti-immunotoxin antibodies. Timing of antibody formation is summarized in Table 1.

Side effects of therapy included transient febrile responses in 6 of 12 (50%) treated dogs and emesis in 3 of 12 (25%), all resolving within 24 hours. These side effects did not seem to be dose related. Episodes of emesis were short-lived in all affected dogs, and no biochemical evidence of organ toxicity affecting the pancreas was noted. Dog 3 experienced gall bladder necrosis, which necessitated a cholecystectomy. The excised tissue was examined with immunostaining and found strongly positive for Leα antigen. It is therefore possible that immunotoxin binding to the gall bladder epithelium led to necrosis, although this could not be confirmed in this case.

Response data are summarized in Table 1. No complete remissions were achieved. Partial response was achieved in three cases, including inflammatory mammary carcinoma (Fig. 1), tonsillar squamous cell carcinoma, and bronchogenic carcinoma (Fig. 2). The partial response seen in one of the dogs with inflammatory mammary carcinoma was first noted after only two infusions of BR96 sFv-PE40 (Fig. 1). Stable disease was observed in four cases, including rectal carcinoma (n = 2) and inflammatory mammary carcinoma (n = 2). Progressive disease occurred in three of the dogs (lacrimal gland adenocarcinoma, bronchogenic, and nasal carcinoma) that met the inclusion criteria. Both of the two dogs that did not meet the study criteria but were treated on a compassionate use basis had progressive disease in spite of therapy.

Antibody titer data (positive, >1:10 or negative, <1:10) were available for all scheduled sample collection times in 11 of the 12 treated dogs. No dogs had pretreatment antibody titers and nine dogs produced antibodies to the PE40 portion of the BR96 sFv-PE40 fusion protein after two to five doses. In five of the first six dogs treated, disease stabilization or clinical response was noted until anti-immunotoxin antibody development occurred after which time the disease became progressive. In four subsequently enrolled dogs, concurrent treatment with dexamethasone did not prevent antibody formation. Finally, the treatment protocol was amended to include CTLA4-Ig as an immunosuppressive agent. Interestingly, both of the dogs treated with CTLA4-Ig developed anti-CTLA4-Ig antibodies rather than anti-immunotoxin antibodies. Timing of antibody formation is summarized in Table 1.

DISCUSSION

In this study, immunostaining techniques were used to identify canine tumor types suitable for therapy with BR96 sFv-PE40. Of the canine carcinomas screened in this study, the types...
most likely to express Le\(^2\) antigen were mammary, bronchogenic, and rectal carcinomas. The evaluation of Le\(^2\) antigen expression in naturally occurring canine carcinomas is an important first step in establishing alternative animal models with which to assess the efficacy of novel Le\(^2\) targeting therapies. In a review article addressing recombinant immunotoxin therapy, Pastan et al. (15) enumerated seven criteria that must be met to develop an immunotoxin for use in people. One of these criteria is that immunotoxin toxicity must be evaluated in animal models expressing the appropriated antigen. They further stated that this generally requires preclinical studies to be done in primates. Based on our identification of spontaneously occurring canine carcinomas that express Le\(^2\), it now seems reasonable that cancer-bearing dogs could serve as alternatives to primates for some preclinical studies.

Toxicity and efficacy of BR96 sFv-PE40 were evaluated not only for the obvious application to veterinary oncology but also because dog tumors may serve as models for similar malignancies in humans (16). Our results, albeit limited, were promising, especially for inflammatory mammary carcinoma in which one of three cases had a partial remission and two had disease stabilization. Inflammatory mammary carcinoma in dogs, as in women, is an extremely aggressive malignancy. In one review of 10 cases of canine inflammatory mammary carcinoma, all dogs had widespread metastatic disease at the time of diagnosis (17). Treatment is generally unrewarding, and most dogs are euthanatized shortly after diagnosis (17, 18). The dramatic initial response noted with BR96 sFv-PE40 treatment in the responder with inflammatory mammary carcinoma was, unfortunately, abrogated when anti-immunotoxin antibodies developed. However, even partial remission for this tumor type with therapy other than radiation is quite encouraging.

As has been the case with other immunotoxins (19–22), antibody formation was a significant limitation to the successful long-term use of BR96 sFv-PE40 in the dogs enrolled in this study. Consistent with previous reports indicating that antibodies against immunotoxins generally develop within 2 weeks of exposure (20, 21), dogs in this study developed antibodies between 2 and 16 days of treatment initiation. In five of the first six dogs treated, disease stabilization or clinical response was noted until anti-immunotoxin antibody development occurred after which time the disease uniformly progressed. Although neutralization assays were not done as part of this study, a previous evaluation of BR96 sFv-PE40 in laboratory dogs indicated that rather than blocking cytotoxicity, development of anti-immunotoxin antibodies resulted in a more rapid clearance of the fusion protein, thus neutralizing the antitumor activity of the agent (13). This parallels what has been noted in people that develop anti-immunotoxin antibodies (21, 22). Treatment with immunosuppressive doses of dexamethasone did not prevent antibody formation. Administration of CTLA4-Ig, a compound that modulates T-cell activation via its inhibitory effects on the costimulatory molecule B7, was used in an attempt to block antibody formation. This genetically engineered product, a fusion protein of human CTLA4 and the IgG1-Fc region, was under preclinical investigation at the time of this study. Preliminary data in cynomolgus monkeys had indicated that a dosing regimen similar to that used in the current study was an effective method to suppress antibody formation. Whereas anti-immunotoxin antibodies were not detected in the two dogs treated with...
CTLA4-Ig, this was of little clinical significance in that they developed antibodies to the foreign fusion protein. The use of foreign proteins that may induce antibody formation is a challenge to developing successful immunotherapy approaches in both human and veterinary oncology. However, the ability to show proof of principle for novel agents in a model of spontaneously developing and inherently heterogeneous cancer is a strength of domestic animal translational research that cannot be duplicated in rodent models (16).

As evidenced by clinical responses in naturally occurring canine cancer, the unique binding and internalization of the BR96 antibody in carcinoma cells make this single-chain immunotoxin an interesting product candidate. Alternative approaches for use of this product in animal models may include combination with other therapies (e.g., administration of chemotherapeutic drugs), which may also decrease the immunogenicity of the immunotoxin. This study provides an example of translational cancer research using canine models of spontaneously occurring cancer to investigate promising therapies for use in both veterinary and human oncology.

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