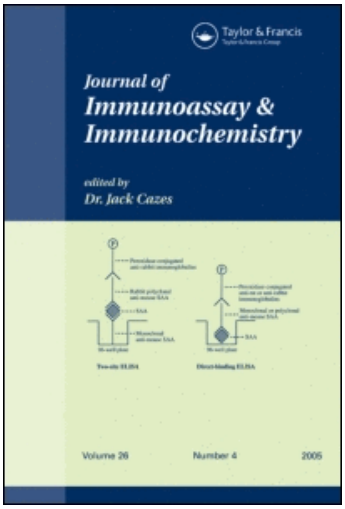

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Development and Validation of an Enzyme Linked Immunosorbent Assay for the Quantification of Carcinoembryonic Antigen in Mouse Plasma

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Abstract: Carcinoembryonic antigen (CEA) is a tumor associated antigen that is over-expressed in colorectal cancer and several other cancers of the gastro-intestinal system. An enzyme linked immunosorbent assay was developed to determine CEA concentrations in mouse plasma. The assay was validated over the standard curve range of 1–20 ng/mL. The intra-assay recoveries ranged from 93–104% with associated percent coefficients of variation (CV%) ranging between 2.5–12.8%. The inter-assay recoveries were in the range of 98.4–105% and their CV% values were between 4.77–10.1%. The assay was used to detect the presence of circulating CEA in the LS174T adenocarcinoma xenograft model and to study the pharmacokinetics of recombinant CEA in athymic mice.

Keywords: Athymic mice, Carcinoembryonic antigen (CEA), ELISA, Plasma, Shed antigen, Xenograft

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INTRODUCTION

Carcinoembryonic antigen (CEA), a complex highly glycosylated membrane bound protein (MW 180 kDa), was first isolated from human colonic tissue extracts by Gold and Freedman.[1,2] It was initially believed to be expressed only in the fetal digestive system and over-expressed in cancers of the gastrointestinal tract; however, it is now known that limited CEA expression is observed in various normal tissues, including apical absorptive cells and goblet cells of the colon, gastric mucous cells, secretory sweat glands and epithelial cells of the pancreas, uterus and prostrate.[3–5] Further work by Gold, Freedman, and co-workers demonstrated the presence of high levels of circulating CEA in the serum of patients suffering from various adenocarcinomas, whereas concentrations <2.5 ng/mL were observed in normal sera.[6] These observations led to an increased interest in the use of CEA as a tumor marker.

Antibodies directed against carcinoembryonic antigen have been increasingly used for the detection and treatment of CEA expressing tumors. LS174T adenocarcinoma cells, which express CEA, are commonly used in mouse models of colorectal cancer. This laboratory is employing the LS174T mouse model to examine a new anti-CEA antibody-mediated drug-targeting approach. It is anticipated that important determinants of the efficacy of the targeting strategy will include rates of CEA production, CEA turnover, and concentrations of CEA in plasma (e.g., following "shedding" of antigen from the xenografts). As such, the investigation of CEA pharmacokinetics is essential for rational development of our, and other similar, targeting strategies.

Radioimmunoassay and enzyme immunoassay methods have been developed to detect carcinoembryonic antigen (CEA) in the serum of patients with colon or rectal cancer.[6,7] Commercial immunoassay and radioimmunoassay CEA assay kits are available to aid prognosis of colorectal cancer. However, to the authors' knowledge, there has been no prior development of validated ELISA to detect and quantify CEA concentrations in mouse plasma.

The anti-CEA antibody, T84.66, is used for detection of carcinoembryonic antigen (CEA) in this assay. This antibody is highly specific for the Gold 1 or A3 domain of CEA,[8] and T84.66 does not react with other members of the CEA family that are present in normal tissues (e.g., non-specific cross reacting antigen and biliary glycoprotein).[9] The assay presented herein was validated with respect to intra-assay and inter-assay precision and accuracy. The assay has been applied to detect circulating CEA in our LS174T xenograft model, and to study the pharmacokinetics of recombinant CEA in athymic nude mice.

EXPERIMENTAL

Production and Purification of T84.66

Hybridoma cells producing anti-CEA antibody, T84.66, were purchased from American Type Culture Collection (ATCC # HB-8747, Manassas, VA). T84.66 is known to bind CEA with an equilibrium affinity constant of 2.6×10^{10} L/M.[10] Hybridoma cells were grown in 1 L spinner flasks containing serum free media (Hybridoma SFM, Invitrogen, NY). Anti-CEA antibody was purified from the supernatant by protein G chromatography (Amersham Biosciences, Uppsala Sweden) through the use of a Bio-Rad medium pressure chromatography system. The loading buffer used was 20 mM Na_2HPO_4 (pH 7.0, Sigma Chemical) and the elution buffer was 100 mM glycine (pH 2.8, Bio-Rad Laboratories, CA). Eluted antibody was collected in glass tubes containing 1 M Tris buffer (pH 9.0) to neutralize the solution and minimize antibody precipitation.

ELISA Procedure

Nunc Maxisorp 96 well plates (Nunc model # 62409-002, VWR, Bridgeport, NJ) were incubated with T84.66 (2 μg/mL in phosphate buffered saline), overnight, at 4°C (250 μL/well). The plates were washed three times with PB-Tween consisting of 0.05% Tween and 0.02 M Na_2HPO_4 (Sigma, no pH adjustment), followed twice with washes of double distilled water. After washing, the plates were incubated with standards and samples in triplicate (50 μL/well), for 2 h at room temperature. At the end of the incubation, plates were washed again, as described above. Wells were then incubated with 50 μl of goat anti-CEA-HRP conjugate (Fitzgerald Industries, Concord, MA) for 1 h at room temperature (1:3000 dilution of the 0.25 mg/mL conjugate with PB-Tween). The plates were washed, followed by incubation with 50 μL of o-phenylene diamine conjugate (Sigma, 1 mg/mL in 0.1 M citrate phosphate buffer containing 0.03% H_2O_2). The reaction was allowed to progress for 8 min, and then stopped by addition of 2 N H_2SO_4. Plates were read at 492 nm using the end-point mode of the plate reader (Spectra Max 250, Molecular Devices; Sunnyvale, CA).

Standards were prepared by diluting stock of recombinant CEA (rCEA, Protein Sciences Corporation, CT) to appropriate concentrations (0, 1, 2.5, 5, 10 and 20 ng/mL) using phosphate buffered saline (pH 7.4) and mouse plasma 50% v/v (Hill Top Lab Animals Inc; Scottdale, PA). Standards were prepared fresh for use in each assay run. The assay was validated for precision and accuracy by determining the recovery of spiked rCEA from mouse plasma quality control

samples (QCs). QCs were prepared in bulk, aliquoted, and stored at 4°C until assayed.

Determination of Shed CEA, Subcutaneous Colorectal Xenograft Model

All animal experimentation was approved by the Institutional Animal Use and Care Committee of the University at Buffalo. Male athymic nude mice (5–6 weeks old, ~25 g in weight) were purchased from Harlan (Indianapolis, IN). Mice were housed in a sterile room and fed standard chow that was previously autoclaved. Nude mice were handled under an aseptic laminar flow hood. LS174T cells in suspension (100 μL, $\sim 5 \times 10^6$ cells) were injected s.c into the right flank of the mice. The mice were examined for tumor growth and their body weight was monitored regularly. Blood samples (100 μL) were collected from retro-orbital plexus using calibrated capillary pipettes (Drummond Scientific Company, Cat # 2-000-020) that were pre-rinsed with EDTA. Samples were collected every third day, starting from the day of tumor cell inoculation, and continuing up to the point when the tumor volumes exceeded 2000 mm^3. Blood was centrifuged at 13,000 rpm for 3 min, and plasma samples were separated and stored at −20°C until analyzed by ELISA.

Determination of Shed CEA, Intra-Peritoneal Colorectal Xenograft Model

Male athymic nude mice (n = 5) were used for this study. 500 μL of LS174T cells ($\sim 2 \times 10^6$ cells) were injected into the peritoneum of the mice. Mice were examined regularly to check for palpable tumor growth, and animal weight was closely monitored. Blood samples (100 μL) were collected from retro-orbital plexus on day 0, 2, 6, 9, 13, 15, 18, 22, 24, 27 and 29. All mice were sacrificed by day 30, by cardiac puncture, and peritoneal ascitis fluid (~300 μL) was collected. The blood was centrifuged at 13,000 rpm for 3 min. The plasma fraction and ascitis fluid were stored at −20°C until analyzed by ELISA.

Pharmacokinetic Studies

Male athymic nude mice (Harlan, Indianapolis, IN) were used for investigation of rCEA pharmacokinetics. Recombinant CEA was administered to mice via intra-penile vein injection at two dose levels: 0.1 mg/kg (n = 5) and 0.2 mg/kg (n = 5). Blood was collected from the retro-orbital plexus using calibrated capillary pipettes (Drummond

Scientific Company, Cat # 2-000-020) that were pre-rinsed with EDTA at 5, 15, 30, 60, 120, 240, 360 min, using a staggered study design. At any given time-point, samples were obtained from at least 3 mice in the group. The blood was then centrifuged at 13,000 rpm for 3 min. The plasma fraction was separated and stored at -20°C until analyzed by ELISA. Non-compartmental pharmacokinetic analyses were performed using WinNonlin, version 5.0 (Pharsight Corporation, Palo Alto, CA)

RESULTS AND DISCUSSION

Assay Validation

The best fit line for the calibration curve was obtained by fitting the data to a quadratic equation; $y = a + bx + cx^2$ (Figure 1). Correlation coefficients of at least 0.995 were obtained during the intra-assay and inter-assay validation. The inter-assay and intra-assay recoveries from plasma samples are listed in Table 1. The intra-assay recovery was in the range of 93–104% and the percent coefficients of variation (CV%) ranged from 2.7–12.8%. The inter-assay recoveries ranged from 98.4–105% and the associated CV% ranged from 4.77–10.1%. The working range of the assay was 1–20 ng/mL. The standards comprised of 50% mouse plasma resulting in a lower limit of quantitation of 2 ng/mL of CEA.

This study is the first report of an intra-assay and inter-assay validated ELISA to measure human carcinoembryonic antigen (CEA) in

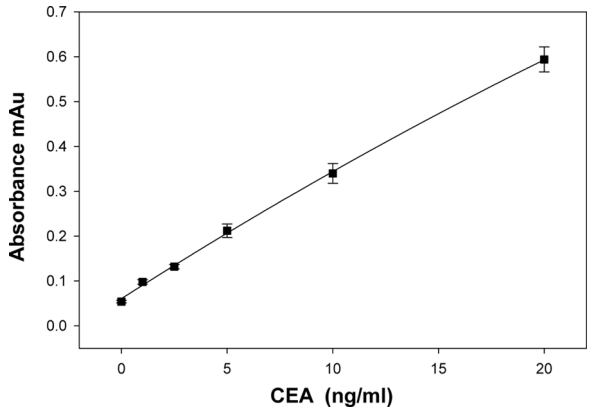

Figure 1. Representative standard curve for CEA over the range of 1–20 ng/mL. The curve is fitted with a cubic equation, and $r^2 = 1$. Error bars represent the standard deviation across the mean of 3 replicates.

Table 1. Variability of ELISA with respect to recovery of carcinoembryonic antigen (CEA) from mouse plasma samples

Actual concentration (ng/mL)	Recovered concentration (ng/mL)	Recovery (%)	CV (%)
Inter-Assay Variability (n = 3)			
1	1.05	105	10.1
2.5	2.52	98.4	8.01
10	10.0	100	4.77
20	20.1	101	7.27
Intra-Assay Variability (n = 3)			
1	1.00	100	12.8
2.5	2.46	98.4	7.40
10	9.30	93.0	2.70
20	20.8	104	3.50

CV = Coefficient of variation.

mouse plasma. Previous xenograft studies used commercially available kits to determine CEA levels in mouse plasma; however these kits are not validated for use with murine samples.

Shed CEA Levels in Subcutaneous Xenograft Model

The plasma CEA levels in all xenograft-bearing mice were below the detectable limits of the assay, i.e., less than 2 ng/mL. Increasing tumor size did not lead to any measurable shedding of CEA from the tumor surface. LS174T cells are known to express large amounts of CEA; however, low CEA concentrations in plasma may be explained by poor absorption of CEA from the subcutaneous site of tumor growth, and/or by rapid rates of CEA elimination following absorption into the systemic circulation.

Shed CEA Levels in Peritoneal Xenograft Model

On autopsy, tumor nodules were detected throughout the peritoneal cavity and metastases in distant organs such as the liver and spleen were observed. Serum CEA levels ranged from <2 ng/mL to 25 ng/mL; however, no correlation was observed between serum levels and tumor burden. These results are in accordance with published literature reports with similar findings in studies performed with LOVO and P116 cell lines.[11,12] Several other studies quote a direct correlation between tumor size and serum CEA levels.[13,14] These conflicting results may be due to

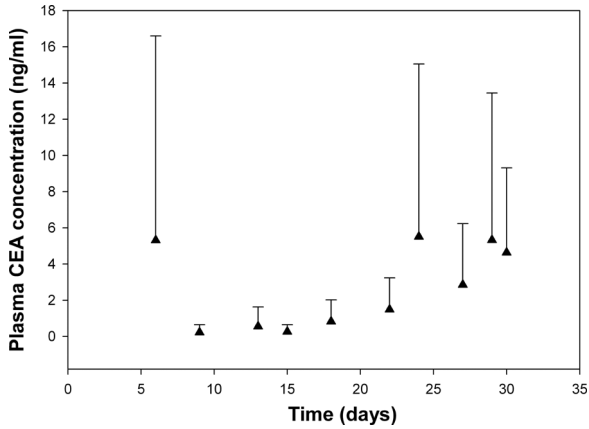

Figure 2. Serum CEA concentrations in mice bearing peritoneal LS174T xenografts (n = 4–5).

the use of different cell lines, with potential for dramatic differences in the rate of CEA expression and shedding. In the current study, serum CEA concentrations were found to be extremely variable between mice bearing tumors of similar volume, and also CEA levels within a given mouse were found to fluctuate by as much as 20-fold during the study period (Figure 2). These wide fluctuations in serum CEA levels and lack of correlation with tumor size could be caused by multiple factors. Increased levels of CEA shedding have been previously linked to cell death and

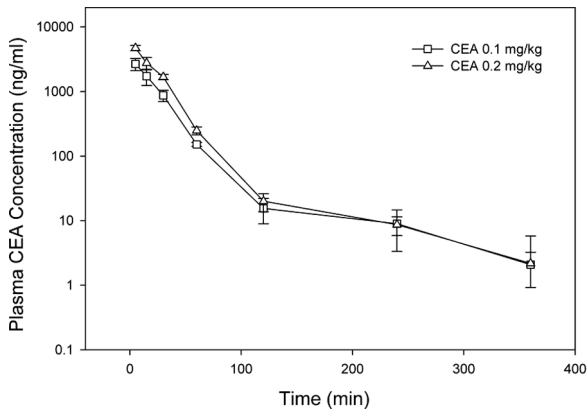

Figure 3. Pharmacokinetics of rCEA following 0.1 mg/kg and 0.2 mg/kg doses in athymic Fox^{nu} mice. Error bars represent standard deviations associated with mean concentrations (n = 3–5).

Table 2. Pharmacokinetic parameters for rCEA administered as single intravenous bolus at dose levels of 0.1 and 0.2 mg/kg

Parameters	0.1 mg/kg	0.2 mg/kg
Vss (mL/kg)	32.0 ± 9.2	32.9 ± 5.1
$t_{1/2}$ (min)	83.1 ± 37	75.1 ± 9.2
CL (mL/min/kg)	1.26 ± 0.26	1.45 ± 0.15

Vss = Steady state volume of distribution.
$t_{1/2}$ = Terminal half-life.
CL = Systemic clearance.

necrosis.[15] It is possible that each tumor in a given host involves different extents of vascular invasion and has a unique sub-population of cells in different phases of cell cycle leading to differential shedding of CEA from tumor surface. Also, CEA is known to be eliminated mainly by the Kupffer cells of liver,[16] and inter-animal variability in hepatic function may lead to variability in serum levels. High concentrations of CEA, 219 ± 26.7 ng/mL, were found in ascitis fluid of 4 of 5 mice used in the study (it was not possible to obtain ascitis fluid from one mouse). This observation is in agreement with a similar study performed in humans demonstrating a significant association between CEA levels in fluids bathing the tumors and colon carcinoma.[17]

LS174T is one of the most widely used cell lines to establish adenocarcinoma xenografts in athymic nude mice. However, there has been no comprehensive study of the presence of circulating or shed carcinoembryonic antigen in this xenograft model. Our study demonstrates an absence of shed CEA in our subcutaneous model, and a lack of correlation between serum CEA concentrations and tumor volume in the peritoneal model.

Pharmacokinetics of rCEA in Athymic Mice

The concentration versus time profile for rCEA in mouse plasma is shown in Figure 3, and demonstrates that the assay is well suited for the determination of CEA pharmacokinetics in mice. Table 2 lists the CL, $t_{1/2}$ and Vss parameter values obtained by non-compartmental analyses.

CONCLUSIONS

A sensitive ELISA assay has been developed and validated for the quantitation of carcinoembryonic antigen (CEA) in mouse plasma. The assay

was successfully applied to detect the presence of circulating antigen in the LS174T xenograft model and to study rCEA pharmacokinetics in mice.

ACKNOWLEDGMENTS

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